CELLULAR SIGNALING NETWORKS IN PLANT HEAT STRESS RESPONSES

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CELLULAR SIGNALING NETWORKS IN PLANT HEAT STRESS RESPONSES

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Comparative Transcriptomics Analysis and Functional Study Reveal Important Role of High-Temperature Stress Response Gene *GmHSFA2* During Flower Bud Development of CMS-Based F_1 in Soybean

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High-temperature (HT) is one of the most important environmental factors that negatively impact the yield of some soybean cytoplasmic male sterility (CMS)-based hybrid (F_1) combinations. The response of soybean to HT, especially at the male organ development stage, is poorly understood. To investigate the molecular mechanisms of the response from soybean CMS-based F_1 male organ to HT, a detailed transcriptomics analysis was performed during flower bud development of soybean HT-tolerant and HT-sensitive CMS-based F_1 combinations (NF_1 and YF_1) under normal-temperature and HT conditions. Obvious HT damage was observed by subjecting YF_1 with HT, such as indehiscent anthers and decreased pollen fertility, whereas the male fertility of NF_1 was normal. In total, 8,784 differentially expressed genes (DEGs) were found to respond to HT stress, which were mainly associated with anther/pollen wall development, carbohydrate metabolism and sugar transport, and auxin signaling. The quantitative real-time PCR (qRT-PCR) analysis and substance content detection also revealed that HT caused male fertility defects in YF_1 by altering pectin metabolism, auxin, and sugar signaling pathways. Most importantly, the sugar signaling-*PIF*-auxin signaling pathway may underlie the instability of male fertility in YF_1 under HT. Furthermore, HT induced the expression of heat shock factor (*HSF*) and heat shock protein (*HSP*) gene families. Overexpression of *GmHSFA2* in *Arabidopsis* can promote the expression of HT protective genes (such as *HSP20*) by binding to the HSE motifs in their promoters, so as to improve the HT tolerance during flowering. Our results indicated that *GmHSFA2* acted as a positive regulator, conferring HT tolerance improvement in soybean CMS-based F_1. *GmHSFA2* may be directly involved in the activation of male fertility protection mechanism in the soybean CMS-based F_1 under HT stress.

Keywords: soybean, CMS-based F_1, HT stress, RNA-seq, *HSFA2*

INTRODUCTION

Temperature is an important ecological factor affecting physiological and biochemical processes in plants. The physiological damage caused by a high-temperature (HT) environment to plants is called HT stress (Puteh et al., 2013). For plants, even an increase of 1°C in the threshold level is considered as HT stress (Teixeira et al., 2013). The development of male organs in plants is extremely sensitive to temperature, and continuous HT stress will cause microspore abortion, anther indehiscence, filament shortening, and abnormal pollen viability or germination (Djanaguiraman et al., 2018; Begcy et al., 2019). In recent years, with the increase of global temperature, HT stress has become a serious factor affecting crop growth and development (Min et al., 2013; Li et al., 2018).

The "cytoplasmic male sterility (CMS)-based" breeding system is composed of the CMS line and its corresponding maintainer line and restorer line, which is one of the most widely used breeding systems in crop hybrid (F_1) seed production (Chen and Liu, 2014). At present, the "CMS-based" matching system has been successfully applied in rice, maize, soybean, and other crops (Chen and Liu, 2014). In the process of hybridization, the sterility of the male sterile line can be restored by the fertility restorer gene of the male restorer line. Due to the genetic effects of cytoplasmic and nuclear interactions between the CMS line and its restorer line, CMS-based F_1 is generally more sensitive to the external environment than conventional materials, especially for gametophyte sterile material, in which only about 50% of its CMS-based F_1 pollen is fertile (Xie, 2008). Under the influence of certain conditions (including HT stress), the percentage of fertile pollen may be greatly reduced for CMS-based F_1 and eventually fail to develop normal seeds (Xie, 2008).

It has been reported that HT is one of the main factors affecting the difference of CMS-based F_1 fertility restoration in some plants, such as cotton and rice (Zhao et al., 2009; Zhang et al., 2019). Continuous HT stress resulted in insufficient anther dehiscence, decreased pollen survival rate, and finally decreased yield of CMS-based F_1. In general, soybeans begin to bloom in late July. However, extreme HT frequently occurred in July and August in the Huanghuai region and South China, the main producing areas of summer-sown soybean in China. Similar to rice and cotton, the male fertility of soybean CMS-based F_1 is also unstable under HT (Nie et al., 2017).

In recent years, it has been reported that fertility-enhancing genes and DNA methylation are involved in the fertility regulation of cotton CMS-based F_1 (Wang, 2019; Zhang et al., 2019). Wang (2019) found that pollen fertility of cotton CMS-based F_1 is related to the restorer gene and fertility-enhancing gene such as *GST* (Zhu, 2005). Under the same nuclear genetic background of the restorer gene, the restorer line with strong adaptability can be selected using different ecological environments (Wang, 2019). Zhang et al. (2019) found that HT-induced DNA methylation maintained the dynamic balance of ATP synthesis and ROS production by upregulating the expression of mitochondrial respiratory chain-related genes, so as to ensure the normal fertility recovery ability of the cotton CMS-D2 system under HT stress. However, no study has focused on the molecular mechanism of HT-induced male fertility instability in soybean CMS-based F_1. In order to better understand the molecular mechanism of difference in male fertility restoration of soybean CMS-based F_1 under HT stress, anther/pollen morphology observation, RNA sequencing (RNA-seq), physiological and biochemical determination, and gene functional verification were performed. Cytological observation showed that soybean HT-sensitive CMS-based F_1 was mainly characterized by anther indehiscence and decreased pollen fertility under HT stress. Based on the analysis of differentially expressed genes (DEGs) and differential metabolites, we found that genes or substances related to anther/pollen wall development and auxin metabolism, carbohydrate metabolism, sugar transport, transcription factors (TFs), and heat shock proteins (*HSP*) may be involved in the fertility regulation of soybean CMS-based F_1 under HT. Most importantly, it was found that *GmHSFA2* can regulate *HSP* and *galactinol synthase* (*GolS*)-related genes to improve HT tolerance of plants.

MATERIALS AND METHODS

Plant Materials and HT Treatment

Two soybean CMS-based F_1 combinations of the CMS system with different degrees of HT stress tolerance in the field were used in this study, namely, NF_1 and YF_1, which are tolerant and sensitive to HT stress, respectively. The hybridization of the CMS line NJCMS1A and its restorer lines N4608 and YY6 was carried out in the field at Dangtu Experimental Station (National Center for Soybean Improvement, Nanjing Agricultural University, Dangtu, Anhui, China) in the summer of 2017. And the F_1 seeds of (NJCMS1A × N4608) and (NJCMS1A × YY6) were harvested in the autumn, which were designated as NF_1 and YF_1, respectively, in this study. The plants were grown in illuminated incubators (RXZ-430D, Ningbo Jiangnan, Ningbo, China) at 26 ± 1/20 ± 1°C (day/night) with a 12-h light/12-h dark photoperiod during seedling. The flowering plants were grown in an illuminated incubator at 30°C/24°C (day/night) considered as a normal-temperature (NT) condition. For temperature gradient treatment, three individual flowering plants (R1 stage) of each combination were incubated at 38/32°C and 34/28°C (day/night) for 7 days. During HT treatment, the flowering plants (R1 stage) were incubated at 38/32°C in an illuminated incubator. Because it is very difficult to judge the precise development stage of pollen from the appearance of the flower buds in soybean as

Abbreviations: ATP-D6P7, ATP-dependent 6-phosphofructokinase 7; CMS, cytoplasmic male sterility; DEGs, differentially expressed genes; DREB, dehydration-responsive element binding; Exo-PG, exopolygalacturonase; Glc, glucose; GolS, galactinol synthase; ER, ethylene responsive; FPKM, fragments per kilobase of transcript per million mapped reads; GFP, green fluorescent protein; GO, Gene Ontology; GUS, β-glucuronidase; HT, high-temperature; HSF, heat shock factor; HSP, heat shock protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; NJ, Neighbor joining; NT, Normal temperature; PCA, principal component analysis; PG, polygalacturonase; PIF, PHYTOCHROME-INTERACTING FACTOR; PL, pectate lyase; PME, pectin methylesterase; qRT-PCR, quantitative real-time PCR; RCA, repeated correlation analysis; SD, standard deviation; SPL, squamosa promoter-binding protein-like; Suc, sucrose; TFs, transcription factors; UDP-GAD, UDP-glucuronic acid decarboxylase; Y1H, yeast one-hybrid; WT, wild-type.

described previously (Ding et al., 2016), after HT treatment for 7 days, flower buds of different sizes were collected from NF_1 and YF_1 plants under NT and HT, respectively, and then immediately frozen in liquid nitrogen and stored at −80°C for RNA isolation. To analyze the expression patterns of *GmHSFA2* (*Glyma.14G096800*) and *GmHSP20a* (*Glyma.12G013100*) genes, flowering plants from N4608 were initially exposed to 40°C for 7 days of HT treatment and then transferred to NT (30°C) for recovery. Flower buds of different sizes were sampled at time points of 0, 1st, 3rd, 5th, 7th day and 1 day after recovery. Flower buds of each genotype were collected from three individual plants as three independent biological replicates for NF_1NT, YF_1NT, NF_1HT, YF_1HT, and N4608.

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used as wild-type (WT) control. The *35S:GmHSFA2*, *pGmHSFA2:GUS*, and *35S:GmHSP20a* transgenic plants were all in the Col-0 background. The seeds were vernalized for 2 days at 4°C and then cultivated on a prefertilized soil mixture (nutritional soil, perlite, and vermiculite at a 3:1:1 ratio) at 23°C with long-day conditions (16 h light/8 h dark) in an illuminated incubator (RXZ-430D, Ningbo Jiangnan, Ningbo, China). To evaluate the HT damage on inflorescence and the expression levels of *GmHSFA2* downstream regulatory genes under HT stress, three *35S:GmHSFA2* transgenic lines and WT were exposed to HT stress at 45/40°C (day/night) for 3 days. The HT treatment on male fertility was performed as Kim et al. (2001) described. The *35S:GmHSFA2* and *35S:GmHSP20a* transgenic plants (two lines for each transgenic type) and WT were held in an illuminated incubator (RXZ-430D, Ningbo Jiangnan, Ningbo, China) at 42°C for 4 h and then transferred to normal growth conditions. All types were grown at 23°C as control.

RNA Isolation and cDNA Library Construction

Total RNA from the flower buds of NF_1NT, YF_1NT, NF_1HT, and YF_1HT (three independent biological replicates for each genotype) was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. In order to obtain mitochondrial and chloroplast-related genes, this study refers to the cDNA library construction of prokaryote, considering that the plant mitochondrial and chloroplast genomes are similar to its ring genome. So after total RNA was extracted, sample mRNA was enriched by removing rRNA by a Ribo-Zero^{TM} Magnetic Kit (Epicentre). Then the enriched mRNA was fragmented into short fragments using a fragmentation buffer and reverse transcribed into cDNA with random primers. A second-strand cDNA was synthesized by DNA polymerase I, RNase H, dNTP, and buffer. Then the cDNA fragments were purified with a QIAquick PCR extraction kit, end repaired, poly(A) added, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina HiSeq^{TM} 2500 by Gene *Denovo* Biotechnology Co. (Guangzhou, China).

Data Analysis of RNA-Seq

Raw reads were filtered to obtain high-quality reads by removing reads containing adapters or more than 10% of unknown nucleotides (N) and more than 50% of low-quality (Q-value ≤ 20) bases. The rRNA mapped reads were removed by a short-reads alignment tool Bowtie 2 (Langmead and Salzberg, 2012). Clean reads (the rRNA removed reads) were subsequently aligned with the soybean Williams 82 reference genome (Wm82.a2.v1) using TopHat2 (version 2.0.3.12, Kim et al., 2013). Gene abundances were quantified by software RSEM (Li and Dewey, 2011), and the gene expression level was normalized by using the fragments per kilobase of transcript per million mapped reads (FPKM) method (Mortazavi et al., 2008).

Subsequent data were analyzed using repeated correlation analysis (RCA) and principal component analysis (PCA). The correlation coefficient between the two replicas was calculated to evaluate repeatability between samples. The closer the correlation coefficient gets to 1, the better the repeatability between two parallel experiments. The PCA was performed with R package models[1]; it is largely used to reveal the relationship of NF_1NT, YF_1NT, NF_1HT, and YF_1HT. To identify DEGs across samples or groups, the edge R package (see text footnote 1) was used. Only genes with $|\ \mathrm{Log_2FC}$ (fold change)$|\ \geq 1$ and false discovery rate (FDR) ≤ 0.05 were identified as significant DEGs. Gene Ontology (GO) enrichment analysis provides all GO terms that are significantly enriched in DEGs compared to genomic backgrounds and maps all DEGs to GO terms in the GO database.[2] GO terms with FDR ≤ 0.05 were considered to be significantly enriched. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed in the KEGG database web server[3] (Kanehisa et al., 2008). Pathways with FDR ≤ 0.05 were defined as significantly enriched pathways in DEGs.

Plant Transformation

Full-length CDS clones of the *GmHSFA2* (*Glyma.14G096800*) and *GmHSP20a* (*Glyma.12G013100*) genes were obtained from SoyBase.[4] Two overexpression constructs were generated by inserting the full-length *GmHSFA2* and *GmHSP20a* CDS fragments into the binary vector *pCAMBIA3301-26* after the CaMV 35S promoter, using a one-step cloning kit (Vazyme, Nanjing, China) and designated as *35S:GmHSFA2* and *35S:GmHSP20a*, respectively. The promoter of *GmHSFA2* (2,000 bp) was amplified by PCR using N4608 DNA and replaced the 35S promoter of *pCAMBIA3301-GUS* using *Hin*dIII and *Nco*I digestion, resulting in a plasmid of *pGmHSFA2:GUS*. All the above overexpression vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 via the freeze–thaw method. *Agrobacterium*-mediated floral dip method was used for *Arabidopsis* transformation (Clough and Bent, 1998). The specific primers used for CDS and promoter cloning are given in **Supplementary Table 1**. Transgenic plants (T_0, T_1, T_2,

[1]http://www.r-project.org/
[2]http://www.geneontology.org/
[3]http://www.kegg.jp/kegg/
[4]https://www.soybase.org/

and T_3) were screened by the Murashige and Skoog medium glufosinate (20 mg/L).

GUS Staining and Plant Trait Investigation

The inflorescence of *pGmHSFA2:GUS* plant materials was GUS stained following the protocol of Jefferson et al. (1987). The morphology of anthers from opened flowers of soybean and *Arabidopsis* was observed under an Olympus CX31 microscope (Japan). Pollen viability of soybean and *Arabidopsis* was analyzed by I_2-KI staining (Nie et al., 2019) and Alexander's staining (Ding et al., 2020), respectively. The stamen and pistil length of *Arabidopsis* was measured with the cellSens software (Olympus, Japan). Nine flower buds/flowers of each genotype/line were collected from three individual plants to measure the length of stamen/pistil and observe the fertility of pollen. Student's *t*-test was performed to compare the trait differences between the experimental group and the control group.

Quantitative Real-Time PCR Analysis

The quantitative real-time PCR (qRT-PCR) was used to validate the gene expression levels in soybean and *Arabidopsis*. All primers (**Supplementary Table 1**) were designed based on the mRNA sequences and synthesized commercially (General Biosystems, Chuzhou, China). Total RNA from the same soybean samples that constructed the cDNA library was used for the validation of RNA-seq. According to the procedures provided in the HiScript Q RT SuperMix for the qPCR kit (+gDNA wiper, Vazyme, Nanjing, China), 1 μg of total RNA was reverse-transcribed using an Oligo(dT) primer. The mRNA qRT-PCR analysis was carried out using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) on a Bio-Rad CFX96 instrument (CFX96 Touch, Bio-Rad, United States). For *Arabidopsis*, all reactions were run with three independent biological replicates, each comprising three individual plants, and *AtActin* (accession number: *NM_001338359.1*) was used as internal control genes. For soybean, *GmTubulin* (accession number: *NM_001252709.2*) was used as internal control genes. The NF_1 and WT under the NT condition were used as the control in qRT-PCR experiments on soybean and *Arabidopsis*, respectively. The relative expression levels of the genes were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Student's *t*-test was performed to compare mRNA expression differences between the experimental group and the control group.

Substance Content and Enzyme Activity Assays

Flower buds of different sizes were collected from NF_1 and YF_1 plants (three independent biological replicates for each genotype) under NT and HT for substance content and enzyme activity assays. The contents of Suc, Glc, starch, and IAA were determined on a UV–vis spectrophotometer (EU-2600D, Onlab, Shanghai, China) using a Suc assay kit (Jiancheng, Nanjing, China), Glc assay kit (Sinobestbio, Shanghai, China), starch assay kit (Sinobestbio, Shanghai, China), and IAA assay kit (Mallbio, Nanjing, China), respectively, by following the manufacturer's protocol. The pectinase activity was measured at 540 nm on a microplate reader (SpectraMax iD5, Molecular Devices, United States) using the pectinase assay kit (Sinobestbio, Shanghai, China) by following the manufacturer's protocol. Three independent biological replicates were assayed, and one-way ANOVA and Duncan's test were performed for statistical analysis.

Subcellular Localization and Yeast One-Hybrid Assay

The open reading frame (ORF) (after removal of the stop codon) of *GmHSFA2* was integrated into the 5′ end of the green fluorescent protein (*GFP*) coding region in the *pCAMBIA3301-GFP* vector using *Bgl*II digestion, resulting in a plasmid of *GmHSFA2-GFP*. Both *GmHSFA2-GFP* and *pCAMBIA3301-GFP* (control) constructs were transformed into tobacco (*Nicotiana benthamiana*) leaves according to the protocol of Sparkes et al. (2006). The treated seedlings of tobacco were grown at 23°C with long-day conditions (16 h light/8 h dark) in an illuminated incubator (RXZ-430D, Ningbo Jiangnan, Ningbo, China) for 3 days and then observed under a confocal laser scanning microsystem LSM780 (Carl Zeiss, Jena, Germany) with 488-nm excitation wavelengths.

The direct interaction between *GmHSFA2* and the promoter of *GmHSP20a* was detected by the yeast one-hybrid (Y1H) assay system. Four tandem *cis*-acting HSE motifs present in the promoter region of *GmHSP20a* were amplified by PCR using N4608 DNA and integrated into the *PAbAi* vector, yielding *pAbAi-pGmHSP20a* as bait, while the full-length CDS of *GmHSFA2* was amplified from the *35S:GmHSFA2* vector and inserted into a *pGADT7* vector, yielding a *pGADT7-GmHSFA2* construct as prey. The primers used are listed in **Supplementary Table 1**. The *pGBKT7-pGmHSP20a* was first introduced into the Y1H gold yeast (Clontech) and cultured on SD/–Ura and SD/–Ura/A medium for self-activating detection. After that, the *PGADT7*, negative control, and positive control vectors were introduced and cultured on SD/–Ura/A for spot assay.

RESULTS

Characterization of Soybean CMS-Based F_1 Male Fertility Under HT Stress

To explore the mechanism of male fertility instability under HT stress, two soybean CMS-based F_1 combinations were used in this study, namely, NF_1 (HT tolerant) and YF_1 (HT sensitive) (**Supplementary Figure 1**). There was no difference in male fertility between the two combinations under NT (30°C) according to gradient temperature treatment (**Supplementary Figures 2A,B** and **Figure 1**). However, obvious HT damage was observed by subjecting YF_1 to gradient temperatures (30, 34, and 38°C, such as worse anther dehiscence and gradually decreasing pollen fertility (**Supplementary Figures 2A,B**). When the temperature reached 38°C (HT treatment in this study), the male fertility of YF_1 was significantly affected, and YF_1 finally displayed forms of anther indehiscence and decreased pollen fertility, while NF_1 performed normally (**Figure 1**).

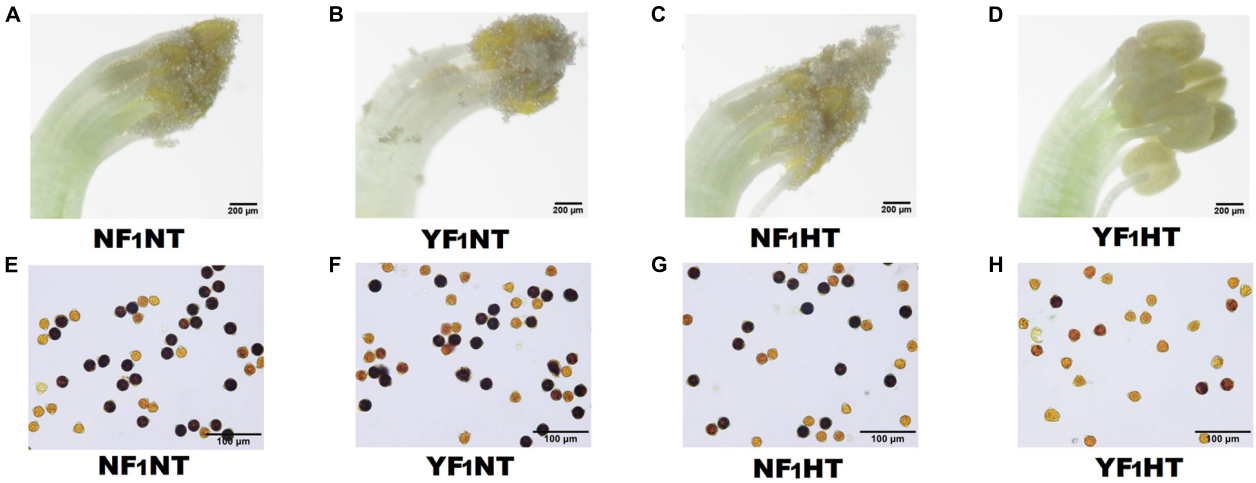

FIGURE 1 | Phenotypic characteristics of soybean male fertility under NT and HT conditions. **(A–D)** Soybean anthers from NF_1 and YF_1 under NT and HT conditions. **(E–H)** Soybean pollens from NF_1 and YF_1 under NT and HT conditions. The sterile pollen was stained bright yellow by I_2-KI solution.

TABLE 1 | Data statistics of cDNA libraries from NF_1NT, YF_1NT, NF_1HT, and YF_1HT.

Sample	Raw reads	Raw reads (bp)	HQ clean reads	HQ clean data (bp)	Clean bases (Gb)	Q20 (%)	Q30 (%)	GC (%)	Mapping ratio (%)
NF_1NT-1	38467484	5770122600	37832070	5596841295	5.60	98.32	94.58	43.82	89.70
NF_1NT-2	59467424	8920113600	58497786	8655405125	8.66	98.44	94.90	43.77	90.43
NF_1NT-3	40597054	6089558100	39934094	5908373737	5.91	98.39	94.79	43.85	90.24
YF_1NT-1	42872316	6430847400	42115742	6224542494	6.22	98.36	94.68	44.14	90.57
YF_1NT-2	30496662	4574499300	29953826	4427793915	4.43	98.28	94.47	43.95	90.49
YF_1NT-3	40171536	6025730400	39432772	5824635146	5.82	98.41	94.85	44.02	90.61
NF_1HT-1	38467484	5770122600	37832070	5596841295	5.60	98.32	94.58	43.82	89.70
NF_1HT-2	59467424	8920113600	58497786	8655405125	8.66	98.44	94.90	43.77	90.43
NF_1HT-3	40597054	6089558100	39934094	5908373737	5.91	98.39	94.79	43.85	90.24
YF_1HT-1	42872316	6430847400	42115742	6224542494	6.22	98.36	94.68	44.14	90.57
YF_1HT-2	30496662	4574499300	29953826	4427793915	4.43	98.28	94.47	43.95	90.49
YF_1HT-3	40171536	6025730400	39432772	5824635146	5.82	98.41	94.85	44.02	90.61
Average	42012079.33	6301811900	41294381.67	6106265285	6.11	98.37	94.71	43.93	90.34

HQ, high quality. Sequence length was 2 × 150 bp, length of each read was 150 bp using double end sequencing.

Transcriptomics Analysis in Flower Buds of NF_1 and YF_1 Under HT Stress

To gain insight into the molecular mechanism of male fertility reduction in soybean HT-sensitive CMS-based F_1 under HT stress, RNA-seq was performed for both HT-tolerant and HT-sensitive F_1 during flower bud development under NT and HT conditions. A total of 50.41 million raw reads were generated from 12 samples with an average read length of 150 bp (**Table 1**). After removal of reads containing adapters, poly(N) greater than 10%, and low-quality sequences, an average of 6.11-Gb clean data were obtained for each sample (**Table 1**). After removal of reads mapped on rRNA, 89.70–90.57% of clean reads were mapped to the soybean reference genome (Gmax_275_Wm82.a2.v1) (**Table 1**).

Principal component analysis was used to analyze the relationship between two genotypes under NT and HT conditions. The first principal component (PC1) accounted for 78.2% of the variance, and the second principal component (PC2) accounted for 12.0% of the variance (**Supplementary Figure 3**). With the exception of NF_1HT, the three biological replicas in each group were clustered closely together. In general, NF_1 and YF_1 were significantly different under NT and HT conditions. As shown in **Supplementary Figure 4**, the correlation coefficients (R^2) between the biological replicates of each group were greater than 0.96 and close to 1, indicating that each group had good repeatability.

Identification of DEGs in Response to Heat Stress

Significantly DEGs were screened between the different samples with the criteria of fold change $\geq$ 2 and FDR $\leq$ 0.05. To determine the genes that were differentially expressed between two genotypes under NT and HT conditions, four comparisons (NF_1NT vs YF_1NT, NF_1NT vs NF_1HT, YF_1NT vs YF_1HT, and

NF_1HT vs YF_1HT) were performed. Under the NT condition, a total of 1,385 (294 upregulated and 1,091 downregulated) DEGs were identified for the comparison of NF_1NT vs YF_1NT (**Figure 2A**). After HT stress, 13,491 genes were differentially expressed in different comparisons. Among these, a total of 10,093 (2,199 upregulated and 7,894 downregulated) and 6,309 (2,162 upregulated and 4,147 downregulated) DEGs were identified for the comparisons of NF_1NT vs NF_1HT and YF_1NT vs YF_1HT, respectively (**Figure 2A**). We identified 4,187 (1,200 upregulated and 2,987 downregulated) DEGs that were in common among these two pairs. A total of 2,181 (1,247 upregulated and 934 downregulated) DEGs were identified for the pair of NF_1HT vs YF_1HT. The Venn diagram showed that the groups NF_1NT vs YF_1NT and NF_1HT vs YF_1HT had only 386 (107 upregulated and 279 downregulated) DEGs in common. However, the DEGs under HT stress accounted for 56.45% of the total DEGs (3,180 DEGs) of these two combinations. This indicated that most DEGs had differential expression changes in response to HT stress. Based on the Venn diagram, we found that 4,519 DEGs showed the same expression pattern between NF_1 and YF_1 and that the remaining 9,359 DEGs were upregulated or downregulated in different comparisons under HT stress (**Figures 2B,C**). Twelve DEGs were randomly selected for qRT-PCR verification, and the coincidence rate between qRT-PCR results and RNA-seq data was 100% (**Supplementary Figure 5**), supporting the reliability of expression patterns revealed by RNA-seq.

Functional Classification of DEGs in Response to Heat Stress

Among the 9,359 DEGs between the NT and HT samples, 2,244 upregulated and 5,965 downregulated genes were identified, and the other 575 DEGs were upregulated or downregulated in different combinations at the same time. In order to understand the potential functions in the list of DEGs, all 8,784 DEGs were further analyzed for GO functional annotations. The results revealed that 4,482 DEGs could be classified into 39 GO terms: 3,024 DEGs participated in biological processes, 4,034 DEGs had molecular functions, and 1,018 DEGs had cellular components (**Figure 2D** and **Supplementary Tables 2–4**). At the biological process level, the DEGs are enriched into 23 biological processes (p.adjust $\leq$ 0.05), including pollination (GO:0009856), reproduction (GO:0000003), phosphorylation (GO:0016310), response to oxidative stress (GO:0006979), and oxidation-reduction process (GO:0055114). In particular, we also observed two DEGs in the GO terms response to heat (GO:0009408) and response to temperature stimulus (GO:0009266). Similarly, large numbers of DEGs were also enriched in the molecular function and cellular component, including pectinesterase activity (GO:0030599), peroxidase activity (GO:0004601), antioxidant activity (GO:0016209), cell wall (GO:0005618), and membrane (GO:0016020).

To explore the biological pathways on the reproductive development of soybean CMS-based F_1 on which HT has an important influence, KEGG pathway analysis was further performed for these DEGs. A total of 13 significant KEGG pathways (Q-value $\leq$ 0.05) were enriched for 1,409 DEGs (**Supplementary Table 5** and **Figure 3A**), including pentose and glucuronate interconversions, phenylpropanoid biosynthesis, and starch and sucrose metabolism (**Figure 3B**). Most importantly, HT stress-induced DEGs were mostly enriched in pentose and glucuronate interconversions, starch and sucrose metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, and circadian rhythm–plant pathways, which belong to carbohydrate metabolism, biosynthesis of other secondary metabolites, and environmental adaptation classes, respectively, for the comparisons NF_1HT vs YF_1HT and YF_1NT vs YF_1HT (**Supplementary Figures 6A,D**). This is consistent with the male sterile phenotype of YF_1 under HT.

HT Caused Anther Defects by Altering Anther/Pollen Wall Development

Based on the expression level, some enzyme genes involved in pectin metabolism were identified, which included *pectate lyase* (*PL*), *pectin methylesterase* (*PME*, also named *pectinesterase*), *polygalacturonase* (*PG*), and *exopolygalacturonase* (*Exo-PG*). Eleven *PL*s, 21 *PME*s, 17 *PG*s, and 4 *Exo-PG*s were downregulated in flower buds of YF_1 compared with those of NF_1 under the HT condition (**Figure 4A** and **Supplementary Table 6**). The results also showed lower expressions of *PL*s, *PME*s, *PG*s, and *Exo-PG*s in flower buds of YF_1 compared with those of NF_1 under the NT condition (**Figure 4A** and **Supplementary Figures 7A–D**). Most importantly, RNA-seq data in Phytozome v12.0 showed that these four type genes were highly expressed in flowers of soybean (**Figure 4B**). This indicated that the pectinase activity of YF_1 anthers was defective under the HT condition, which led to abnormal formation of the anther cell wall and finally affected anther dehiscence (**Figure 1D**). To further confirm this result, the pectinase activity was assessed under NT and HT conditions (**Figure 4C**). However, pectinase activity in the YF_1 decreased only slightly compared with that in NF_1 under HT, which may be due to the pectinase being composed of *PG*, *PL*, and *PME* (Li et al., 2019) that have different activities in the pollen-related tissues and need to be further studied.

The RNA data and qRT-PCR also revealed that three *cellulose synthase proteins* (*GmCESA*s) were downregulated in flower buds of YF_1 compared with those of NF_1 under the HT condition (**Supplementary Figure 7E**), and they were also involved in the pollen wall development of plants (Wang et al., 2011). In addition, three *pollen-specific protein* (*GmCDPK*) DEGs were downregulated in flower buds of YF_1 under both NT and HT conditions (**Supplementary Figure 7F**). These results indicated that pectinase, cellulose, and *CDPK* are associated with anther defects in YF_1 under HT.

Carbohydrate Metabolism and Sugar Transport in Flower Buds of Soybean HT-Sensitive CMS-Based F_1 Were Disrupted Under HT

A lot of DEGs involved in carbohydrate metabolism during soybean CMS-based F_1 flower bud development under HT were found. Among them, there were 79, 117, and 31 DEGs that

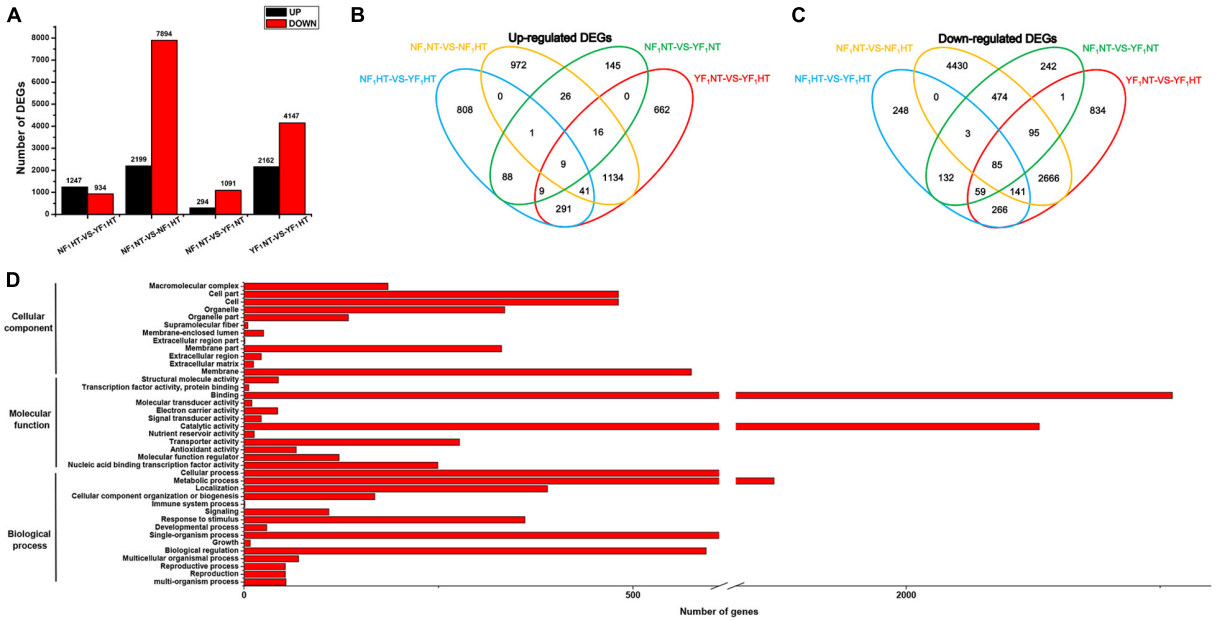

FIGURE 2 | Analysis of DEGs between NF_1 and YF_1 under NT and HT conditions. **(A)** Number of DEGs that were up- and down-regulated under NT and HT conditions. **(B,C)** Venn diagram of common up- and down-regulated DEGs under NT and HT conditions. **(D)** GO classification of DEGs. The *x*-axis indicates the number of DEGs, and the *y*-axis indicates the GO groups.

participated in pentose and glucuronate interconversions, starch and sucrose metabolism, and galactose metabolism pathways, respectively (**Supplementary Table 5** and **Figure 3**). Further analysis indicated that many genes related to carbohydrate metabolism and sugar transport were downregulated, such as *PLs*, *PMEs*, *PGs*, *Exo-PGs*, *beta-glucosidase 13*, *ATP-dependent 6-phosphofructokinase 7* (*ATP-D6P7*), *UDP-glucuronic acid decarboxylase 2* (*UDP-GAD2*), *sugar transport protein 11* (*STP11*), *bidirectional sugar transporter SWEET5* (*SWEET5*), and *sucrose transport protein SUC8-like* (*SUC8*) (**Supplementary Tables 6**, **7**). This result was also confirmed by qRT-PCR analysis (**Supplementary Figures 7, 8**, and **Figures 4A,D**). Most importantly, most of them were highly expressed in flowers of soybean (**Figures 4B,E**).

Carbohydrate analysis revealed that sucrose (Suc) and glucose (Glc) accumulation in flower buds of YF_1 was reduced compared with NF_1 under HT (**Figures 4F,G**). Interestingly, Suc and Glc content in either NF_1 or YF_1 leaves showed no difference under HT compared with NT (**Figures 4F,G**). Based on starch content detection, starch accumulation in YF_1 flower buds was also lower than that in NF_1 flower buds under HT (**Figure 4H**). All these results revealed that abnormal carbohydrate transport and accumulation affected pollen development, which was consistent with the results of KEGG analysis and reduction of pollen fertility in YF_1HT.

HT Caused Instability of Male Fertility in YF_1 by Altering Auxin Signaling

During soybean CMS-based F_1 flower bud development under HT, many DEGs were found to be involved in plant hormone signal transduction (**Supplementary Table 5**). Among them, genes encoding proteins involved in auxin biosynthesis (*YUCCA11* and *GH3.1*), auxin response protein (*IAA29*), and auxin-induced genes (except for *AUX10A5* and *AUXX15*) were downregulated (**Supplementary Figure 9**). In addition, the expression of IAA regulator *PHYTOCHROME-INTERACTING FACTOR* genes (*PIF1* and *PIF4*) were upregulated (**Supplementary Table 8** and **Figure 5A**). Furthermore, the concentration of endogenous IAA in YF_1 flower buds was lower than that of NF_1 under HT (**Figure 5B**). These results suggested that the reduction in auxin concentration is caused by a decrease in auxin metabolism gene expression, which may lead to anther defects such as anther indehiscence in YF_1 under HT afterward.

TFs and *HSP* May Participate in the Male Fertility Regulation of Soybean CMS-Based F_1 Under HT

Our transcriptomics analysis indicated that numerous HT-responding genes encoding TF are involved in heat signal transduction, such as *heat shock factor* (*HSF*), *ethylene-responsive TF RAP2-2* (*ER RAP2-2*), *myb-related protein 305* (*MYB305*), *dehydration-responsive element-binding protein 3* (*DREB3*), *NAC*, *squamosa promoter-binding-like protein 8* (*SPL8*), and *WRKY25* (**Figure 5C**). As shown in **Supplementary Figure 10**, both transcriptomics and qRT-PCR analyses indicated that *ER RAP2-2*, *MYB305*, *DREB3*, and *SPL8* were downregulated in YF_1HT compared with NF_1HT, and *NAC* and *WRKY25* were activated by HT in YF_1.

FIGURE 3 | KEGG enrichment analysis of the DEGs. **(A)** KEGG analysis with heat map. **(B)** Top 20 of pathway enrichment. The *x*-axis indicates the rich factor corresponding to each pathway, and the *y*-axis indicates name of the KEGG pathway. The color of the point represents the *P*-values of the enrichment analysis. The size and color of bubbles represent the number and degree of enrichment of DEGs, respectively.

Remarkably, the rapid response to heat triggered downregulation of a substantial number of *HSF* and *HSP* genes in YF_1HT (**Figure 5D**). The results showed that 55 DEGs about *HSF*s and *HSP*s were identified in the NT and HT comparison (**Supplementary Table 9** and **Supplementary Figure 11**). Five *GmHSF*s, namely, four *HSFA* and one *HSFB* genes, were induced in NF_1 but repressed in YF_1 under HT. In this study, a total of 50 *GmHSP* genes were identified to be upregulated in NF_1HT, including 25 small *GmHSP* (*GmsHSP*), 6 *GmHSP20*, 8 *GmHSP40* (DnaJ protein, Georgopoulos et al., 1980), 8 *GmHSP70*, and 3 *GmHSP90* genes.

Both *GmHSFA2* and Its Downregulated Gene *GmHSP20a* Overexpression Conferred Tolerance to HT Stress During Flowering in *Arabidopsis*

According to the RNA-seq and qRT-PCR analyses, *GmHSFA2* (*Glyma.14G096800*) was induced and inhibited by HT in NF_1 and YF_1, respectively (**Supplementary Table 9**). Its role in HT response was further analyzed. Bioinformatics analysis showed that *GmHSFA2* had high sequence identity with *AtHSFA2* and *SoHSFA2*, which contained a 1,095-bp ORF and predicted to encode 364 amino acids (**Figures 6A,B**). The alignment revealed that the *GmHSFA2* has the typical domains of *HSFA2*, including a conserved DNA binding domain (DBD), an oligomerization domain (OD) with two adjacent hydrophobic heptad repeats (HR-A/B), a nuclear localization signal (NLS), and an AHA motif (**Figure 6B**). Subcellular localization analysis showed that the *35S:GmHSFA2-GFP* fusion protein was exclusively localized in the nucleus, which was consistent with the predicted NLS domain between the OD and AHA motif (**Figures 6B,C**). GUS staining of three *pGmHSFA2:GUS*-transformed *Arabidopsis* lines confirmed that *GmHSFA2* was expressed only in early-stage anthers of inflorescence during flowering (**Figure 6D**). The expression patterns of *GmHSFA2* under the HT condition (40°C) were evaluated by qRT-PCR using RNA samples extracted from flower buds of soybean, and the NT condition (30°C) was used as a

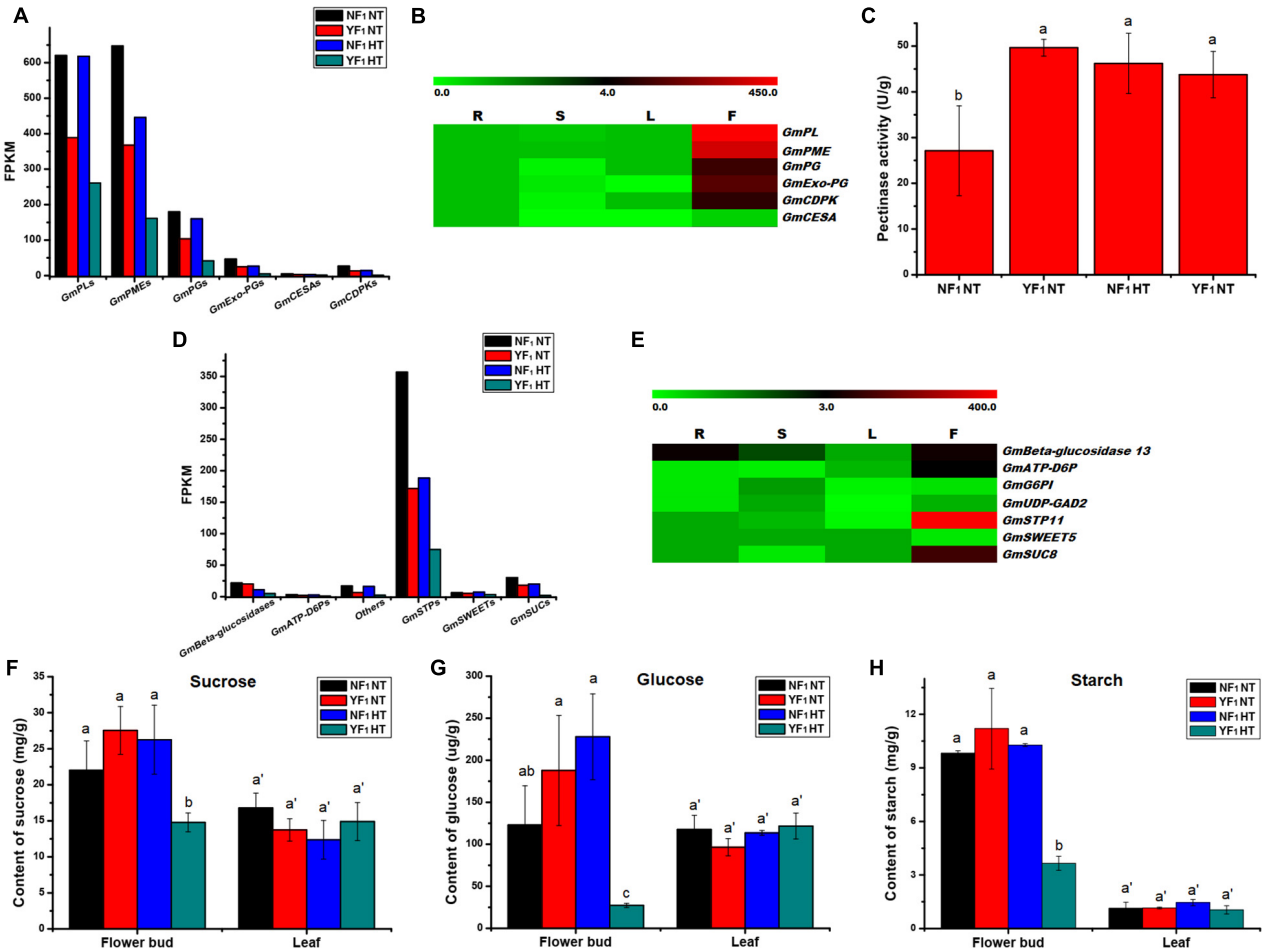

FIGURE 4 | Effect of HT on anther/pollen wall development, carbohydrate metabolism and sugar transport. **(A)** DEGs related to anther/pollen wall development in RNA-seq data. **(B)** Heat map of the DEGs related to anther/pollen wall development in four different tissues. The color scale represents the relative transcript abundance of the DEGs in four soybean tissues. The heat map was conducted using MeV 4.9 software. The FPKM values were obtained from the RNA-seq data in Phytozome v12.0. R, root; S, stem; L, leaf; F, flower. **(C)** Pectinase activity in flower buds of NF_1 and YF_1 under NT and HT conditions (n = 3). Data are presented as means ± standard deviation (SD) from independent biological replicates. Values with different letter indicates statistical differences (one-way ANOVA, Duncan, $P < 0.05$). **(D)** DEGs related to carbohydrate metabolism and sugar transport in RNA-seq data. **(E)** Heat map of the DEGs related to carbohydrate metabolism and sugar transport in four different tissues. The color scale represents the relative transcript abundance of the DEGs in four soybean tissues. The heat map was conducted using MeV 4.9 software. The FPKM values were obtained from the RNA-seq data in Phytozome v12.0. R, root; S, stem; L, leaf; F, flower. **(F–H)** Suc, glc, and starch contents in flower buds and leaves of NF_1 and YF_1 under NT and HT conditions (n = 3). Data are presented as means ± standard deviation (SD) from independent biological replicates. Values with different letter indicates statistical differences (one-way ANOVA, Duncan, $P < 0.05$).

control. The expression level of *GmHSFA2* increased significantly with time and peaked at the seventh day and then decreased rapidly after recovery with NT for 1 day (**Supplementary Figure 12**). This implies that *GmHSFA2* was extremely sensitive to HT during flowering in soybean reproductive tissues.

To further confirm the role of *GmHSFA2* in HT tolerance during flowering, three lines of *Arabidopsis* overexpressing *GmHSFA2* with different expression levels were selected for HT treatment (**Figure 7A**). In the HT tolerance assay (45°C for 3 days) during flowering, the top of the inflorescence of transgenic plants basically kept normal growth while that of the WT wilted (**Supplementary Figure 13**). Most importantly, HT treatment increased the stability of *35S:GmHSFA2* transgenic plants under HT stress (42°C for 4 h), which showed anther dehiscence and only a little pollen abortion after HT treatment for 2 and 6 days, respectively (**Figures 7C,D**), while the rate of stamen length/pistil length in both WT and transgenic lines decreased after 2 days of HT treatment (**Figure 7E**). However, the WT showed pollen shrinkage with anther indehiscence and male sterility (no pollen grains or most pollen abortion) after 2 and 6 days of HT treatment, respectively (**Figures 7C,D,F**).

The expression levels of *GmHSFA2* downstream regulatory genes (*AtsHSP*, *AtHSP20*, *AtHSP40*, *AtHSP70*, *AtHSP90*, *AtGolS1*, and *AtGolS2*) under HT stress during flowering in *35S:GmHSFA2* plants were compared by qRT-PCR analysis. The transcripts of most of them except *AtHSP70* and *AtHSP90* were all higher than those in the WT under the NT condition (**Figure 8A**). After HT treatment, all of these downstream regulator genes

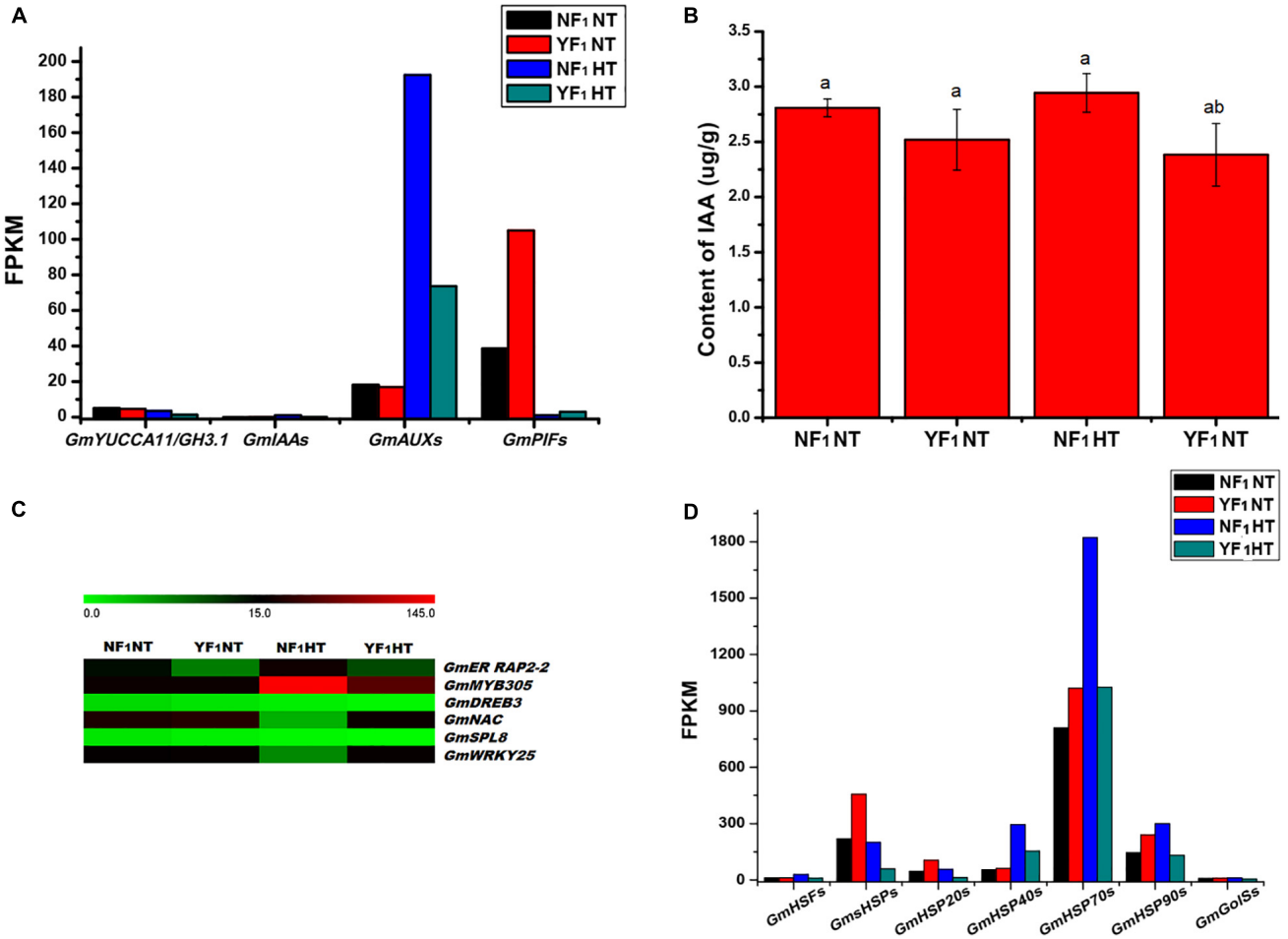

FIGURE 5 | Effect of HT on auxin signaling, transcription factors, *HSP* and *GolS*. **(A)** DEGs related to auxin signaling in RNA-seq data. **(B)** IAA content in flower buds of NF_1 and YF_1 under NT and HT conditions ($n = 3$). Data are presented as means ± SD from independent biological replicates. Values with different letter indicates statistical differences (one-way ANOVA, Duncan, $P < 0.05$). **(C)** Heat map of the DEGs related to transcription factors in flower buds of NF_1 and YF_1 under NT and HT conditions. The color scale represents the relative transcript abundance of the DEGs in flower bud of soybean. The heat map was conducted using MeV 4.9 software. **(D)** DEGs related to *HSF*, *HSP*, and *GolS* in RNA-seq data.

were upregulated in *35S:GmHSFA2* plants compared with WT (**Figure 8A**). Moreover, the fold changes in the expression levels of almost all genes (except *AtHSP40*) between the two materials under HT were higher than that under the NT condition (**Figure 8A**). Most importantly, both RNA-seq data and qRT-PCR analysis showed that their homologous genes in soybean NF_1 were upregulated by HT induction (**Figure 8B** and **Supplementary Figures 11D–J**).

Bioinformatics analysis showed that perfect and imperfect HSE motifs are distributed within promoter regions of selected *HSP*s and *GolS*s in both soybean and *Arabidopsis* (**Figure 8C**). Furthermore, we investigated a direct link between *GmHSFA2* and the promoter of a selected *GmHSP20a* (*Glyma.12G013100*) by Y1H assay (**Figure 8D**). In addition, the expression trend of *GmHSP20a* was consistent with that of *GmHSFA2* under HT stress (**Supplementary Figure 12**). Most importantly, the *35S:GmHSP20a Arabidopsis* transgenic lines also improved HT tolerance during flowering (**Supplementary Figure 13** and **Figures 7B–D,F**). All these results show that *GmHSFA2* might improve the HT tolerance of soybean CMS-based F_1 and transgenic *Arabidopsis* by regulating the expression changes of *HSP* and *GolS*.

DISCUSSION

The CMS-based hybridization method has been widely used in plant hybrid breeding due to its effective way of hybrid seed production by use of the CMS line, maintainer line, and restorer line. However, increasing evidence has indicated that male fertility of CMS-based F_1 is affected by climate conditions such as HT stress (Zhao et al., 2009; Zhang et al., 2019; Nie et al., 2017). In this study, two soybean CMS-based F_1 combinations, NF_1 and YF_1, were employed, and it was found that the male fertility of YF_1 was obviously damaged by HT, such as anther indehiscence and decreased pollen fertility, thereby decreasing

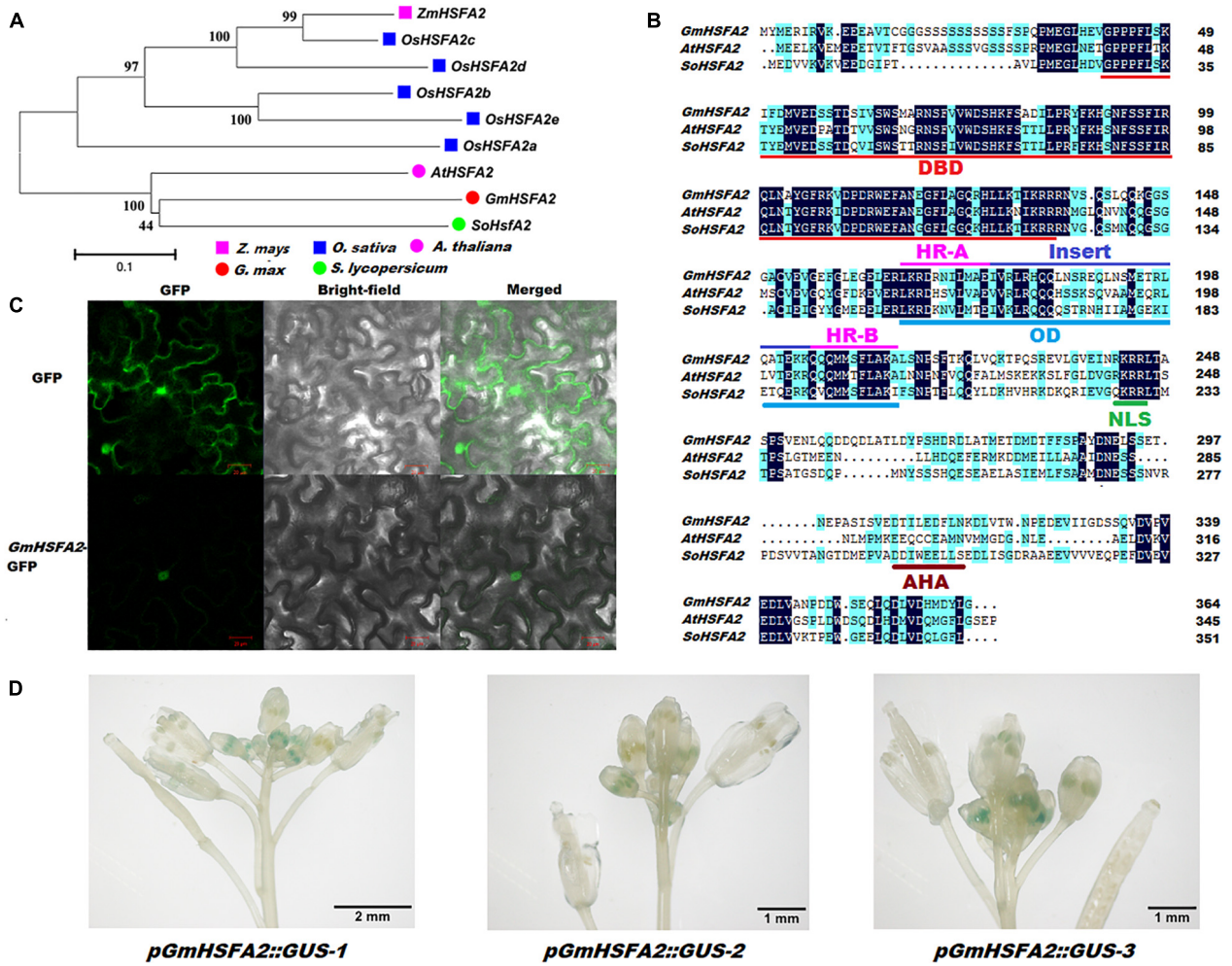

FIGURE 6 | Identification of *GmHSFA2*. **(A)** Phylogenetic tree analysis of *HSFA2* in five plant species. The phylogenetic tree was constructed using MEGA 5.02 based on the Neighbor-joining (NJ) method. Bootstrap values in percentage (1000 replicates) are labeled on the nodes. **(B)** Amino acids sequence comparison and conserved domains of *GmHSFA2* and its closest orthologs *AtHSFA2* and *SoHSFA2* in *Arabidopsis* and tomato, respectively. Sequence alignment was performed with DNAMAN. Dark blue and light blue regions indicate identical and similar amino acids among the three sequences, respectively. DBD, DNA-binding domain; OD, oligomerization domain; HR, hydrophobic heptad repeats; NLS, nuclear localization signal; AHA motif, activator motif. **(C)** Subcellular localization of *GmHSFA2*. **(D)** *pGmHSFA2:GUS* expression pattern in inforescence with developing flower buds during flowering in *Arabidopsis*.

soybean yield (**Supplementary Figure 1**). Furthermore, RNA-seq and functional study of *GmHSFA2* were adopted to globally identify the DEGs and pathways participating in male fertility regulation of soybean CMS-based F_1 under HT.

Abnormal Anther/Pollen Development Is Related to Male Fertility Instability of HT-Sensitive F_1 Under HT

In our RNA-seq, many *PL*, *PME*, *PG*, and *Exo-PG* genes showed differential expression between NF_1 and YF_1 under the HT condition (**Figures 4A–C**). Among them, pectinase (*PL*, *PME*, and *PG*) is a key enzyme involved in the degradation of plant pectin and participates in the regulation of anther/pollen development (Micheli, 2001; Ogawa et al., 2009; Corral-Martínez et al., 2016; Li et al., 2019). It has been shown that pectinase activity was decreased in anthers of Qx-115 (anther indehiscent phenotype material of chrysanthemum) during anther development (Li et al., 2019). Pectinase has been extensively studied in many plants. Wei et al. (2019) found that *PL*, *Exo-PG*, and *PME* were related to the fertility restorer of the CMS line in pepper. In *Brassica campestris*, downregulation of *BcPLL9* and *BcPLL10* results in disorder of pectin metabolism in pollen and finally leads to male semi-sterility (Jiang et al., 2014a,b). Also, in *B. campestris*, Huang et al. (2009) found that a *PG* gene (*BcMF2*) was specifically expressed in the tapetum and pollen and that its inhibition led to pollen deformity with abnormal intine development. Except *PL*s and *PG*s, *PME*s are also important for pollen development in plants. Recently, a CRISPR/Cas9 system-induced *BcPME37c* mutant has been characterized, and its mutation caused the abnormal thickening of the pollen intine in *B. campestris* (Xiong et al., 2019). The

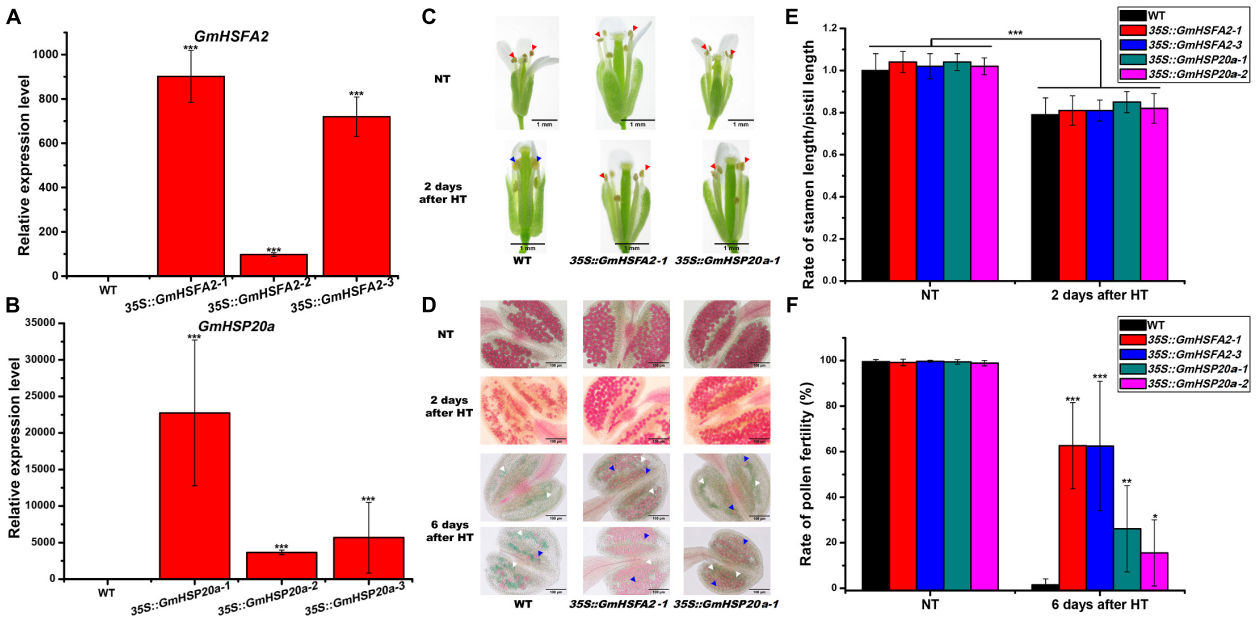

FIGURE 7 | Effect of HT on male fertility in WT, *35S:GmHSFA2* and *35S:GmHSP20a* lines. **(A,B)** *GmHSFA2* and *GmHSP20a* expression levels in WT, *35S:GmHSFA2* and *35S:GmHSP20a* lines. The WT under NT condition was used as the control and qRT-PCR data are expressed as the mean values ± SD of three independent biological replicates. **(C)** Phenotype of anthers in WT, *35S:GmHSFA2* and *35S:GmHSP20a* lines under NT and HT conditions. The red and blue arrows indicate dehiscent and indehiscent anthers, respectively. **(D)** Phenotype of pollen in WT, *35S:GmHSFA2* and *35S:GmHSP20a* lines under NT and HT conditions. The red pollens pointed by blue arrows and green pollens pointed by white arrows indicate fertile pollens and sterile pollens, respectively. **(E,F)** Calculation of rate of stamen length/pistil length and rate of pollen fertility in WT, *35S:GmHSFA2* and *35S:GmHSP20a* lines under NT and HT conditions ($n = 9$). Asterisk indicates statistical differences, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

downregulation of pectinase genes in YF_1HT may reduce the degradation of pectin, thus changing the maintenance of the anther wall, leading to anther indehiscence in YF_1HT, and needs further research.

Carbohydrate Undersupply and Sugar Transport Blockage Are Two of the Main Causes for Male Fertility Instability in YF_1HT

Our RNA-seq analysis found that compared with NF_1HT, the expression of hundreds of DEGs related to carbohydrate metabolism in YF_1HT was downregulated, including *PL*s, *PME*s, and *beta-glucosidase* (**Supplementary Table 7**). In addition, many sugar transport-related DEGs, such as *STP11*, *SWEET5*, and *SUC8*, are also decreased in expression (**Supplementary Table 7**). Based on the determination of Suc, Glc, and starch contents, it is speculated that their reduction affected the male fertility of YF_1 under HT stress (**Figure 9**). Moreover, similar results were found in tomato and cotton, where a decrease in sugar affected their male reproductive development under HT (Firon et al., 2006; Min et al., 2014).

Many studies have shown that genes related to either carbohydrate metabolism or sugar transport are associated with male sterility in plants. For example, our previous study found that male sterility of the soybean CMS line is associated with alterations in carbohydrate metabolism (Li et al., 2015). In cucumber, the downregulation of sugar transporters *CsHT1* and *CsSUT1* inhibits pollen germination and causes male sterility, respectively (Cheng et al., 2015; Sun et al., 2019). At the same time, they also protect against HT stress during pollen development (Frank et al., 2009; Min et al., 2013, 2014). Min et al. (2014) found that HT disrupted anther carbohydrate metabolism in cotton, including starch and Suc metabolism pathways, leading to abnormal male fertility development in H05 (HT-sensitive line) under HT. Further investigation demonstrated that *GhCKI* caused pollen abortion and anther indehiscence in cotton via inactivating starch synthase (Min et al., 2013). In tomato, HT-induced expressions of carbohydrate metabolism and sugar transport genes, such as sucrose phosphate synthase and sorbitol transporter, were involved in the HT response during pollen development (Frank et al., 2009). Thus, we speculate that carbohydrate undersupply and sugar transport blockage are two of the main causes for male fertility instability in YF_1 under HT and need to be verified in future studies.

Sugar Signaling-*PIF*-Auxin Signaling Pathway May Underlie Instability of Male Fertility in YF_1 Under HT

Sugar plays a vital role as a protector defending against HT stress during male reproductive organ development (Min et al., 2013, 2014). And auxin is also closely related to instability of male fertility in plants under HT stress (Sakata et al., 2010; Higashitani, 2013; Min et al., 2014; Ding et al., 2017). In barley

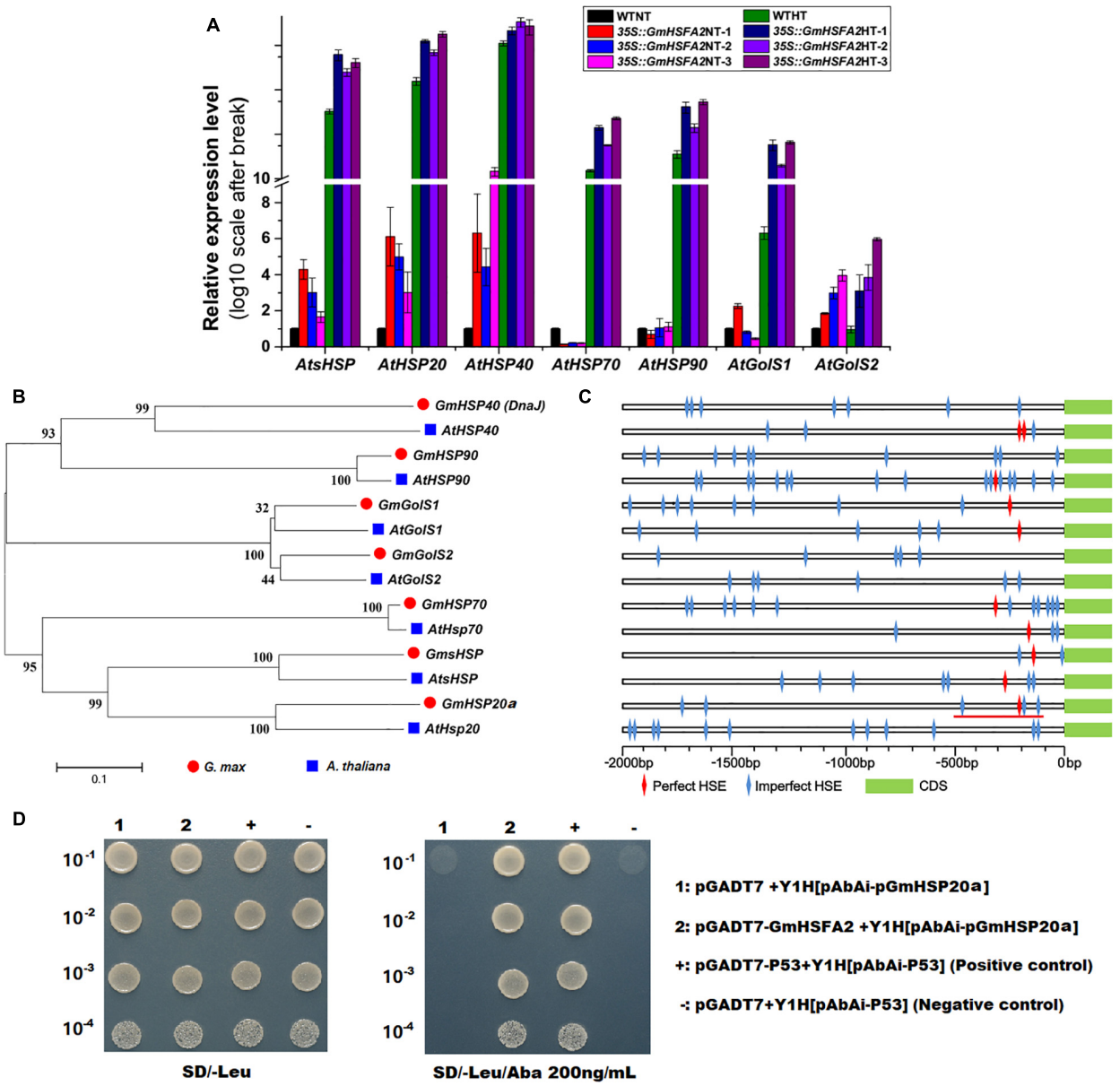

FIGURE 8 | Effect of *GmHSFA2* on its downstream regulatory genes. **(A)** Expression levels of *HSP* and *GolS* in *35S:GmHSFA2 Arabidopsis* transgenic and WT plants under NT and HT conditions. The WT under NT condition was used as the control and qRT-PCR data are expressed as the mean values ± SD of three independent biological replicates. **(B,C)** Phylogenetic tree and schematic of *HSP* and *GolS*. The phylogenetic tree was constructed using MEGA 5.02 based on the NJ method. Bootstrap values in percentage (1000 replicates) are labeled on the nodes. Red line labeled cis-acting HSE motifs (–500 to –101 bp relative to the translational start codon of *GmHSP20a*) were used for cloning and Y1H assay. **(D)** The interaction between *GmHSFA2* and the promoter of *GmHSP20a* in yeast. The relevant bacterial solution was diluted in gradient of 1:10, 1:100, 1:1000, and 1:10,000, and grown on medium.

and *Arabidopsis*, HT can induce downregulation of genes related to auxin biosynthesis (*YUC2*, *YUC6*, and *TAA1/TIR2*), resulting in a sharp decrease in endogenous auxin level and eventually anther abortion (Sakata et al., 2010). Furthermore, exogenous auxin could completely reverse the male sterility of barley and *Arabidopsis* under HT stress (Sakata et al., 2010). Previous research has uncovered a pathway where sugar signaling is involved in plant growth by regulating auxin metabolism through the *PIF* protein (Stewart et al., 2011; Min et al., 2014). The *PIF* protein is an IAA regulator (Sun et al., 2012, 2019), and it is also involved in the HT response (Leivar and Quail, 2011). Furthermore, *PIF* expression was induced by low content of sugar, which altered auxin metabolism afterward and led to male sterility in cotton and cucumber eventually (Min et al., 2014; Sun et al., 2019). Similar to the cotton male sterility induced by HT stress, the sugar content and the expression levels of *PIF*s (*GmPIF1* and *GmPIF4*) in flower buds of YF_1 were also altered under HT (**Supplementary Table 8**). Meanwhile, downregulated

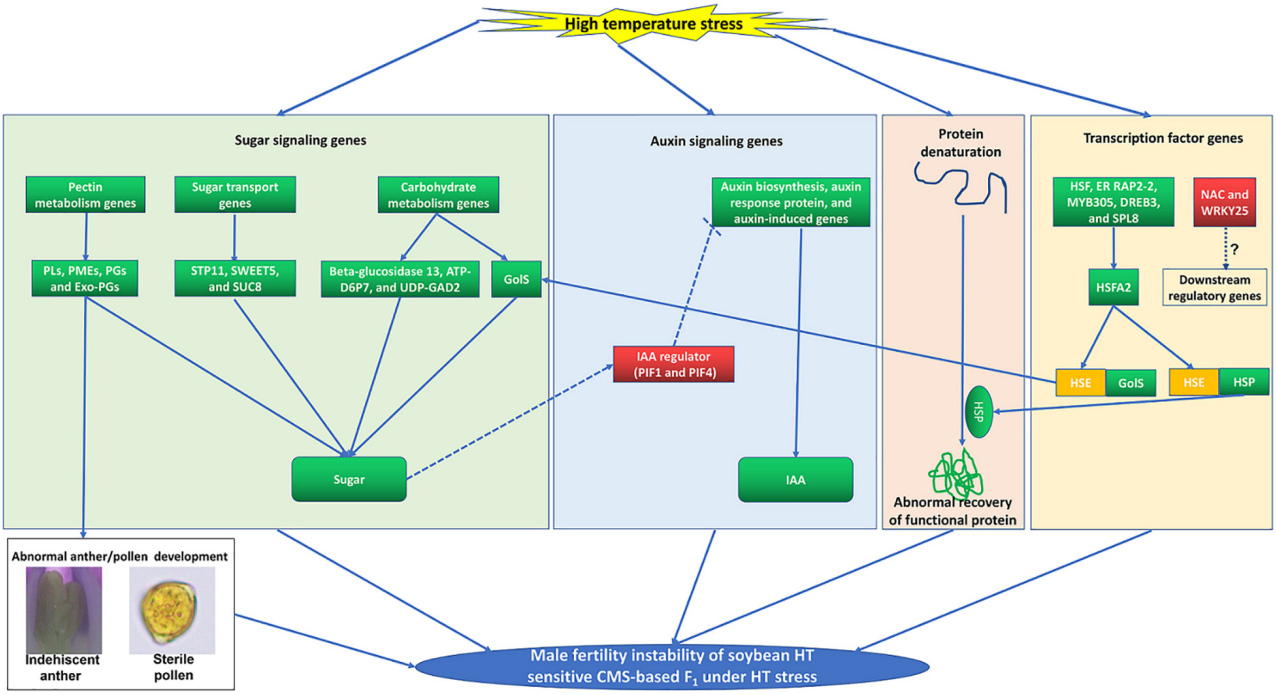

FIGURE 9 | A proposed model incorporating the main processes that involved in the male fertility instability of soybean HT sensitive CMS-based F_1 under HT stress. The up-regulated and down-regulated of gene or metabolite content are in red and green backgrounds, respectively.

auxin signaling genes and content were observed in flower buds of YF_1 under HT, indicating that *PIF* might act as a negative regulator of IAA biosynthesis, which is consistent with the results in *Arabidopsis* and cucumber (Sairanen et al., 2012; Sun et al., 2019). However, Min et al. (2014) showed that *PIF* acts as a positive regulator of HT-induced IAA biosynthesis in cotton. It appears that the sugar signaling-*PIF*-auxin signaling pathway acts as a master switch role during the male organ development under HT stress in soybean CMS-based F_1, which needs further study (**Figure 9**).

TFs Is Required for Enhanced Activation of HT Stress Response and Increased Thermotolerance in Soybean CMS-Based F_1

Transcription factors are central regulators of gene expression affecting plant HT responses (Li et al., 2018). Many TF families, including *ER*, *MYB*, *DREB*, *SPL*, and *HSF*, are involved in HT stress response and enhanced tolerance in both model and crop plants (Hong et al., 2009; El-Kereamy et al., 2012; Wan et al., 2014; Chao et al., 2017; Li et al., 2018). In our study, some TF family members were upregulated in NF_1HT related to YF_1HT, including *GmER RAR2-2*, *GmMYB305*, *GmDREB3*, *GmSPL8*, and *HSFA2*, which may confer tolerance to NF_1 under HT stress (**Figure 5C** and **Supplementary Table 9**). However, some TF family members may play as negative regulators, such as *GmNAC* and *GmWRKY25*, which were upregulated in YF_1HT compared with NF_1HT (**Figure 5C**). In *Arabidopsis*, a NAC-like gene (*AtAIF*) was found to be an inhibitor that controls anther dehiscence (Shih et al., 2014). Similarly, the overexpression of *GhWRKY22*, *GmWRKY45*, and *AtWRKY27* in *Arabidopsis* displayed the male fertility defect with decreased pollen viability (Mukhtar et al., 2017; Wang et al., 2019; Li et al., 2020). Most importantly, Dang et al. (2018) found that the overexpression of *CaWRKY27* in *Arabidopsis* inhibited the scavenging of H_2O_2 and played a negative regulator role in HT stress.

Although great progress has been made in deciphering the response of TFs such as *HSF* to HT stress in *Arabidopsis*, maize, tomato, tall fescue, and other plants (Charng et al., 2007; Giorno et al., 2010; Fragkostefanakis et al., 2016; Wang et al., 2017; Gu et al., 2019), few *HSF* genes have been elucidated in soybean, especially on the stability of male fertility. Four *GmHSFA2* genes (*Glyma.13G105700*, *Glyma.14G096800*, *Glyma.17G053700*, and *Glyma.17G227600*) were found in this study (**Supplementary Table 10**), and one of them (*Glyma.17G227600*) was overexpressed in *Arabidopsis*, showing the characteristics of HT and drought resistance during seedling in previous studies (Li et al., 2014). In this study, only *GmHSF-30* (*Glyma.14G096800* and *GmHSFA2* in this study) was induced by HT in soybean CMS-based F_1 flower buds during flowering at the mRNA level (**Supplementary Figure 11A**). Fragkostefanakis et al. (2016) found that *HSFA2* is an important coactivator of *HSFA1a* during HT to control pollen viability by regulated *HSP101* and *HSP17.7C-CI* in tomato. In rice, *HSF* and *HSP* genes including *HSFA2a* and *HSP17.9A* are highly induced in HT-tolerant material rather in HT-sensitive varieties during anthesis under HT

stress (González-Schain et al., 2016). In tomato, HT induced expressions of *HSFA2*, *sHSP* genes, *HSP70*, and *HSP101* during pollen development. In this study, both *HSFA2* and *HSP* (*sHSP*, *HSP20*, *HSP40*, *HSP70*, and *HSP70*) were induced by HT stress during flower bud development. Most importantly, a functional study found that *HSFA2* was directly involved in HT stress response and that inhibition of *HSFA2* reduces the viability and germination rate of tomato pollen under HT (Giorno et al., 2010; Fragkostefanakis et al., 2016). It has been reported that HT stress causes male sterility by affecting anther dehiscence and pollen production at a specific stage in *Arabidopsis* (Kim et al., 2001), and similar results were obtained in this study. Most importantly, ectopically expressing *GmHSFA2* enhanced HT tolerance in *Arabidopsis*, suggesting that it positively regulated HT tolerance during flowering in plants.

Our results suggest that *GmHSFA2* is a key regulator in response to HT stress. However, its regulatory molecular mechanism in soybean is still unknown. Many studies have shown that *HSF* promotes HT tolerance by binding to the HSE motifs in the promoter of *HSP* and *GolS* (Busch et al., 2005; Kotak et al., 2007; von Koskull-Doering et al., 2007; Fragkostefanakis et al., 2016; Wang et al., 2017; Gu et al., 2019). Frank et al. (2009) found that HT induced expressions of *HSF* and *GolS* during pollen development in tomato. However, the relationship among them during pollen development under HT stress is still unknown, especially in soybean. In our study, multiple *HSP* and *GolS* genes, including *sHSP*, *HSP20*, *HSP40*, *HSP70*, *HSP90*, *GolS1*, and *GolS2*, were upregulated by overexpression of *GmHSFA2* in *Arabidopsis* compared with WT under HT (**Figure 8A**). Most importantly, their homologous genes in soybean were also upregulated in NF_1HT related to YF_1HT, according to the RNA-seq and qRT-PCR analyses (**Figure 5D**). Furthermore, multiple HSE motifs were found in their promoters, and the Y1H assay revealed that there was a direct link between *GmHSFA2* and the promoter of *GmHSP20a*, indicating that *GmHSFA2* could regulate these genes (**Figure 9**). And *HSP* and helper molecular chaperones can help inactivated proteins reassemble into active high-level structures and maintain normal cell functions (Scharf et al., 2012). Most importantly, overexpression of *GmHSP20a* in *Arabidopsis* also conferred plant HT tolerance during flowering (**Figures 7C,D,F**). However, its HT tolerance was lower than that of *35S:GmHSFA2* transgenic plants under HT stress, indicating that *GmHSP20a* was only one of the downstream regulator genes of *GmHSFA2*. All the above results revealed that a complex TF regulatory network exists in soybean CMS-based F_1 (**Figure 9**). As a key regulator in response to HT stress, the regulation mechanism of *GmHSFA2* in soybean needs to be explicated further.

DATA AVAILABILITY STATEMENT

The datasets generated by this study can be found in the NCBI using accession number PRJNA677945.

AUTHOR CONTRIBUTIONS

XD and SY conceived and designed the experiments. XD, QG, and QL performed the experiments. XD wrote the manuscript. SY and JG revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.600217/full#supplementary-material

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RNA-Seq Highlights Molecular Events Associated With Impaired Pollen-Pistil Interactions Following Short-Term Heat Stress in *Brassica napus*

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The global climate change is leading to increased frequency of heatwaves with crops getting exposed to extreme temperature events. Such temperature spikes during the reproductive stage of plant development can harm crop fertility and productivity. Here we report the response of short-term heat stress events on the pollen and pistil tissues in a commercially grown cultivar of *Brassica napus*. Our data reveals that short-term temperature spikes not only affect pollen fitness but also impair the ability of the pistil to support pollen germination and pollen tube growth and that the heat stress sensitivity of pistil can have severe consequences for seed set and yield. Comparative transcriptome profiling of non-stressed and heat-stressed (40°C for 30 min) pollen and pistil (stigma + style) highlighted the underlying cellular mechanisms involved in heat stress response in these reproductive tissues. In pollen, cell wall organization and cellular transport-related genes possibly regulate pollen fitness under heat stress while the heat stress-induced repression of transcription factor encoding transcripts is a feature of the pistil response. Overall, high temperature altered the expression of genes involved in protein processing, regulation of transcription, pollen-pistil interactions, and misregulation of cellular organization, transport, and metabolism. Our results show that short episodes of high-temperature exposure in *B. napus* modulate key regulatory pathways disrupted reproductive processes, ultimately translating to yield loss. Further investigations on the genes and networks identified in the present study pave a way toward genetic improvement of the thermotolerance and reproductive performance of *B. napus* varieties.

Keywords: *Brassica napus*, heat stress, heatwaves, pollen-stigma interaction, plant reproduction, pollen, pistil, canola

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INTRODUCTION

Brassica napus L. (canola/rapeseed) is the third most important oilseed crop produced globally. Rising demand for canola/rapeseed oil for human consumption, industrial uses and bio-diesel production has led to a continuous expansion of its cultivating areas to comparatively drier regions, thereby increasing the exposure to unfavorable weather events (Jaime et al., 2018). The critical

growth temperatures for canola range from 27 to 30°C (Kirkegaard et al., 2018; Lohani et al., 2020a). Like other crops, *Brassica* requires a specific number of heat units or growing degree days (GDD) for the onset of the flowering phase (Kjellström, 1993). *B. napus*, a temperate crop is especially susceptible to high-temperature events, particularly during reproductive stages (Angadi et al., 2000; Young et al., 2004; Aksouh-Harradj et al., 2006). Accumulation of excessive heat units during the anthesis results in decreased reproductive fitness, thus making it a crucial developmental period for yield determination in *B. napus* (Morrison and Stewart, 2002; Uppal et al., 2019). With frequently occurring heatwaves predicted in the future global climate change scenarios, it is becoming increasingly important to understand the severity of likely damage to reproductive fitness and yield in crop plants.

The intensity, timing, rate of temperature change, and duration of heat stress exposure controls the sensitivity of sexual reproduction to high temperature. High temperature disrupts the stages involved in sexual reproduction by initiating a series of physiological, molecular, cellular, and biochemical changes in crops (Lohani et al., 2020b). A survey of the heat stress regimes utilized for studying the pollen thermotolerance highlight a need for focusing on the impact of short-term heat stress events on *B. napus* and other crops (Mesihovic et al., 2016). Since even short-lived high-temperature spikes can have significant adverse effects on reproductive success, it is crucial to understand the underlying molecular basis short-term heat stress response during pollen-pistil interactions (Frank et al., 2009; Begcy et al., 2019). Exposure of plants to shorter pulses of heat stress during reproductive processes will not only help to understand the heat stress response during various reproductive stages but also the extent of vulnerability of the developing reproductive organs to heat stress regimes.

While high temperature is expected to affect male and female reproductive tissues concurrently, the available data mainly focusses on the effects of heat stress on pollen development and function. Nevertheless, there is a recent focus on the female reproductive organ sensitivity to high temperature in crops like sorghum, rice, maize, wheat, and tomato (Gonzalo et al., 2020; Jagadish, 2020; Lohani et al., 2020b). The outcomes of these studies reveal a considerable variation in heat stress response of pistil (stigma or ovary) across crops. The female reproductive organ or the pistil comprises of the stigma, style, and ovary. The stigma (receptive part) and style (transmitting tract) play crucial roles in triggering, promoting, and guiding the growth of pollen tubes toward female gametes within the ovule. The exposure to high temperatures can disrupt pollen-pistil interactions and fertilization, leading to decline in seed set and crop productivity. However, the molecular basis of heat stress response in the female reproductive tissues remains largely unexplored.

To address gaps in our understanding of effects of short-term heat stress events on reproduction, we investigated the effects of heat stress at 40°C for 4 h on pollen-pistil interactions by performing reciprocal crosses between non-stressed, and heat-stressed pollen and pistil. We further explored the changes in transcriptional patterns of mature pollen and pistil to unravel the molecular signatures in response to heat stress (40°C for 30 min). We have also attempted to integrate physiological effects with the corresponding transcriptome level changes in the gene expression patterns upon short term heat stress exposure. Our findings show that heat stress impairs both the pollen viability and the ability of the pistil to support pollen germination and tube growth. Our RNA-seq analysis highlights differential regulation of specific genes involved in pollen cell wall organization, water channel activity, ROS metabolism, fatty acid metabolism, phenylpropanoid biosynthesis, and genes involved in pollen-pistil interactions.

MATERIALS AND METHODS

Plant Growth Conditions and Temperature Treatments

Brassica napus var. Garnet (AV Garnet) was chosen for this study is mid- to early-maturing variety is grown commercially across Australia. The plants were grown in a Thermoline growth cabinet (model TPG-2400-TH) at the Plant Growth Facility of The University of Melbourne, Australia. Control growth conditions were 23/18°C day/night; a photoperiod of 16/8 h light/dark, 200 $\mu molm^{-2}s^{-1}$ light intensity and 60% humidity. The plants bearing secondary inflorescence (50 das) were exposed only once to 40°C for 4 h. The high-temperature treatment started 2 h after the beginning of the day. Each experiment had three biological replicates. Throughout each experiment, plants were spatially randomized weekly and were kept well-watered to minimize any effects associated with drought stress. Soluble nutrient fertilizer was applied directly to the soil once a week for each plant.

Pollen Viability and Germination Assay

Pollen viability was evaluated by double staining with Fluorescein Diacetate (FDA) and Propidium Iodide (Regan and Moffatt, 1990). Anthers isolated from the buds of appropriate size (6–7 mm) were macerated gently to release pollen grains in the staining solution (20 μL: 10% sucrose, 1 μL: 2 mg/mL FDA and 2 μL: 1 mg/mL PI). The samples were kept in the staining solution in the dark at room temperature for 20 min and observed under a fluorescence microscope (Olympus BX60). The viability was presented as percentage (%) calculated by counting a minimum of 200 pollens from each sample.

For pollen germination, pollen grains were collected from flowers with freshly dehisced anthers after each treatment. The pollen grains were allowed to hydrate for 30 min after which they were brushed onto the surface of the freshly prepared solid pollen germination medium (100 g sucrose, 25 mg boric acid, 90 mg calcium chloride, 50 mg potassium nitrate and 100 mg of Tris dissolved in 500 mL of water; 1% agar was used for solidifying it) (Singh et al., 2008). The pollen grains were germinated for 4 h under high humidity (>70%) and light (200 $\mu molm^{-2}s^{-1}$). After 4 h, the plates were observed under a microscope for scoring.

Reciprocal Crosses

At least 20 immature flowers (those due to open the next day) were emasculated from two plants per replicate (total three

replicates) for each reciprocal cross. Next day the appropriate numbers of plants were exposed to heat stress at 40°C for 4 h. Manual pollination was performed immediately after heat stress treatment. The following four combinations of reciprocal crosses of non-stressed (NS) and heat-stressed (HS), pistil (♀) and pollen (♂) were carried out: NS♀ × NS♂, NS♀ × HS♂, HS♀ × NS♂ and HS♀ × HS♂. After pollination, the plants were allowed to mature under control conditions until the completion of seed filling to observe seed set.

Pistils from each reciprocal cross were also collected 24 h after pollination and were fixed in Carnoy's solution overnight. Then washed with distilled water, softened with 8 M NaOH solution overnight and stained with aniline blue solution (0.1% aniline blue in 0.1 M K_2HPO_4-KOH buffer, pH 11) for 3 h in complete darkness (Kho and Baer, 1968). The stained pistils were observed with a fluorescence microscope (Olympus BX60).

Statistical Analysis

All experiments were performed in triplicates. Results were expressed as the mean ± SD of n replicates available per treatment. The data were analyzed using GraphPad Prism 8.2.1 (1992–2020 GraphPad Software, Inc.) software. Data analysis was done by performing Welch's *t*-test to compare a time point at a given temperature with the control conditions, and the variance was considered unequal for the comparison. Significant differences among the treatment were considered at $p < 0.05$.

Isolation of Mature Pollen Grains and Pistil (Stigma + Style) for RNA Seq

Mature flower buds (6–7 mm) were collected from non-stressed, and heat-stressed (40°C for 30 min) plants and transferred to a petri dish containing 0.5 × B5 medium and were kept on ice. Anthers carefully dissected out from the buds were lightly macerated in B5 medium. The crushed suspension was then filtered through a 44μm nylon mesh into 1.5 mL tubes. The filtrate was centrifuged at 150 g for 3 min at 4°C. The supernatant was discarded, the pellet was washed using 0.5 × B5 medium and centrifuged at 150 g for 3 min at 4°C. This step was repeated twice. After removing the supernatant, the pellet was immediately frozen in liquid nitrogen and stored at -80°C. An aliquot from each isolation was analyzed by nuclei staining with DAPI to check the purity of the sample for containing tri-nucleate pollen grains. While dissecting the buds for collecting anthers, the pistils were dissected out from the buds. An incision was made at the bottom of style using a scalpel for separating the upper part of the pistil from the ovary. The isolated stigma + style samples were collected in a 1.5 mL tube which was suspended in liquid nitrogen and then stored at -80°C (**Figure 1**). Twenty-five to thirty buds were collected from three plants as one biological replicate, and three independent biological replicates were prepared for each sample. To avoid confusion, the term "pistil" is used to refer to "stigma + style" samples throughout the article.

For RNA sequencing, heat-stressed pollen and pistil were collected from 50 days old plants immediately after exposure to heat stress at 40°C for 30 min. A 30 min heat stress treatment was selected to capture early heat stress-responsive changes in the transcriptome. Non-stressed pollen and pistil were collected from plants grown at control growth conditions.

RNA Extraction, Library Preparation, and RNA-Sequencing

Total RNA was isolated from non-stressed and heat-stressed mature pollen as well as non-stressed and heat-stressed pistil samples (three biological replicates) using mirVana microRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's instructions. The RNA samples were then shipped on dry ice to the BGI TECH SOLUTIONS (HONGKONG) CO., LIMITED where they underwent additional testing, according to their quality control pipeline for RNA Sequencing. The integrity of the RNA samples was analyzed using an Agilent Bioanalyzer 2100 before being confirmed as suitable to be run on a BGISEQ-500 platform for PE100 strand-specific mRNA sequencing with an expected output of 30 million raw reads per sample. After sequencing, the raw reads were filtered. Data filtering included removing adaptor sequences, contamination, and low-quality reads from raw reads. The read statistics of the RNA-Seq libraries are provided in **Supplementary Table S1a**.

Transcriptome Analysis Pipeline

Quality checks for the raw fastq files were conducted using FastQC v0.11.8 (Andrews, 2010). Reference transcriptome file for *Brassica napus* was downloaded from Genoscope[1]. Transcript expression was quantified using Kallisto v0.44.0 (Bray et al., 2016). The transcript expression levels were converted to gene expression levels using tximport (Soneson et al., 2015) v1.6.0 (countsFromAbundance = "no"). The low-count genes were prefiltered by keeping only those genes that have at least 5 counts in total. The DESeq2 R package v1.28.1 was used to perform differential expression analysis. The tximport data was loaded into DESeq2 with DESeqDataSetFromTximport thus creating offset and correcting for changes to the average transcript length across samples (Love et al., 2019). Principal component analysis (PCA) was conducted to determine the relatedness of the biological replicates (**Supplementary Figure S1A**). Pairwise contrasts were performed between control and heat-stressed samples to identify differentially expressed genes (DEGs) in response to heat stress. To generate more accurate $\log_2$ foldchange estimates lfcShrink (type = "apeglm") function was used. The thresholds for differential expression were set at fold change 1.5 and p-adjusted value cut off 0.01 (lfcthreshold = 0.585, altHypothesis = "greaterAbs," alpha = 0.01, pAdjustMethod = "BH") for the alternate hypothesis, BH: Benjamini-Hochberg (Love et al., 2019; Zhu et al., 2019).

Functional Annotation

The DEGs were functionally annotated and enriched for GO terms using online tool PlantRegMap (Tian et al., 2020). This tool employs TopGO and incorporates GO annotations from TAIR 10, UniProt-GOA, InterProScan prediction and RBH-based experimental annotation transfer. The annotated GO terms

[1]http://www.genoscope.cns.fr/brassicanapus/data/

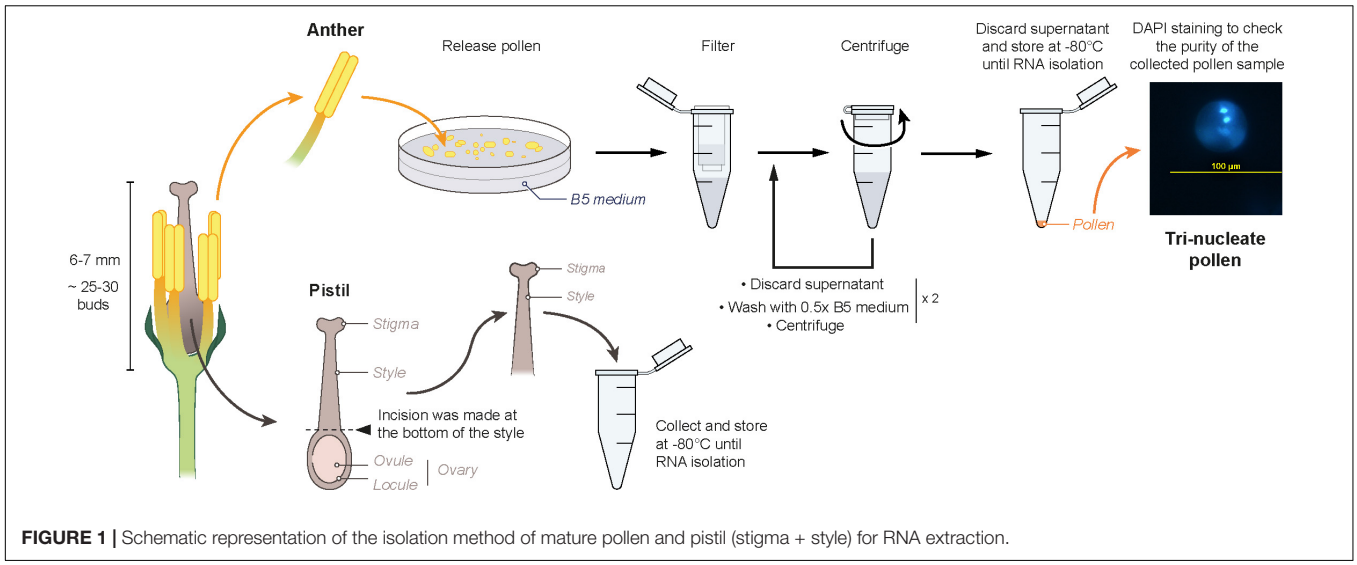

FIGURE 1 | Schematic representation of the isolation method of mature pollen and pistil (stigma + style) for RNA extraction.

were plotted using WEGO Tool, and the redundant enriched GO terms were removed and visualized by REViGO (Supek et al., 2011; Ye et al., 2018). Subsequently, pathway enrichment analysis of DEGs was carried out using the KOBAS 3.0 database (Xie et al., 2011). A GO term and a KEGG pathway were considered significantly enriched only when the corrected p-value for that pathway was <0.01 after applying Fisher's exact test and false discovery rate (FDR; BH method) correction. Visualization of significantly enriched functional pathways was performed by ggplot2 R package (Wickham, 2016). Differentially expressed gene sets were used as input into SeqEnrich (Becker et al., 2017). SeqEnrich is a program adapted for *B. napus*, and it contains the most current information on *B. napus* TFs, promoter motifs, and gene ontology (GO) available, and uses these data to produce predictive regulatory networks. Networks produced with SeqEnrich were visualized in Cytoscape (Smoot et al., 2011). Based on the gene descriptors, the logos of TF binding motifs were downloaded from JASPAR2020 database[2] (Fornes et al., 2020). Homologous *B. napus* genes, in comparison to the Arabidopsis proteome, were identified using the BlastP program with an E-value ≤ 1e-05. R packed Complexheatmap package was used to generate heat maps of gene expression (Gu, 2015).

RESULTS AND DISCUSSION

Short-Term Heat Stress Events Negatively Regulate Reproductive Fitness in *B. napus*

To study the impact of short-term heat stress (4 h) on mature pollen, we exposed the *Brassica napus* plants bearing secondary inflorescences to 40°C for 4 h during the day. The pollen grains of non-stressed plants exhibited ∼90% pollen viability and ∼62% *in vitro* pollen germination (**Figure 2A**). Exposure to a short high temperature spike drastically reduced the pollen viability to ∼25% and pollen germination to ∼10%. Reciprocal crosses between non-stressed (NS), and heat-stressed (HS; 40°C for 4 h) pollen (♂) and pistil (♀): NS♀ × NS♂, NS♀ × HS♂, HS♀ × NS♂ and HS♀ × HS♂ revealed drastic reduction in pollen adhesion and germination on the stigma surface when heat-stressed (HS) pistils were pollinated with heat-stressed (HS) pollen, led to the failure of fertilization and seed set (**Figures 2B–D**). The highest seed set was recorded in, non-stressed (NS) pistil × non-stressed (NS) pollen cross. On the other hand, no significant reduction in seed set was noticed in NS pistil × HS pollen cross. The higher rate of seed set in NS pistil × HS pollen indicate that even ∼25% viable pollen grains (heat-stressed at 40°C for 4 h) were sufficient to fertilize almost all of the ovules. However, the seed set was reduced by ∼70% in HS pistil × NS pollen cross (**Figure 2B**). Relatively few non-stressed pollen grains, were successful in attaching to the heat-stressed stigma surface and then further germinating (**Figure 2C**) suggesting that non-stressed pollen is exposed to a unfavorable environment on the heat-stressed stigma surface leading to impaired interaction and reduced fertility. Thus, heat-induced changes in the pistil likely exert considerable influence over pollen performance and thereby the overall seed yield. Since plants produce abundant pollen, only a small percentage of viable pollen is sufficient for successful fertilization (Larden and Triboi-Blondel, 1994). On the other hand, due to relatively small number of ovules, heat stress sensitivity of pistil can have far severe implications for seed set and yield.

Differential Transcriptional Response to Heat Stress in the Upper Part of the Pistil and Mature Pollen

To explore the gene networks underlying heat stress responses in pollen and pistil, we performed heat stress treatments at 40°C for 30 min and employed a high-throughput strand-specific RNA-seq approach. The clean reads were uniquely aligned (**Supplementary Figure S1B**) to the reference assembly with

[2]http://jaspar.genereg.net/

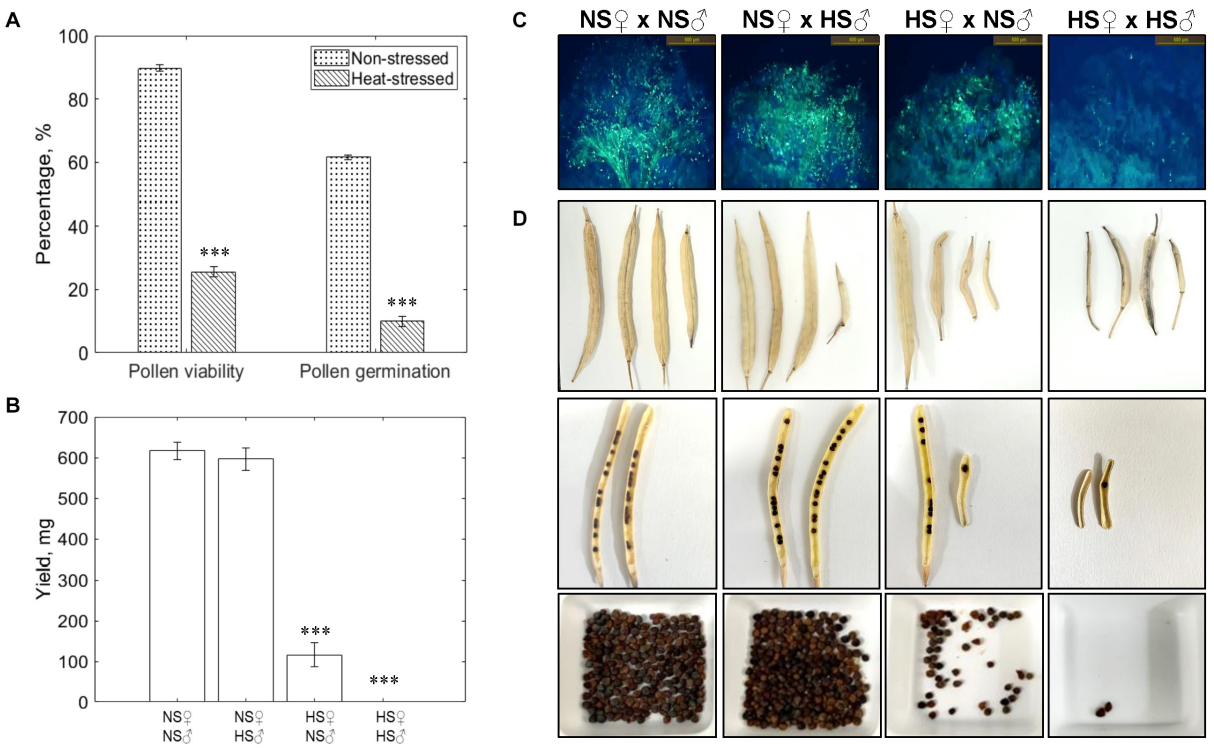

FIGURE 2 | Impact of short-term heat stress on pollen-pistil interactions. **(A)** Pollen viability (%) and pollen germination (%) of non-stressed, and heat-stressed (40°C for 4 h) pollen. **(B)** Seed yield, mg produced from the fertilization of pistils pollinated during reciprocal crosses. **(C)** Aniline blue staining of reciprocal crosses (NS♀ × NS♂, NS♀ × HS♂, HS♀ × NS♂ and HS♀ × HS♂) to observe pollen-pistil interactions. **(D)** Siliques, seed filling of siliques, total seeds produced by the reciprocal crosses. Data are represented as the mean of three replicates ± SEM (standard error of the mean). The asterisk (*) represents a significant difference between heat stress treatment and non-stressed control (***$P \leq 0.001$).

a rate of 72.1% (median). The biological replicates for each sample clustered together and four clusters representing non-stressed pollen, heat-stressed pollen, non-stressed pistil, and heat-stressed pistil were identified in the PCA plot (**Supplementary Figure S1A**). The PCA plot highlighted the similarity of gene expression in the biological replicates for each sample. The relative transcript abundance in terms of TPM (transcripts per million) across all four samples (three biological replicates) are provided in **Supplementary Table S1b** (non-stressed and heat-stressed pollen) and **Supplementary Table S1c** (non-stressed and heat-stressed pistil). The threshold for differential expression was set at fold change 1.5 ($\log_2$foldchange = 0.585) and the adjusted p-value cut off 0.01 for an alternate hypothesis. Heat stress treatment of pollen led to differential expression of 1,524 genes comprising 939 up- and 585 down-regulated genes (**Supplementary Table S1d**). On the other hand, heat stress treatment of pistil revealed a total of 7,133 differentially expressed genes, including 4,333 up- and 2,800 down-regulated genes as compared with control treatment (**Supplementary Table S1e**). *B. napus* is an allotetraploid formed as a result of spontaneous hybridization between its two diploid progenitors: *B. rapa* (A genome, AA, 2n = 2x = 20) and *B. oleracea* (C genome, CC, 2n = 2x = 18). The genome composition of *B. napus* is AACC (2n = 4x = 38). Subtle differences in the distribution of DEGs on sub-genome A_n and sub-genome C_n in both heat-stressed pollen and pistil was observed (**Figure 3A**). Recent reports are suggesting a sub-genome bias in *B. napus* favoring the expression of A_n sub-genome (Khan et al., 2020; Li et al., 2020). However, a further detailed investigation is required to improve the understanding of observed sub-genome expression dominance in *B. napus*.

Interestingly, heat stress-responsive up-regulated transcripts also demonstrated a greater relative change than down-regulated genes (**Figure 3B**). Comparison of the DEGs in response to heat stress in pollen and pistil, highlighted 764 shared DEGs (**Figure 3C**), out of which 755 DEGs showed similar regulation patterns (700 up- and 55 down-regulated in both). In contrast, nine shared DEGs were up-regulated in heat-stressed pistil and down-regulated in heat-stressed pollen (**Figure 3D** and **Supplementary Table S2a**).

Heat stress results in structural changes in proteins, accumulation of misfolded proteins, and various downstream ramifications for cellular homeostasis and development. To counter these effects, heat stress response through chaperone/heat shock protein (HSP)–heat shock factor (HSF) complexes is activated (Bokszczanin et al., 2013; Berz et al., 2019). Transcriptional reprogramming at higher temperatures is regulated by heat stress transcription factors (HSFs) leading to activation of a heat stress response. Based on the reports identifying the members of HSF

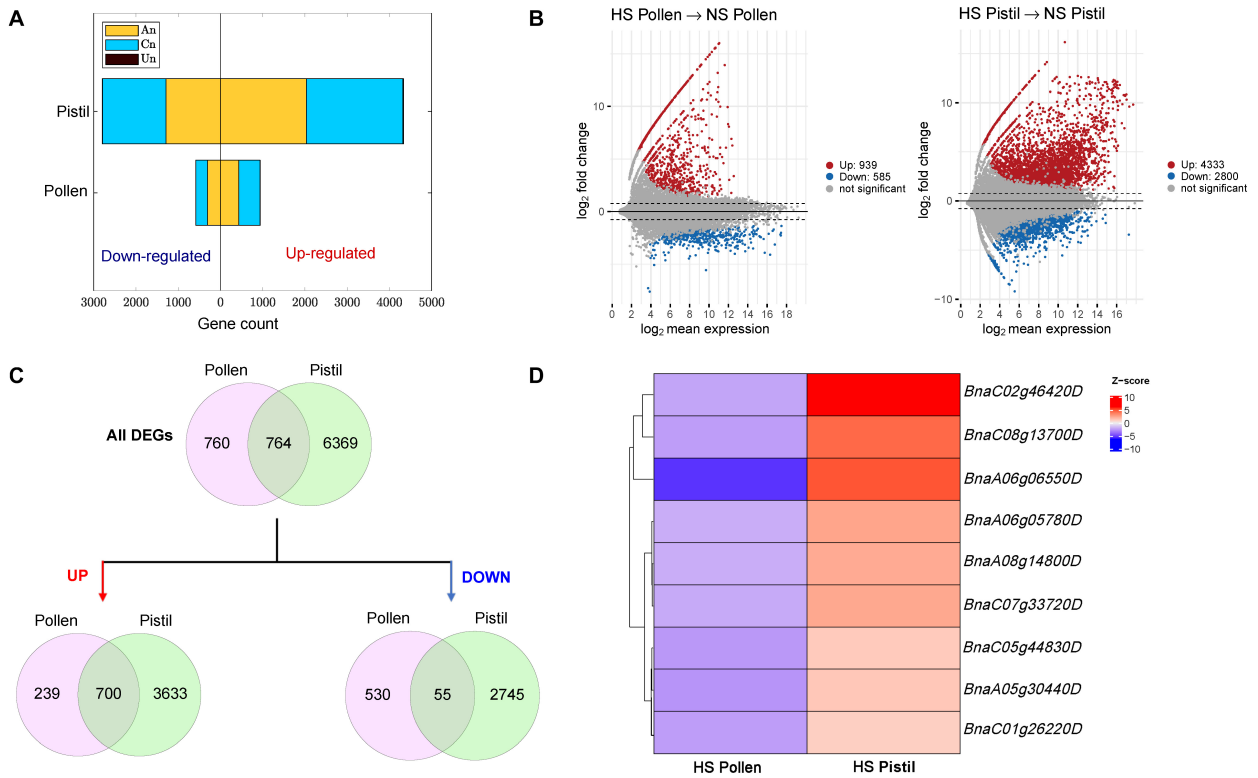

FIGURE 3 | Differential gene expression analysis. **(A)** Number of up and down-regulated gene in heat-stressed pollen and pistil and their distribution on the A_n and C_n subgenome (Un represents genes located on uncertain chromosomes. **(B)** MA Plot of the differentially expressed genes in heat-stressed pollen and pistil; red dots represent upregulated genes, blue dot represent downregulated genes; and gray dots represent non-significant genes. HS, heat stressed; NS, non-stressed. **(C)** Venn diagrams showing all shared DEGs, only upregulated shared DEGs and only down-regulated shared DEGs between heat-stressed pollen and pistil. **(D)** Details and expression levels of the shared DEGs with upregulated expression in heat-stressed pistil and downregulated expression in heat-stressed pollen.

gene family in *B. napus* (Zhu et al., 2017; Lohani et al., 2019), we detected the up-regulation of several members of the *B. napus* HSF gene family in heat-stressed pollen and pistil, except for HSFC1 (*BnaC07g07130D*) which was downregulated in pistil upon heat stress exposure. *B. napus* HSFA2 (*BnaC03g26940D, BnaA03g22890D*), HSFA7 (HSF7a: *BnaA03g41550D, BnaC07g32600D, BnaA03g41540D*, HSF7b: *BnaA09g40360D*) and HSFB2a (*BnaC03g52080D*) were the key HSFs upregulated in both pollen and pistil with >100-fold change in expression levels. Further, several differentially regulated HSPs and other chaperone encoding genes (ClpB1, DnaJ-domain chaperone, BAG6, ClpB3, ClpB4) were detected in heat-stressed pollen and pistil (**Supplementary Tables S2b,**). In pollen, all the identified HSPs and chaperones were upregulated by heat stress. Similarly, in the heat-stressed pistil, majority of HSPs and chaperones were upregulated except *BnaC06g39440D* (Chaperone DnaJ-domain superfamily protein), *BnaA06g28870D* (Prefoldin chaperone subunit family protein), *BnaA02g11050D*, *BnaA03g12820D* (BAG family molecular chaperone regulator 1) and *BnaC05g29580D* (HSP). Small heat shock proteins (15.7, 17.6, 17.8, 18.5, 21, 22, 23.6, and 26.5 kDa HSPs) along with Hsp70b were among the upregulated HSPs in both heat-stressed pollen and pistil. Thus, the accumulation of HSFs, HSPs (sHSP, HSP70, HSP90, and HSP100) and chaperones in response to heat stress, illustrate the ability of pollen and pistil to activate "classical" heat stress-responsive mechanisms (Bokszczanin et al., 2013; Fragkostefanakis et al., 2015; Lohani et al., 2020b).

Functional Annotation

Gene ontology (GO) analysis of the DEGs was performed to unravel their role in heat stress response (**Supplementary Figure S2**). Notably, 1,041 (642 up- and 499 down) and 5,192 (3,091 up- and 2,101 down) differentially regulated genes were assigned GO annotations in pollen and pistil, respectively. Analysis of enriched GO terms ($q < 0.01$) revealed that the upregulated genes in heat-stressed pollen and pistil were associated with abiotic stress associated GO terms like "response to stress," "response to heat," "response to stimulus," and "protein folding" (**Figure 4**).

Further, the most significantly enriched downregulated GO terms in pollen were associated with "ion transmembrane transport," "carbohydrate metabolic process," "localization," and "glycosylation" (**Figure 5A**). In heat-stressed pistil, the downregulated GO terms were related to "fatty acid metabolism," "cell wall organization," "response to abiotic stimulus," "cuticle development," and "lipid transport" (**Figure 5B**). Heat responsive down-regulated genes involved in cellular transport, carbon

GO.ID	Term	Pistil_Genes	Pollen_Genes
GO:0050896	response to stimulus	491	147
GO:0006950	response to stress	296	122
GO:0009628	response to abiotic stimulus	192	77
GO:0042221	response to chemical	243	67
GO:0009266	response to temperature stimulus	103	58
GO:1901700	response to oxygen-containing compound	129	51
GO:0009408	response to heat	74	49
GO:0006457	protein folding	125	47
GO:0010035	response to inorganic substance	93	35
GO:0009642	response to light intensity	30	26

FIGURE 4 | Top significantly enriched common GO terms associated with upregulated genes in heat-stressed pollen and pistil ($q < 0.001$).

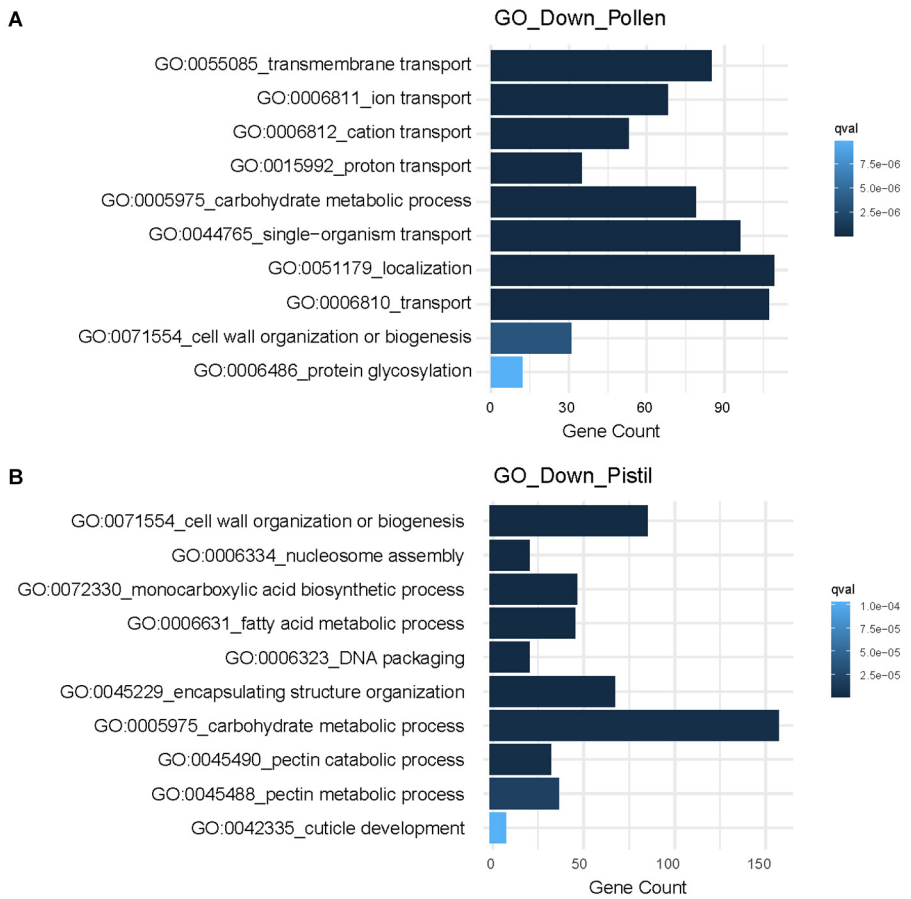

FIGURE 5 | GO enrichment analysis of downregulated DEGs showing top 10 significantly. **(A)** Enriched GO terms associated with downregulated genes in heat-stressed pollen. **(B)** Enriched GO terms associated with downregulated genes in the heat-stressed pistil.

and nitrogen metabolism, cell organization and growth, and metabolic processes determine long-term adverse effects and are reported to differ significantly depending on the developmental stage or tissue exposed to heat stress (Janni et al., 2020).

Further, the analysis of enriched KEGG pathways in both pollen and pistil revealed differential upregulation of diverse pathways including protein processing in the endoplasmic reticulum (111 genes in pollen, 192 genes in pistil), spliceosome (40 genes in pollen, 106 genes in pistil), metabolic pathways (166 genes in pollen, 668 genes in pistil), and plant-pathogen interaction (24 genes in pollen, 68 genes in pistil) among other pathways (**Figure 6**). Many of these enriched functional gene categories and pathways have been linked with heat stress response or thermotolerance across different tissues in crops

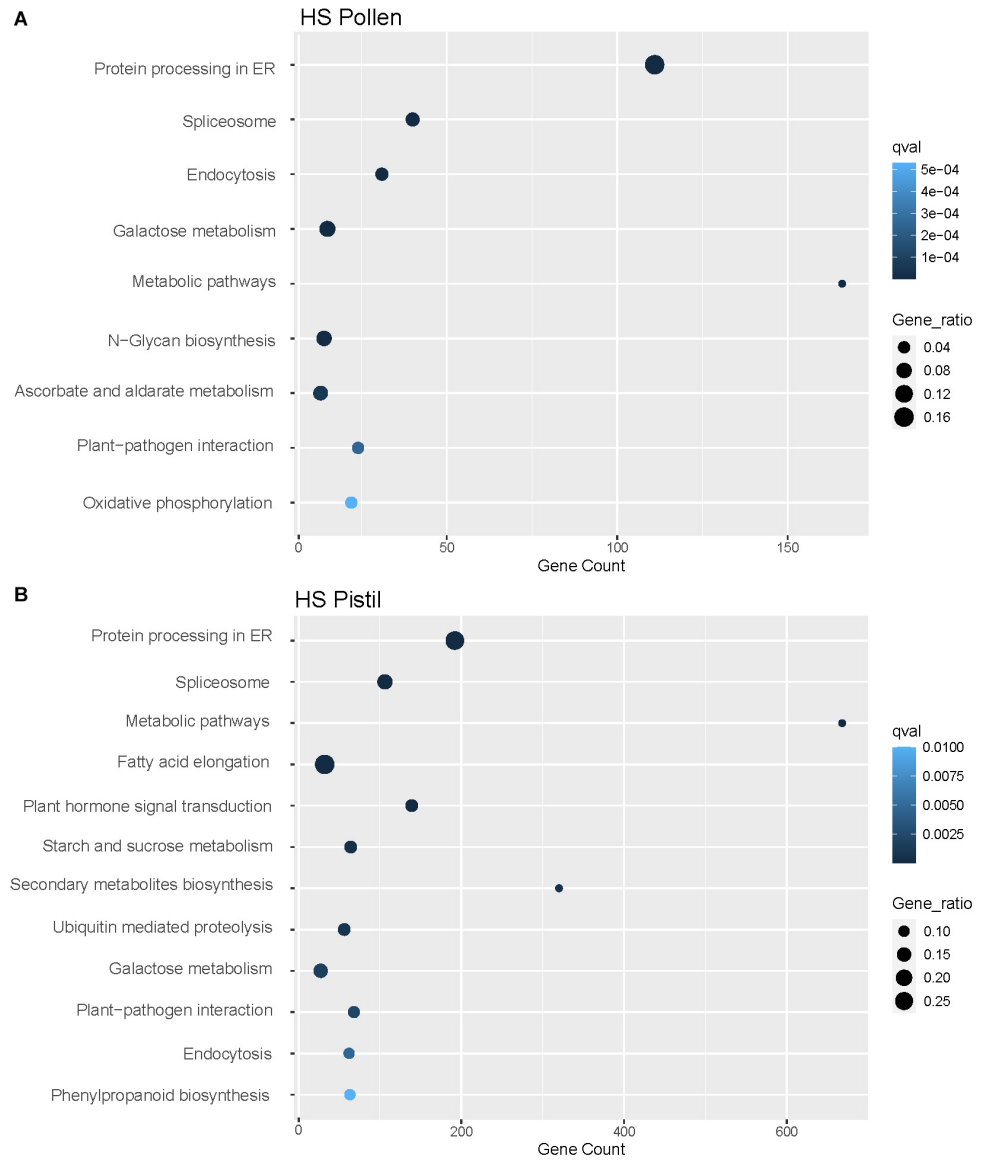

FIGURE 6 | (A) Enriched KEGG pathways ($p < 0.01$) associated with the DEGs in heat-stressed (HS) pollen. **(B)** Enriched KEGG pathways ($p < 0.01$) associated with the DEGs in heat-stressed (HS) pistil.

such as rice, wheat, maize, and tomatoes, which indicates a conserved mechanism of heat stress response in plants (Janni et al., 2020). We further explored the role of the functionally annotated DEGs associated with different metabolic and cellular processes to explore their role in regulating reproductive fitness and pollen-pistil interactions.

Heat Stress Altered Cellular and Metabolic Processes Impair Pollen-Pistil Interactions

The acceptance of compatible pollen by the receptive pistil and the subsequent steps leading to successful fertilization involves interactive processes. The result of reciprocal crosses (NS♀ × NS♂, NS♀ × HS♂, HS♀ × NS♂ and HS♀ × HS♂) in our study highlighted that heat stress has a negative influence on the pollen-pistil interaction resulting in reduced seed set. In our datasets, the DEGs involved in the GO category "recognition of pollen" and "pollen-pistil interactions" were downregulated in the heat-stressed pistil (**Figure 7**). These heat-responsive genes might interfere with the attachment of pollen on to the stigma surface. For instance, the S-locus related gene, *SLR1* (*BnaA03g32070D*) belonging to the downregulated category, is reported to play a role in pollen adhesion (Luu et al., 1997, 1999). Further, genes involved in "plant-pathogen interaction (bna04626)" and "phenylpropanoid biosynthesis (bna00940)" were also differentially regulated in heat-stressed pollen and pistil. The differential regulation of phenylpropanoid biosynthesis pathway

B. napus Gene ID	Arabidopsis homolog	Gene Description	LFC
BnaA03g32070D		SLR1	-3.42
BnaA09g46530D	*AT1G11330.2*	G-type lectin S-receptor-like serine/threonine-protein kinase	-3.37
BnaA09g46530D		Brassica-specific	-3.37
BnaC06g27660D	*AT4G21380.1*	SD18, Receptor-like serine/threonine-protein kinase SD1-8	-3.08
BnaA04g23210D	*AT4G21380.1*	SD18, Receptor-like serine/threonine-protein kinase SD1-8	-2.45
BnaC05g08740D	*AT1G11340.1*	RKS1, G-type lectin S-receptor-like serine/threonine-protein kinase RKS1	-2.23
BnaC08g42090D	*AT1G11330.2*	G-type lectin S-receptor-like serine/threonine-protein kinase	-2.06
BnaC08g14850D	*AT1G11330.2*	G-type lectin S-receptor-like serine/threonine-protein kinase	-2.00
BnaA04g05920D	*AT1G65800.1*	SD16, Receptor-like serine/threonine-protein kinase SD1-6	-1.96
BnaA04g14930D	*AT1G11280.2*	S-locus lectin protein kinase family protein	-1.91
BnaC08g14740D	*AT1G61360.1*	Serine/threonine-protein kinase	-1.55
BnaAnng22710D	*AT4G21380.2*	SD18, Receptor-like serine/threonine-protein kinase SD1-8	-1.42
BnaC02g44050D	*AT4G21380.1*	SD18, Receptor-like serine/threonine-protein kinase SD1-8	-1.39
BnaA08g25300D	*AT1G11340.1*	RKS1, G-type lectin S-receptor-like serine/threonine-protein kinase RKS1	-1.33

FIGURE 7 | Details of the DEGs associated with the GO terms "recognition of pollen" and "pollen-pistil interactions" in the heat-stressed pistil. LFC, log_2foldchange.

leads to accumulation of the intermediates (**Supplementary Figure S3**) which has been suggested to result in incompatible pollen-pistil interactions (Elleman and Dickinson, 1999).

Following pollen adhesion, the success of pollination and pollen tube growth depends on pollen hydration. In pollen very-long-chain fatty acids (VLCFA) are essential components of the pollen coat, facilitating interactions between pollen and stigma (Heizmann et al., 2000) and genes belonging to 3-ketoacyl-CoA synthase family are involved in their biosynthesis (Han et al., 2001). In heat-stressed pollen, five and in the heat-stressed pistil, twenty-nine genes homologous to 3-ketoacyl-CoA synthase genes were downregulated, suggesting downregulation of VLCFA synthesis due to heat stress (**Supplementary Tables S3a, S4a**).

Since lipid biosynthesis is essential for all aspects of pollen development, germination and pollen tube penetration of pistil tissues (Evans et al., 1992; Piffanelli et al., 1997), the observed alteration of genes expression involved in the fatty acid synthesis and lipid transport in heat-stressed pollen and pistil has apparent implications for pollen fitness and pollen-pistil interactions.

Additionally, in heat-stressed pollen the GO categories "water channel activity" and "water transmembrane transport" were downregulated; especially genes homologous to Aquaporin TIP1-3 (*BnaA09g51590D*, *BnaCnng01570D*), indicating possible interference of the pollen hydration process (**Supplementary Table S3b**). In Arabidopsis TIP1-3 (*At4g01470*) aquaporin, is selectively expressed in pollen (Soto et al., 2008). A possible role of aquaporin-like genes in pollen hydration has also been previously suggested in *Brassica* (Ikeda et al., 1997). Here, we also observed the upregulation of genes homologous to Aquaporin PIP2-7, TIP1-1, and TIP2-1 in the heat-stressed pistil (**Figure 8A** and **Supplementary Table S4b**). PIP aquaporin genes are expressed in the stigma of *Brassica* (Dixit et al., 2001). In tobacco, PIP genes are suggested to be involved in the movement of water between pollen and stigma (Bots et al., 2005). However, the role of TIP1-1 and TIP2-1 in pistil remains elusive (Pérez Di Giorgio et al., 2016).

The stigma in *B. napus* is dry, and the stigmatic papillae are protected from dehydration and environmental conditions by waxy cuticle of the highly modified epidermal cells. Mutations that disrupt the formation of cuticles can have a profound impact on the ability of stigma to support pollen tube growth (Ingram and Nawrath, 2017). In heat-stressed pistil, genes involved in cuticle formation were also downregulated (**Figures 5B**, **8A** and **Supplementary Table S4c**). Our data suggest that the downregulation of the genes associated with the biosynthesis of cuticle constituents in response to heat stress in pistil can interfere with pollen hydration and further germination.

Following hydration, pollen germinates on the stigma surface penetrating the stigma cells and then growing within the style's transmitting tract. During growth toward ovary, the pollen tubes interact intimately with the extracellular matrix (ECM) components of the transmitting tract (Cheung et al., 1995). Hydroxyproline-rich glycoproteins (HRGPs), such as arabinogalactan proteins, AGPs (Cheung and Wu, 1999) in the extracellular matrix (ECM) have been suggested to regulate pollen tube growth and pollen tube guidance in the style tissue and regulate other functions, such as maintaining cellular integrity and cell-cell communications (Palanivelu and Preuss, 2000; Losada et al., 2017). Accordingly, we identified several differentially regulated AGPs like AGP4, AGP12, AGP15 and AGP18 in the heat-stressed pistil (**Figure 8A** and **Supplementary Table S4d**). Further research is required to explore the impact of heat stress on the HRGP mediation of pollen-pistil interactions in styler tissues.

Pollen Cell Wall Organization and Cellular Transport-Related Genes Possibly Regulate Pollen Fitness Under Heat Stress

A significant percentage of downregulated genes in heat-stressed pollen was associated with the cell membrane

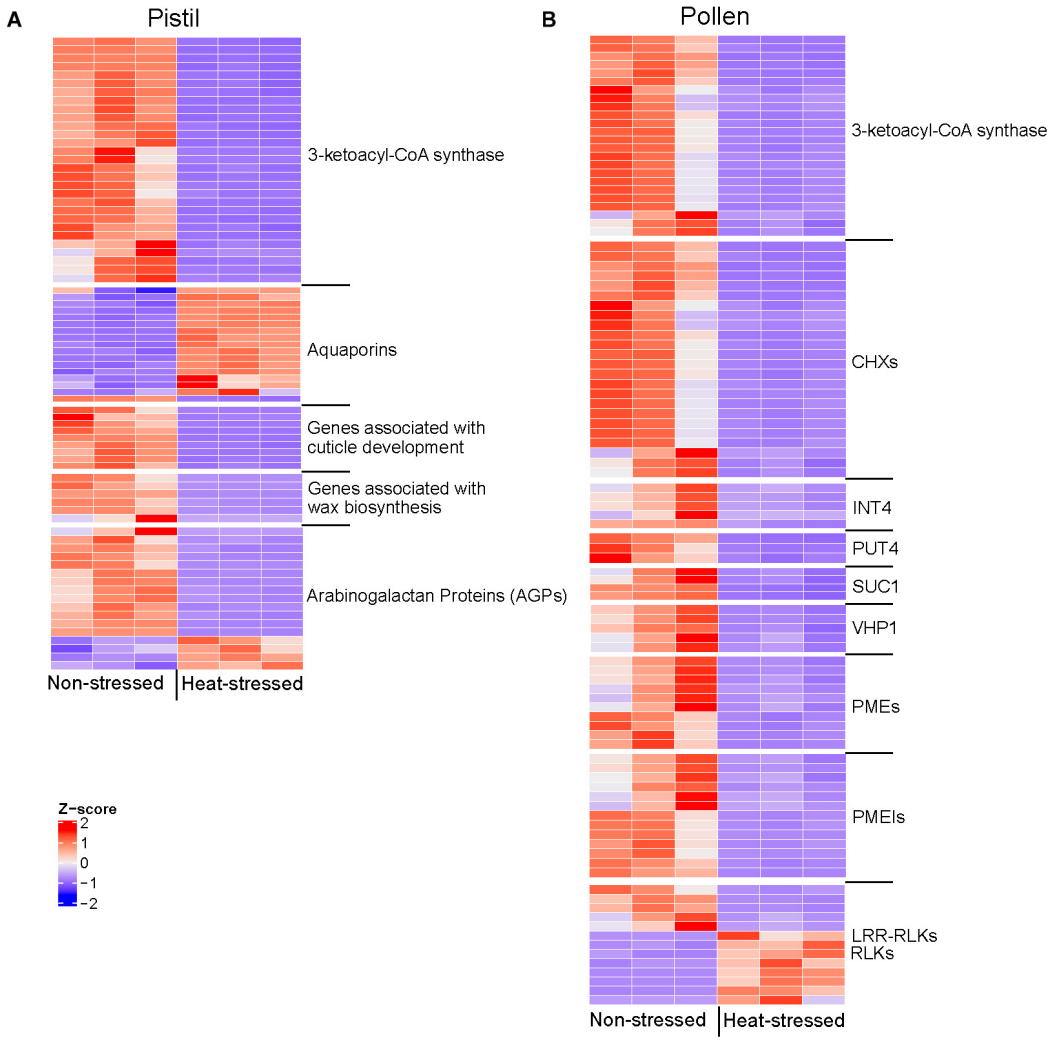

FIGURE 8 | (A) Expression of selected genes differentially regulated in heat-stressed pistil possibly involved in pollen-pistil interactions. **(B)** Expression of selected genes differentially regulated in heat-stressed pistil possibly involved in pollen-pistil interactions. See the summary in **Supplementary Tables S3a–f, S4a–f** for log_2foldchange.

components in comparison to heat-stressed stigma/style tissues. Key gene families involved in cellular transport such as cation/H^+ antiporters, cation/H^+ exchangers (CHXs), inositol transporters (INT4), sucrose transporter, SUC1, Na^+/Ca^{2+} exchanger, proline transporter (PUT4), plasma membrane-associated ATPase, vacuolar H^+ pyrophosphatases (VHP1), metal transporters and K^+ uptake permease were downregulated among other transporters in heat-stressed pollen (**Figure 8B** and **Supplementary Table S3c**). While the possible role of CHX transporters in pollen function is not fully characterized, the Arabidopsis, *cxh17/18/19* mutants display a disordered architecture of the pollen wall, reduced male fertility and seed set (Chanroj et al., 2013). Similarly, *chx21/23* double mutant display male-sterility as the pollen tube is incapable of reaching the ovules (Lu et al., 2011). There is a recent report of the downregulation of these transporters in *in vitro* germinated Arabidopsis pollen exposed to heat stress (Poidevin et al., 2020). Sucrose transport gene *AtSUC1* shows high expression in pollen. The Arabidopsis, At*suc1* mutant pollen is defective *in vivo* with slower rates of pollen germination *in vitro* (Sivitz et al., 2008). Recently, the role of plasma membrane-associated ATPases in maintaining plant fertility was reported as these transporters control the ion balance by controlling downstream pH-dependent mechanisms essential for pollen tube growth (Hoffmann et al., 2020). Thus, based on our analysis and previous reports, we suggest that the downregulation of key genes involved in transmembrane transport is one of the key reasons for the loss of pollen function under heat stress conditions (Frank et al., 2009; Giorno et al., 2010; Fragkostefanakis et al., 2016; Keller et al., 2018; Gonzalo et al., 2020).

We also investigated heat-responsive pollen DEGs associated with cell wall organization to explore their role in pollen fitness. In our analysis, *BnaA02g34360D* encoding gene homologous to cellulose synthase CESA-6 is downregulated in heat-stressed

pollen. In Arabidopsis, subunits of cellulose synthase complex CESA1-, CESA3-, and CESA6-related genes are involved in cellulose synthesize at the plasma membrane. Exclusion of any of these components leads to gametophytic lethality, indicating that primary-wall cellulose synthesis is crucial for pollen development (Persson et al., 2007). Thus, the downregulation of CESA-6 in heat-stressed pollen points toward inhibition of cellulose biosynthesis as one of the heat-induced pollen defects.

B. napus genes encoding probable pectin methyl esterases (PMEs) and PME inhibitors (PMEIs) were differentially regulated in heat-stressed pollen (**Figure 8B** and **Supplementary Table S3d**). PME and PMEI are known to regulate the stability of certain pollen tube wall domains during its elongation (Guan et al., 2013). Furthermore, in pollen crucial role of serine-threonine kinases and leucine-rich repeat receptor-like kinases (LRR-RLKs) in regulating pollen germination, pollen tube growth, and/or pollination has been suggested (Kim et al., 2004) and several members of the RLK gene family were also differentially regulated in heat-stressed pollen. Interestingly, we observed downregulation of genes homologous to *Arabinosyl transferase RRA3* (*BnaA06g13470D, BnaA06g13630D*) in heat-stressed pollen. In Arabidopsis, *RRA3* is involved in glycosylation of cell wall proteins which is suggested to be a crucial step in cell wall assembly for root hairs. This gene is also suggested (Velasquez et al., 2011) to be possibly involved in a similar modification of cell wall proteins involved in pollen tube growth but has not been experimentally validated. We also identified one *expansin* gene (*BnaCnng40260D*) downregulated by heat stress in pollen and pistil, which might play a role in cell wall loosening and assisting in penetration of stigma by the pollen tube (Valdivia et al., 2009). We conclude that downregulation of genes associated with cell wall organization and cellular transport adversely impact the pollen fitness and integrity, leading to reduced reproductive success and seed set.

High Temperature-Induced Oxidative Stress Interferes With Reproductive Functions

In heat-stressed pollen and pistil, we identified genes associated with GO categories "response to hydrogen peroxide," "response to reactive oxygen species" (**Supplementary Tables S3e, S4e**). Majority of the ROS responsive DEGs in pollen were also differentially regulated in pistil except for *BnaC03g73200D, BnaA09g36810D, BnaAnng06520D, BnaA02g06590D, BnaA06g18310D*, and *BnaC03g55730D*. Interestingly, homologs of NADPH oxidases (Respiratory burst oxidase homolog H and J), *BnaA02g06590D* (*RbohJ*), *BnaA06g18310D* (*RbohJ*), and *BnaC03g55730D* (*RbohH*) were downregulated only in heat-stressed pollen. In Arabidopsis, defective pollen tube growth which interrupts adequate fertilization was reported in the *rbohHrbohJ* double mutant, suggesting the role of these two genes in pollen germination (Boisson-Dernier et al., 2013). Furthermore, *RbohH* and *RbohJ* are involved in enzymatic production of ROS, which is crucial for pollen germination (Kaya et al., 2014). Thus, the downregulation of these genes negatively impacts the pollen function. Furthermore, the interaction network of RBOHs, ROS and Ca^{2+} concentrations is also possibly involved in pollen tube growth, and the negative regulation of this interaction by heat stress can cause interference with reproduction. Additionally, Ca^{2+} signaling is reported to play a crucial role in pollen tube growth and development (Konrad et al., 2018). The identified DEGs in heat-stressed pollen and pistil with a possible role in regulating Ca^{2+} gradient and signaling were homologous to Calcium-dependent lipid-binding (CaLB) family proteins, Calmodulin- binding family proteins, CML47, Calmodulin 1, Calcium-binding EF-hand family protein, Calcium-dependent protein kinase (CDPK), sodium/calcium exchangers, Calcium-transporting ATPase, alpha-mannosidase 3, Cyclic nucleotide-gated channel-16 (CNGC16) and others (**Supplementary Tables S3f, S4f**). We observed downregulation (log_2foldchange 1.8) of a probable CNGC16 *B. napus* gene (*BnaCnng14420D*) in heat-stressed pollen. CNGC16 is crucial pollen expressing gene playing a role in heat stress-responsive Ca^{2+} signaling and downstream transcriptional heat stress response (Tunc-Ozdemir et al., 2013). The transcriptome analysis of the Arabidopsis mutant *cngc16* pollen highlighted the defect in triggering or maintaining heat stress-responsive transcriptome, indicating the importance of CNGC16 in pollen heat stress response (Ishka et al., 2018).

In heat-stressed pollen and pistil, aldehyde oxidase genes homologous to *glyoxal oxidase 1* (*Glox1*) were downregulated. In Arabidopsis, *Glox1* possibly play a vital role in the tapetum and pollen development (Krishnamurthy, 2015). It can be suggested that GLOX1 might negatively regulate pollen fitness and also play a damaging role in heat stress exposed pistil as well. Additionally, in heat-stressed pollen and pistil homologs of *galactinol synthase 1* (*GloS1*) and raffinose synthase family proteins were upregulated. These genes are involved in the raffinose family of oligosaccharides (RFOs) synthesis and have a possible role in oxidative stress response (Sengupta et al., 2015). *B. napus* genes homologous to ROS scavenging genes APX1 (Ascorbate Peroxidase 1; *BnaA06g04380D, BnaC05g05550D*) and APX2 (Ascorbate Peroxidase 2; *BnaA01g32160D, BnaC01g39080D*) were upregulated in pollen and pistil. The role of GLOS1 and raffinose synthase family proteins, APXs in heat stress response in reproductive tissues is not clear.

Transcriptional Cascades Involved in Heat Stress Response in Pollen and Pistil

Transcription factors (TFs) are fundamental to the regulation of gene expression. The transduction of stress signal to stress-responsive gene expression is mediated by TFs, which then interact with cis-acting elements located in the promoters of several target stress-responsive genes (Ohama et al., 2017). Around 4.9% (74/1,524; up: 74) and 7.9% (561/7,133; up: 305, down: 256) DEGs in heat-stressed pollen and pistil, respectively, were identified as encoding TFs (**Figures 9A**, **10A**). Further, the SeqEnrich analysis, DNA sequence motifs or transcription factor binding sites (TFBS) significantly ($p < 0.001$) enriched in promoters of query genes are associated with TFs within the same query gene list capable of binding to that DNA sequence motif. Thus, we further identified the enriched transcriptional module,

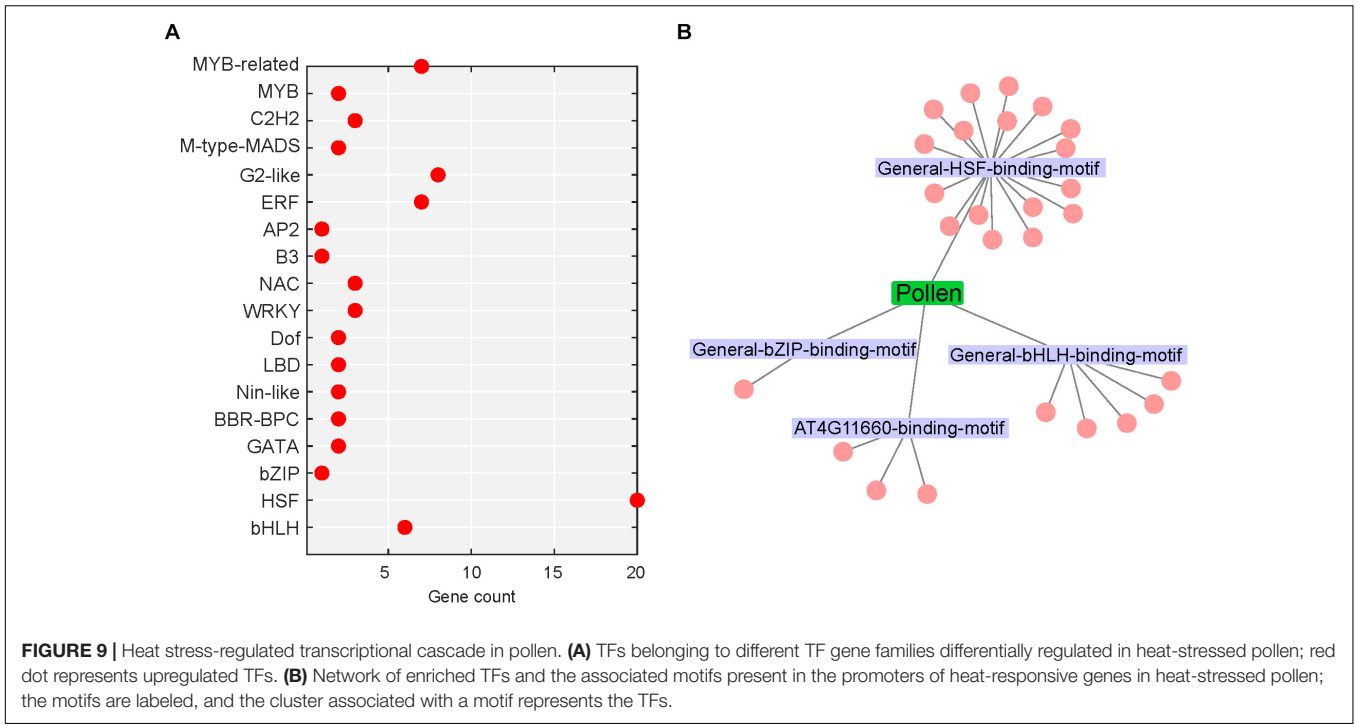

FIGURE 9 | Heat stress-regulated transcriptional cascade in pollen. **(A)** TFs belonging to different TF gene families differentially regulated in heat-stressed pollen; red dot represents upregulated TFs. **(B)** Network of enriched TFs and the associated motifs present in the promoters of heat-responsive genes in heat-stressed pollen; the motifs are labeled, and the cluster associated with a motif represents the TFs.

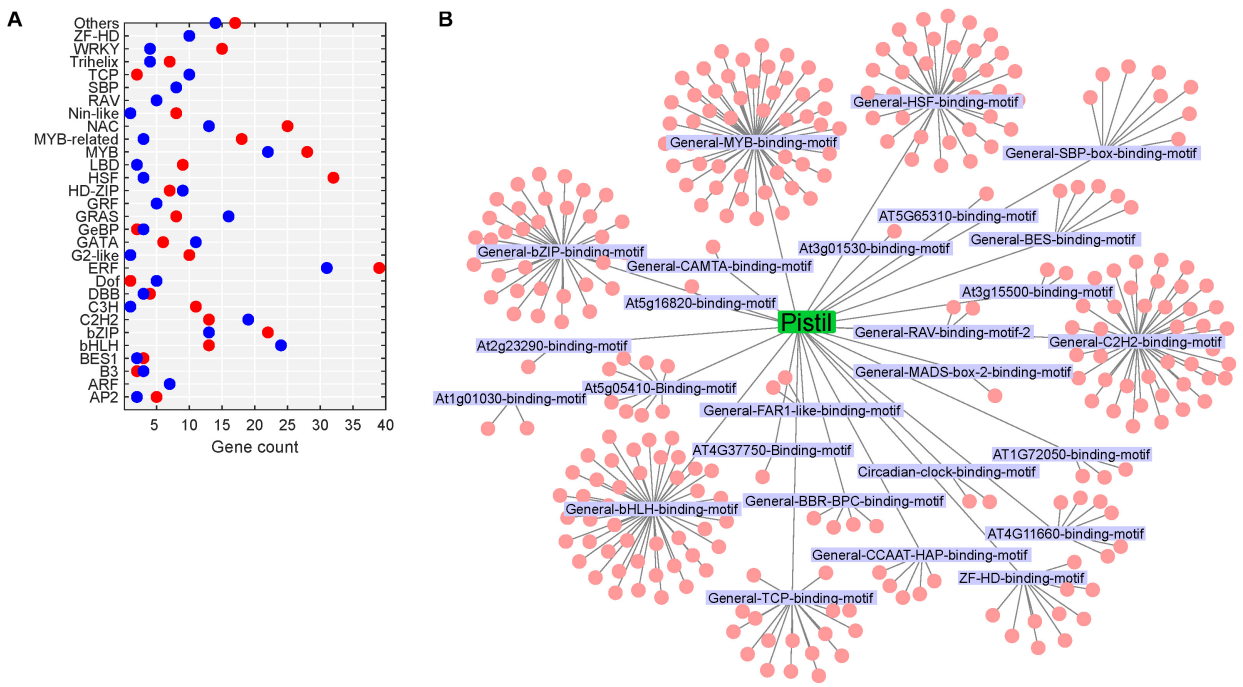

FIGURE 10 | Heat stress-regulated transcriptional cascade in the pistil. **(A)** TFs belonging to different TF gene families differentially regulated in heat-stressed pistil; red dot represents upregulated TFs, and the blue dot represents downregulated genes. **(B)** Network of enriched TFs and the motifs present in the promoters of heat-responsive genes in heat-stressed pistil; the motifs are labeled, and the cluster associated with a motif represents the TFs.

including TFs and their binding sites active in heat stress response in pollen and pistil (**Figures 9B**, **10B**).

In heat-stressed pollen and pistil, 13 and 47 significantly enriched TFBS were identified in the 1kb promoter region of the differentially expressed genes, respectively (**Supplementary Tables S5, S6**). The HSF binding motifs, bHLH binding motif, bZIP binding motif, GATA/TIFY binding motifs, MYB binding motifs and CAMTA binding motif were among the

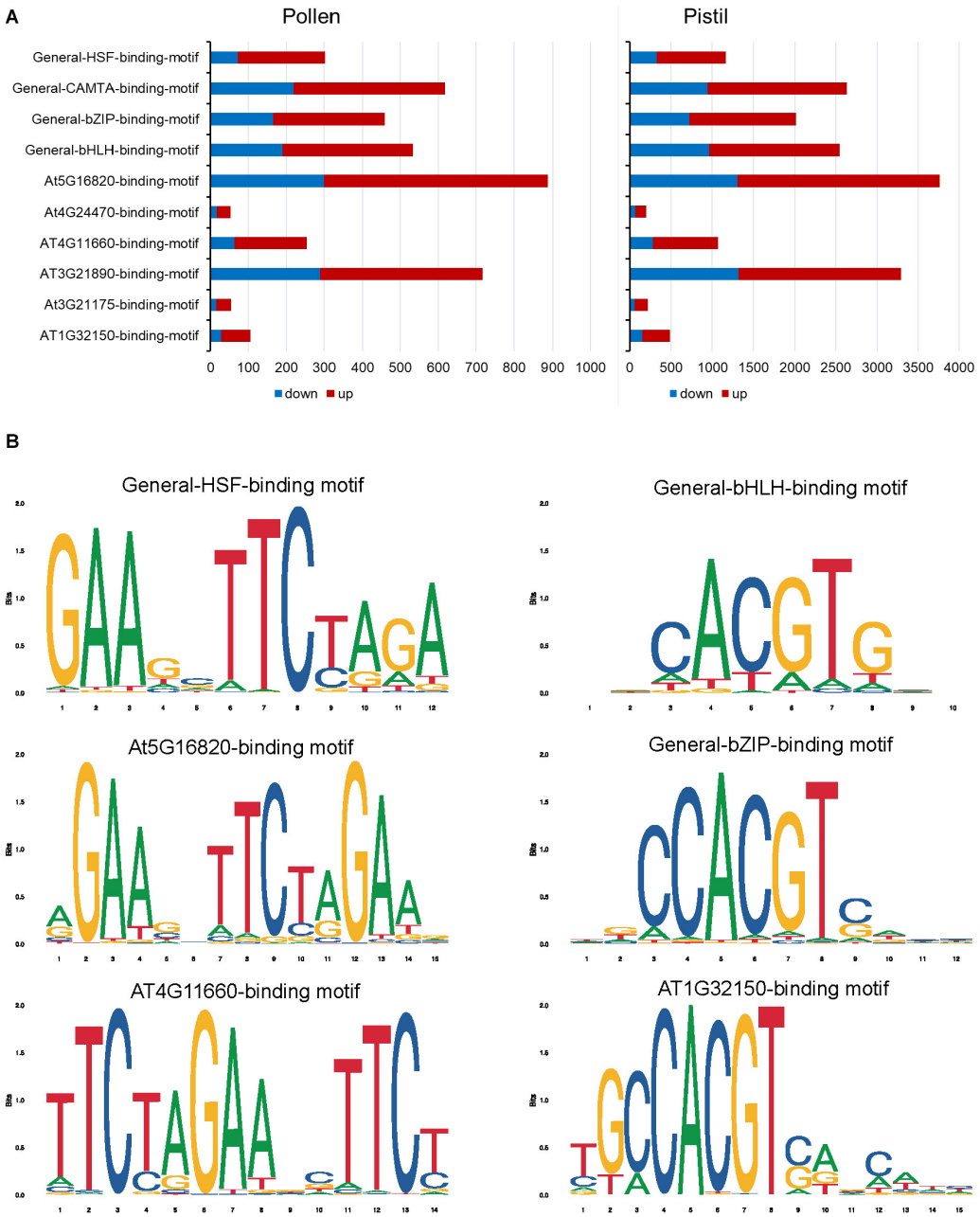

FIGURE 11 | Heat stress-responsive transcription factor binding sites present in the 1 kb promoter region of the differentially regulated genes in heat-stressed pollen and pistil. **(A)** Top significantly enriched of DEGs in heat-stressed pollen and pistil. **(B)** Consensus sequence of HSF (General-HSF-binding-motif, At5g16820-binding-motif, At4G11660-binding-motif), bHLH and bZIP (General-bZIP-binding-motif, At1G32150-binding-motif) transcription factor binding sites.

most significant TFBS predicted to be present in the target DEG's promoters in heat-stressed pollen and pistil (**Figure 11**). The HSF binding sites (General-HSF-binding-motif, At5g16820-binding-motif, At4G11660-binding-motif) were present in 68 and 62% target genes in heat-stressed pollen and pistil, respectively. HSFs are well known to bind to the heat stress elements (HSEs: 5′-nGAAnnTTCnnGAAn-3′ or 5′-nTTCnnGAAnnTTCn-3′) present in the promoters of their target genes (Guo et al., 2016). In heat-stressed pollen and pistil, almost 35% of the target DEGs had the bHLH TFBS in their promoters. The bZIP TBFS (General-bZIP-binding-motif, At1G32150-binding-motif) were present in 32 and 31% DEGs in heat-stressed pollen and pistil. The consensus sequences of bZIP and bHLH TFBS had an ACGT core, and the bZIP and bHLH TFs are generally thought to recognize ACGT core containing ABRE sequences such as the G-box motif with flanking regions varying to some degree (Mehrotra et al., 2013; Ezer et al., 2017). The transcription factors bZIPs and bHLHs can act antagonistically,

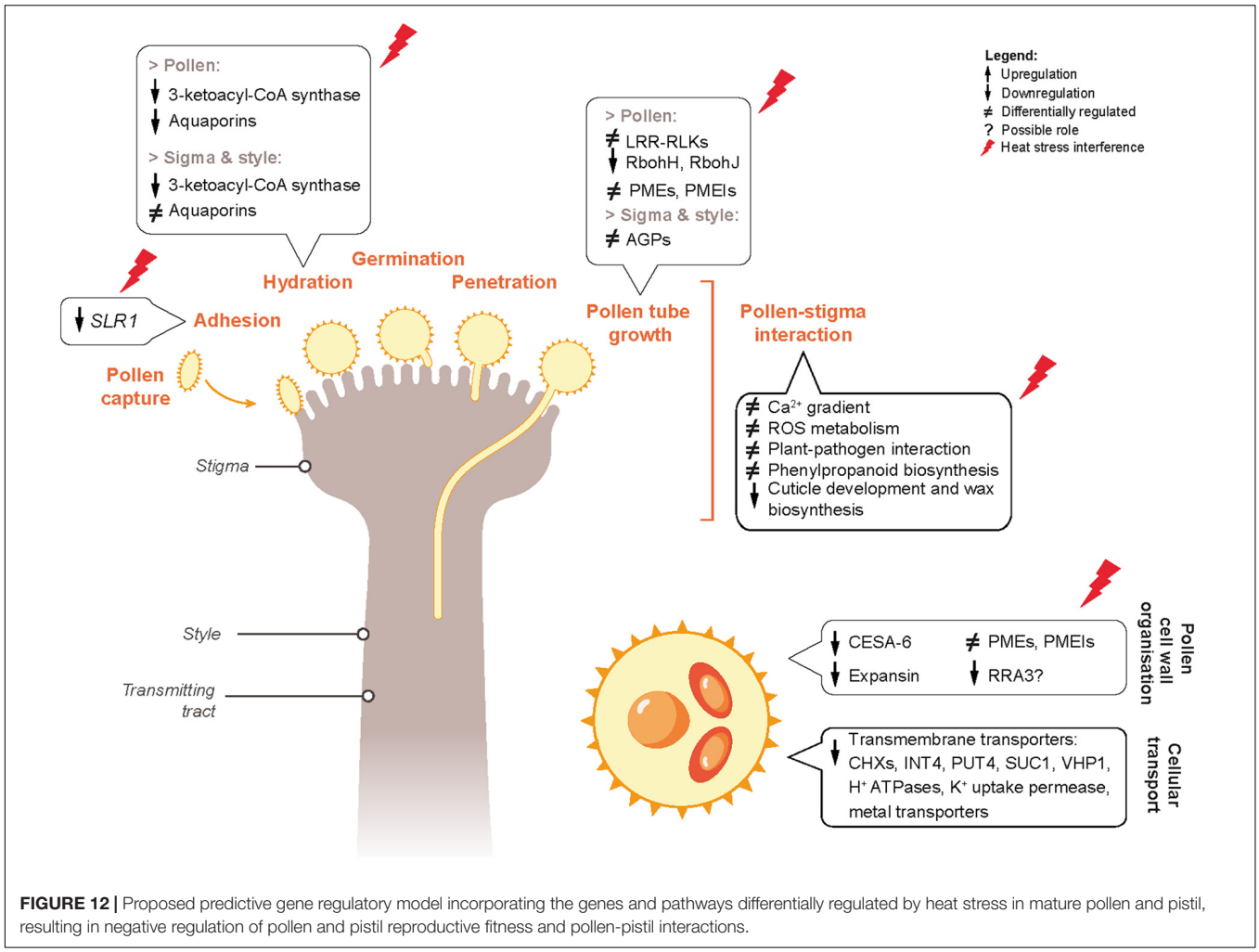

FIGURE 12 | Proposed predictive gene regulatory model incorporating the genes and pathways differentially regulated by heat stress in mature pollen and pistil, resulting in negative regulation of pollen and pistil reproductive fitness and pollen-pistil interactions.

competing for binding to the same sites. Thus, understanding how bZIP and bHLH regulate their target genes is vital to explore pathways that can be engineered for imparting reproductive thermotolerance (Ezer et al., 2017).

In heat-stressed pollen, four significant TFBS were associated with 26 significantly enriched TFs. Among the enriched TFs, 19 genes belonged to the HSF family; five genes belonged to the bHLH family and one candidate to the bZIP family (**Supplementary Table S7**). *BnaC03g26940D*, *BnaA09g40360D*, *BnaC07g32600D*, *BnaA03g41550D*, and *BnaC03g52080D* with >11 log2 fold change in expression were among the top upregulated TFs. One transcription factor *BnaA04g09700D* showing association with the HSF-binding motif had no significant similarity with any Arabidopsis transcription factor. Interestingly no downregulation of any TF in heat-stressed pollen was observed.

In heat-stressed pistil 284 (up 144, down: 140) significantly enriched TFs were identified. The enriched temperature-responsive TFs in heat-stressed pistil belonged to diverse TF families such as bHLH, bZIP, HSF, DREB2A, AP2/ERF, MYB, NAC, WRKY, C2H2-zinc-finger, HD-zip (homeodomain-leucine zipper), FAR1-like (far-red impaired response-like) and transcription factors involved in brassinosteroids signaling, among others (**Supplementary Table S8**). Majority of the genes belonged to the MYB, bHLH, bZIP and HSF gene family. MYB (50 TFs) and MYB-related (21 TFs) TF members were significantly overrepresented in the heat-stressed pistil. Genes encoding MYB and MYB-related TFs were reported to be strongly induced by heat stress in maize pollen (Begcy et al., 2019). While the functions of MYBs in heat stress response in reproductive tissues are not clear, it has been reported that over-expression of wheat MYB transcription factor gene *TaMYB80* in Arabidopsis led to enhanced heat and drought stress tolerance (Zhao et al., 2017).

As discussed earlier, our results show that the *B. napus* HSFA2 (*BnaC03g26940D, BnaA03g22890D*), HSFA7 (HSF7a: *BnaA03g41550D, BnaC07g32600D, BnaA03g41540D*, HSF7b: *BnaA09g40360D*) and HSFB2a (*BnaC03g52080D*) were the key HSFs upregulated in both pollen and pistil with >100-fold change in expression levels. HSFA2 is reported to regulate thermotolerance during male reproductive development in tomato (Giorno et al., 2010; Fragkostefanakis et al., 2016). The heat stress exposure during tomato pollen development leads to enhanced *HsfA7* expression only in the tetrad stage,

while enhanced expression of *HsfA2* was noticeable in all developmental stages (Keller et al., 2018). Class B HSFs acts as repressors of the expression of other HSFs. HSFB2a has been suggested to play a role in female gametophyte development (Wunderlich et al., 2014). In the present study, heat-stressed pollen, and pistil, bHLH TF family member, PIF6 (*BnaA09g39670D, BnaC08g32020D*) homologs showed >64-folds upregulation. In heat-stressed pollen, only *BnaC08g32020D* was upregulated, whereas in pistil both copies of PIF6 were upregulated. While PIF6 has been reported to act as a positive regulator of photomorphogenesis in plants and unlike other PIFs and it also plays a role in controlling seed dormancy (Pham et al., 2018), its role in thermo-sensing is not clear and remains an interesting area for future investigations.

In our data, heat-stressed pistil, down-regulated TFs, homologs of MYB75 (*BnaCnng28030D*), NYF subunit A3 (*BnaC04g27090D*), C2H2 type Zn finger protein (*BnaCnng02770D*), bHLH51 (*BnaA04g22490D*), and HSFC1 (*BnaC07g07130D*) were the top candidates with >40-fold decrease (**Supplementary Table S8**). In *Arabidopsis*, downregulation of gene expression of MYB75 (Georgii et al., 2017) and HSFC1 (Swindell et al., 2007) in response to heat stress has been reported. However, the causative relationship between the downregulation of these genes with heat stress response has not been established. Further, down-regulation of TFs in pistil as seen in our analysis, suggests that the heat stress-induced repression of transcription might be an adaptive mechanism that is markedly different between pollen and pistil. The significant transcriptional changes in gene expression in pollen and pistil in response to heat stress are largely due to the differential regulation of TF expression. Our study highlights the need for further investigations to understand the role of the network involving different HSFs along with other significant TFs in regulating the plant's reproductive heat stress response in an organ/stage-specific manner.

CONCLUSION

In this study, the effects of short-term heat stress events during flowering on the success of reproduction in *B. napus* have been described. We have shown that exposure to heat stress impairs the function of both pollen and pistil, leading to loss of fertility and seed set. The genes with possible involvement in negative regulation of pollen fitness and pollen-pistil interactions have also been identified. Based on the heat-responsive DEGs in pollen and pistil associated with heat-sensing, signaling cascades, cellular and metabolic processes; we propose a molecular mechanism through which heat stress possibly alters pollen-pistil interactions in *B. napus* (**Figure 12**). Our data reveal that detailed exploration of the molecular basis of these pathways is warranted. Dynamic transcriptomic analysis of non-stressed and heat-stressed pollen and pistil provided the basis of highlighting the distinctive mechanisms involved in heat stress response in these reproductive tissues. Further investigations on the DEGs identified in this study could be directed toward improving the performance and thermotolerance of *B. napus* varieties. Overall, data reported in our study provides a framework for further studies to explore key components involved in early heat sensing and response mechanisms during the reproductive phase of the Brassica crop.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. The RNA-Seq data generated for the analysis are deposited at the NCBI Sequence Read Archive (BioProject ID: PRJNA666230, BioSample IDs: SAMN16286967 and SAMN16286968). The output files generated by Kallisto analysis, containing the information of abundance estimates (counts and TPM), and transcript length information length for all samples analyzed in this study are provided in **Supplementary Tables S9a–d**.

AUTHOR CONTRIBUTIONS

NL conducted the experiments, analyzed the sequencing data, and prepared a draft of the manuscript. PB and MS conceived the research, supervised, and extensively edited the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.622748/full#supplementary-material

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Heat-Induced Oxidation of the Nuclei and Cytosol

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The concept that heat stress (HS) causes a large accumulation of reactive oxygen species (ROS) is widely accepted. However, the intracellular compartmentation of ROS accumulation has been poorly characterized. We therefore used redox-sensitive green fluorescent protein (roGFP2) to provide compartment-specific information on heat-induced redox changes of the nuclei and cytosol of Arabidopsis leaf epidermal and stomatal guard cells. We show that HS causes a large increase in the degree of oxidation of both compartments, causing large shifts in the glutathione redox potentials of the cells. Heat-induced increases in the levels of the marker transcripts, heat shock protein (*HSP*)*101*, and ascorbate peroxidase (*APX*)*2* were maximal after 15 min of the onset of the heat treatment. RNAseq analysis of the transcript profiles of the control and heat-treated seedlings revealed large changes in transcripts encoding HSPs, mitochondrial proteins, transcription factors, and other nuclear localized components. We conclude that HS causes extensive oxidation of the nucleus as well as the cytosol. We propose that the heat-induced changes in the nuclear redox state are central to both genetic and epigenetic control of plant responses to HS.

Keywords: epigenetics, heat shock proteins, reactive oxygen species, redox-sensitive green fluorescent protein, oxidation

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INTRODUCTION

Heat stress (HS) is a major threat to current and future agriculture. Future increases in atmospheric CO_2 levels and associated global warming are predicted to amplify the impact of HS on crops such as wheat, rice, and maize that form the basis for human nutrition. Climate change models predict that mean ambient temperatures will increase between 1.8 and 5.8°C by the end of this century as well as the frequency of heat waves. Ensuing the sustainability yield of all major crops in the global warming era is a major challenge faced by plant scientists (Easterling and Apps, 2005). Plant responses to HS are very well characterized. HS has drastic impact on seed germination, radicle emergence, plumule growth, and seedling growth (Toh et al., 2008; Piramila et al., 2012). Reproductive organs, especially the male reproductive tissues (Giorno et al., 2013) are highly sensitive to HS-induced loss of function, leading to a significant reduction in yield (Warland et al., 2006; Ahamed et al., 2010). Exposure to HS causes flower abortion, spikelet sterility, and decreased pollen germination (Sato et al., 2006). Photosynthesis is also sensitive to heat-induced inhibition (Berry and Bjorkman, 1980; Wise et al., 2004; Rossi et al., 2017; Wang et al., 2018). In particular, the activation of ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) by Rubisco activase is heat sensitive (Portis, 2003; Sage et al., 2008; Perdomo et al., 2017), as are starch and sucrose synthesis (Sumesh et al., 2008). Thylakoid membrane stabilization is also

an important response to HS (Zhang and Sharkey, 2009; Niinemets, 2018), as is the induction of molecular chaperones, the accumulation of solutes, and changes in gene expression through mitogen-activated kinase and calcium-dependent protein kinase cascades (Wahid et al., 2007; Dietz et al., 2016).

The accumulation of reactive oxygen species (ROS) coupled to the induction of heat shock proteins (HSPs) are characteristic features of the heat shock response (Suzuki and Katano, 2018). The accumulation of ROS is a key trigger for plant stress responses that can lead either to acclimation or programmed cell death depending on the signaling pathways involved (Baxter et al., 2014; Noctor et al., 2018). The HS-induced accumulation of H_2O_2 is a key trigger in regulation of the expression of HSPs. The expression of HSPs is governed by heat shock factors (Hsfs), which are transcription factors that bind to the heat shock promoter elements (HSEs) in the *HSP* genes (Liu and Charng, 2013). Arabidopsis has 21 Hsfs of which the heat-inducible class A Hsfs, *HsfA2*, *HsfA7a*, and *HsfA3*, and class B Hsfs, *HsfB1*, *HsfB2a*, and *HsfB2b*, play an essential role in acquired thermotolerance (Meiri and Breiman, 2009). ROS accumulation induces the expression of Hsfs and HSPs accumulation (Zhang et al., 2009; Banti et al., 2010). Hsfs such as HsfA1b and HsfA2 are involved in the regulated expression of the marker gene, *ascorbate peroxide 2* (*APX2*), which is a marker for the HS response (Schramm et al., 2006; Ogawa et al., 2007). Thermotolerance is associated with changes in phytohormones such as salicylic acid (SA) that amplify H_2O_2 production and the expression of *HSP* genes.

In natural environments, the exposure to HS often occurs in combination with other stresses such as high light and drought (Choudhury et al., 2017). However, the combination of HS and high light leads to an increase in jasmonic acid (JA) levels and the subsequent activation of transcriptional responses that are distinct from the responses to the single stress treatments (Balfagón et al., 2019). Moreover, *APX2* expression was only shown to be regulated by high light if there was a simultaneous increase in temperature (Huang et al., 2019).

Although there is overwhelming evidence in support of HS-induced ROS accumulation, little information is available concerning the status of these compounds in the cellular compartments where ROS are produced. The ROS-induced oxidation of the cellular antioxidant reduced glutathione (GSH) to glutathione disulfide (GSSG) causes a shift of the glutathione redox potential (EGSH) toward less negative values, a process that is considered to be hallmark of exposure to stress (Noctor et al., 2011). The application of *in vivo* imaging techniques such as redox-sensitive green fluorescent proteins (roGFPs) provides compartment-specific information on the degree of oxidation and the glutathione redox potential (Meyer et al., 2007; Schwarzländer et al., 2008; García-Quirós et al., 2020). Genetically-encoded roGFP2-based sensors are robust and highly reliable probes for monitoring cellular oxidation. The fusion of human glutaredoxin-1 to roGFP2 (Grx1-roGFP2) increases the speed of roGFP2 oxidation by GSSG (for EGSH), while fusing the yeast glutathione peroxidase, Orp1, to roGFP2 enhances the specificity to H_2O_2. The H_2O_2-specific roGFP2-Orp1 probe has been used to analyze H_2O_2 dynamics in the intracellular compartments of Arabidopsis cells (Nietzel et al., 2019; Ugalde et al., 2020). Moreover, studies using Grx1-roGFP2 and roGFP2-Orp1 have demonstrated that exposure to the oxidizing herbicide methyl viologen causes rapid oxidation of the chloroplasts, cytosol, and mitochondria (Ugalde et al., 2020). We have previously used the roGFP2 probe to analyze changes in EGSH in the leaves (Schnaubelt et al., 2015) and flowers (García-Quirós et al., 2020) of mutants that are deficient in glutathione, as well as redox changes occurring in the cell cycle in the primary roots of wild-type (WT) Arabidopsis and mutants that are deficient in the antioxidant, ascorbic acid, and the effects of chloroplast inhibitors on EGSH in the nuclei and cytosol of leaf epidermal cells and stomatal guard cells (Karpinska et al., 2017a). We therefore extended this analysis to explore the effects of HS on the redox states of the cytosol and nuclei of *Arabidopsis thaliana* (Arabidopsis) seedlings in order to provide to further explore the effects of HS on the redox state of different cellular compartments. We provide evidence of extensive heat-induced oxidation of the nuclei as well as the cytosol and discuss how the regulated oxidation of both cellular compartments can influence the cell signaling in both cellular compartments to achieve thermotolerance and survival of HS.

MATERIALS AND METHODS

Plant Material

Seeds of WT *A. thaliana* accession Columbia-0 (Col-0), Col-0 expressing roGFP2 (de Simone et al., 2017), were surface sterilized (70% ethanol, 2% {v/v} bleach, and 0.1% Tween 20) and washed twice with sterile water. The seeds were plated onto half strength Murashige-Skoog (MS) medium containing 1% sucrose and 0.8% agar (pH 5.8). After stratification in a cold room (4°C) for 48 h, the plates were transferred to a growth room maintained at 22 ± 1°C with 16:8 h light and dark regime and a light intensity of 120 μmol $m^{-2}s^{-1}$. Agar-grown seedlings were used in the present study because this method has been used in previous studies on HS (Silva-Correia et al., 2014; Tiwari et al., 2020).

HS Treatments

Heat stress was applied to 5-days-old Arabidopsis seedlings growing on petri plates. The plates were sealed using Petriseal (Diversified Biotech, United States) and exposed to 42°C for 60 min in a water bath. Batches of control seedlings were maintained at 22°C. Each biological replicate consisted of 100 control or heat-stressed seedlings. Three biological replicates were used per timepoint and each experiment was repeated at least three times. Samples were harvested from control and heat-stressed seedlings at the time points shown in the figure legends or immediately after treatment had ended and used in the analysis below.

Confocal Laser Scanning Microscopy and Image Analysis

For quantitative monitoring of EGSH, we used the roGFP2 variant derived from the original EGFP containing the S65T

mutation that has a main excitation peak at 490 nm (Hanson et al., 2004). The roGFP2 form has advantages in less reducing conditions, as occur, for example, in partially glutathione-deficient mutants (Meyer et al., 2007). Control and heat-treated seedlings were placed on a slide in a drop of sterile water. roGFP2 was then interrogated using a Carl Zeiss confocal microscope LSM700 equipped with lasers for excitation at 405 and 488 nm to image oxidized and reduced form of ro-GFP2, respectively. Images were taken with 40x/1.3 Oil DIC M27 lens in a multitrack mode. Ratiometric analyses were performed using ImageJ software[1]. The roGFP2 signal was calibrated at the end of each experiment using standard conditions of incubation with either 2.5 mM dithiothreitol (reduced) or 2 mM H_2O_2 (oxidized). Samples for fully reduced and oxidized controls were treated for 10 min with dithiothreitol or H_2O_2, respectively. The oxidation degree and glutathione redox potential values were calculated as described by Meyer et al. (2007) and de Simone et al. (2017).

Nitro Blue Tetrazolium (NBT) and 3,3′-Diaminobenzidine-HCl (DAB) Staining

Staining with nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) was performed essentially as described by Groten et al. (2005). For NBT staining, whole seedlings were vacuum-infiltrated in 10 mM NaN_3 and stained in 0.5 mg ml^{-1} NBT (Sigma–Aldrich) prepared in 10 mM potassium phosphate buffer (pH 7.8) for 30 min. The reaction was stopped by transferring the seedlings into 90% ethanol at 70°C until all the chlorophyll was removed. The seedlings were photographed under a stereomicroscope (Leica M165-FC; Leica Microsystems, Wetzlar, Germany). DAB (5 mg ml^{-1}) in dimethyl sulfoxide (DMSO), diluted 1: 10 with 10 mM sodium phosphate buffer (pH 7.8), was used to detect H_2O_2, using a similar protocol.

[1]http://rsbweb.nih.gov/ij/

RNA Seq

Control and heat-stressed seedlings were harvested in liquid nitrogen and immediately stored at −70°C before further analysis. Total RNA was isolated from 5-days-old Arabidopsis seedlings using RNA purification kit (Sigma–Aldrich, United States) according to the manufacturer's instructions. RNA quality and quantity was examined by Nanodrop and on the basis of RIN value (RNA integrated number). 1 μg of total RNA was used for the construction of RNAseq libraries. Single-end RNAseq libraries were constructed independently using the RNA Sample Preparation Kit (Illumina, Foster City, CA, United States) according to the manufacturer's instructions. Sequencing was performed on the Illumina GAII platform. Unpaired 100 bp Illumina reads were aligned using TopHat (v2.0.10) against a Bowtie2 (v2.2.8) index built from the TAIR10 reference sequence to create sequence alignment/map (SAM) files. SAM files were then sorted and converted into binary alignment/map (BAM) alignment files using Samtools (v1.3). The aligned read replicates were counted using feature Counts to create gene-count matrix and tested for differential gene expression using EdgeR in R/Bioconductor. Differentially expressed genes were defined as those showing fold changes of greater than 2 and a false discovery rate (FDR) corrected *p*-value of 0.05 or less (**Supplementary Table 1**). The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, accession no: PRJNA669354.

Real-Time PCR

Real-time qPCR was performed as described previously (de Simone et al., 2017). The abundance of transcripts was measured

TABLE 1 | Sequences used for qPCR analysis.

Oligo name	Sequence	Gene ID	Annotation
Act2.F	5′-ACCTTGCTGGACGTGACCTTACTGAT-3′	At3g18780	ACTIN 2
Act2.R	5′-GTTGTCTCGTGGATTCCAGCAGCTT-3′		
Apx1.F	5′-GTCCATTCGGAACAATGAGGTTTGAC-3′	At1g07890	ASCORBATE PEROXIDASE 1, cytosolic
Apx1.R	5′-GTTGTCTCGTGGATTCCAGCAGCTT-3′		
Apx2.F	5′-CCCATCCGACCAAACACATCTCTTA-3′	At3g09640	ASCORBATE PEROXIDASE 2, cytosolic
Apx2.R	5′-CCCATCCGACCAAACACATCTCTTA-3		
Apx3.F	5′-CCCAAAATCACATACGCAGACCTGTA-3	At4g35000	ASCORBATE PEROXIDASE 3, microsomal
Apx3.R	5′-AGTTGTCAAACTTCAGCGGCTCTTG-3′		
Apx4.F	5′-CTACTAAATCCGGGGGGAGCCAATG-3′	At4g09010	ASCORBATE PEROXIDASE 4, microsomal
Apx4.R	5′-CTCTGTTGCATCACTCCTTCCAAAAT-3′		
Apx-s.F	5′-TGCTAATGCTGGTCTTGTGAATGCTT-3′	At4g08390	ASCORBATE PEROXIDASE, stromal
Apx-s.R	5′-CCACTACGTTCTGGCCTAGATCTTCC-3′		
Apx-t.F	5′-CAGAATGGGACTTGATGACAAGGAAA-3′	At1g77490	ASCORBATE PEROXIDASE, thylakoidal
Apx-t.R	5′-ATGCAGCCACATCTTCAGCATACTTC-3′		
Apx6.F	5′-TGCAAAACGAAATAAGGAAAGTGGTG-3′	At4g32320	ASCORBATE PEROXIDASE 6, cytosolic
Apx6.R	5′-CACTCAGGGTTTCTGGAGGTAGCTTG-3′		
HSP101.F	5′-TTATGACCCGGTGTATGGTG-3′	At1g74310	HEAT SHOCK PROTEIN 101, cytosolic and nuclear
HSP101.R	5′-AGCGCCTGCATCTATGTAAA-3′		

in samples harvested during the HS treatment at the time points indicated in the figure legends. Total RNA was extracted as described above. RNA reverse transcription and qPCR were performed on an Eppendorf Realplex2 real-time PCR system by one-step RT-PCR using Quantifast SYBR Green RT-PCR Kit (Qiagen), following manufacturer's instructions. The expression of the genes of interest was initially normalized using four house-keeping transcripts. We have reported the data in terms of one

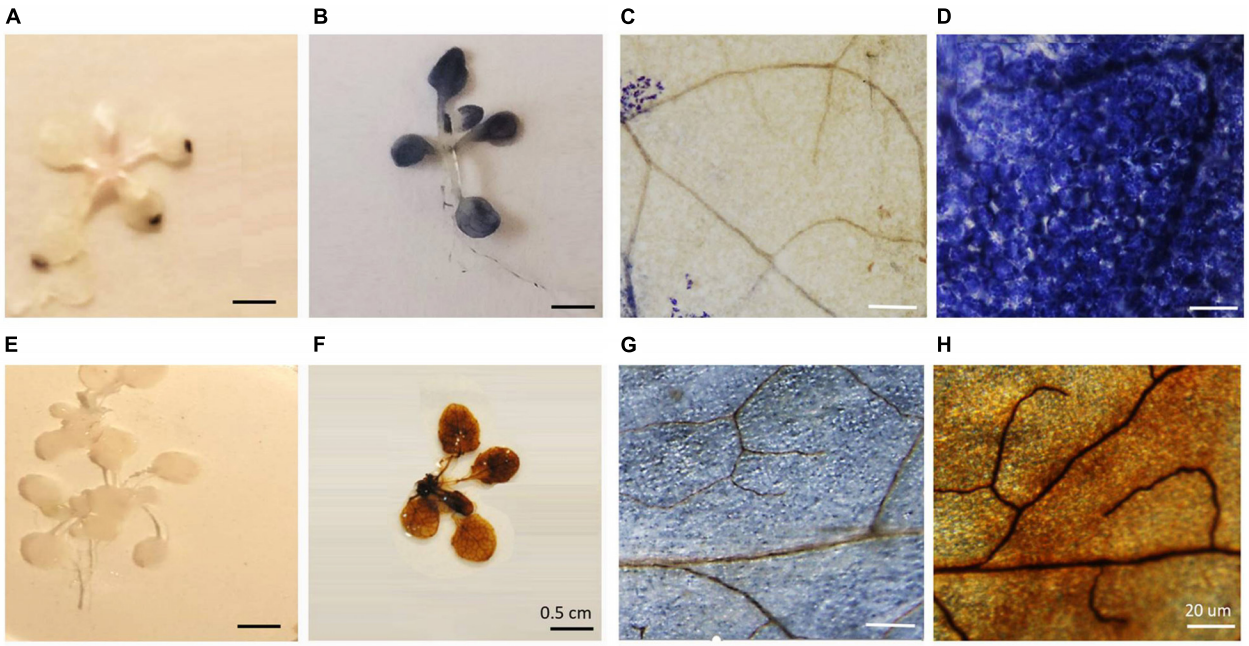

FIGURE 1 | The effects of heat stress on nitrobluetetrazolium **(A–D)** and 3,3′–diaminobenzidine **(E–H)** staining of leaves.

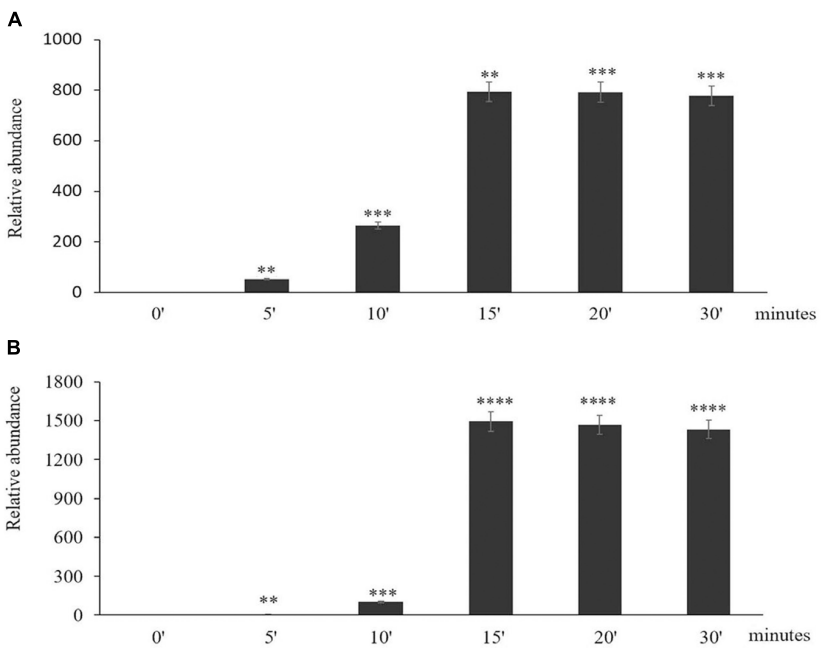

FIGURE 2 | The time course of expression of *Heat Shock Protein* (*HSP*) *101* **(A)** and *Ascorbate Peroxidase* (*APX*)*2* **(B)** in response to heat stress treatment. Asterisks indicate statistical significance ($P \leq 0.05$) between the treatments. Seedlings were exposed to heat stress for 0 (control), 5, 10, 15, 20, and 30 min. Values are means ± SD ($n = 3$ independent biological replicates each consisting of 100 seedlings). Asterisks indicate significant differences between control and heat-stressed plants according to the Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$).

reference gene, actin, which proved to be the most stable in response to the HS treatment. However, the other reference genes gave very similar results. Accessions and primer sequences used are given in **Table 1**.

RESULTS

Seven-days-old agar-grown seedlings were subjected to HS by placing the sealed plates in a water bath at 42°C for 1 h (Silva-Correia et al., 2014; Tiwari et al., 2020). Control seedlings were treated in the same way but the water bath was maintained at 22°C. The survival of the HS treatment seedlings was 98%, a value similar to the control and heat-stressed seedlings maintained at 22°C. Following HS, the seedlings were stained with either NBT or DAB. While both the NBT and DAB staining techniques have limitations, as we have discussed previously, these techniques have been widely used to indicate changes in the level of oxidation experienced by stressed organs and tissues (Noctor et al., 2016). In control seedlings (**Figure 1A**), blue staining was only observed in the tips of the rosette leaves. In contrast, following the HS treatment, the blue staining was observed throughout the rosette leaves (**Figure 1B**). A higher resolution image of the stained leaves revealed that while relatively few cells in the control leaves

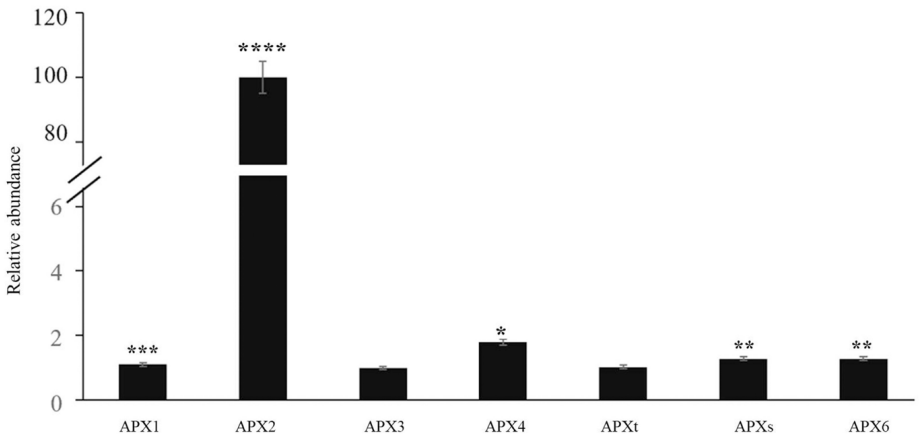

FIGURE 3 | The abundance of transcripts encoding all the Arabidopsis ascorbate peroxidase (APX) family members in control and heat-stressed seedlings. The APX gene family included three *cytosolic* (*APX1*, *APX2*, *APX6*) forms, two chloroplast forms (a stromal form APX_s and a thylakoid form APX_t), and two microsomal (*APX3*, *APX4*) forms. Values are means ± SE (n = 3 independent biological replicates each consisting of 100 seedlings). Asterisks indicate significant differences between control and heat-stressed plants according to the Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$).

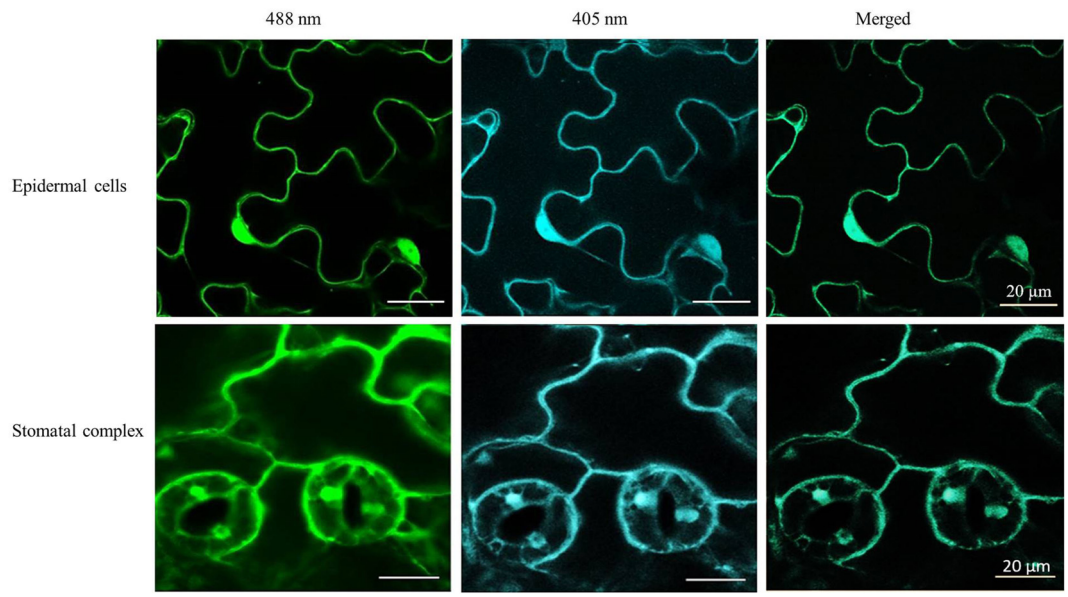

FIGURE 4 | Examples of typical ro-GFP2 fluorescence images of the epidermal cells and stomatal guard cells on leaves exposed to heat stress (42°C) for 1 h. Scale bar is 20 µm.

had blue staining (**Figure 1C**), all of the cells contained blue staining after HS (**Figure 1D**). No DAB staining was observed in the rosettes in the absence of HS (**Figure 1E**), but the leaves of the heat-stressed leaves showed staining (**Figure 1F**). Higher resolution images of the stained leaves showed that while some cells in the control leaves had brown staining (**Figure 1G**) but brown staining was greatly increased around the veins after HS (**Figure 1H**).

The time course of expression of the marker genes *HSP101* (**Figure 2A**) and *APX2* (**Figure 2B**) showed that the levels of transcripts increased rapidly after the onset of the HS treatment and was maximal after 15 min, remaining at a high level

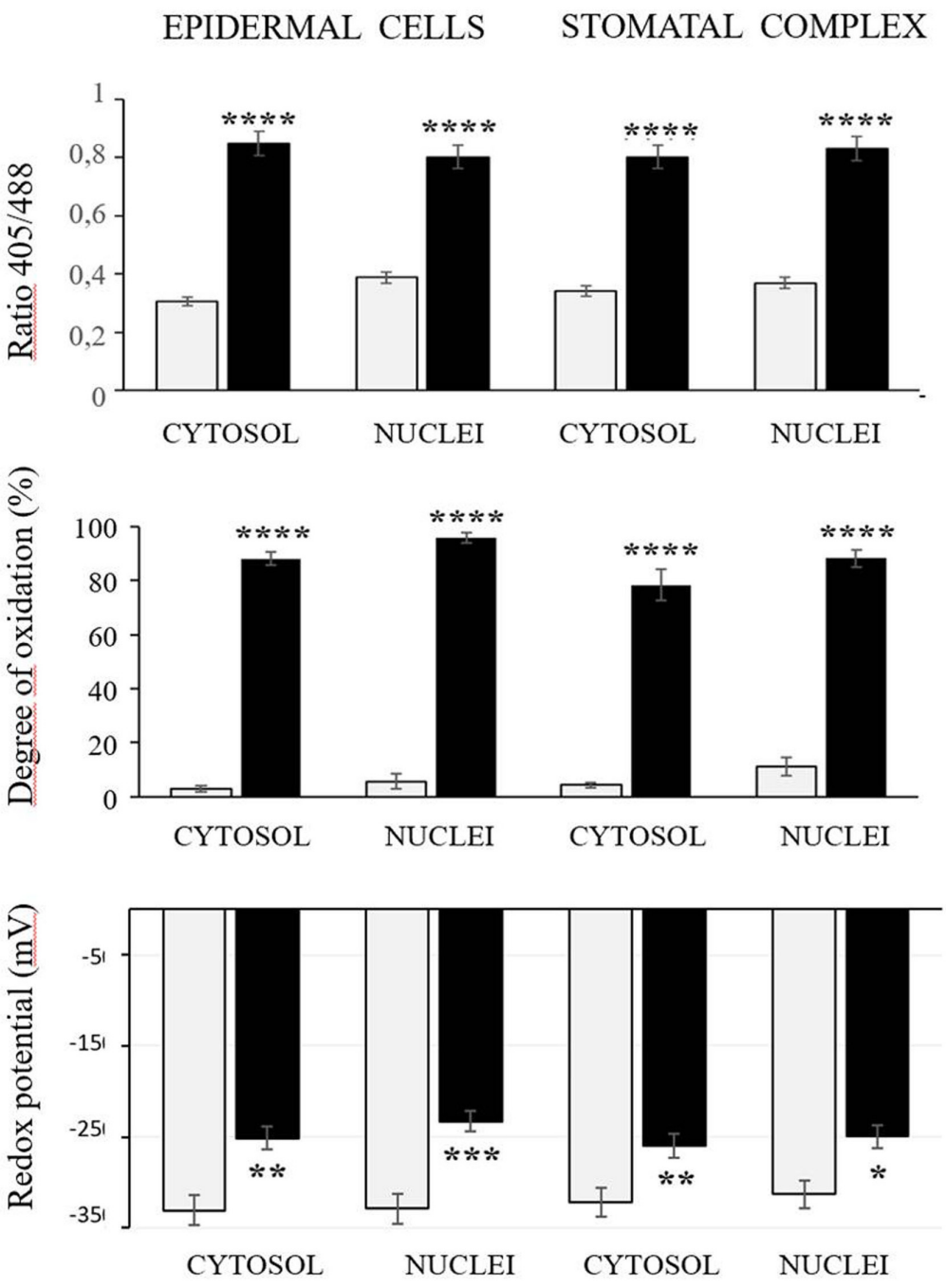

FIGURE 5 | The 405/488 ratios, the degree of oxidation, and the glutathione redox potential in the cytosol and nuclei of the epidermal and stomatal guard cells of the control (white bars) and heat treated (black bars) seedlings. Asterisks indicate significant differences between control and heat-stressed plants according to the Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$).

thereafter. Moreover, an analysis of the abundance of transcripts of seven members of the Arabidopsis APX family revealed that only the levels of the *APX2* transcripts were increased as a result of the HS treatments and the levels of other APX transcripts were nearly similar in control and heat-stressed seedlings (**Figure 3**).

The roGFP fluorescence was readily detected in the cells of the control and heat-treated leaves (**Figure 4**). Large changes in the 405/488 ratios of the cytosol and nuclei of the epidermal and stomatal guard cells were observed after the heat treatment (**Figure 5**). The degree of oxidation was low in the cytosol and nuclei of the epidermal and stomatal guard cells in the absence of HS (**Figure 5**), suggesting that the glutathione pool is highly reduced in these circumstances. However, the degree of oxidation was greatly increased in the nuclei and cytosol of both cell types following the HS treatment (**Figure 5**), demonstrating that HS caused substantial oxidation of the nuclei and cytosol. Similarly, the glutathione redox potentials of the cytosol and nuclei of the epidermal and stomatal guard cells were greatly changed as a result of HS (**Figure 5**).

RNAseq analysis revealed that large numbers of transcripts associated with different mitochondrial functions were increased (**Supplementary Figure 1**). Other transcripts involved in plant defense, secretory pathways, and vacuolar functions were decreased in abundance (**Supplementary Figure 2**) in response to the HS. These trends can be clearly seen from the transcripts that were most abundant after HS (**Figure 6A**) and the transcripts that encode transcription factors and markers for phytohormone-dependent pathways that were significantly decreased in response to the HS (**Figure 6B**). In particular, transcripts encoding markers proteins such as the vegetative storage protein (VSP)1 and VSP2 for JA signaling pathways were decreased in abundance following exposure to HS (Hickman et al., 2017). The list of transcripts that were most highly expressed in response to HS includes those encoding the mitochondrially-localized HSP23.6, HSP70B proteins (**Figure 6A**), and HSP90.1 (**Supplementary Figure 3**). Moreover, transcripts that encode respiratory proteins and related mitochondrial functions were much less abundant

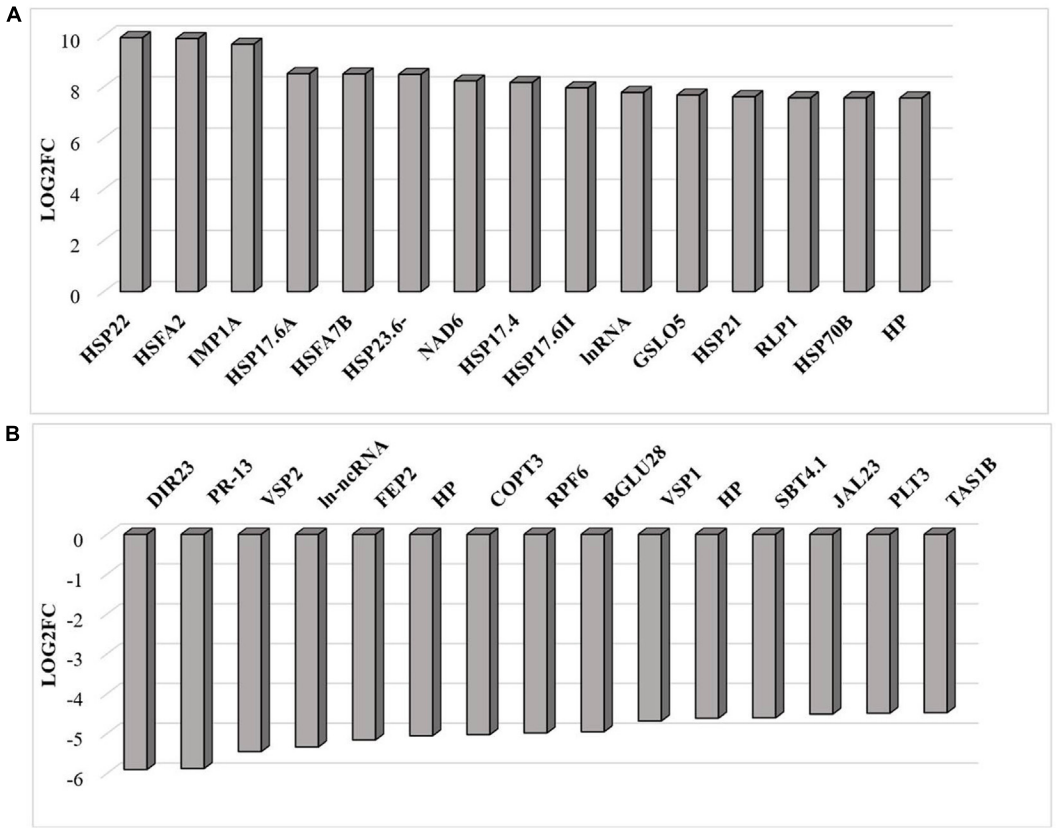

FIGURE 6 | The transcripts that were most differentially changed in abundance after heat stress. The relative expression (log2) fold change-range of transcripts was annotated from their gene accession numbers from the TAIR website. Differentially expressed genes were those showing fold changes of (log2FC > 2 for up-regulated genes and log2FC < −2) for down-regulated genes, an FPKM > 1 and FDR-corrected *p*-value of 0.05 or less. DIR23 (AT2G2100), PR-13 (AT1G72260), VSP2 (AT5G24770), lncRNA (AT3G12502), FEP2 (AT1G47395), HP-hypothetical protein (AT3G06435), COPT3 (AT3G46900), RPF6 (AT1G63130), BGLU28 (AT2G44460), VSP1 (AT5G24780), HP-hypothetical protein (AT2G19970), SBT4.1 (AT5G59120), JAL23 (AT2G39330), PLT3 (AT2G18480), TAS1B (AT1G50055), HSP22 (AT4G10250), HSFA2 (AT2G26150), HSP17.6C (AT1G53540), HSP17.6A (AT5G12030), HSFA7B (AT3G63350), HSP23.6 (AT4G25200), HSP17.6A (AT5G12030), NAD6 (ATMG00270), HSP17.4A (AT3G46230), HSP17.6 (AT5G12020), natRNA (AT3G07365), CALS12 (AT5G03550), HSP21 (AT4G27670), RLP1 (AT1G07390), HSP17.8 (AT1G16030), HP-hypothetical protein (AT2G07779). **(A)** Increased in abundance and **(B)** decreased in abundance.

in seedlings after exposure to HS (**Figure 7**). In addition to HS-induced decreases in transcripts encoding cytochrome C oxidase (COX) components (*COX1*, *COX2*) and a large number of components of the nicotinamide adenine dinucleotide [NAD(P)H] dehydrogenase complexes, transcripts involved in mitochondrial RNA editing and maturation were decreased in response to HS. Given the observed changes in transcripts that will ultimately limit mitochondria electron transport functions, it is surprising that exposure to HS was not found to elicit responses in mitochondrial retrograde responsive genes such as those encoding the mitochondrial alternative oxidases, particularly alternative oxidase 1a (Van Aken and Whelan, 2012).

Similar to transcripts encoding APX2, transcripts encoding the Zinc finger protein ZAT12, which is involved in oxidative stress signaling (Nguyen et al., 2012), were highly expressed in response to HS (**Figure 8**). It has previously been shown that HSFA4A binds to the promoters of transcription factors such as WRKY30 and ZAT12 leading to enhanced tolerance to heat through reduction of oxidative damage (Andrási et al., 2019). Exposure to HS also increased the levels of transcripts encoding glutathione transferase (GST) U3 and LIFEGUARD4 (LF4), which is a BCL2 associated X, apoptosis regulator (BAX)-inhibitor1 family protein (**Figure 8**). Exposure to HS results in the movement of the glycolytic enzyme cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) to the nucleus, where it mediates HS responses through association with NF-YC10 (Kim et al., 2020). Overexpression of GAPC1 enhanced the expression of a HS genes including LF4 (Kim et al., 2020). The nuclear localized calmodulin binding protein Bcl-2 associated athanogene (BAG)6, which is a regulator of programmed cell death, was highly expressed in response the HS (**Figure 8**). BAG6 is a HSP70-binding protein that suppresses the expression of Fes1A and plays a positive role in thermotolerance in Arabidopsis (Fu et al., 2019). However, the levels of *Fes1A* transcripts were found to be more abundant after HS treatment in the current experiments (**Figure 9**). The heat-induced expression of

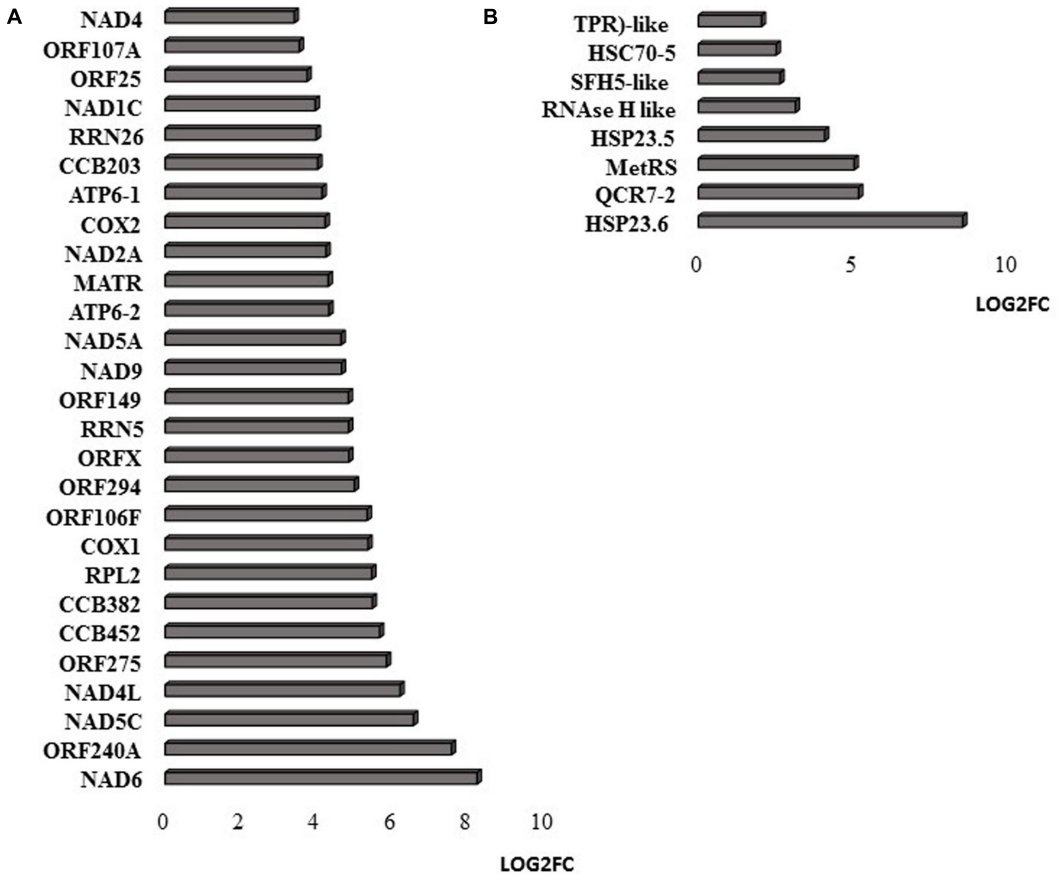

FIGURE 7 | Mitochondrial-encoded transcripts **(A)** and nuclear-encoded transcripts targeted to mitochondria **(B)**. The abundance is expressed as relative expression of (log2) fold change (LOG2FC) with an FPKM > 1 and FDR-corrected *p*-value of 0.05 or less. NAD6 (ATMG00285), ORF240A (ATMG00382), NAD5C (ATMg00070), NAD4L (ATMG00660), ORF275 (ATMG00690), CCB452 (ATMG00270), CCB382 (ATMG00960), COX1 (ATMG01380), ORF106F (ATMG01170), ORF294 (ATMG01330), ORFX (ATMG00570), RRN5 (ATMG01390), ORF149 (ATMG00670), NAD9 (ATMG00090), NAD5A (ATMG00513), ATP6-2 (ATMG01200), MATR (ATMG00640), NAD2A (ATMG00285), COX2 (ATMG00180), ATP6-1 (ATMG00410), CCB203 (ATMG01130), RRN26 (ATMG00665), NAD1C (ATMG00520), ORF25 (ATMG00650), ORF107A (ATMG00030), NAD4 (ATMG00580), COX3 (ATMG00730), NAD7 (ATMG00510), ORF107H (ATMG01360), HSP23.6 (AT4G25200), QCR7-2 (AT5G25450), MetRS (AT5G10695), HSP23.5 (AT5G51440), RNAse H like (AT5G42965), SFH5-like (AT4G27580), HSC70-5 (AT5G09590), TPR-like (AT3G24000).

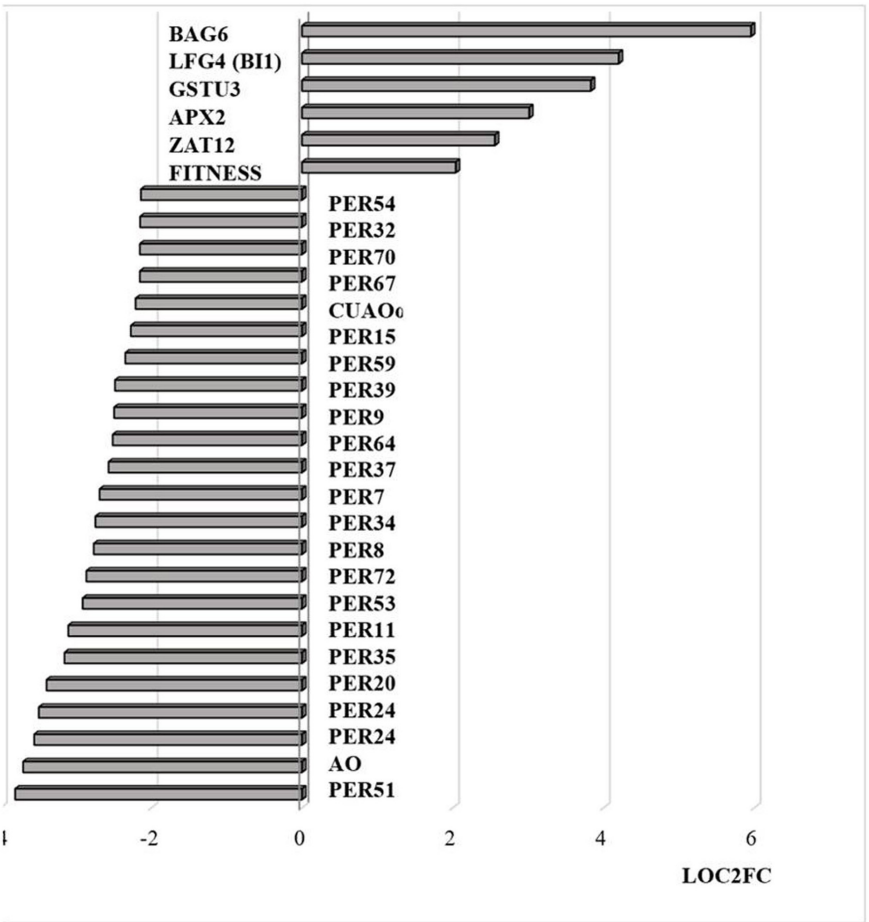

FIGURE 8 | Transcripts encoding proteins associated with cell death and peroxidases that are differentially regulated by heat stress and targeted to the nucleus. The abundance is expressed as relative expression of (log2) fold change (LOG2FC) with an FPKM > 1 and FDR-corrected *p*-value of 0.05 or less. PER54 (AT5G06730), PER32 (AT3G32980), PER70 (AT5G64110), PER67 (AT5G58390), PER15 (AT2G18150), PER59 (AT5G19890), PER39 (AT4G11290), PER64 (AT5G42180), PER37 (AT4G08770), PER34 (AT3G49120), PER8 (AT1G34510), PER72 (AT5G66390), PER53 (AT5G06720), PER11 (AT1G68850), PER35 (AT3G49960), PER20 (AT2G35380), PER24 (AT2G39040), PER24 (AT2G39040), AO (AT5G21100), PER51 (AT4G37530), FITNESS (AT1G07050), ZAT12 (AT5G59820), APX2 (AT3G09640), GSTU3 (AT2G29470), LFG4(BI1) (AT1G03070), BAG6 (AT2G46240), PER7 (AT1G30870), CUAOα2 (AT1G31690), PER9 (AT1G34245).

HSP18.2 and *HSP25.3* in *bag6* mutants was found to correlate with enhanced thermotolerance, suggesting that BAG6 restricts the induction of some sHSPs, limiting the extension of the HS response through regulation of the transcriptional cascade (Echevarría-Zomeño et al., 2016). In contrast to the above genes, transcripts encoding large numbers of apoplastic peroxidases and cell wall associated ascorbate oxidases (AOs) were less abundant after exposure to HS (**Figure 8**).

Transcripts encoding transcription factors and related proteins that are localized in the nucleus were more abundant after HS treatment (**Figure 9**). In particular, transcripts encoding HSFA2, HASFA7, FKBP62 (ROF1, that interacts with HSP90.1 and modulates HsfA2), and the transcriptional co-activator multiprotein bridging factor 1 (MBF)1c were greatly increased in abundance after the HS treatment. These proteins are key regulators of thermotolerance in Arabidopsis. For example, MBF1c is a transcriptional regulator of HS response genes, including the DRE-binding protein 2A (DREB2A), HSFs, and zinc finger proteins. In addition, the levels of transcripts encoding the E3 ligases SNIPER1, which modulates plant immune responses through ubiquitination, and PUB48 that regulates plant abiotic stress responses, were more abundant after HS treatment (**Figure 9**). In addition, exposure to HS resulted in marked changes in the levels of transcripts encoding components that are involved in the epigenetic regulation of gene expression (**Figure 10**).

DISCUSSION

The exposure to heat triggers a general accumulation of ROS in plant organs, together with the activation (or inactivation) of multiple redox-regulated proteins that often contain thiol-disulfide switches. HS-induced ROS production and associated redox signaling are intimately associated with the HS responses that underpin thermotolerance, particularly the expression of

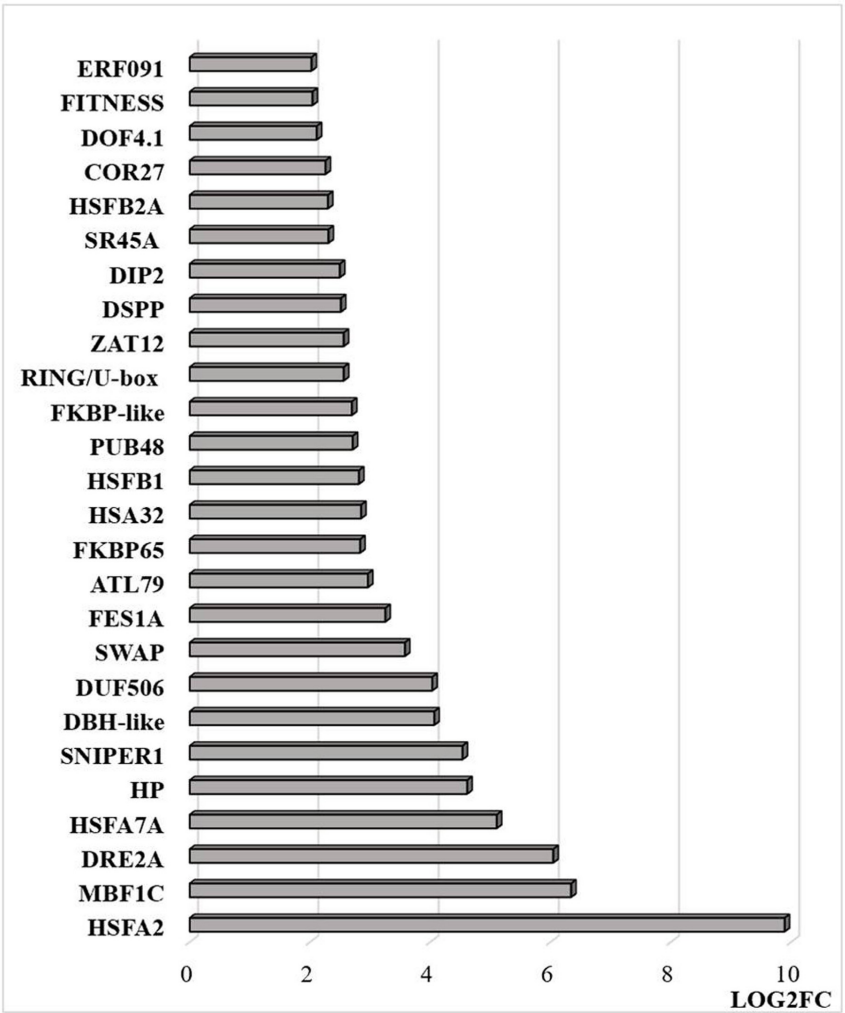

FIGURE 9 | Transcripts that are differentially regulated by heat stress and targeted to the nucleus. The abundance is expressed as relative expression of (log2) fold change (LOG2FC) with an FPKM > 1 and FDR-corrected *p*-value of 0.05 or less. The thresholds were (LOG2FC > 2) and (LOG2FC < −2) for up- and down-regulated genes, respectively. HSFA2 (AT2G26150), MBF1C (AT4G25490), DRE2A (AT5G05410), HSFA7A (AT3G51910), HP—hypothetical protein (At1g21550), SNIPER1 (AT1G14200), DBH-like (AT5G35320), DUF506 (AT3G25240), SWAP (AT3G49130), FES1A (AT3G09350), ATL79 (AT5G47610), FKBP65 (AT5G48570), HSA32 (AT4G21320), HSFB1 (AT4G36990), PUB48(AT5G18340), FKBP-like (AT5G03990), RING/U-box (AT5G05530), ZAT12 (AT5G59820), DSPP (AT1G07330), DIP2 (AT5G03210), SR45A (AT1G07350), HSFB2A (AT5G62020), COR27 (AT5G42900), DOF4.1 (AT4G00940), FITNESS (AT1G07050), ERF091 (AT4G18450).

HSFs and HSPs (Driedonks et al., 2015). Heat directly and indirectly (via ROS) stimulates HSF activity. In turn, HSFs stimulate the expression of HSP chaperones and ROS-processing proteins and antioxidant-related transcription factors such as ZAT10 and HSFA1D, which stimulates the *APX2* expression. The HS-induced changes in the transcriptome profile reported here are similar to previous reports, with the increase in transcripts encoding HSPs and significant downregulation of membrane proteins such as transporters (Poidevin et al., 2020).

While HS is likely to trigger different levels of ROS accumulation in each intracellular and inter(extra)cellular compartment, relatively little information is available on the compartment-specific redox changes that are induced in response to HS. Although the exact mechanisms by which the separate redox pools in different compartments interact and orchestrate cell signaling remain unclear, oxidation in each cellular compartment is likely to transmit specific signals that facilitate an appropriate change in gene expression (Noctor and Foyer, 2016). For example, H_2O_2 produced in the apoplast/cell wall can be directly sensed by membrane receptor kinases such as HPCA1 (also called CANNOT RESPOND TO DMBQ1; CARD1), which triggers an influx of calcium ions into the cell, leading to the activation of MAP kinases and other signaling pathways (Wu et al., 2020). The data presented here show that the levels of transcripts encoding apoplastic H_2O_2-producing enzymes such as the Respiratory Burst Homologs (RBOH) and cell wall peroxidases are decreased in response to heat, transcripts encoding AO. The heat-induced activation of the

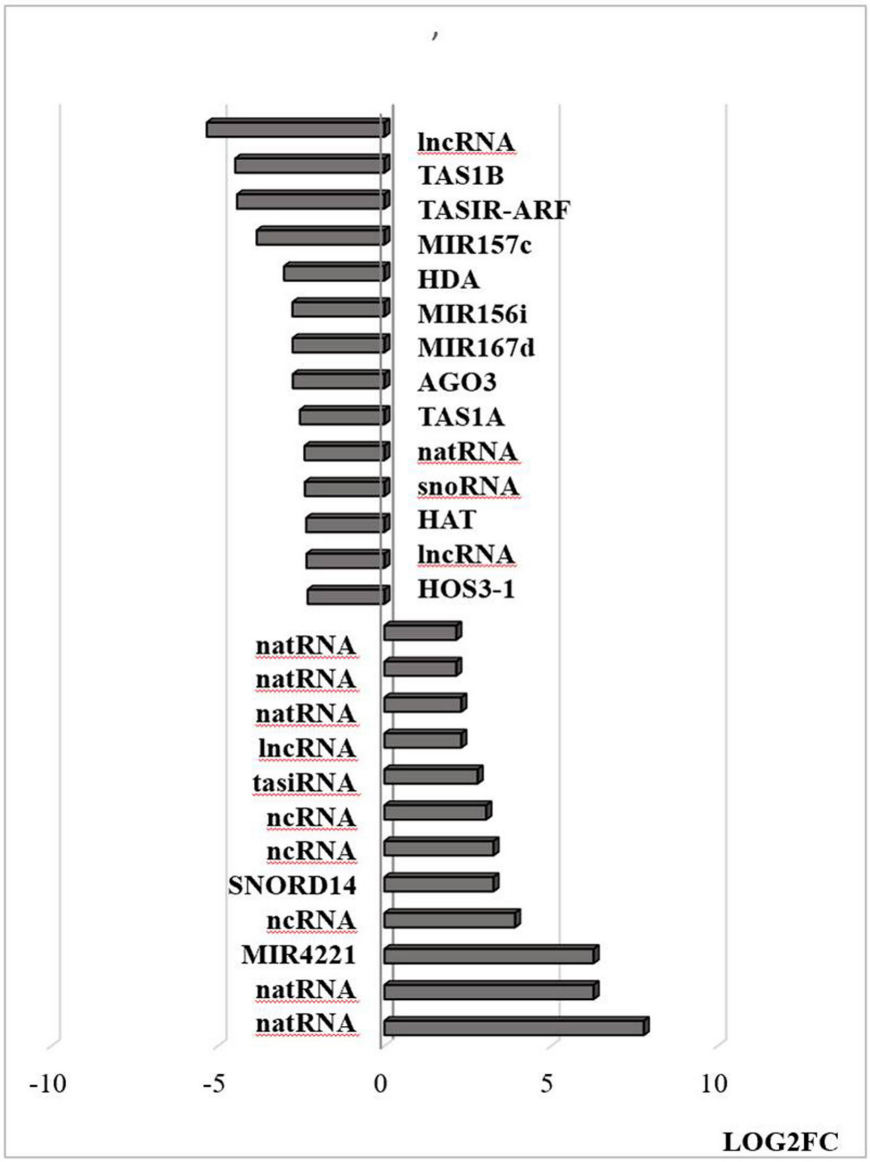

FIGURE 10 | Transcripts associated with small RNAs that are differentially regulated by heat stress. Examples of this class include Argonaute complexes (AGO) and histone deacetylase (HDAC). The abundance is expressed as (log2) fold change (LOG2FC). natural antisense RNAs (AT3G07365, AT3G04685, AT3G04685, AT1G04767, AT1G04767, AT5G03185); trans-acting siRNAs [AT1G63150, AT1G31130 (TAS1A), AT5G57735 (TASIR-ARF), AT1G50055 (TAS1B); AT1G75166 (snoRNA)], non-coding RNA (AT3G03215, AT3G41761, AT2G05205, AT4G06265, AT3G05655, AT5G03185, AT3G12502); microRNAs [AT4G06260 (MIR4221), AT1G31173 (MIR167D), AT1G07867 (MIR167D), AT1G31173 (MIR156i), AT3G18217 (MIR157C)] and proteins; AT1G31290 (AGO3), AT1G24145 (HDAC).

respiratory burst oxidase homolog 1 (RBOH1) was reported to be important in the apoplastic oxidative burst that triggers MAP kinase signaling cascades leading to thermotolerance (Zhou et al., 2014). However, the observed changes in transcripts encoding cell wall peroxidases and AO reported here would favor decreased apoplastic H_2O_2 production and a more reduced state of the apoplast, as illustrated in **Figure 11**. The decrease in AO transcripts may lead to a higher level of ascorbate in the apoplast and ultimately influence the overall process of acclimation to HS, in a similar manner to the role of AO in acclimation to high light (Karpinska et al., 2017a).

Like mitochondria, chloroplasts play an important role in heat-induced ROS accumulation and in the resultant expression of nuclear heat-response genes (Hu et al., 2020). The data presented here demonstrate that the cytosol and nuclei become highly oxidized in response to HS-induced ROS production. Relatively, little attention has been paid to how direct oxidation will influence nuclear proteins and their roles in the acclimation process (Martins et al., 2018). The high temperature-induced accumulation of ROS is often discussed in terms of oxidative stress, cell injury, and death (Nishad and Nandi, 2020). However, ROS production is essential for plant growth and

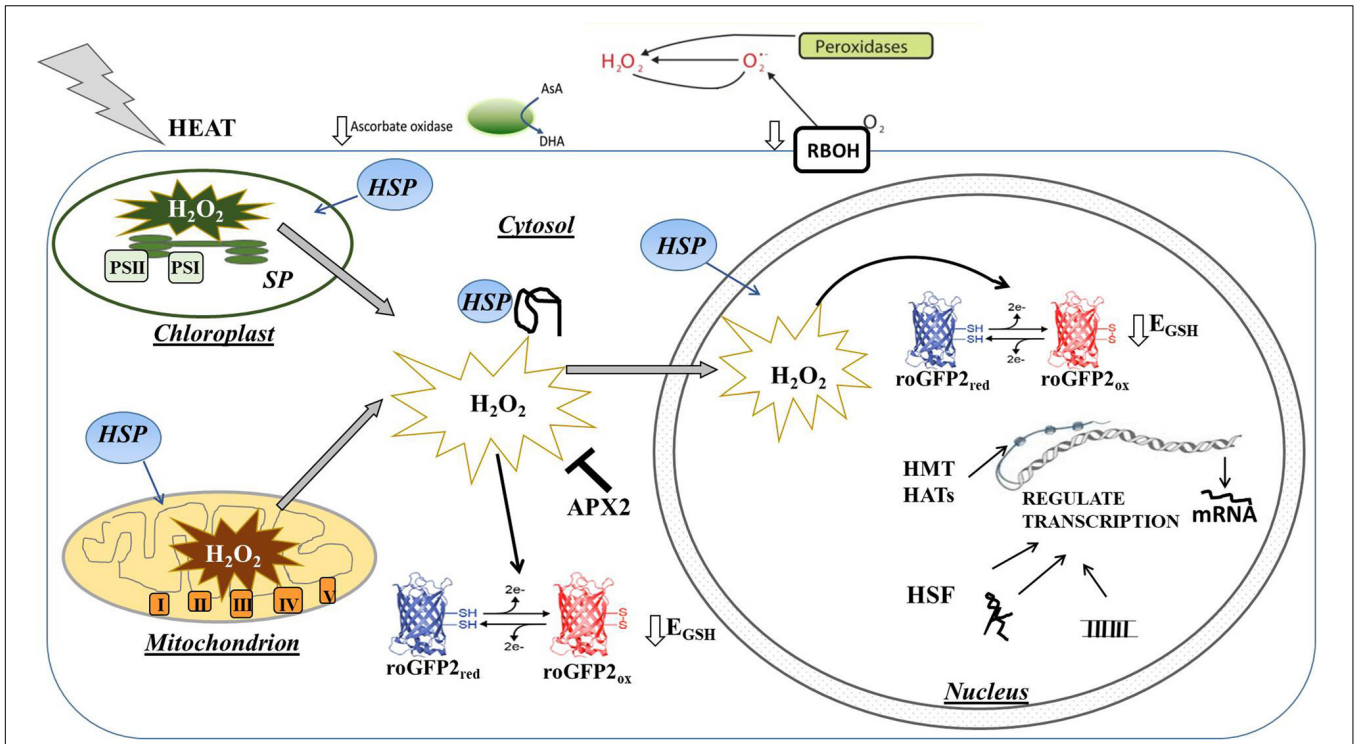

FIGURE 11 | Simplified model illustrating the effect of heat stress on H_2O_2 production and associated oxidation of the cytosol and nucleus on the regulation of gene expression. The levels of transcripts encoding apoplastic H_2O_2 producing enzymes such as the Respiratory Burst Homologs (RBOH) and cell wall peroxidases were decreased in response to heat, as were transcripts encoding ascorbate oxidase (AO), which catalyzes the first step of the ascorbate degradation pathway. Heat increases the production of H_2O_2 in chloroplasts and mitchondria leading to oxidation of the cytosol and nucleus. This oxidation causes a highly specific expression of antioxidant genes such as APX2 that are important in the regulation of signal transduction, as well as heat shock factors (HSF) that regulate heat shock-induced signaling process. Transcription is also rapidly regulated by epigenetic factors such as histone methyltransferases (HMT) and histone acetyltransferases (HAT) as well as by the production of microRNAs (miRNA) and long non-coding RNA (lncRNA).

development (Considine et al., 2017; Mittler, 2017) and ROS accumulation is required for the activation of Hsfs and thermotolerance (Hasanuzzaman et al., 2013; Rezaei et al., 2015). The HS transcriptome signature reported here demonstrates that relatively transcripts associated with antioxidant status are increased in abundance, suggesting that there is no generic antioxidant response to counteract the HS-induced oxidation. In contrast, the only antioxidant transcript that was increased is APX2, suggesting a very specific redox-processing response to HS. In addition, transcripts encoding ZAT12 were increased in the seedlings after HS, suggesting altered oxidative stress signaling (Nguyen et al., 2012).

The nucleus also contains glutathione, glutaredoxins, thioredoxins, and thiols reductases to process ROS, as well as proteins with redox-regulated cysteines that regulate nuclear functions, such as gene expression, transcription, epigenetics, and chromatin remodeling (Martins et al., 2018). The oxidation of redox-regulated transcription factors in the nucleus will have a direct influence on gene expression (He et al., 2018). For example, the AP2/ethylene response factor (ERF) transcription factors are subject to redox regulation (Vogel et al., 2014) as are the ROXY proteins that interact with TGA transcription factors (Dietz, 2014; Delorme-Hinoux et al., 2016). The heat-induced oxidation of the nucleus observed in the present study will undoubtedly regulate the functions of these redox-sensitive transcription factors (Rouhier et al., 2015; Waszczak et al., 2015). Oxidation of the cytosol can also trigger the movement of redox-sensitive proteins into the nucleus (Foyer et al., 2020). In particular, the oxidative of the cytosol triggers the movement of GAPC to the nucleus, where it associates with NF-YC10 to mediate HS responses (Kim et al., 2020). Other examples of oxidation-induced protein translocation to the nucleus include HSFA1D and HSFA8 (Giesguth et al., 2015; Dickinson et al., 2018) and the NON-EXPRESSOR OF PR GENES1 (NPR1), which is an important component of the SA-dependent transcriptional response (Kneeshaw et al., 2014).

In addition, the epigenome is re-modeled by heat-induced priming (Liu et al., 2015). Many of the enzymes involved in histone methylation are subject to redox regulation, which affects both positive and negative histone marks (e.g., H3K4me2, H3K4me3, H3K79me3, H3K27me2, and H3K9me2) which control recombination in meiosis and other processes (Niu et al., 2015). Histone acetylation is regulated by redox changes in the nucleus of mammals, to alter chromatin conformation and transcription (Doyle and Fitzpatrick, 2010). While this type of regulation has not yet been described in plants, reactive nitrogen species such as nitric oxide (NO) induce inhibition of histone deacetylases (HDACs) leading to the stress-induced

regulation of gene transcription (Mengel et al., 2017). Histone H3 is glutathionylated in mammals on a conserved and unique Cys residue. The level of H3 glutathionylation increases during cell proliferation and aging, leading to a more open chromatin structure (García-Giménez et al., 2013, 2014, 2017). Moreover, members of the Arabidopsis DICER-LIKE (DCL) and RNASE THREE-LIKE (RTL) endonucleases families are glutathionylated on a conserved Cys, a process that changes their RNase III activities (Charbonnel et al., 2017). Therefore, the synthesis of small RNA and their subsequent regulation of gene expression are under the control of the cellular redox environment (Charbonnel et al., 2017).

DNA methylation is a major mechanism of epigenetic regulation of gene expression in plants. Redox regulation of the enzymes of the S-adenosyl methionine (SAM) cycle, which provide precursors for DNA and histone methylation, may be important in the regulation of these processes. Other likely targets for redox regulation are the DNA demethylases called Repressor of Silencing 1 (ROS1) and the Demeter-like (DME, DML2, and DML3) enzymes, which remove methylated bases from the DNA backbone (Zhu, 2009). Moreover, the cytosolic Fe-S cluster assembly enzymes such as MET18 and AE7 that are involved in DNA methylation might be subject to redox regulation because they affect nuclear DNA demethylases Fe-S cluster metabolism. In addition, miRNA-mediated epigenetic changes constitute an additional regulatory mechanism of activating HS responses. HS-regulated miRNAs and their target genes that are associated with thermotolerance were recently characterized in wheat (Ravichandran et al., 2019). The target genes of miRNA156, miR159, miR166, and miR398 were shown to be conserved between species such as wheat, other cereals, and dicotyledonous plants (Ravichandran et al., 2019). Chloroplast and mitochondria localized pentatricopeptide repeat-containing and mitochondrial transcription termination factor-like proteins were regulated through miRNA-guided cleavage (Ravichandran et al., 2019).

CONCLUSION

The data presented here shed new light on the intracellular compartmentation of heat-induced redox changes with plant cells and highlight the significant oxidation of the nucleus as well as the cytosol following HS, as illustrated in **Figure 11**. Few studies to date have described direct stress-induced oxidation of the nucleus and considered how the resultant oxidation of the nuclear proteins influences the observed genetic and epigenetic changes. Rather than regarding the accumulation of ROS as essentially a harmful consequence of HS, the data presented here suggest that specific redox processing and signaling pathways are triggered, as evidenced by the differential transcriptional responses in the APX and peroxidase genes. The compartment-specific increases in oxidation, together with the relative changes in redox state between the different cellular compartments are crucial to the signaling that underpins the HS response. Moreover, considerations of the oxidative regulation of nuclear proteins may identify important mechanisms of epigenetic regulation of thermotolerance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, accession nos: PRJNA669354 and SRP287927.

AUTHOR CONTRIBUTIONS

RB, BK, AG, and CF planned the experiments. RB and BK undertook the experimental work and data analysis. BK produced the figures. CF co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.617779/full#supplementary-material

Supplementary Figure 1 | Pie diagrams of transcripts showing heat-induced increases in abundance according to GO enrichment. All RNAs were analyzed for their respective Gene Ontology (GO) terms and fold enrichment through the GO consortium and PANTHER classification system, using *Arabidopsis thaliana* as a reference genome. GO enrichment that is over-represented in up-regulated set of transcripts was analyzed using to the PANTHER Classification System which contains up-to-date GO annotation data for Arabidopsis and other plant species (https://arabidopsis.org/tools/go_term_enrichment.jsp). Pie diagrams represent GO-terms for biological processes, molecular functions, and cellular components.

Supplementary Figure 2 | Pie diagrams of transcripts showing heat-induced decreases in abundance according to GO enrichment for biological processes, molecular functions, and cellular components. All RNAs were analyzed for their respective gene ontology (GO) terms and fold enrichment through the GO consortium and PANTHER classification system, using *Arabidopsis thaliana* as a reference genome. GO enrichment that is over-represented in up-regulated set of transcripts was analyzed using to the PANTHER Classification System which contains up-to-date GO annotation data for Arabidopsis and other plant species (https://arabidopsis.org/tools/go_term_enrichment.jsp). Pie diagrams represent GO-terms for biological processes, molecular functions, and cellular components.

Supplementary Figure 3 | Transcripts encoding heat shock proteins and heat shock factors that are differentially changed in abundance in response to heat stress.

Supplementary Table 1 | RNAseq data used for the analysis of heat stress responses.

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REVIEW
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Plant Hormone-Mediated Regulation of Heat Tolerance in Response to Global Climate Change

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Agriculture is largely dependent on climate and is highly vulnerable to climate change. The global mean surface temperatures are increasing due to global climate change. Temperature beyond the physiological optimum for growth induces heat stress in plants causing detrimental and irreversible damage to plant development, growth, as well as productivity. Plants have evolved adaptive mechanisms in response to heat stress. The classical plant hormones, such as auxin, abscisic acid (ABA), brassinosteroids (BRs), cytokinin (CK), salicylic acid (SA), jasmonate (JA), and ethylene (ET), integrate environmental stimuli and endogenous signals to regulate plant defensive response to various abiotic stresses, including heat. Exogenous applications of those hormones prior or parallel to heat stress render plants more thermotolerant. In this review, we summarized the recent progress and current understanding of the roles of those phytohormones in defending plants against heat stress and the underlying signal transduction pathways. We also discussed the implication of the basic knowledge of hormone-regulated plant heat responsive mechanism to develop heat-resilient plants as an effective and efficient way to cope with global warming.

Keywords: heat stress, phytohormone, heat response, heat tolerance, signal transduction

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INTRODUCTION

The world population is growing at an alarming rate and is forecast to reach nearly 10 billion by the middle of this century. Global food security has become a serious concern over recent years. Increasing agricultural crop productivity is a sustainable approach to feeding the future world population.

Plant growth and geographic distribution are severely limited by various abiotic stresses, such as drought, salinity, cold, and heat (Zhu, 2016). In particular, extreme seasonal heat caused by global warming substantially disturbs normal crop growth and yield around the world, which further exacerbates food insecurity and malnutrition. It is estimated that a 1°C increase in seasonal temperature may directly cause 2.5–16% staple crop yield losses in tropical and subtropical regions (Battisti and Naylor, 2009). Heat stress has deleterious influences on plant growth and development.

Biochemical and physiological consequences following heat stress include excess accumulation of reactive oxygen species (ROS) that induces oxidative stress, irreversible denaturation of proteins that leads to protein misfolding and aggregation, and alterations to the lipid membrane that result in injured membrane permeability and raft disruption (Goraya et al., 2017; Lippmann et al., 2019). In addition, the photosynthesis system is highly sensitive to heat stress (Allakhverdiev et al., 2008; Wang et al., 2017a; Hu et al., 2020). High temperature induces a variety of damage to photosynthesis, ranging from moderate stress that principally attenuates photosynthetic rate to permanent impairment that eliminates photosynthetic capacity.

As sessile organisms, plants immediately sense nearby dangers but cannot escape from harmful environments. Plants have evolved an arsenal of adaptive mechanisms to achieve tolerance in order to survive under heat stress. Plants change their metabolism to increase antioxidant capacity to maintain cellular redox balance and homeostasis upon sensing stress (Nadarajah, 2020). The expression and accumulation of heat-shock proteins (HSPs) are enhanced as chaperones to protect proteins against heat-induced irreversible damage (Jacob et al., 2017; Ul Haq et al., 2019). Accordingly, cellular signaling cascades and transcriptional activities are activated to coordinate physiological and biochemical processes by gene expression changes in response to elevated temperature (Qu et al., 2013).

Phytohormones are the endogenous signal molecules that play an important role in almost every aspect of plant development, growth, and defense processes (Verma et al., 2016; Kumar et al., 2019; Emenecker and Strader, 2020; Jang et al., 2020; Küpers et al., 2020). In recent years, studies have found that exogenous application of phytohormones significantly ameliorated heat-induced damage and improved plant heat tolerance, which indicates that phytohormones actively participate in plant response to heat stress. The phytohormone biosynthetic and signaling pathways have been thoroughly elucidated, mainly in the model plant *Arabidopsis thaliana*. Investigation of the underlying molecular processes of plant hormone-mediated heat response may provide opportunities to generate thermotolerant varieties and to grow agriculturally important crop cultivars in response to changing climate (Grover et al., 2013). In this review, we summarize and discuss recent progress on the versatile roles and the molecular mechanisms of phytohormones involved in plant heat tolerance and how agricultural translational research may transfer the emerging knowledge to ensure global food security.

ROLES OF PHYTOHORMONES IN PLANT RESPONSE TO HEAT STRESS

The Stress Hormone Abscisic Acid Improves Plant Tolerance to Heat Stress

Abscisic acid (ABA) is a phytohormone crucial for plant growth and regulates plant stress responses. In general, ABA limits plant growth in order to coordinate plant adaptation to stressful conditions, e.g., salinity, drought, cold, and heat (Suzuki et al., 2016).

Air temperatures exceeding certain threshold levels cause excessive oxidative stress and membrane damage, which collectively reduce plant photosynthetic and transpiration efficiencies (Bita and Gerats, 2013; Hasanuzzaman et al., 2013). Heat shock elicits a rapid and transient increase in endogenous ABA levels (Larkindale et al., 2005). ABA confers heat tolerance by increasing ROS levels to enhance antioxidant capacity. ABA induces the expression of plant NADPH oxidases, known as respiratory burst oxidase homologs (RBOHs), to induce ROS. RBOHs are plasma membrane proteins. By structural and phylogenetic analysis, 10 *RBOH* genes (*AtRBOHA-AtRBOHJ*) were identified in the *Arabidopsis* genome (Suzuki et al., 2011; Kaya et al., 2019). Transcriptional analysis revealed that only the expression of AtRBOHD, the main NADPH oxidase in *Arabidopsis*, was up-regulated in leaves upon heat stress (Suzuki et al., 2011). The At*RBOHD* loss-of-function mutant displayed impaired heat stress tolerance as measured by seed germination and seedling survival capacities (Larkindale et al., 2005; Silva-Correia et al., 2014). Exogenous application of ABA increases hydrogen peroxide (H_2O_2) accumulation. H_2O_2 mediates ABA-induced thermotolerance by elevating ROS scavenging enzymes and antioxidant substances. In the ABA biosynthesis-deficient mutant plants that lack ABA production, heat-inducible H_2O_2 accumulation is abolished. Consequently, the ABA-deficient mutant plants show impaired heat tolerance, which can be reversed by exogenous addition of ABA (Larkindale and Knight, 2002). Similarly, treatment with ABA synthesis inhibitor impairs heat response by reducing ROS levels in plants (Larkindale et al., 2005). Both ABA biosynthetic and signaling pathways are involved in heat stress response. In addition to ABA synthetic mutants, plants with mutation in ABA signaling components fail to establish thermotolerance and display increased sensitivity to heat stress (Larkindale et al., 2005). However, the molecular mechanism by which ABA mediates heat-induced expression of antioxidant related genes to enhance heat tolerance in plants is largely unclear.

Abscisic acid may also serve as a thermo-priming hormone that enables plants to respond more rapidly and efficiently to heat stress. ABA improves drought acclimation in plants. Exogenous application of ABA confers *Arabidopsis* resistance more rapid and effective to drought-triggered dehydration stress by priming a transcriptional memory (Virlouvet et al., 2014). ABA mediates plant tolerance to a variety of abiotic stressors and is also required for priming across different stressors (Sah et al., 2016). A mild and transient drought treatment (drought priming) enhanced heat tolerance in tall fescue (*Festuca arundinacea* Schreb.) and *Arabidopsis* (Zhang et al., 2019). Indeed, both drought priming and pretreatment of ABA could improve heat tolerance in tall fescue. ABA is required for drought priming-induced heat tolerance (Zhang et al., 2019), and the priming effect is compromised in ABA-deficient *Arabidopsis* mutant plants or in ABA-synthesis inhibitor-treated tall fescue plants.

Interestingly, ABA may modulate levels of carbohydrates and energy status through accelerated transport and enhanced metabolism of sucrose to strengthen plant thermal tolerance

(Rezaul et al., 2019; Santiago and Sharkey, 2019). The expression of genes involved in sucrose transport and metabolism, such as sucrose transporters, sucrose synthase, and invertase, is activated by ABA under heat stress. However, sucrose alone may contribute to plant thermal adaption by providing energy and/or acting as a regulatory signal (Wind et al., 2010). Induction of gene expression, protein production, and ROS scavenging during heat responses are high energy cost processes. Therefore, ABA and sucrose show synergistic effects on improving plant heat tolerance.

Heat-shock proteins function as molecular chaperones to defend plants against heat stress by maintaining protein in functional conformations. Upon heat stress, HSPs are rapidly induced through the transcriptional activity of heat stress transcription factors (HSFs). ABA also improves plants' heat tolerance through the regulation of HSFs and HSPs. For instance, exogenous ABA application alleviates heat-induced detrimental effects and enhances heat tolerance of tall fescue (Wang et al., 2017b). ABA treatment increases the expression levels of tall fescue heat stress transcription factor A2c (FaHSFA2c). Notably, the tall fescue ABA-responsive element binding protein 3 (FaAREB3), a master regulator of the ABA-responsive pathway, directly binds to the *cis*-elements in the promoter of the *FaHSFA2c* gene.

Heat stress extensively up-regulates transcript levels of the wheat (*Triticum aestivum* L.) heat shock factor HsfA6f (TaHsfA6f) (Bi et al., 2020). *Arabidopsis* transgenic plants overexpressing the *TaHsfA6f* gene showed increased accumulation of ABA and subsequently improved tolerance to various environmental stresses, including heat. Further transcriptomic analysis revealed that, in addition to a number of heat-protective genes, several ABA biosynthesis and signaling genes are differentially expressed in *TaHsfA6f* transgenic plants when compared with non-transgenic plants. Under heat stressed conditions, ABA activates *TaHsfA6f* expression, and TaHsfA6f in return enhances ABA accumulation, forming a positive feedback circuit to strengthen heat response. Regulatory components of this circuit may serve as valuable targets for molecular breeding and genetic engineering to develop heat-resistant crops for securing future food production (**Figure 1**).

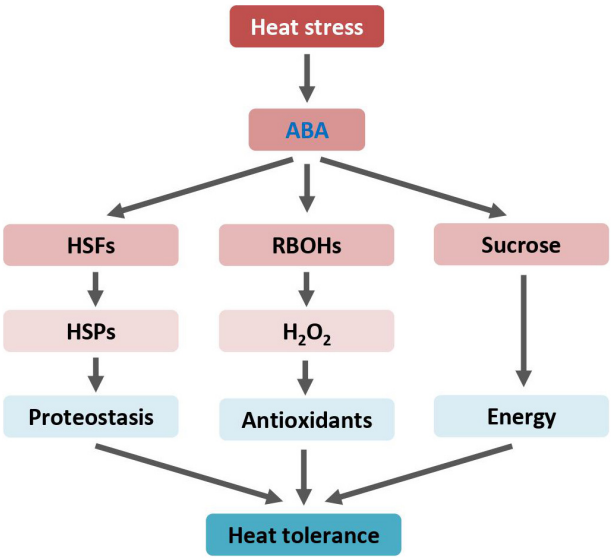

FIGURE 1 | ABA enhances heat tolerance by mediating HSP and RBOH genes expression, and sucrose metabolism. Arrows indicate positive regulation. ABA, abscisic acid; H_2O_2, hydrogen peroxide; HSFs, heat shock transcription factors or heat shock factors; HSPs, heat shock proteins; RBOH, respiratory burst oxidase homologue.

The Growth Hormone Auxin Mediates Heat-Induced Plant Thermomorphogenesis

As an adaptive response to higher ambient temperatures, plants exhibit dramatic morphological and architectural changes termed thermomorphogenesis. The phytohormone auxin plays an important role in heat stress-induced thermomorphogenesis, including stem (hypocotyl) elongation and leaf hyponasty (Küpers et al., 2020). The heat-induced growth response is drastically restrained in auxin signaling mutants or transgenic plants expressing the bacterial *IAA-lysine synthase* (*iaaLys*) gene, which contains a relatively lower level of free IAA (Gray et al., 1998). Correspondingly, auxin concentration is significantly increased in seedlings grown under heat stress. However, exogenous auxin application does not trigger hypocotyl elongation at normal growth temperatures, suggesting that auxin accumulation is required but not sufficient for temperature-induced thermomorphogenesis (Gray et al., 1998).

The PIN-LIKES (PILS) proteins are putative auxin carriers at the endoplasmic reticulum (ER), where they are implicated in intracellular auxin distribution and limit nuclear auxin availability, and consequently confound auxin signaling output (Sauer and Kleine-Vehn, 2019). PILS6 is temperature-sensitive. Heat shock diminishes the PILS6 protein levels, resulting in subcellular auxin re-distribution and increase in auxin signaling response (Feraru et al., 2019). The Auxin Response Factors (ARFs) are involved in auxin-responsive hypocotyl elongation. ARFs activate auxin-responsive gene expression. The ARF-deficient plants displayed a decreased response to high temperatures (Reed et al., 2018).

HSP90 is required for plant thermomorphogenesis (Xu et al., 2012; di Donato and Geisler, 2019). Application of HSP90 inhibitor affects heat-induced hypocotyl elongation. HSP90 is required for the induction of auxin-responsive genes and the depletion of transcriptional repressors Aux/IAAs. In the auxin signaling pathway, Aux/IAAs interact with and restrain the transcriptional activity of ARFs. The HSP90 chaperone system stabilizes the auxin co-receptor F-box protein TIR1 at high temperatures (Wang et al., 2016; Watanabe et al., 2016).

Genetic studies showed that stem elongation and leaf hyponasty responses to heat stress require the activity of the basic helix-loop-helix (bHLH) transcriptional regulators Phytochrome Interacting Factor 4 (PIF4) and PIF7 (Koini et al., 2009; Fiorucci et al., 2020). The *Arabidopsis* PIF family contains eight members, namely, PIF1–8, which can interact with at least one of the

phytochrome photoreceptors (Leivar and Monte, 2014; Pham et al., 2018). High-temperature-mediated thermomorphogenesis was abolished in *PIF4* and *PIF7* loss-of-function mutants. PIF4 and PIF7 activity depend on each other by forming heterodimers, whereas other PIFs play a neglectable, if any, role in *Arabidopsis* heat stress response. Auxin levels did not increase in *pif4* mutant plants at high temperatures (Franklin et al., 2011; Sun et al., 2012). The *pif4* mutants displayed dramatically reduced levels of auxin biosynthesis enzymes, such as members of YUCCA, aminotransferase, and cytochrome P450s, involved in temperature response. The expression of PIFs is also induced when plants are subjected to heat stress. An *in vitro* study showed that PIF4 directly binds to the promoter region of *YUCCA8* gene, a rate-limiting enzyme of auxin synthesis, and activates its expression (Sun et al., 2012). Therefore, PIFs play a major role in auxin-mediated thermomorphogenesis by controlling expression of auxin biosynthesis genes (**Figure 2**). In addition, PIFs also require components of the auxin signaling pathway to regulate high-temperature-induced hypocotyl growth. Interestingly, the chromatin-modifying enzyme Histone Deacetylation 9 (HDA9) is stabilized under high temperatures (van der Woude et al., 2019). HDA9 mediates histone deacetylation at *YUCCA8* nucleosomes to promote H2A.Z depletion and finally facilitates binding of a transcriptional regulator, such as PIF4, to the *YUCCA8* promoter (van der Woude et al., 2019).

Phytochrome interacting factor 4 is also involved in photomorphogenesis (Choi and Oh, 2016). The blue-light receptor cryptochrome 1 (CRY1) suppresses temperature-induced hypocotyl elongation through physical interaction with PIF4 and deterring the transcription activity of PIF4 (Ma et al., 2016). Heat induced auxin-responsive gene expression was abolished in *CRY1* ectopic expression plants. The potential thermosensor for thermomorphogenesis, phyB, phosphorylates PIF4, leading to the PIF4 protein degradation *via* the 26S proteasome pathway (Huq and Quail, 2002). Most recently, the epidermal auxin response was reported to be crucial for hypocotyl growth phenotype (Procko et al., 2016). Indeed, the endogenous PIF4 protein levels were increased particularly in epidermal cells by high temperatures (Kim et al., 2020). Ectopic expression of *PIF4* under the epidermis-specific promoter, but not under the vasculature-specific promoter, can restore the heat-induced hypocotyl growth in the *pifs* null mutants, indicating that epidermal PIF4 is required for thermomorphogenesis (Kim et al., 2020). Both auxin synthesis, perception, and signaling pathway are involved in heat-induced thermomorphogenesis in plants; thus, auxin enables a chance to generate climate-smart plants to ensure crop and food productivity in the context of global climate change.

Brassinosteroid Regulates ROS Homeostasis and HSP Accumulation to Alleviate Heat Stress

Mazorra et al. (2011) examined how endogenous brassinosteroid (BR) content influences heat stress tolerance by assessing the ion leakage, lipid peroxidation, and survival rate after heat shock. The BR-deficient and -overproduction seedlings represented similar thermal tolerance, indicating that thermotolerance is independent of BR homeostasis, but downstream of BR signaling (Mazorra et al., 2011). In the spring barley (*Hordeum vulgare* L.), heat stress enhances the expression of HSPs. Compared to wild-type plants, proteins of the HSP group were less produced in the BR-deficient or BR-signaling mutants under heat stress (Sadura et al., 2020a).

Interestingly, the BR-induced HSP90 protein in turn interacts with two homologous transcription factors, BRI1-EMS-suppressor 1 (BES1) and brassinazole-resistant 1 (BZR1), of the BR signaling pathway (Shigeta et al., 2014, 2015; Samakovli et al., 2020). HSP90 may affect the stability of BES1 protein to facilitate BR-dependent gene expression (Samakovli et al., 2020). BR treatment leads to a significant increase in basic thermotolerance. Translation initiation and elongation factors of the translational machinery are present at significantly higher levels in BR-treated seedlings (Dhaubhadel et al., 2002). *In vivo* protein synthesis assay unraveled that increased accumulation of HSPs in BR-treated plants results from higher protein synthesis (Dhaubhadel et al., 2002). BR was also involved in regulating heat-induced accumulation of membrane proteins, such as proton-pumping ATPase and aquaporins (Sadura et al., 2020b). Heat stress triggers the translocation of *Arabidopsis* transcription

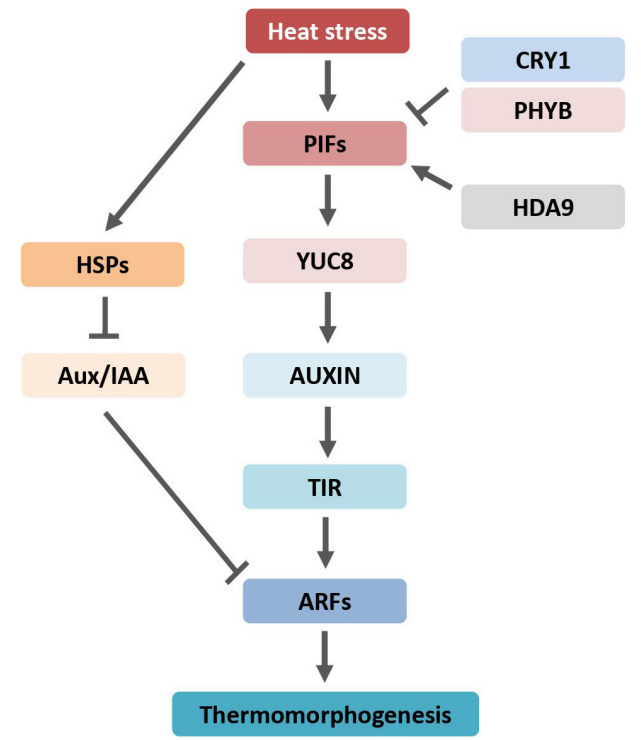

FIGURE 2 | Auxin regulates plant morphogenesis through transcription factor PIFs and is counteracted by phyB and CRY1. Arrows indicate positive regulation, blunt-ended lines indicate negative regulation. ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; HDA9, HISTONE DEACETYLASE 9; HSPs, heat shock proteins; phyB, phytochrome B; PIFs, PHYTOCHROME INTERACTING FACTORs; CRY1, cryptochrome 1; TIR1, TRANSPORT INHIBITOR RESPONSE 1; YUC8, YUCCA8.

factors bZIP17 and bZIP28 from the ER membrane into the nucleus, where they activate ER chaperone and BR signaling gene expression (Che et al., 2010).

Low levels of ROS may serve as second signals and thus play a regulatory role in plant stress response. Expression and activity of antioxidant enzymes are induced by exogenous BR treatment under heat stress (Nie et al., 2013). Like ABA, BR treatment in tomato (*Solanum lycopersicum* L.) leads to increases in *RBOH1* gene expression and H_2O_2 accumulation in leaf apoplast. Virus-induced gene silencing of *RBOH1* resulted in reduced H_2O_2 accumulation and compromised heat stress tolerance. Interestingly, H_2O_2 produced by RBOH1 activates MPK2, which in turn enhances *RBOH1* gene expression (Zhou et al., 2014). Therefore, BR-regulated heat stress tolerance includes a positive feedback loop among RBOH1, H_2O_2, and mitogen-activated protein kinase 2 (MPK2). However, the molecular mechanism by which BR induces *RBOH1* gene expression in not clear.

Brassinazole-resistant 1 is an important transcription factor of the BR signaling pathway (He et al., 2005). Following heat stress, BZR1 accumulates in the nucleus, where it regulates expression of growth-promoting genes (Ibañez et al., 2018). Yin et al. found that BZR1-like protein in tomato regulates heat response by directly controlling the receptor-like kinase FERONIA (FER) homologs (Yin et al., 2018). The promoter region of *FER2* and *FER3* contains several putative BZR1-binding sites. BZR1 binds to the promoters of *FER2* and *FER3* gene and activates their expression. The tomato *BZR1* loss-of-function mutant (*slbzr1*) was generated using CRISPR/Cas9 gene editing technology. Transcriptional analysis showed that *FER2* and *FER3* transcripts were induced by both BR and heat stress in the WT but not in the *slbzr1* mutant. Induction of RBOH1, production of apoplastic H_2O_2, and heat stress tolerance were impaired in the *FER2* and *FER3* gene-silenced plants (Yin et al., 2018). Consequently, BR-induced stress tolerance was diminished in those *FER2* and *FER3* gene-silenced plants.

Under heat stress, BZR1 was recruited to the promoter of *PIF4* gene and activated its expression (Ibañez et al., 2018). Furthermore, BZR1 was found to interact with the heat-activated transcription factor PIF4 in a transient bimolecular fluorescence complementation (BiFC) assay (Oh et al., 2012). Global chromatin immunoprecipitation sequencing (ChIP-Seq) analysis showed that BZR1 and PIF4 bind to common genomic targets. BZR1–PIF4 interaction regulates a core transcriptional network that integrates endogenous hormonal signals and environmental stimuli to modulate plant morphological development (Oh et al., 2012). The BR-receptor protein kinase BRI1 regulates root response to high temperatures (Martins et al., 2017). Elevated ambient temperatures specifically affect BRI1 levels at a post-transcriptional level to downregulate BR signaling and prompt root elongation.

The Systemic Cytokinin Levels Positively Affect Heat Stress Tolerance

Numerous studies provide evidence that temperatures modulate cytokinin (CK) responses and CK levels are involved in plant adaptive mechanisms to temperature stress (O'Brien and Benková, 2013; Pavlů et al., 2018).

Hot ambient temperatures unusually cause pre-anthesis abortion in flower primordia of passion fruit (*Passiflora edulis*) during summers (Sobol et al., 2014). CK application showed an increased resistance in response to hot ambient temperatures. Genotypes isolated with higher CK in leaves can reach anthesis during summer. This result suggests that CK has a protective role for developing flowers exposed to heat stress and may have important implications in future crop breeding and field application to enhance crop production. CK applications can alleviate heat stress injury on creeping bentgrass (*Agrostis stolonifera* L.) (Wang et al., 2012). CK enhances antioxidant metabolism, by inducing activities of antioxidant enzymes superoxide dismutase, ascorbate peroxidase, and guaiacol peroxidase in roots under heat stress.

Heat stress treatment reduces panicle CK abundance and number of spikelets per panicle in rice. The heat stress severely decreases the xylem sap flow rate and CK transportation rate. Number of spikelets and CK content are positively correlated with CK translocation rates through xylem. CK applications alleviate the adverse impact of high temperatures on panicle differentiation and spikelet formation (Wu et al., 2017). Treatment of CK oxidase/dehydrogenase inhibitor showed a positive effect on heat stress tolerance in the model plant *Arabidopsis* (Prerostova et al., 2020). In addition, ectopic expression of the CK biosynthetic gene *isopentenyltransferase* (*ipt*) from the *Agrobacterium tumefaciens* increases CK levels, resulting in plant tolerance to heat stress (Skalák et al., 2016). A quantitative proteomic analysis was carried out to identify protein profiles in leaves and roots of *ipt* transgenic lines in response to heat stress. Expression of *ipt* resulted in protein changes involved in multiple functions, such as energy metabolism, protein compartmentation and storage, and stress defense. The identity of proteins altered in transgenic plants in response to heat stress provides further insights into the biochemical and molecular mechanisms of CK-regulated heat tolerance in plants (Xu et al., 2010).

A dramatic increase in CK levels and a transient decrease in ABA levels, therefore a higher CK/ABA ratio, were observed when shoots or whole plants were targeted to heat stress. The ABA levels in plants subjected to heat stress are under rigorous and dynamic control. Heat stress applied to part of plant elicits a rapid expression of components of CK signaling pathway in the non-exposed tissues. Heat-induced CK activates transcription of genes involved in photosynthesis and carbohydrate metabolism (Dobrá et al., 2015). Recently, an elegant proteomic study of *Arabidopsis* plants in response to high temperatures in the presence and absence of exogenous CK was performed to identify heat stress response proteins regulated by CK. A large proportion of the heat responsive proteome seems to be co-regulated by CK, indicating that heat stress and CK signaling pathway might be interconnected and CK directly involved in heat signaling in plants. Interestingly, the heat and CK response proteomes are preferentially targeted to the chloroplasts, which may play a major role in heat stress response (Cerný et al., 2014). Constitutive expression of a maize small *HSP* (*ZmsHSP*)

in *Arabidopsis* under the control of CaMV 35S promoter causes lower endogenous CK content and higher sensitivity to CK during early developmental stage, indicating that *ZmsHSP* plays a role in CK response in plants (Cao et al., 2010).

Although climate change and global warming pose threats to forests, so far, research on the physiological and biochemical mechanisms that underlie heat stress response in woody trees remains scarce. An integrated physiological and phytohormonal profile of heat-induced thermotolerance in conifer, *Pinus radiata*, revealed that early heat shock and later heat tolerance exhibited differential dynamics patterns. CK plays important roles during long-term temperature acclimation and changes in plant developmental program to recover chloroplast function and photosynthetic ability (Escandón et al., 2016).

SA Reduces Heat-Induced Growth and Physiological Damage

The role of SA in protecting plants against heat-induced damage was repeatedly reported. Exogenous SA treatment on alfalfa (*Medicago sativa* L.) seedlings notably alleviates heat shock-induced adverse effects. SA application prior to heat stress generally improved the plant growth and physiological activities, such as plant height, biomass, and photosynthetic efficiency (Wassie et al., 2020). Accordingly, SA reduces heat stress-induced membrane damage and modulates the activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD). Similarly, exogenous SA enhances tomato heat tolerance through improving photosynthesis efficiency and scavenging of reactive oxygen species by induction of antioxidants (Shah Jahan, 2019). However, SA has little, if any, influence on photosynthesis at normal growth temperatures. SA pretreatment alleviates the decrease of the net photosynthesis rate by protecting photosystem II function and maintaining higher Rubisco activities under heat stress (Wang et al., 2010). In addition, the chloroplast HSP21 proteins showed higher levels in both mock- and SA-treated leaves when stressed with heat shock. During the recovery period, the levels of HSP21 in SA-pretreated leaf samples remain high. SA not only relieves the decrease of photosynthesis rates under heat stress but also facilitates the recover of photosynthesis after stress, mainly due to maintaining higher levels of HSP21 chaperones in chloroplast.

Heat stress treatment induces the production of proline, which was further increased with exogenous SA application (Khan et al., 2013). The production of proline is an adaptive response that plants thrive for survival under adverse conditions (Verbruggen and Hermans, 2008; Szabados and Savouré, 2010). Proline acts as an antioxidant. SA significantly increases the activities of proline biosynthesis enzymes while inhibiting the activities of proline-metabolizing enzymes (Lv et al., 2011; **Figure 3**). Correlation between SA-induced protection of photosynthesis and SA-induced production of proline under heat stress implies that SA application ameliorates heat stress-induced oxidative stress apparently through maintaining a higher proline accumulation. On the contrary, using transgenic *Arabidopsis* plants overproducing proline by ectopically expressing a proline biosynthesis gene, it was found that increased proline production decreases plant thermotolerance under heat stress (Lv et al., 2011). The growth of transgenic *Arabidopsis* was more severely inhibited than that of control plants after heat shock. The inhibitory effect is attributed to the production of proline. The discrepancy between SA-induced endogenous proline accumulation and transgene-mediated proline overproduction on plants' heat response is yet unclear.

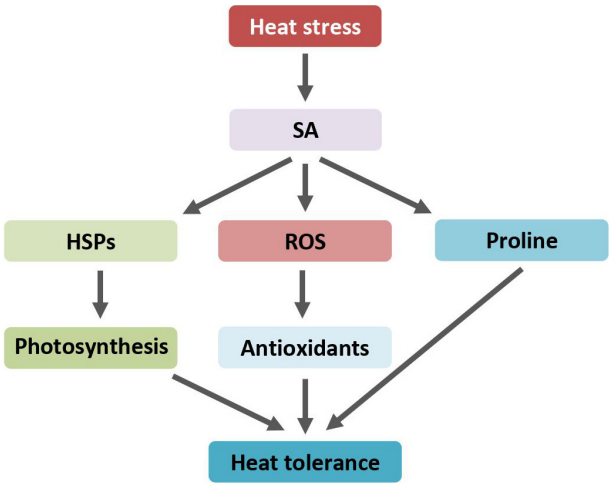

FIGURE 3 | SA regulates heat response through induction of HSPs accumulation, ROS scavengers, and proline biosynthesis. Arrows indicate positive regulation. ROS, reactive oxygen species; SA, salicylic acid; HSPs, heat shock proteins.

Recently, it was reported that simultaneous application of both SA and melatonin mitigated the effects of heat stress by restoring relative water contents and increasing antioxidant enzyme activities in the aromatic herbs such as mint (*Mentha* × *piperita* and *Mentha arvensis* L.) that are cultivated worldwide, mainly in subtropical and tropical regions (Haydari et al., 2019). In the meantime, SA and melatonin treatment improves essential oil yields. The results could be considered for future applications in managing plants that are suffering from temperature extremes in these areas in the wake of global warming.

Diverse Roles for JA and ET in Plant Heat Response

The gaseous hormone ethylene (ET) and the oxylipin-based hormone jasmonate (JA) together play multifaceted roles in plant response to biotic and abiotic stresses (Zhu, 2014). Both ET and JA are necessary for the activation of defense response against necrotrophic pathogens. The ET- and JA-mediated defense signaling pathways act synergistically to induce the expression of pathogen defense gene plant defensin 1.2 (PDF1.2) (Penninckx et al., 1998). Mutation in either ET- or JA-biosynthetic pathway renders plants hypersensitive to necrotrophic pathogens, such as *Botrytis cinerea* (Thomma et al., 1998, 1999). However, the ET and JA pathway may also act antagonistically to regulate plant adaptation to various abiotic stresses (Li et al., 2019).

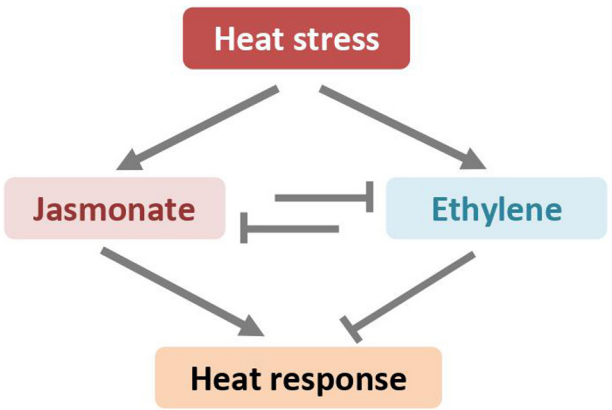

FIGURE 4 | High-temperatures induced jasmonate and ethylene antagonistically regulates plant heat response through unknown mechanisms.

The abovementioned studies in the model plant *A. thaliana* also showed that high temperatures led to accumulation of both JA and ET (Larkindale and Knight, 2002; Larkindale et al., 2005). JA and ET show reverse effects on plant heat response (**Figure 4**). The *constitutive expresser of PR1* (*cpr5-1*) mutant, in which the signaling pathways of SA, JA, and ET are constitutively active, displays enhanced tolerance of heat stress (Clarke et al., 2009). However, the thermotolerance become compromised when *cpr5-1* crossed with mutants deficient in JA biosynthesis pathway (i.e., *jar1-1*) or in JA signaling pathway (i.e., *coi1-1*), demonstrating that at least JA is required for facilitating heat tolerance (Clarke et al., 2009). Indeed, the *coi1-1* mutant plants are thermosensitive and more susceptible to heat stress, as manifested by higher electrolyte leakage and severer chlorosis. Exogenous application of JA to wild-type plants before heat stress reduces heat-induced adverse damage, indicating that JA directly protects plants from heat stress (Clarke et al., 2009). However, the expression of HSPs, the well-established markers for thermotolerance, is neither induced by exogenous JA nor impaired in the JA signaling mutant. Although the role of JA in plant heat tolerance is well documented, the underlying mechanisms are not well understood (Sharma and Laxmi, 2015). Several lines of evidence suggest that JA might regulate plant heat response through a subset of JA-inducible transcription factors of the WRKY superfamily (Li et al., 2010, 2011; Dang et al., 2013).

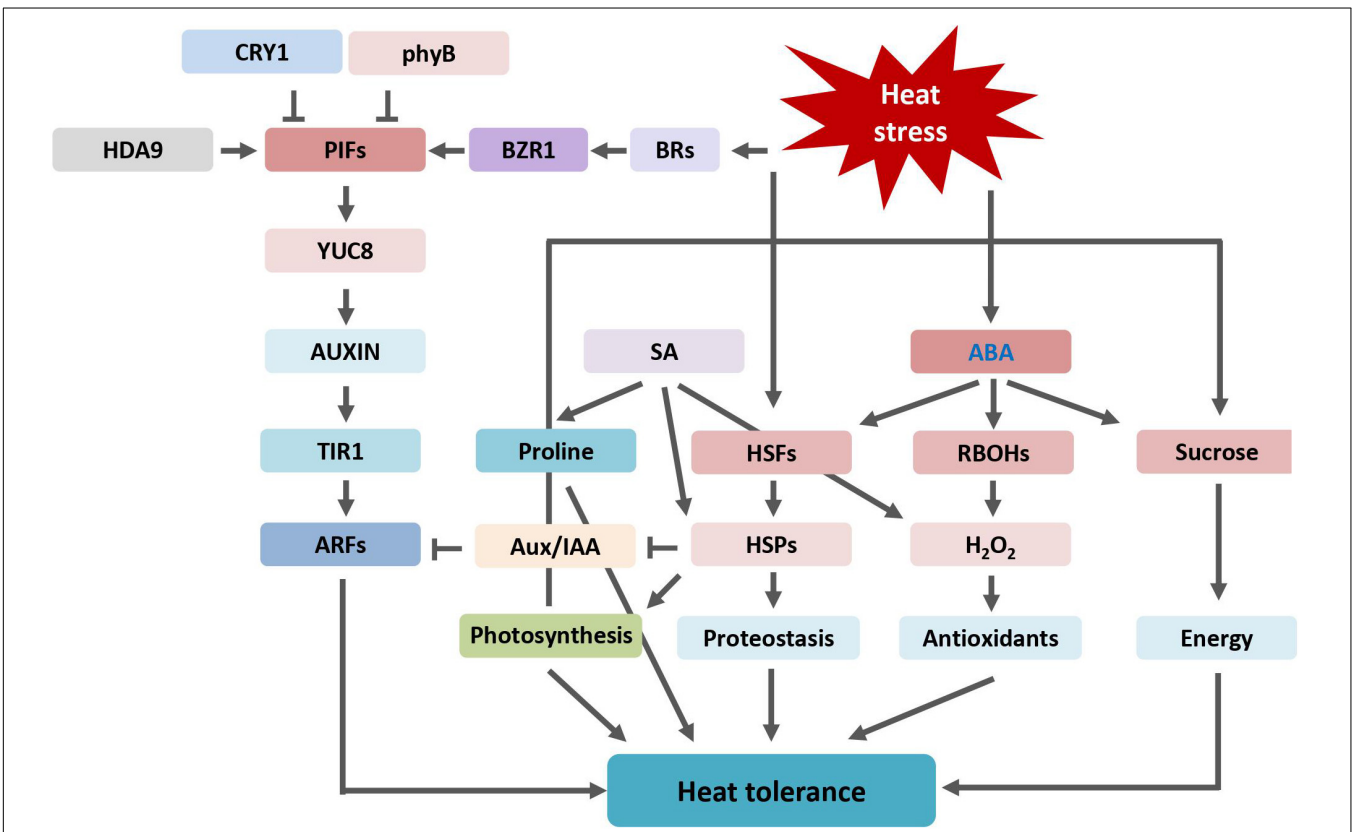

FIGURE 5 | Overview of phytohormones regulated heat stress tolerance described and discussed in this review. ABA, abscisic acid; ARF, AUXIN RESPONSE FACTOR; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; CRY1, BRs, brassinosteroids; BZR1, BRASSINAZOLE-RESISTANT 1; cryptochrome 1; HDA9, HISTONE DEACETYLASE 9; H_2O_2, hydrogen peroxide; HSFs, heat shock transcription factors or heat shock factors; HSPs, heat shock proteins; phyB, phytochrome B; PIFs, PHYTOCHROME INTERACTING FACTORs; RBOHs, respiratory burst oxidase homologues; SA, salicylic acid; TIR1, TRANSPORT INHIBITOR RESPONSE 1; YUC8, YUCCA8.

In addition, JA represses stomatal development and induces stomatal closure, which is not suitable to sustain leaf cooling *via* transpiration at high ambient temperatures. It is also interesting to evaluate how heat stress may influence JA signaling pathway. Notably, JA has been reported to play a vital role in *Arabidopsis* cold response by regulating the C-repeat binding factor (CBF) pathway (Hu et al., 2013, 2017).

In contrast to JA, the ET appears to be a negative regulator on plant heat stress response in the model plant *Arabidopsis*. The *Arabidopsis* ET-insensitive mutant, *ethylene-insensitive 2-1* (*ein2-1*), which lacks a central regulator gene of ethylene signaling pathway, exhibits enhanced tolerance in response to heating (Clarke et al., 2009). Although heat stress elicits ethylene production, the ethylene-initiated and EIN2-mediated signaling pathway might repress plant heat response. Recently, Pan et al. provided evidence that ET biosynthesis and signaling are required for CO_2-induced heat stress response in tomato (Pan et al., 2019). The airborne ET may reduce thermotolerance of holm oak (*Quercus ilex*) plants to heat stress by deterring antioxidant defenses (Munné-Bosch et al., 2004).

High temperatures during the reproductive stage cause severe threats to crop seed production. In pea (*Pisum sativum* L.), ethylene biosynthesis is differentially regulated in floral and fruit tissues upon heat stress in order to optimize resource allocation in reproductive tissues (Savada et al., 2017). In rice (*Oryza sativa* L.), ethylene confers thermotolerance and ameliorates heat-induced adverse effects (Wu and Yang, 2019). Therefore, the physiological, biochemical, and molecular functions of ET in plant in response to heat stress varied in plant species and tissues. A fuller understanding of the role of ET in plant thermotolerance must await further studies.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The implication of phytohormones in plant heat tolerance has been well-documented. An overview of phytohormones and pathway components involved in plant heat stress tolerance is shown in **Figure 5**. High temperatures stimulate the biosynthetic pathways resulting in higher accumulation of those hormones. Auxin and auxin pathway regulate plant thermomorphogenesis in response to heat stress to coordinate plant growth and stress defense. Both ABA and SA alleviate the negative effects of heat stress on plants by reducing oxidative damage and maintaining photosynthesis. In addition to SA, JA contributes to thermotolerance in *Arabidopsis* by physiological protection from heat-induced damage. CK alters antioxidant metabolism by inducing activities of antioxidant enzymes to alleviate heat stress injury. The role of ET on plant heat response is complicated and varies in different plant species. BR enhances plant thermotolerance by increasing photosynthetic rate and elevating the expression level of HSPs. BR signaling pathway induces the expression of PIFs and coordinates plant architectural changes under thermal stress condition. Heat stress inevitably causes accumulation of ROS. Thus, most hormones modulate plant ROS homeostasis, by production of antioxidants and scavenging of ROS, to improve heat tolerance.

Overwhelming evidences support the fact that plant hormones play important roles in plant biochemical, physiological, and architectural responses to high temperatures. The molecular mechanisms by which phytohormones regulate those defensive response are hitherto poorly understood. The signal transduction pathway leading to activation of hormone biosynthesis at high temperatures remains elusive. Crop plants are always exposed to a complex of environmental stresses in the field. Moreover, those hormones do not work along or act in a linear pathway to regulate plant growth, development, and defense. Intensive crosstalk between SA and ET/JA signaling pathways has been revealed in plant defensive response to pathogenic stress. The interaction and communication between multiple hormones in order to precisely coordinate plant defense response to heat stress deserve further investigation. For instance, it is unclear how JA and SA overcome ET-rendered negative effect to enhance heat tolerance in the *cpr5* mutant plants.

In the past decades, the heat-related responses in plants have been intensively studied (Bokszczanin et al., 2013; Hasanuzzaman et al., 2013; Ohama et al., 2017). So far, the molecular breeding and genetic modification strategies of developing heat-resilient agricultural crops are most unsuccessful, in a larger part due to limited knowledge on the molecular mechanism underlying plant heat response. Considering the elevated environmental temperature following global climate change that threatens plant growth, crop yield, and food productivity worldwide, there is a pressing need to thoroughly investigate the thermal-responsive hormone signal transduction pathway and sophisticated crosstalk between different signaling pathways to elucidate phytohormone function in plant heat response.

AUTHOR CONTRIBUTIONS

NL, L-JH, and WK: conceptualization. NL and ZL: literature review. NL, DE, and L-JH: writing—original preparation. ML and WK: writing—review and editing. NL, DE, JC, and ZL: design and revision of the images. All authors have read and agreed to the final version of the manuscript.

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Physiological and Gene Expression Changes of *Clematis crassifolia* and *Clematis cadmia* in Response to Heat Stress

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Clematis is a superior perennial ornamental vine known for varied colors and shapes of its flowers. *Clematis crassifolia* is sensitive to high temperature, whereas *Clematis cadmia* has a certain temperature adaptability. Here we analyzed the potential regulatory mechanisms of *C. crassifolia* and *C. cadmia* in response to heat stress by studying the photosynthesis, antioxidant parameters, amino acids, and gene expression patterns under three temperature treatments. Heat stress caused the fading of leaves; decreased net photosynthetic rate, stomatal conductance, superoxide dismutase, and catalase activity; increased 13 kinds of amino acids content; and up-regulated the expression of seven genes, including C194329_G3, C194434_G1, and C188817_g1, etc., in *C. crassifolia* plants. Under the treatments of heat stress, the leaf tips of *C. cadmia* were wilted, and the net photosynthetic rate and soluble protein content decreased, with the increase of 12 amino acids content and the expression of c194329_g3, c194434_g1, and c195983_g1. Our results showed that *C. crassifolia* and *C. cadmia* had different physiological and molecular response mechanisms to heat stress during the ecological adaptation.

Keywords: ***Clematis crassifolia*, *Clematis cadmia*, heat stress, physiological analysis, gene expression**

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INTRODUCTION

Clematis is a large genus that belongs to Ranunculaceae and has great ornamental value. There are various species of *Clematis*, which have rich variations in colors and shapes of flower. *Clematis* can be widely used in flower stands, corridors, lamp pillars, fences, arches, and other configurations to form an independent landscape, and it occupies a very important position in landscaping (Lehtonen et al., 2016). *Clematis* also has a certain medicinal value, which can be used as anti-inflammatory, antirheumatism, and analgesic agents (Hao et al., 2013; Li et al., 2017). There are approximately 355 species of *Clematis* in the world and 147 species in China (Pringle, 1971; Liu et al., 2018). In terms of adaptability to the environment, *Clematis* prefers a cool climate and is often associated with shrubs in the wild. At present, most cultivars of *Clematis* on the market are susceptible to high temperature in summer (Gao et al., 2017; Jiang et al., 2020). After the heat stress, the leaves will fade, wilt, and fall off, stem will wither, and there will be other heat damage symptoms, which severely affects the ornamental characteristics of *Clematis*.

With the global warming caused by the greenhouse effect, the heat stress has become one of the most important abiotic stresses that restrict plant growth (Berry and Bjorkman, 1980). In recent years, the frequent occurrence of extreme high temperature posed a severe challenge to the ability of plants to withstand high temperatures (Hasanuzzaman et al., 2013; Borrell et al., 2020). Therefore, the regulatory mechanism of plants in response to heat stress and the cultivation of heat-resistant varieties have become a focus of attention. Plants have produced a series of adaptation mechanisms to resist heat stress in the process of evolution (Wahid et al., 2007). One is heat resistance, which strengthens plants by changing leaf orientation, increasing leaf trichomes, and increasing xylem cells (Tozzi et al., 2013; Bickford, 2016). The other is heat tolerance mechanism, which is involved in a series of signal pathways, including ion transporters, osmoprotectants, free radical scavengers, signal cascades, and transcription factor regulation (Rodríguez et al., 2008; Ohama et al., 2017).

Previous studies have shown that heat stress changes the components and structure of plant cell membranes, reduces cell membrane integrity, increases cell membrane permeability, and causes the ion leakage (Wise et al., 2004; Bita and Gerats, 2013; Lohani et al., 2020). Heat stress can cause plants to accumulate excess reactive oxygen species (ROS), break the balance of ROS in cells, inhibit the photosynthetic electron transport chain, and cause irreversible damage to photoresponse system II while intensifying membrane lipid peroxidation (Ahmad et al., 2010; Gururani et al., 2015; Choudhury et al., 2017). Plants have evolved both enzymatic and nonenzymatic systems to remove ROS to maintain the growth of plants. The currently reported enzymatic antioxidant systems include superoxide dismutase (SOD), catalase (CAT), and ascorbic peroxidase (APX), etc. Nonenzymatic antioxidants are antioxidants in plants such as proline, ascorbic acid, mannitol, and glutathione, etc (Hameed et al., 2012; Tripathy and Oelmüller, 2012). In *Lilium longiflorum*, the antioxidant enzyme activities including SOD, peroxidase (POD), CAT, APX, and glutathione reductase (GR) were stimulated after 10 h of high-temperature treatment at 37°C and 42°C, and the concentrations of ascorbic acid (AsA) and glutathione (GSH) were maintained at a high level, resulting in the decrease of ROS content, so as to mitigate the damage caused by heat stress (Yin et al., 2008). The accumulation of osmotic regulation substances is also an important physiological mechanism for plants to respond to heat stress (Kaplan et al., 2004).

The up-regulated expression of genes has been reported to help plants adapt to heat stress (Atkinson and Urwin, 2012). Heat shock proteins (HSPs) are a type of stress protein that is induced in organisms under the heat stress (Jacob et al., 2017). They are involved in protein synthesis, folding, cell localization, protein transmembrane transport, and target protein degradation to maintain the stability of the plant homeostasis (Qu et al., 2013; Xu et al., 2016). Heat shock transcription factors (HSFs) are the key regulator of plant response to heat stress and play a critical role in the regulation of plant heat stress response (Guo et al., 2016). HsfA1 was an important transcription factor for *Arabidopsis thaliana* to obtain heat resistance (Ohama et al., 2016). The transcription factor FaHsfA2c of *Festuca arundinacea* was upregulated in leaves and roots under heat stress, which could enhance the heat resistance of *F. arundinacea* (Wang et al., 2017). In tomato (*Solanum lycopersicum*), Mishra founded that plants with silenced of *HsfA1a* were more sensitive to heat stress than wild type in each developmental stage (Mishra et al., 2002). HsfB1 was a transcriptional inhibitor, and it could also be used as coactivator of HsfA1a to inhibit the expression of *HsfA1b* and *HsfA2* (Röth et al., 2017; Zhou et al., 2018).

As an excellent ornamental vine, the market demand for *Clematis* is constantly rising in the world. However, the continuous loss of wild resources and the limited heat tolerance of horticultural varieties put forward a severe test to the cultivation of *Clematis*. How to effectively improve the heat resistance and reduce the damage of heat stress is the emphasis work of cultivation and breeding of *Clematis*. *Clematis crassifolia* is a kind of evergreen species, which blooms in winter and is mostly distributed in dense forests or sparse forests with an altitude of 100–300 m in China. *Clematis cadmia* is a potential material for resistance breeding because of its strong resistance and abundant flowers. In preliminary study, we found that *C. cadmia* has a certain temperature adaptability, whereas *C. crassifolia* was more sensitive to temperature in summer, so it is necessary to analyze the physiological and biochemical differences between *C. crassifolia* and *C. cadmia*. In this study, in order to understand the effects of heat stress on *Clematis*, we explored the response of photosynthesis, antioxidant enzyme activity system, amino acid levels, and gene expression in *C. crassifolia* and *C. cadmia*, which is expected to provide a theoretical foundation for the cultivation and breeding of *C. crassifolia* and *C. cadmia*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants of *C. crassifolia* and *C. cadmia* were grown under different heat stress and conducted in the Zhejiang Institute of Subtropical Crops (120°37′53″E, 28°0′8″N), China. Two-year-old healthy and homogenous plants were grown in a grown chamber under a 16/8-h long-day cycle at 25°C/20°C, 65% humidity for 2 weeks. After 2 weeks of pretreatment, in order to carry out the heat stress, the pretreated plants of *C. crassifolia* and *C. cadmia* were transferred to the grown chamber for cultivation at 25°C/20°C, 35°C/30°C, and 45°C/40°C temperature, respectively. Heat stress treatments lasts for 4 days; during the treatment period, water was poured every 2 days, 500 mL each time, to ensure sufficient soil moisture. Take the mature leaves of two to five positions after 4 days of treatments, respectively. Fresh leaves were tested for physiological indicators, and other samples were frozen in liquid nitrogen and stored at −80°C for the analysis of amino acid and gene expression. Experimental treatments were repeated three times.

Leaf Gas Exchange Parameters

Healthy and fully developed leaves from each treatment were randomly chosen for photosynthetic parameter measurements, using LI-6400 XT portable photosynthesis system (Li-Cor Inc., Lincoln, NE, United States), and equipped with a 6400-18 RGB

LED light source. The measurements were carried out from 9:00 to 11:00 AM, the photosynthetic photon flux density was 1200 μmol m^{-2} s^{-1}, the CO_2 concentration was 400 ppm, and the relative humidity was 65%.

Photosynthetic Pigments

The finely cut and well-mixed leaf samples (100 mg) were transferred to a 10 mL tube. Then, 8 mL of 80% acetone was added to the test tube and mixed. Chlorophyll was extracted at 4°C in the dark. The absorbance of supernatant was measured at 663, 645, and 470 nm with a spectrophotometer (Shimadzu UV-2550, Kyoto, Japan). The total chlorophyll content was calculated and was expressed as mg g^{-1} FW. The total chlorophyll content was measured according to the described method by Lichtenthaler and Buschmann (2001).

Measurement of MDA Content, Hydrogen Peroxide Content, and Soluble Protein Content

The malondialdehyde (MDA) content was determined as previously described (Ouyang et al., 2010).

The H_2O_2 content and soluble protein content were measured according to the method as previously described (Luo et al., 2012).

Determination of SOD, CAT, and POD Activity

For antioxidant enzyme activity analysis, fresh leaves (0.1 g) were ground in liquid nitrogen and then suspended in 8.0 mL solution containing 50 mM phosphate buffer (pH 7.4). The homogenate was centrifuged 10,000 rpm for 15 min at 4°C, and the supernatant was collected to obtain crude enzymes.

The SOD activity was analyzed by measuring the inhibiting rate of the enzyme to O_2^- produced. One-unit SOD activity (U) was defined as the amount of enzyme that resulted in 50% inhibition of reduction of nitrite in 1 mL reaction solution. The SOD activity was determined at 550 nm after 40 min of reaction at 37°C and expressed as U g^{-1} FW (Ma et al., 2017).

The CAT activity was determined by the hydrolysis reaction of hydrogen peroxide (H_2O_2) with CAT; the reaction could be terminated rapidly by molybdenum acid (MA) to produce yellow MA-H_2O_2 complex (Li et al., 2013). The CAT activity was calculated in absorbance at 405 nm. One unit was defined as the amount of enzyme that resulted in the decompose of 1 μmol H_2O_2 per second in 1.0 g fresh tissue.

The POD activity was measured based on the change of absorbance at 470 nm by catalyzing H_2O_2 (Zheng et al., 2018). One unit was defined as the amount of enzyme that resulted in the change of 0.01 at 470 nm per minute by 1.0 g fresh tissues in the reaction system.

Amino Acid Contents Analysis

Samples (10 mg) were weighed, mixed with 1 mL methanol, and subsequently homogenized in an ultrasonic instrument for 3 min, tubes were static for 5 min at room temperature, and 10,000 rpm centrifuged for 15 min at 4°C. The supernatant was diluted 10 times. One hundred microliters of dilution was transferred to heat-resistant tubes, and 100 μL of internal standard solution (100 ppb) was added; the mixture was filtered through a 0.45-μm membrane and then injected into the Ultra Performance Liquid Chromatography (UPLC) for analysis. The 24 kinds of standards were weighed accurately. Stock solutions were prepared using methanol or water, and a series of mixed working standard solutions were properly prepared and diluted with water. The standard solutions were stocked under 0°C.

UPLC separation was performed on an Acquity UPLC system (Waters, United Kingdom) equipped with an ACQUITY UPLC® BEH HILIC (1.7 μm, 2.1 × 100 mm, Waters) column. The temperature of the column was set at 40°C. The sample injection volume was 5 μL. Eluents consisted in water/methanol (90:10) with 0.1% (vol/vol) formic acid (eluent A) and water/methanol (50:50) with 0.1% (vol/vol) formic acid (eluent B). The gradient elution started with 10% B for 0 min, ramped to 30% of B within next 6.5 min, ramped to 100 % of B in 7 min, kept at 100% of B until 8 min and dropped to 10% B in 8.5 min at a flow rate of 0.3 mL/min, and finally kept at 10% of B until 12.5 min at a flow rate of 0.4 mL/min.

The MS analysis was performed using a AB 4000 mass spectrometer (AB, United States) equipped with an ESI source in the positive-ion mode working in the multiple reaction monitoring mode. An ion source voltage of 5.5 kV and a source temperature of 500°C were used. Collision gas and the curtain gas were set at 6 and 30 psi, respectively, whereas both of the atomization gas and auxiliary gas were 50 psi.

Gene Expression

Isolation of total RNA from the leaf tissues and real-time quantification of transcriptional expression of the genes were the same as that reported previously (Ma et al., 2019). All of the primers used for quantitative reverse transcriptase–polymerase chain reaction (PCR) are listed in **Supplementary Tables 1**, **2**. All of the PCR products were confirmed by sequencing.

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) using the SPSS 10 program (SPSS Inc., Chicago, IL, United States). Different letters on the histograms between different treatments indicate their statistical difference at $P \leq 0.05$.

RESULTS

High-Temperature Stress Caused Leaf Discoloration and Crimping of *C. crassifolia* and *C. cadmia*

Under different temperature treatments, the leaves damage degree of *C. crassifolia* and *C. cadmia* showed significant differences. In comparison with the normal-temperature treatment (25°C), *C. crassifolia* mainly showed water loss, leaf carnification degree reduced, and leaf turned yellow (**Figure 1B**). *C. cadmia* exhibited partial curling with the yellow leaf tips

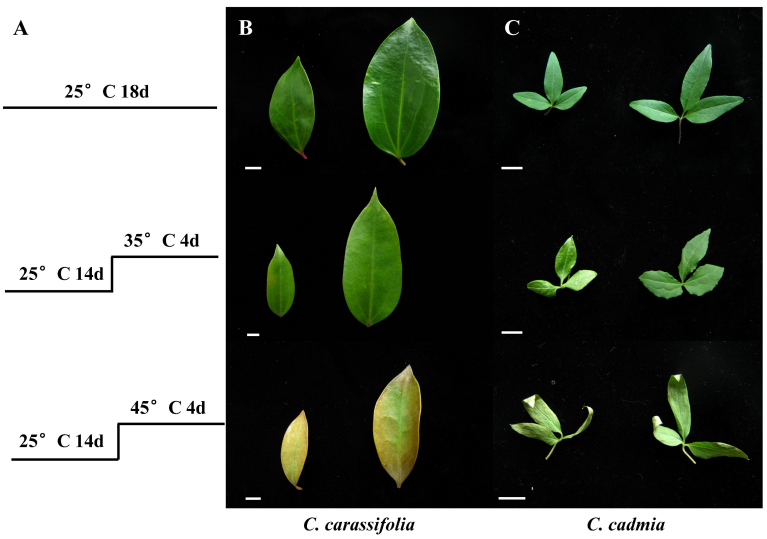

FIGURE 1 | Effect of heat stress on the morphology of the leaves of *C. crassifolia* and *C. cadmia*. **(A)** Treatment conditions. **(B)** The phenotype of *C. crassifolia* leaves. **(C)** The phenotype of *C. cadmia* leaves. Bar means 1 cm.

(**Figure 1C**). In addition, after 45°C heat stress treatment, the MDA content of *C. crassifolia* was increased 443.97% compared with normal-temperature treatment (25°C). No significant differences were observed in *C. cadmia* between two heat stress treatments (**Supplementary Figure 1**).

Heat Stress Inhibited the Photosynthesis of *C. crassifolia* and *C. cadmia*

The heat stress can aggravate the degradation of chlorophyll and inhibit its synthesis, so the change of chlorophyll content can reflect the damage degree of high temperature to plants (Zhou and Leul, 1999). According to the heat stress treatments, the Chla, Chlb, and carotenoid content of *C. crassifolia* were decreased, instead of the increased Chla/b ratio. Even at the same heat stress condition, *C. cadmia* plants have high photosynthetic pigments than *C. crassifolia*; the Chla and Chlb content under the moderate (35°C) temperature increased by 31.59% and 24.10%, respectively, compared with those under the normal temperature (25°C) in *C. cadmia*, and decreased by 21.59% and 14.79% under high (45°C) temperature compared with the normal temperature (25°C), respectively (**Figure 2**).

The photosynthetic parameters of *C. crassifolia* and *C. cadmia* were also strikingly affected by heat stress treatments. The net photosynthetic rate (P_n) of *C. crassifolia* and *C. cadmia* showed significant decrease in high-temperature treatment (45°C), which were only 1.29% and 3.25% of normal-temperature treatment (25°C), respectively (**Figure 3A**). With the increase of temperature, the variation trends of stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (TR) of *C. crassifolia* and *C. cadmia* were similar. *C. crassifolia* plants grown under 45°C condition have extremely low Gs and TR values, decreased 71.58% and 65.80%, respectively, compared with the plants under normal temperature (25°C). However, the Gs, Ci, and TR of *C. cadmia* has no significant difference between 25°C and 45°C treatments (**Figures 3B–D**).

The Antioxidant System of *C. crassifolia* Was More Influential Than *C. cadmia* Under Heat Stress

The activities of POD and SOD in *C. cadmia* leaves were significantly affected by heat stress treatments. The POD activity was 1.95 times that in the normal-temperature treatment (25°C).The SOD activity was decreased significantly with different heat stress, by 27.33% and 32.79%, respectively. In *C. crassifolia*, there was no significant difference of POD activity among three temperature degrees, but SOD activity decreased gradually with the increase of temperature (**Figure 4**).

The CAT activity of *C. crassifolia* was significantly inhibited by heat stress. Under the condition of 45°C heat stress, the activity of CAT decreased by 91.76%, whereas there was no significant change in *C. cadmia* plants (**Figure 5A**), while the H_2O_2 content in *C. crassifolia* was increased obviously by gradient with the increase of temperature, increased by 60.64% and 215.54%, respectively, under moderate (35°C) and high (45°C) temperature compared with the normal temperature (25°C). The *C. cadmia* plants exposed to high-temperature treatment (45°C) showed 150.60% increase of the H_2O_2 content than those under normal (25°C) treatment (**Figure 5B**).

The Content of Soluble Protein and Amino Acid in *C. crassifolia* and *C. cadmia* Showed Difference Patterns Under Heat Stress

After different heat treatments, the soluble protein content of *C. cadmia* decreased 45.67% under high-temperature treatment (45°C) compared with normal-temperature treatment

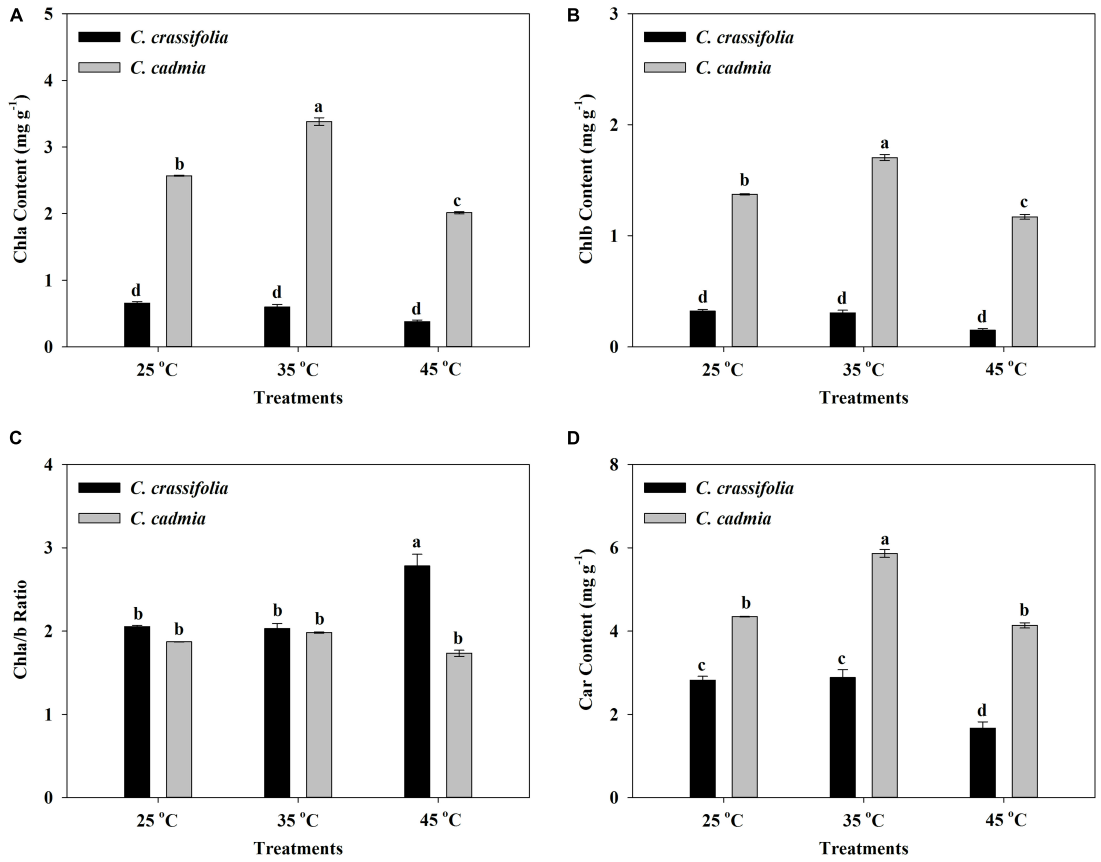

FIGURE 2 | Chlorophyll a (Chla), chlorophyll b (Chlb), chlorophyll a/b ratio, and carotenoid (Car) in *C. crassifolia* and *C. cadmia* grown under three different temperatures including normal temperature (25°C), moderate temperature (35°C), and high temperature (45°C). **(A)** Chla content. **(B)** Chlb content. **(C)** Chla/b ratio. **(D)** Car content. Error bars indicate SE (n = 5 plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

(25°C); however, there was no significant change of soluble protein content in *C. crassifolia* plants under heat-stressed conditions (**Figure 6**).

The amino acid content was assessed in *C. crassifolia* and *C. cadmia* plants under different heat stress treatments (**Table 1**). Isoleucine, glutamine, histidine, and tryptophan were significantly increased in both *C. crassifolia* and *C. cadmia* plants under moderate-temperature (35°C) and high-temperature (45°C) treatments compared with normal-temperature treatment (25°C). Ornithine hydrochloride was only detected in *C. crassifolia* plants, and glycine was only detected in *C. cadmia* under 45°C heat treatment. Proline, valine, threonine, lysine, phenylalanine, and tyrosine were up-regulated at 45°C condition in *C. crassifolia* and at 35°C treatment in *C. cadmia* (**Table 1**).

Gene Expression Pattern in *C. crassifolia* and *C. cadmia* Under Heat Stress

To explain the response of *C. crassifolia* and *C. cadmia* plants to heat stress, we examined the expression of 12 genes under 45°C culture condition, including HSP, HSF, photosystem, and POD genes, which were descripted in transcriptome profiling (**Figure 7**). The transcript levels were significantly different in *C. crassifolia* and *C. cadmia*. The expression levels of HSP and HSF genes including c194329_g3 and c194434_g1 in *C. cadmia* were remarkably higher (>2.0-fold) after heat stress, and those two genes have significant up-regulation in *C. crassifolia* except endure the heat stress for 12 h (**Figures 7A,B**). Four genes associated with heat stress, including c188817_g1, c200811_g3, c187075_g1, and c194962_g2, which were involved in biosynthesis of photosystem and chlorophyll, have high transcript levels after heat stress in *C. crassifolia* plants, and only c188817_g1 and c208712_g3 showed a small amount of increase after 6 h of heat stress treatment in *C. cadmia*. For antioxidant enzyme genes, the transcript level of c199977_g2 has substantially increased in *C. crassifolia* after heat stress, and the expression of c202620_g2, c195983_g1 and c198009_g1 varied slightly in *C. cadmia* (**Figures 7C,D**).

DISCUSSION

With the aggravation of greenhouse effect and the rise of global temperature, heat stress is the main environmental stress that restricts plant growth and development. Plants respond

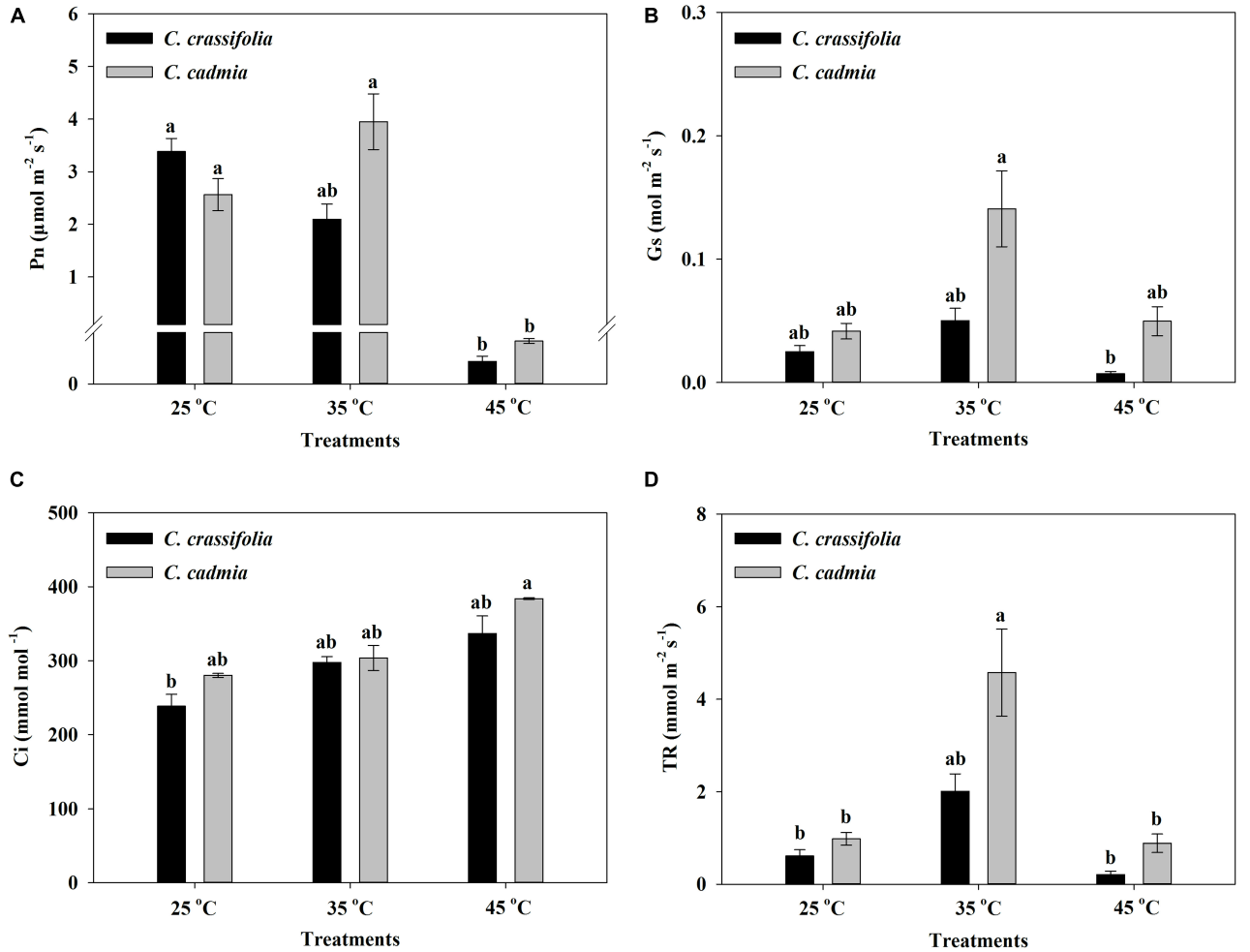

FIGURE 3 | Photosynthetic parameters of *C. crassifolia* and *C. cadmia* grown under three different temperatures including normal temperature (25°C), moderate temperature (35°C), and high temperature (45°C). **(A)** Net photosynthetic rate (P_n). **(B)** Stomatal conductance (Gs). **(C)** Intercellular CO_2 concentration (C_i). **(D)** Transpiration rate (TR). Error bars indicate SE (n = 5 plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

to heat stress through changes in physiological, biochemical, and transcriptional regulatory systems (Baniwal et al., 2004; Rizhsky et al., 2004; Wu et al., 2018). This study presents the leaves phenotype, physiological mechanism, and gene expression pattern of *C. crassifolia* and *C. cadmia* in response to different heat stress. The damage to plant organs, tissues, and cells caused by heat stress was multifold. Plants exposed to heat stress damage showed discoloration or peeling of leaves, damage of flowers and fruits, poor seed germination rate, and inhibition of plant growth (Rodríguez et al., 2005). With the increase of temperature, *C. crassifolia* and *C. cadmia* manifested different symptoms of heat damage. The fading and wilting leaves were observed in *C. crassifolia*, whereas *C. cadmia* leaves showed rolling and drying in leaf tip and leaf margins (**Figure 1**).

Photosensitive pigment regulation is an important regulation mechanism of photosynthesis protection in plants under heat stress. Increasing the chlorophyll content with a certain range can improve the absorption and transformation of light energy by plants (Shi et al., 2006). In this study, the content of Chla and Chlb decreased under the 45°C heat stress and caused a 35.49% increase in Chla/b and a 40.84% decrease in *C. crassifolia* (**Figures 2A–C**), indicating that Chlb was more sensitive to heat stress than Chla in *C. crassifolia*. Heat stress affects the early stage of photosynthesis and mainly changes the membrane properties of chloroplasts and the uncoupling of energy transfer mechanism, but protein denaturation under continuous heat stress can cause irreversible damage (Fan et al., 2018). The reasons for the decrease of Pn in plant leaves are mainly divided into stomata limiting factors and non–stomata-limiting factors. The previous studies have observed that the heat stress can cause plant stomatal closure and reduce the photosynthetic rate (Crafts-Brandner and Salvucci, 2002; Pnueli et al., 2003).

In this study, the Pn, Gs, and Tr of *C. crassifolia* were significantly reduced under heat stress of 45°C, indicating that heat stress caused the decline in photosynthesis of *C. crassifolia* by

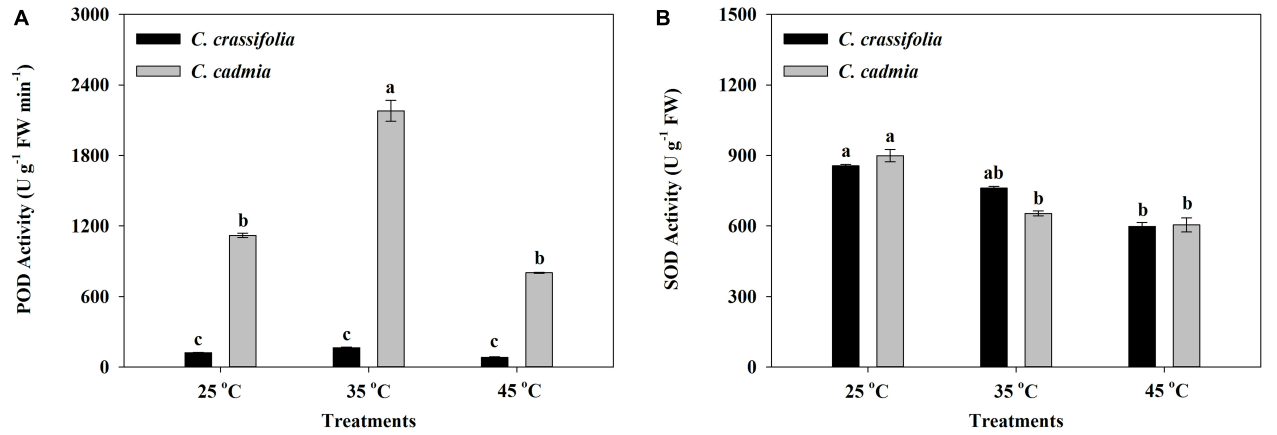

FIGURE 4 | Effect of heat stress on the peroxidase (POD) activity and the superoxide dismutase (SOD) activity of *C. crassifolia* and *C. cadmia* grown under three different temperatures. **(A)** POD activity. **(B)** SOD activity. Error bars indicate SE (n = 5 plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

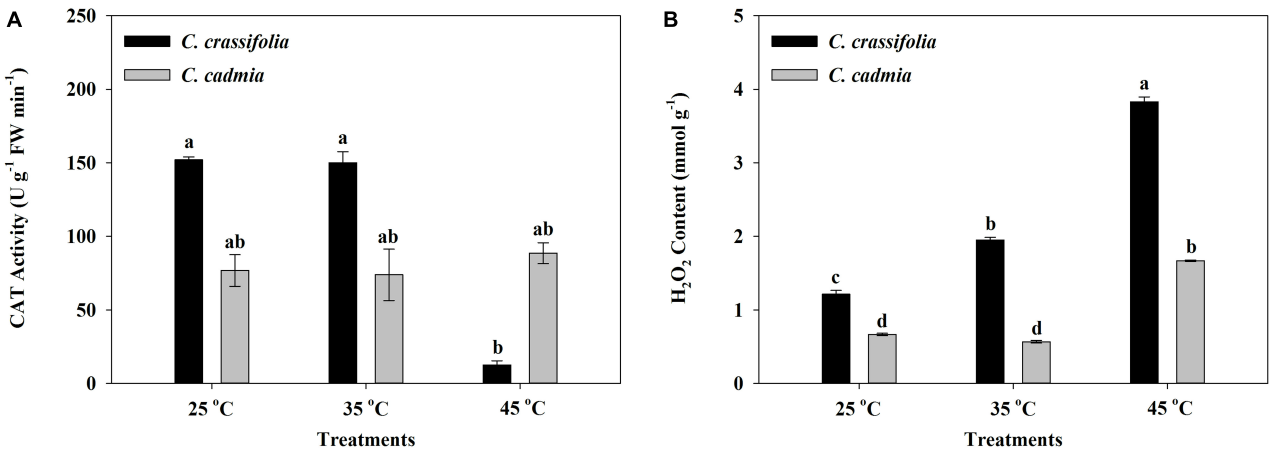

FIGURE 5 | Effect of heat stress on the Catalase (CAT) activity and the peroxide (H_2O_2) content of *C. crassifolia* and *C. cadmia* grown under different temperatures. **(A)** CAT activity. **(B)** H_2O_2 content. Error bars indicate SE (n = 5 plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

both stomata-limiting factors and non–stomata-limiting factors. The total chlorophyll content of *C. cadmia* decreased significantly at 45°C heat stress, and there were no significant differences in Gs and Tr, whereas the increased of Gs and Tr were observed in *C. cadmia* plants under 35°C, resulting in a 54.02% increase in Pn. These results indicated that *C. cadmia* can adapt to the environment by increasing the chlorophyll content and Gs under moderate temperature conditions, and the decrease of photosynthetic activity under heat stress was the main reason for the reduction of photosynthesis.

The injury of plants under adversity is closely related to membrane lipid peroxidation induced by the accumulation of ROS (Asada, 2006). MDA is one of the most important products of membrane lipid peroxidation. Therefore, MDA level can be measured as an indirect measure of injury to membrane lipid and stress resistance of plants. In this study, we observed that 45°C heat stress led to a significant increase in MDA content and H_2O_2 content of *C. crassifolia*; however, there were no significant changes in *C. cadmia* under different temperature treatments (**Supplementary Figure 1** and **Figure 5B**). The results suggested that the high temperature could result in the accumulation of excessive H_2O_2 in *C. crassifolia*, which would cause the destruction of cell membrane. But *C. cadmia* could maintain a relatively stable cell homeostasis under heat stress.

Plant in long-term evolution formed in the process of enzymatic reaction system in order to eliminate the oxidative stress caused by heat stress and enhance the protection ability of plants, including POD, CAT, and SOD, etc., which play a key role in the regulation of ROS homeostasis in the cell (Larkindale and Vierling, 2008; Vidya et al., 2018). The stress tolerance of tomatoes at heat is closely associated with its antioxidant mechanism (Zhou et al., 2019). In our study, it was shown that *C. crassifolia* and *C. cadmia* could respond to heat stress by

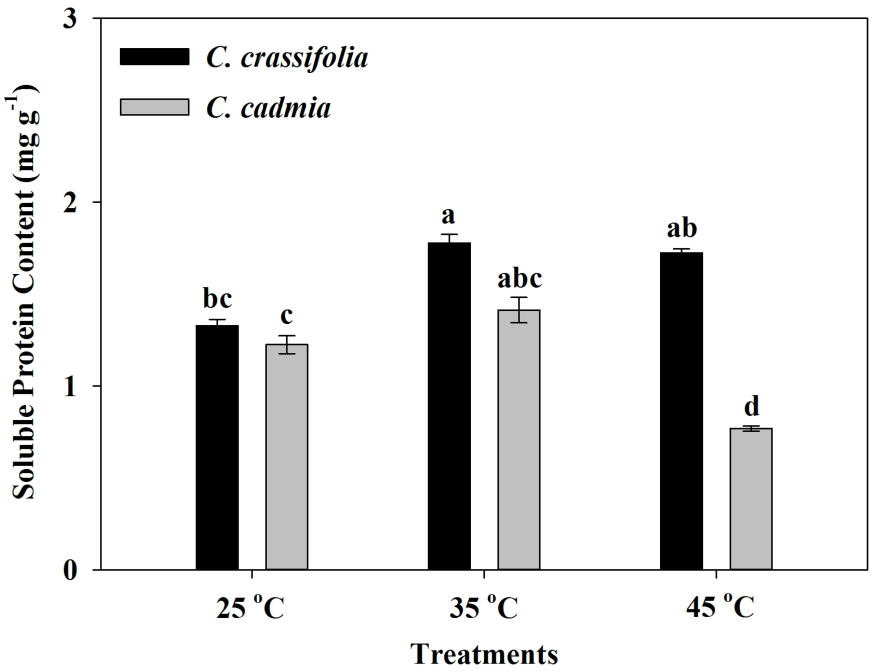

FIGURE 6 | Soluble protein content of *C. crassifolia* and *C. cadmia* grown under three different temperatures. Values are the means ± standard error (n = 5 plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

TABLE 1 | Amino acid contents (data shown are the mean) of *C. crassifolia* and *C. cadmia grown* under different temperature treatments.

Amino acid	Absolute content (μg g^{-1})						Relative content to normal temperature			
	C. crassifolia			*C. cadmia*			*C. crassifolia*		*C. cadmia*	
	25°C	35°C	45°C	25°C	35°C	45°C	35°C	45°C	35°C	45°C
Glycine	–	–	–	–	–	604.04	–	–	–	I
Alanine	22.20	20.00	30.61	125.00	310.10	434.34	0.90	1.38	2.48	3.47
Aminobutyric acid	10.24	38.29	19.13	58.65	75.56	231.31	3.74	1.87	1.29	3.94
Serine	27.80	44.95	21.74	313.54	582.83	520.20	1.62	0.78	1.86	1.66
Proline	10.56	12.67	135.48	114.58	352.53	142.42	1.20	12.83	3.08	1.24
Valine	5.08	5.70	69.22	108.33	533.33	635.35	1.12	13.61	4.92	5.86
Threonine	72.71	82.10	594.78	146.88	500.00	483.84	1.13	8.18	3.4	3.29
Isoleucine	3.58	7.81	81.39	60.63	433.33	549.49	2.18	22.76	7.15	9.06
Leucine	11.39	9.37	129.39	112.50	200.00	495.96	0.82	11.36	1.78	4.41
Asparagine	608.47	163.05	30.26	776.04	12828.28	955.56	0.27	0.05	16.53	1.23
Ornithine hydrochloride	0.22	0.14	0.48	–	–	–	0.65	2.15	/	/
Aspartic acid	58.98	39.43	10.56	63.75	361.62	160.61	0.67	0.18	5.67	2.52
Glutamine	121.02	321.90	688.70	902.08	7565.66	97676.77	2.66	5.69	8.39	108.28
Lysine	10.00	11.10	66.09	68.96	145.45	46.36	1.11	6.61	2.11	0.67
Glutamic acid	618.64	382.86	89.04	1052.08	1636.36	1545.45	0.62	0.14	1.56	1.47
Histidine	0.50	1.17	5.53	5.59	107.07	69.60	2.33	11.02	19.14	12.44
Phenylalanine	7.00	11.45	87.48	78.96	543.43	91.21	1.64	12.5	6.88	1.16
Arginine	0.74	0.70	5.01	18.75	63.13	–	0.94	6.76	3.37	D
Tyrosine	18.98	29.90	311.30	–	95.96	169.70	1.58	16.4	I	I
Tryptophan	6.49	14.13	70.61	23.54	234.34	218.18	2.18	10.88	9.95	9.27

D ≤1.0 1–2 2–4 ≥4.0 I

D, detected in normal temperature but not in moderate- and high-temperature treatments; I, detected in moderate and high temperature but not detected in normal-temperature treatments; —, indicates not detectable.

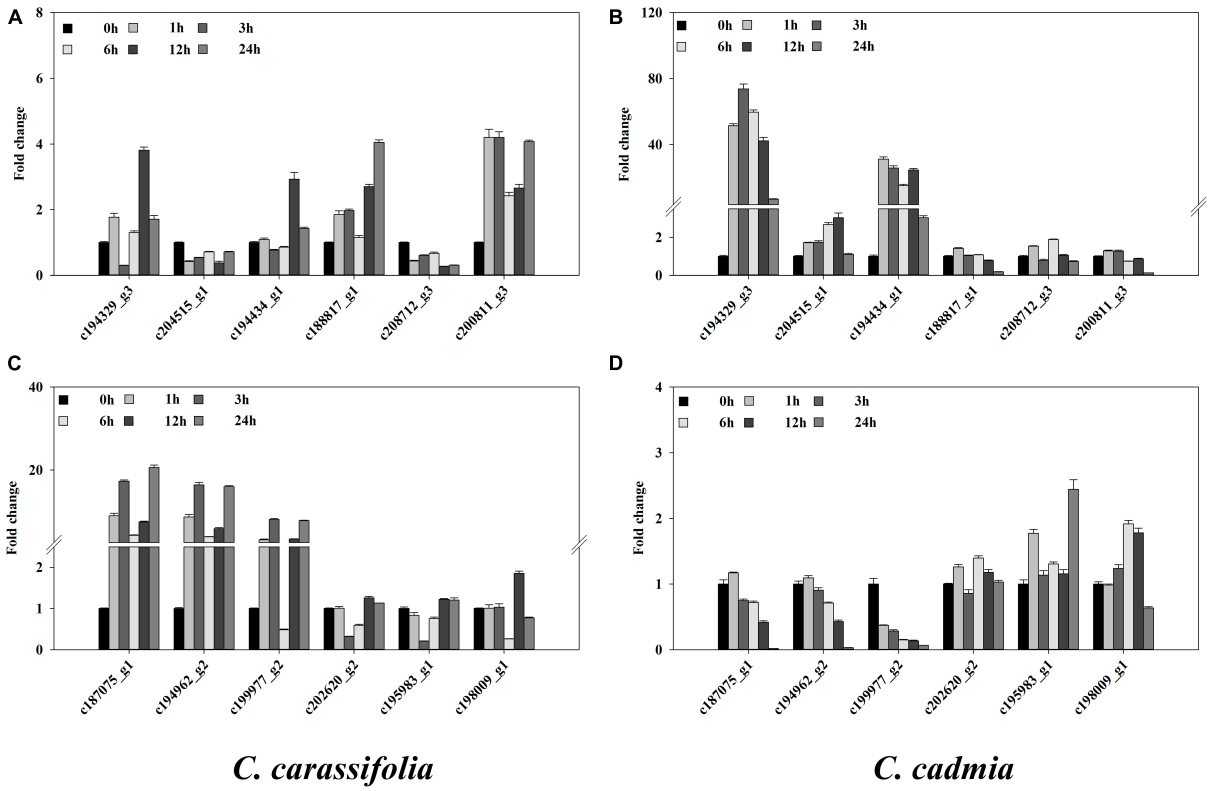

FIGURE 7 | Effect of heat stress on the gene expression in *C. crassifolia* and *C. cadmia* leaves. Time course of heat in *C. crassifolia* and *C. cadmia* shifted to 45°C. **(A,C)** *C. carassifolia*. **(B,D)** *C. cadmia*. Soluble protein content. Bars indicate SE (n = 3).

regulating the antioxidant mechanism, but sustained heat stress would reduce the enzyme activity.

Amino acids are a class of important physiological active substances. Substances such as amino acids or polyamines synthesized with amino acids as precursors can accumulate under heat stress, stabilize proteins, and maintain cell osmotic pressure (Bowlus and Somero, 1979). Glycine, as an important amino acid, is a synthetic substrate of glycine betaine (GB), which can protect photosystem II, stabilize membranes, and reduce oxidative damage (Sita et al., 2018; Alhaithloul et al., 2020). γ-Aminobutyric acid (GABA) is a nonprotein amino acid widely present in plants. Under heat stress, GABA can improve the activity of antioxidant enzymes such as POD and CAT to reduce peroxidation damage (Nayyar et al., 2014). Exogenous application of GABA could enhance accumulation of osmolytes such as proline and trehalose due to increase in the activities of their biosynthetic enzymes and improved the leaf turgor, carbon fixation, and assimilation processes to protect the reproductive function from heat stress in mungbean (Priya et al., 2019). In this study, amino acids such as Pro, Val, Thr, Ile, Leu, Glu, Lys, His, and Tyr were increased to relieve osmotic pressure of leaf cell in *C. crassifolia*. *C. cadmia* sufficiently increased the content of amino acids such as Gly, GABA, Glu, and Tyr; maintained the activity of antioxidant enzymes; and reduced the content of MDA, thereby enhancing the stability of cell membrane structure and alleviating the damage caused by heat stress.

Plant response to heat stress involves a complex gene regulatory network, and the damage caused by stress can be alleviated by regulating the expression of related genes (Krasensky and Jonak, 2012). In the previous study, a total of 81 SRAP and 133 EST-SSR polymorphic loci were detected in *Clematis* (Li et al., 2018). HSPs, especially small HSPs, antioxidant enzymes (e.g., APX), and galactosyl alcohol synthesis enzymes play key roles in the heat resistance of grapes (Liu et al., 2012). The HSF HsfA1 plays an important role in transcriptional regulatory networks in promoting the expression of heat stress–related genes, to regulate intracellular protein activity, rehabilitate denatured proteins, degrade misfolded proteins, and alleviate the damage caused by heat stress (Yoshida et al., 2011). We screened out the expression patterns of 12 genes associated with heat stress (**Figure 7**). The expressions of c194329_g3, c204515_g1, c194434_g1, and c195983_g1 were up-regulated in *C. cadmia* within a short period of heat stress, whereas the response time of *C. crassifolia* was longer. c194329_g3 and c194434_g1 were up-regulated 12 h later. The results showed that *C. cadmia* could rapidly respond to heat shock stress and promote the synthesis of related enzymes and metabolites by enhancing the expression of small HSP, HSFs, and APX genes, thus alleviating the damage caused by heat stress. PsaH is a membrane peripheral protein located at the surface of PSI, and PSBY protein is located in the thylakoid membrane, both of which play an important role in photosynthetic system composition and electron transport

(Obokata et al., 1993; Ozawa et al., 2018). *C. crassifolia* leaves photosynthetic system response was more sensitive to heat stress than *C. cadmia*, by increasing the expression of photosynthesis-related genes in the early stage of heat stress. It increased the excitation energy transferred to the PSII core complex and promoted the increase of electron transfer efficiency. But continuous heat stress will lead to a decrease in the chlorophyll content and net photosynthetic rate.

In summary, these investigation results indicated *C. crassifolia* and *C. cadmia* exhibited different photosynthetic characteristic, metabolic characteristics, antioxidant system, and gene expression patterns, which were related to their suitable living environment and genetic evolution. And we found that the photosynthetic system and enzymatic system may be the key links in the response to heat stress of *C. crassifolia* and *C. cadmia*, respectively. The hypothesis will be tested in future work. This study will pave the way to research the response and tolerance molecular mechanisms in clematis under heat stress.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

QH performed the experiments, analyzed the data, and completed the manuscript. RQ and YZ helped to perform the experiment. XZ and XM revised the manuscript. JZ approved the final version. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.624875/full#supplementary-material

Supplementary Figure 1 | MDA content of *C. crassifolia* and *C. cadmia grown* under three different temperatures. Values are the means ± standard error ($n = 5$ plants). Different letters indicate significant differences based on two-way ANOVA followed by Tukey multiple comparisons ($P \leq 0.05$).

Supplementary Table 1 | The unigene annotations in the KEGG pathway database.

Supplementary Table 2 | Primer sequences for gene expression analysis.

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The Role of Endoplasmic Reticulum Stress Response in Pollen Development and Heat Stress Tolerance

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Endoplasmic reticulum (ER) stress is defined by a protracted disruption in protein folding and accumulation of unfolded or misfolded proteins in the ER. This accumulation of unfolded proteins can result from excessive demands on the protein folding machinery triggered by environmental and cellular stresses such as nutrient deficiencies, oxidative stress, pathogens, and heat. The cell responds to ER stress by activating a protective pathway termed unfolded protein response (UPR), which comprises cellular mechanisms targeted to maintain cellular homeostasis by increasing the ER's protein folding capacity. The UPR is especially significant for plants as being sessile requires them to adapt to multiple environmental stresses. While multiple stresses trigger the UPR at the vegetative stage, it appears to be active constitutively in the anthers of unstressed plants. Transcriptome analysis reveals significant upregulation of ER stress-related transcripts in diploid meiocytes and haploid microspores. Interestingly, several ER stress-related genes are specifically upregulated in the sperm cells. The analysis of gene knockout mutants in Arabidopsis has revealed that defects in ER stress response lead to the failure of normal pollen development and enhanced susceptibility of male gametophyte to heat stress conditions. In this mini-review, we provide an overview of the role of ER stress and UPR in pollen development and its protective roles in maintaining male fertility under heat stress conditions.

Keywords: endoplasmic reticulum stress, unfolded protein response, plant reproduction, pollen development, male gametophyte, heat stress, pollen, sperm cell

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INTRODUCTION

The endoplasmic reticulum (ER) is a large, structurally complex organelle whose membrane can constitute half of a eukaryotic cell's total membranes. ER is a main production site for lipids and many proteins. Each cell carries two types of the ER: smooth ER (SER) and rough ER (RER). The SER is a site of lipid and sterol biosynthesis. In contrast, RER with its outer cytosol-facing surface studded with ribosome plays a crucial role in biosynthesis and productive post-translational processing and folding of secretory and transmembrane proteins. Nearly one-third of protein production and folding occurs in this organelle (Schubert et al., 2000). This highly active process requires finely tuned regulation of ER homeostasis. The protein homeostasis in the

ER is maintained by chaperone functioning, folding, quality control (QC), and degradation systems. Following assembly on membrane-bound ribosomes, the unfolded polypeptides enter into the ER lumen for a chaperone-assisted folding to their correct three-dimensional conformation (Hetz et al., 2020) to enable them to perform their assigned biological functions. Other post-translational modifications in the ER include N-linked glycosylation and disulfide bond formation. Proteins that get folded successfully leave the ER and move towards their final destination through the secretory pathway.

The protein folding is an intrinsically error-prone process with nearly 30% of the newly synthesized protein folded inappropriately (Balchin et al., 2016). When the folding fails, misfolded polypeptides are retained in the ER by QC mechanisms (**Figure 1**). The terminally misfolded and aggregated proteins are retrotranslocated into the cytosol to be degraded by endoplasmic-reticulum-associated degradation (ERAD) machinery (Li et al., 2017a). ERAD is an essential component of the ER QC system that clears toxic misfolded proteins via an ER-specific ubiquitin/proteasome system involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and 26S proteasome system. Selective autophagy of ER termed ER phagy, which includes vacuolar degradation of cytoplasmic components, is another component of the ER QC process. Activation of autophagy leads to the *de novo* formation of double-membrane vesicles termed as autophagosomes at the ER that envelop damaged or superfluous cell components and traffic them to vacuoles for degradation to simple molecules for recycling them back into the cytosol (Wirawan et al., 2012; Marshall and Vierstra, 2018; Bao and Bassham, 2020).

Endoplasmic reticulum protein folding, export and degradation machinery can manage the protein folding demands under the homeostatic state. However, under certain physiological conditions or environmental stresses, the ER gets overwhelmed with misfolded proteins leading to ER stress (**Figure 1**). To cope with the ER stress, the ER triggers an adaptive program, the unfolded protein response (UPR) (Buchberger et al., 2010; Liu and Howell, 2010; Angelos et al., 2017). In contrast, a cytosolic protein response (CPR) involving specific heat shock factors functions to maintain protein homeostasis in the cytosol (Sugio et al., 2009). The CPR involves heat shock caused activation HSFs leading to enhanced expression of genes encoding heat shock proteins (HSPs). HSPs function as molecular chaperones to counteract protein aggregation and target misfolded proteins for degradation (Buchberger et al., 2010; Hartl et al., 2011; Li et al., 2017a). The compartmentalization of CPR and the ER UPR, with their own distinct chaperones, permits independent responses to disorders in protein folding processes.

The UPR response signature is enhanced expression of genes encoding ER chaperones and the components of the ERAD system (Martínez and Chrispeels, 2003). The UPR activation to prevent the accumulation of unfolded or misfolded proteins in the ER includes ER-localized sensor protein inositol requiring enzyme 1 (IRE1) (Deng et al., 2011). IRE1 functions as an RNA splicing factor whose principal substrate in plants is mRNA encoding the transcription factor bZIP60. Upon its activation by ER stress, IRE1 splices bZIP60 mRNA and produces a form that encodes a bZIP60 protein lacking transmembrane domain (Li and Howell, 2021). Another arm of the UPR pathway involves stress-induced membrane-associated transcription factors, bZIP17 and bZIP28. Molecular chaperones in the ER, including immunoglobulin-binding protein (BiP), a heat shock protein (Hsp 70) family chaperone, Calnexin (CNX), and Calreticulin (CRT), play important roles in ER QC. CNX and CRT mediate folding of Asn-linked glycoproteins trafficking through the ER (Leach and Williams, 2003). Heat shock 70-kDa proteins transiently bind to their client proteins through an ATP hydrolysis and ATP rebinding cycle (Mayer and Gierasch, 2019). This cycle is regulated by DnaJ/Hsp40 (heat shock protein 40) proteins, which contain J-domain a ∼70 amino acid signature sequence region through which they bind to Hsp70s (Kampinga and Craig, 2010).

ENDOPLASMIC RETICULUM STRESS AND MALE REPRODUCTIVE DEVELOPMENT

Plants, being sessile, cannot move to avoid adverse effects of heat waves that are increasing in duration and frequency due to current global climate change conditions. Though heat stress adversely impacts all plant growth stages, the plant reproductive development remains the most vulnerable stage of the life cycle. This vulnerability at the reproductive stage leads to a significant reduction in seed set and crop yields. The pollen development and pollination are particularly vulnerable to heat stress events as elevated temperatures during pollen development can trigger pollen abortion (Reiu et al., 2017; Begcy et al., 2019; Lohani et al., 2020, 2021). Recent investigations have uncovered the crucial role of the UPR for ensuring normal pollen development and successful fertilization (Deng et al., 2013, 2016; Fragkostefanakis et al., 2016). An active UPR pathway is required to meet the high demands of secretory proteins during normal development even in the absence of exogenous stresses (Deng et al., 2013). The knockout mutations in the UPR signaling pathway genes result in pollen developmental abnormalities primarily resulting in male sterility (**Table 1**). In this review, we focus on the ER stress pathways concerning pollen vulnerability to heat stress conditions.

ENDOPLASMIC RETICULUM STRESS PATHWAYS AND POLLEN DEVELOPMENT

The process of pollen development from meiocytes to microspores involves intense protein biosynthesis and trafficking of secretory proteins through ER and Golgi apparatus. Arabidopsis mutants for genes involved in the ER to Golgi trafficking exhibit male sterility phenotype (Conger et al., 2011; Tanaka et al., 2013; Deng et al., 2016). High requirement for secretory proteins in developing pollen triggers ER stress constitutively. This constitutive functioning of UPR has been

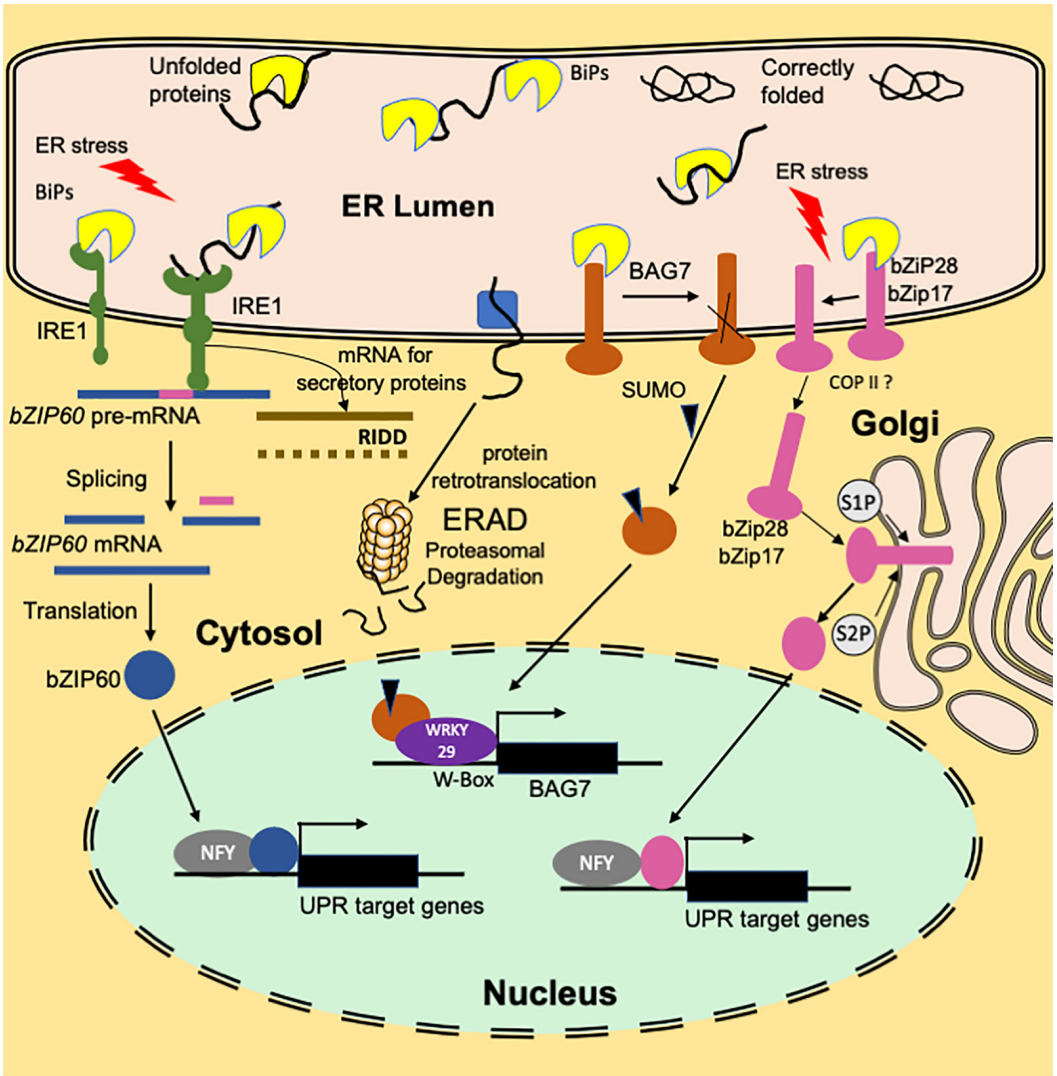

FIGURE 1 | An overview of the functioning of plant ER stress signaling in response to heat stress. A branch of the UPR signaling pathway involves ER transmembrane sensor, IRE1, that propagates the UPR signal from ER to the cytosol. Association with luminal BIP keeps IRE1 in an inactive monomer form. Under ER stress conditions, BIP dissociates from the sensor end of IRE1 to facilitate the folding of the accumulated unfolded proteins. The binding of unfolded proteins to the luminal domain of IRE1 triggers dimerization (or oligomerization) and activation of RNase activity that cleaves bZIP60(u) mRNA resulting in a spliced variant bZIP60(s). Translation of the spliced variant leads to the synthesis of active bZIP60 TF protein whose transport to nucleus activates the stress-responsive genes. Another function of IRE1 is IRE1-Dependent RNA Decay (RIDD) that involves degradation of ribosome-associated RNAs encoding secretory proteins. Dissociation of BIP from ER-anchored transcription factors bZIP28/17 results in their mobilization to Golgi. In the Golgi, these TFs are processed to bZIP17(p) and bZIP28 (p) by S1P and S2P proteases to release cytosolic facing domains that are further transported to the nucleus. In the nucleus, bZIP28/17 bind to ER stress response elements to upregulate the transcription of UPR genes. Another branch of UPR involves an ER-resident transcription factor, BAG7. BAG7 is involved in UPR in response to heat and cold stress conditions by acting as a co-chaperone to prevent the accumulation of unfolded proteins. Under heat stress conditions, BAG7 is sumoylated, released from ER by protease and then translocated to the nucleus where it interacts with WRKY29 to regulate *BAG7* and other chaperone expression.

reported to be essential for pollen development (Deng et al., 2016). This conclusion is also supported by the presence of spliced forms of bZIP60 in *Arabidopsis* male reproductive tissues under normal conditions (Iwata et al., 2008; Deng et al., 2016). The transcriptome-wide mining of male meiocytes and microspores from *Arabidopsis* plants growing under normal conditions reveals highly elevated expression of most of the ER stress and UPR component genes (**Figure 2**). Many of these genes show the highest expression levels in diploid meiocytes (microspore mother cells) undergoing meiosis. ER stress component genes highly expressed in the meiocytes belong to UPR, ERAD, and the ER autophagy programs. Among most conspicuous ER phagy genes are those encoding members of Respiratory Burst Oxidases Homolog (RBOH) family, which comprises 10 NADPH oxidase genes in Arabidopsis (Chang et al., 2016). Seven out of 10 gene members show highly elevated expression in meiocytes. The majority of the knockout mutants of *Arabidopsis* ER stress-related genes involved in UPR, ERAD,

TABLE 1 | A summary of Arabidopsis ER stress response genes and roles as determined by fertility phenotypes in gene knockout mutants.

Gene name	Arabidopsis Gene id	Gene Product Localization	Arabidopsis Mutant	Pollen Development Phenotype at Normal Temperature	Pollen Development Phenotype under Heat Stress	References
bZIP28; BASIC REGION/LEUCINE ZIPPER MOTIF 28	AT3G10800	ER membrane, Cytoplasm, Nucleus	*bzip28 bzip60* double mutant	Normal fertility	Reduced fertility, silique lengths in *bzip28 bzip60* double mutant plants were largely reduced compared with the wild-type plants	Zhang et al., 2017
bZIP60; BASIC REGION/LEUCINE ZIPPER MOTIF 60	AT1G42990	ER membrane and nucleus				
IRE1a; INOSITOL REQUIRING 1A	AT2G17520	ER membrane	*ire1a ire1b* double mutant	Normal viable pollen	Temperature-sensitive male sterility, improper deposition of pollen coat materials possibly due to tapetal defects, shortened siliques generally devoid of seeds	Deng et al., 2016
IRE1b; INOSITOL REQUIRING 1B	AT5G24360					
CNX1; CALNEXIN HOMOLOG 1	AT5G61790	ER membrane	*cnx1 crt1 crt2 crt3*	Diverse effect on pollen viability and pollen tube growth, leading to a significant reduction pollen mediated transmission		Vu et al., 2017
CRT1; CALRETICULIN1	AT1G56340	ER and vacuole membrane, secretory vesicles				
CRT2; CALRETICULIN2	AT1G09210	ER and vacuole membrane, secretory vesicles	*cnx1 cnx2 crt1 crt2 crt3*	Lethal—no pollen mediated transmission		
CRT3; CALRETICULIN3	AT1G08450	ER lumen				
BiP1; ER localized member of HSP70 family	AT5G28540	ER lumen and nucleus	*bip1 bip2* double mutant	Significant reduction in pollen tube growth activity		Maruyama et al., 2010
BiP2; LUMINAL BINDING PROTEIN	AT5G42020	ER lumen and nucleus	*bip1 bip2 bip3* triple mutant	Lethality of pollen due to defects in mitosis1, bicellular stage that contained one or two abnormal microspores with one nucleus		Maruyama et al., 2014
BiP3; HSP70 FAMILY PROTEIN	AT1G09080	ER lumen and nucleus				
SHD/HSP90; SHEPHERD, HEAT SHOCK PROTEIN 90-7	AT4G24190	ER lumen	*shd*	Defects in pollen—tube elongation or penetration into the style	Increased the severity of the defects	Ishiguro et al., 2002
ERdj2A/SEC. 63-1;J-Domain protein	AT1G79940	ER membrane	*aterdj2a-1, aterdj2a-2*	Defects in pollen germination but not pollen development		Yamamoto et al., 2008

(Continued)

TABLE 1 | Continued

Gene name	Arabidopsis Gene id	Gene Product Localization	Arabidopsis Mutant	Pollen Development Phenotype at Normal Temperature	Pollen Development Phenotype under Heat Stress	References
ERdj3A/TMS1; THERMOSENSITIVE MALE STERILE 1	AT3G08970	ER lumen	*tms1-1*	The fertility of tms1-1 plants was slightly affected, with some ovules in the lower part of the siliques unfertilized	Greatly retarded pollen tube growth in the transmitting tract, resulting in a significant reduction in male fertility	Yang et al., 2009
ERdj3B; J-Domain protein	AT3G62600	ER lumen	*erdj3b*	Normal flower development and fertility	Produced few seeds at high temperatures due to anther development defects, abnormal enlargement of tapetum cells with vacuolated and aborted microspores, defective pollen release from the anthers	Yamamoto et al., 2020
			atp58ipk aterdj3b	Defects in male gametophyte		Yamamoto et al., 2008
Sec62; protein with similarity to yeast Sec62p.	AT3G20920	ER membrane	*atsec62* (T-DNA and amiRNAi)	Smaller and round depressed pollens, defects in pollen development, smaller, aborted, and lesser number of siliques		Hu et al., 2020;
			atsec62	Aborted and mostly empty siliques, delayed anther and pollen development, less pollen released from mutant anthers and reduced pollen germination	Pollen hardly germinated	Mitterreiter et al., 2020
PDI9; PROTEIN DISULFIDE ISOMERASE 9	AT2G32920	ER lumen	*pdi9*	Normal viable pollen	Disruptions in the reticulated pattern of the exine and an increased adhesion of pollen grains	Feldeverd et al., 2020
PDI10; PROTEIN DISULFIDE ISOMERASE 10	AT1G04980	ER lumen	*pdi9 pdi10* double mutant	Normal viable pollen	Completely lost exine reticulation	
POD1; POLLEN DEFECTIVE in GUIDANCE 1	AT1G67960	ER lumen	*pod1*	Pollen tubes fail to target the female gametophyte, defective in micropylar pollen tube guidance leading to zygotic lethality		Li et al., 2011
UTR1, UDP-GALACTOSE TRANSPORTER 1	AT2G02810	ER and golgi membranes	*utr1 utr3* double mutant	Abnormalities in both male and female germ line development, haploid atutr1 atutr3 combination is a fully penetrant lethal mutation for the male gametophyte and is partially penetrant for the female gametophyte		Reyes et al., 2010
UTR3, UDP-GALACTOSE TRANSPORTER 3	AT1G14360					
STT3a; STAUROSPORIN AND TEMPERATURE SENSITIVE 3-LIKE A	AT5G19690	ER membrane	*stt3a-1 stt3b-1* double mutant	Gametophytic lethal		Koiwa et al., 2003

(Continued)

TABLE 1 | Continued

Gene name	Arabidopsis Gene id	Gene Product Localization	Arabidopsis Mutant	Pollen Development Phenotype at Normal Temperature	Pollen Development Phenotype under Heat Stress	References
SERK1; SOMATIC EMBRYROGENESIS RECEPTOR-LIKE KINASE 1	AT1G71830	ER and cell membrane	*serk1 serk2*	Completely male sterile due to a failure in tapetum specification, double mutant anthers lack development of the tapetal cell layer leading to the microspore abortion and male sterility		Albrecht et al., 2005; Colcombet et al., 2005
SAR1; SECRETION ASSOCIATED RAS 1	AT1G56330	ER-, COPII vesicle coat and golgi apparatus	*sar1b sar1bsar1c* double mutant	Malfunctioning tapetum, leading to male sterility. Microspores in sar1b pollen sacs started to degenerate. The plasma membrane (PM) of microspores in sar1b pollen sacs was detached from the cell wall, and at anther dehiscence, sar1b pollen sacs contained only a pile of cellular debris Microspores aborted at anther developmental stage 10, arrest of pollen development at Pollen Mitosis I		Liang et al., 2020
PDR2, PHOSPHATE DEFICIENCY RESPONSE 2	AT5G23630	ER membrane	*mia* mutants	Male gametogenesis impaired anthers (*mia*) show severe reduction in fertility. Mutant microspores fail to separate from tetrads and fragile pollen grains with an abnormal morphology and altered cell wall structure.		Jakobsen et al., 2005
AEP1; ASPARAGINYL ENDOPEPTIDASE 1	AT2G25940	Protein storage vacuole, Vacuole	β*vpe*	Abnormal degradation of the tapetum, incomplete pollen cytoplasm development, with few oil bodies and an indistinct generative cell. Some of the pollen grains were shrunken and abnormally shaped, immature pollen still contained numerous small vacuoles		Cheng et al., 2020
CEP1; CYSTEINE ENDOPEPTIDASE 1	AT5G50260	ER and vacuole	*cep1*	Aborted tapetal PCD, reduced male fertility due to impaired pollen development and abnormal pollen exine		Zhang et al., 2014
RBOHE; Riboflavin Synthase-Like Family Protein	AT1G19230	Multi pass membrane protein	*rbohe-2*	Delayed degeneration of tapetum, reduced pollen viability, abnormal pollen grain shape and exine layer		Xie et al., 2014
RBOHJ; RESPIRATORY BURST OXIDASE HOMOLOG J	AT3G45810	Multi pass membrane protein	*rbohH rbohJ* double mutant	Pollen tip growth severely impaired due to impaired ROS accumulation		Kaya et al., 2014
RBOHH; RESPIRATORY BURST OXIDASE HOMOLOG J	AT5G60010	Multi pass membrane protein				

and ER-autophagy processes show loss of fertility phenotypes in plants grown under non-stressed conditions (**Table 1**). These observations highlight the essential role of ER homeostasis in permitting normal pollen development.

ROS generated by these NADPH-dependent oxidases (Nox) complex control various developmental processes including programmed tapetal cell death and is an essential component of developing pollen–tapetum interaction network (Xie et al., 2014). Other ER autophagy-related genes showing a high level of expression in diploid meiocytes include Metacaspase 5 (MC5) and Bcl-2-associated athanogene 7 (BAG7). MC5 and BAG7 are involved in the programmed cell death (PCD) network. Metacaspases are cysteine proteases involved in PCD that are distantly related to animal caspases (Kørner et al., 2015). MC5 has been shown as a positive regulator of ER stress-induced PCD (Sobri and Zulfazli, 2016). AtBAG7 is a member of the Arabidopsis BAG family encoding plant homologs of mammalian regulators of apoptosis (Li et al., 2017b). These ER-localized proteins play a crucial role in maintaining UPR in response to external stresses (Li et al., 2017b; Nawkar et al., 2018).

ENDOPLASMIC RETICULUM STRESS DURING POST-MEIOTIC DEVELOPMENT AND POLLEN TUBE GROWTH

In post-meiotic stages of pollen developmental progression, several ER stress component genes show expression at the unicellular microspore and bicellular stage. These include genes encoding BiPs, both IRE1 isoforms (IRE1a and IRE1b), CRT, CNX, site-specific proteases (S1P and S2P), and NAC family of membrane-bound transcription factors.

Another noticeable feature of the developing pollen transcriptome is high-level expression of *ERAD* genes. Gene encoding UBC32, a ubiquitin-conjugating enzyme (E2) localized in the ER membrane, is expressed constitutively in the male meiocytes. Its expression, although much lower in microspores, shows an increase during the pollen development. The Arabidopsis genome contains five J-domain encoding genes (AtERdj3A, AtERdj3B, AtP58^{IPK}, AtERdj2A, and AtERdj2B) that encode Hsp40 family co-chaperones of Hsp70. The Arabidopsis Thermosensitive Male Sterile 1 (TMS1) encoding a J-domain protein identical to AtERdj3A plays a significant role in determining thermotolerance of pollen and vegetative tissues (Ma et al., 2015). Arabidopsis plants carrying a knockout mutation in TMS1 grown at 30°C were reported to show a significant decrease in male fertility resulting from retarded pollen tube growth in the stylar transmitting tract (Yang et al., 2009). Recently, Yamamoto et al. (2020) reported that a second ER-resident Arabidopsis J-domain protein, AtERdj3B, also plays a critical role in another development at elevated temperatures. The *erdj3b* mutant showed a significantly reduced seed set at an elevated temperature of 29°C. This reduced seed set phenotype could be rescued in mutants by introducing *ERDJ3B* gene expressing under its promoter. Interestingly, this defect could be rescued by overexpression of *ERDJ3A* gene regulated by the *ERDJ3B* promoter. The *erdj3b* mutant plants grown at 29°C revealed collapsed pollen with abnormalities in their pollen coats. Furthermore, the authors addressed whether pollen-coat abnormality in erdj3 at elevated temperatures is caused by the effect of the mutation in tapetal cells. The transformation of *erdj3b* mutant plants with *ERDJ3B* gene expressing under a tapetum-specific promoter led to partial suppression of the reduced seed set phenotype in mutant plants growing at 29°C. Interestingly, this study could not observe fertility defects in *erdj3a-1* or *erdj3a-2* mutants grown at 29°C. It was further proposed that among ER-localized three J proteins, heat stress-sensitive fertility defect results only from defective interaction of only EEdj3B with BiP. Three Hsp70 chaperone proteins (BiP1, BiP2, and BiP3) are localized in ER of *Arabidopsis thaliana* (Yamamoto et al., 2008; Ma et al., 2015). BiP1 and BiP2 are 99% identical and have been reported to be expressed ubiquitously. Interestingly, BiP1 and BiP2 expression is significantly upregulated in Arabidopsis meiocytes (**Figure 2**). BiP3 that shows less identity with the other two paralogs is expressed only under ER stress conditions (Maruyama et al., 2015). Maruyama et al. (2010) have shown that the *Arabidopsis bip1/bip2* double mutant shows normal pollen viability but retarded pollen tube growth both *in vitro* and *in vivo*. Since the secretion of cell wall proteins is crucial for pollen tube growth, the reduced BiP level led to retarded pollen tubes growth rates due to decline in protein translocation, protein folding, and ER QC activities.

Recently, Poidevin et al. (2020) used a Riboprofiling technique to unravel the effect of heat stress on transcriptome and translatome of mature and *in vitro* germinated Arabidopsis pollen grains. Riboprofiling (Ribo-seq) allows accurate comparison of cellular transcriptome with translatome (Ingolia et al., 2009; Hsu et al., 2016). Riboprofiling data showed transcriptional and translational level upregulation of DNA-J chaperones and ER stress related in germinated pollen, induced by the heat stress. These upregulated genes include Hsp70 BiPs (BiP1, BiP2, and BiP3), the DNAJ chaperones (ERDJ3A, ERDJ3B, and P581PK), Calnexin and Calreticulin (CNX1, CRT1a, and CRT1b), and proteins involved in ERAD pathway such as DER1 and DER2.1. The key transcription factors, bZIP28, bZIP60, and NF-YC2, are transcribed and translated in pollen tubes (Poidevin et al., 2020). The authors conclude from their data that the Arabidopsis pollen can respond to heat stress by enhancing the expression of thermotolerance genes. However, their data are based on transcriptome and translatome profiling of *in vitro* germinated pollen grains. The transcriptional repertoire of pollen tubes penetrating the stigma and styler tissues is very different from that of *in vitro* germinated pollen tubes with *in vivo* growing tubes expressing a substantially larger fraction of the genome (Qin et al., 2009). The analysis of transcriptome data of Arabidopsis pollen tubes growing *in vivo* shows default upregulation of expression of ER stress genes in the absence of external stress (**Figure 2**).

The transcriptional activity of ER stress-related genes in the pollen germinating *in vitro* is comparable to mature ungerminated pollen with no significant change in expression levels. However, the pollen tubes growing in the styler transmitting tissues show significant upregulation of several

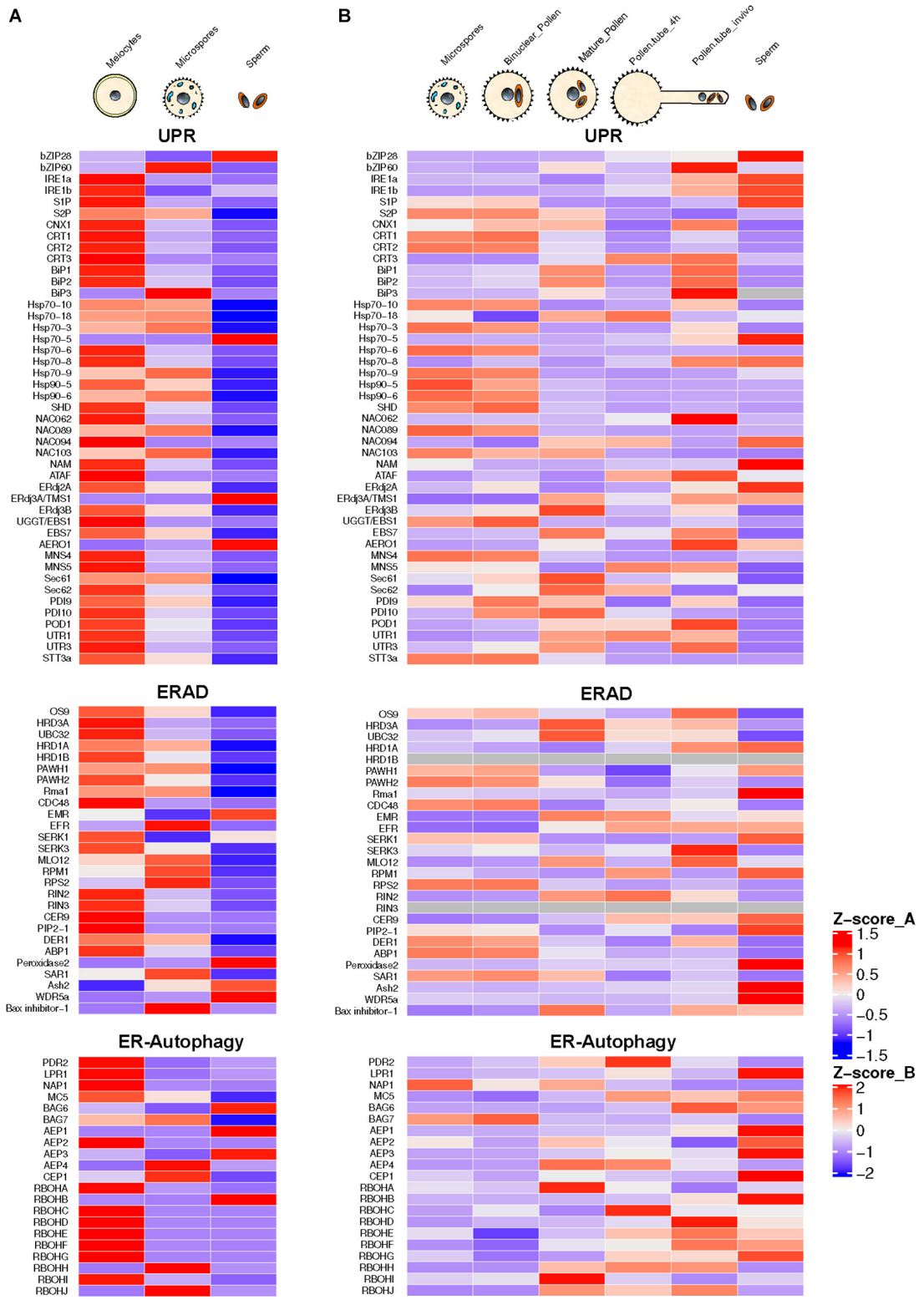

FIGURE 2 | Annotation and expression of ER stress component genes in Arabidopsis developing pollen, pollen tubes, and sperm cells. The data were compiled using the gene expression obtained from RNA-seq based analysis **(A)** or microarrays **(B)**. RNA-seq data sets from previously published literature were downloaded from the NCBI Sequence Read Archive database. For Microarray data, the expression values were downloaded from the Arabidopsis Heat Tree Viewer (http://arabidopsis-heat-tree.org/). The gray color in the heatmap denotes missing values. Full details available in **Supplementary File 1**.

ER stress component genes such as bZIP60, both isoforms of IRE1, S2P, NAC062, J-domain protein ERdj2a, and AERO1 (ER oxidoreductin 1). The observed stark differences in the gene expression patterns in pollen tubes growing *in vitro* or *in vivo* are predictable as pollen tubes attain about 135 μm length *in vitro* (Dickinson et al., 2018), while pollen tubes growing *in vivo* have to traverse stigma/style length. Rapidly growing pollen tubes show high trafficking with secretory vesicles providing membrane components (Campanoni and Blatt, 2007). High demands for secretory proteins likely trigger UPR in the pollen tubes. Interestingly, Arabidopsis mutants for many UPR genes display pollen germination and pollen tube growth defects (Ishiguro et al., 2002; Yamamoto et al., 2008; Maruyama et al., 2010; Deng et al., 2013).

The ERAD component genes expressing highly in pollen tubes include genes encoding OS9, HRD3A, PAWH1, Rma1, MLO12, CER9, EBS7, and Bax-inhibitor-1. High ER-associated protein degradation appears to be a hallmark of rapidly elongating pollen tubes. ERAD involves modifying target unfolded/misfolded proteins with ubiquitin, removal from the ER, followed by degradation by the cytoplasmic 26S proteasome (Preston and Brodsky, 2017). AtOS9 is an Arabidopsis homolog of mammalian ER luminal lectin OS9 with binding specificity for asparagine-linked glycan on misfolded proteins. An interesting feature of the pollen tube transcriptome is a high expression of ER phagy-related genes encoding BAG6, MC5, AEP1, RBOHH, and RBOHJ (Respiratory Burst Homologs). RBOHH and RBOHJ encode NADPH oxidases containing Ca^{2+} binding EF-hand motifs and possessing Ca^{2+}-induced ROS production activity. While Arabidopsis single mutants, *rbohH* and *rbohJ*, attain pollen tube growth comparable to wild type, the double mutant showed severe impairment of pollen tube growth (Kaya et al., 2014). Also, *in vitro* grown pollen tubes of the *rbohH* and *rbohJ* double mutants rupture easily (Boisson-Dernier et al., 2013).

EXPRESSION OF ER STRESS-RELATED GENES IN SPERM CELLS

Pollen is largely made up of vegetative cell that forms a pollen tube, which acts as a conduit to transmit male germline, the non-motile sperm cells, into the female gametophyte to execute double fertilization, a defining feature of flowering plants (Singh and Bhalla, 2007). The male germline is initiated by asymmetric division of the microspore leading to the formation of much smaller generative cell enveloped within the larger vegetative cell. The generative cell divides once again to produce two sperm cells required for double fertilization (Russell and Jones, 2015). In the mature pollen, sperm cells may comprise much less than 1% of the pollen volume (Russell and Strout, 2005; Russell and Jones, 2015). For a long time, the inconspicuous generative and sperm cells were considered passive carriers of male genetic lineage. However, it was later shown that both these cells are largely transcriptionally and translationally distinct from much larger vegetative cells (Xu et al., 1999; Singh and Bhalla, 2007). Transcriptomic analysis using either microarrays or RNA-seq approaches have highlighted the highly divergent nature of gene expression in generative and sperm cells compared to that of the vegetative cells (Okada et al., 2007; Singh et al., 2008; Russell et al., 2012; Russell and Jones, 2015). A survey of ER stress-related gene expression in Arabidopsis sperm isolated from mature pollen reveals constitutive expression of several ER stress-related genes relating to UPR, ERAD, and ER phagy with remarkably high expression of UPR genes encoding bZIP 28, ERdj3A/TMS1, and AERO-1. ERAD-related genes showing significant expression in sperm cells include SERK1, Peroxidase 1, Ash2, and WDR5a. ER autophagy-related genes with significant expression in sperm cells include BAG6, AEP1, AEP3, and RBOHB. While the data from microarray and RNA-seq experiments cannot be compared directly, there appears to be a good overall cross-platform concordance particularly among genes showing high expression levels. The quantitative expression pattern of ER stress related in sperm cells is quite distinct from that of total pollen (**Figure 2**). However, it remains an open question whether the pattern of ER stress gene expression show further changes in sperm cells following heat stress or due to pollen tube growth in female tissues.

FUTURE PERSPECTIVES

Despite an increasing number of publications on plant reproduction and ER stress response in recent years reporting intriguing findings, there remain open questions about the activation of ER stress and the role this response plays in protecting pollen development and pollination processes from detrimental effects of excessive heat exposure. The cellular trigger for high constitutive activity of ER stress-related genes in diploid meiocytes warrants further investigation. An intriguing possibility is the potential role of cellular hypoxia in triggering UPR in meiocytes. Studies on animal systems have revealed that the activation of UPR is an adaptive response to hypoxic stress (Bartoszewska and Collawn, 2020). Earlier, Kelliher and Walbot (2012) have proposed that meiotic fate in the archesporial cells in the immature anthers is triggered by hypoxia. Future investigations can focus on the potential crosstalk between hypoxia conditions in the anther cavity and triggering of UPR in resident meiocytes.

It has been recognized that pollen development is one of the situations where high demands for secretion triggers ER stress under normal conditions without externally imposed stress (Howell, 2017). Thus, it can be postulated that the protein homeostasis maintained by enhanced protein folding capacity allows normal pollen development to proceed. There is no evidence for the direct interaction between the pollen ER stress response and the transcription factors and downstream pathways linked to regulation of cell fate determination and developmental progression. The constitutive ER stress response leading to near-capacity functioning of the ER protein folding and trafficking machinery likely diminishes ER's adaptive capacity to adjust to external stresses, resulting in the high sensitivity of pollen to heat stress events. Thus, future studies can be expected to focus on

investigating the overexpression of key ER signaling components and chaperones as a tool to enhance pollen thermotolerance. This would open new opportunities for engineering crop plants that can offer yield stability in the face of increased frequency of heat waves with crops getting exposed to extreme temperature events.

AUTHOR CONTRIBUTIONS

NL analyzed the sequencing data. MS conceived the research. MS and PB drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.661062/full#supplementary-material

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Exogenous Methyl Jasmonate Improves Heat Tolerance of Perennial Ryegrass Through Alteration of Osmotic Adjustment, Antioxidant Defense, and Expression of Jasmonic Acid-Responsive Genes

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Perennial ryegrass (*Lolium perenne* L.) is an important cool-season grass species that is widely cultivated in temperate regions worldwide but usually sensitive to heat stress. Jasmonates (JAs) may have a positive effect on plant tolerance under heat stress. In this study, results showed that exogenous methyl jasmonic acid (MeJA) could significantly improve heat tolerance of perennial ryegrass through alteration of osmotic adjustment, antioxidant defense, and the expression of JA-responsive genes. MeJA-induced heat tolerance was involved in the maintenance of better relative water content (RWC), the decline of chlorophyll (Chl) loss for photosynthetic maintenance, as well as maintained lower electrolyte leakage (EL) and malondialdehyde (MDA) content under heat condition, so as to avoid further damage to plants. Besides, results also indicated that exogenous MeJA treatment could increase the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), thus enhancing the scavenging ability of reactive oxygen species, alleviating the oxidative damage caused by heat stress. Heat stress and exogenous MeJA upregulated transcript levels of related genes (*LpLOX2*, *LpAOC*, *LpOPR3*, and *LpJMT*) in JA biosynthetic pathway, which also could enhance the accumulation of JA and MeJA content. Furthermore, some NAC transcription factors and heat shock proteins may play a positive role in enhancing resistance of perennial ryegrass with heat stress.

Keywords: perennial ryegrass, heat tolerance, methyl jasmonic acid, gene expression, growth regulator

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is native to Asia, Europe, and northern Africa and becomes a crucial grass species widely cultivated in temperate regions as high-quality forage and turfgrass (Yu et al., 2015). It has many desirable agronomic qualities, such as long growing season with strong adaptability, rapid establishment as a primary turf species, and high forage yield under suitable

environments and conditions (Casler et al., 1976). However, it is reported that perennial ryegrass is generally sensitive to heat stress, so that it does not withstand hot weather, especially in warmer regions (Yu et al., 2013; Zhang et al., 2017). High temperature always affects the balance of growth and development by accelerating and redirecting metabolic processes (Wang et al., 2017; Li et al., 2020). Furthermore, the regions planting perennial ryegrass even experience high temperature over 38°C in summer, which greatly exceeds the temperature of these cool-season grasses for growth and reduces the forage yield and lawn quality (Wang et al., 2015).

Plant adaptation to adversity is controlled by both genetic and hormonal factors. To date, the use of plant hormones such as jasmonic acid (JA) to improve the growth of turfgrass and enhancing resistance is increasingly concerning in recent years. JA and its derivatives including methyl jasmonic acid (MeJA) together are called jasmonates (JAs). Many studies have shown that JA substances have a wide range of physiological effects on plant growth, development, and abiotic tolerance, such as inhibiting plant growth and pollen germination, promoting leaf senescence, and fruit maturation (Sembdner and Parthier, 1993; Creelman and Mullet, 1995; Arooran et al., 2019). Besides, as an endogenous signal molecule, it is involved in plant resilience to mechanical injury, pests and diseases, drought, high salinity, low temperature, and other conditions (Jiang et al., 2017). Previous study has shown that the increasing level of MeJA induces a JA-dependent defense response relating to the enhancement of secondary metabolism (Cheong and Yang, 2003). When plant is injured, the amount of MeJA increases significantly, which promotes the biosynthesis of some substances related to environmental stress (such as hormone and proline) and induces the expression of a series of genes related to stress tolerance, thus enhancing the plant resistance (Wasternack, 2014). JAs are derived from α -linolenic acid (α-LeA, 18:3), whose formation in plastids is catalyzed by fatty acid desaturase (*FAD*) and phospholipase A1 (*PLA*). Subsequently a-LeA is converted to cis-(+)-12-oxophytodienoic acid (*OPDA*) by enzymes including lipoxygenase (*LOX*), allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*), and 12-oxo-phytodienoic acid reductase (*OPR*), then undergoes three rounds of β-oxidation to form JA (Turner et al., 2002). The expression of several key enzyme genes in the JA synthesis pathway has a great impact on JA level in plants. In addition, trauma and other stress factors that induce the JA response could also promote the expression of these genes, and the activation of these gene transcription occurs at the site of JA synthesis (Dhondt et al., 2000; Halitschke and Baldwin, 2003; Stenzel et al., 2003; Matsul, 2006; Tomoyuki et al., 2008).

NAC genes play various roles as transcription factors in multiple plants during growth and developmental processes, as well as diverse defense responses (Olsen et al., 2005). It has been reported that overexpression of *OsNAC10* enhanced rice (*Oryza sativa* L.) resistance to drought, high salinity, and low temperature significantly (Jeong et al., 2010; Sun et al., 2013) and also responded to salicylic acid and MeJA treatment (Zhou et al., 2013; Liang et al., 2014). For a cell to survive under heat stress, it is quite important to prevent upholding the proteins in their functional conformation and foreign proteins collection (Nakamoto and Vígh, 2007). Heat shock proteins (Hsps) are reported to be proteins that are usually expressed when responding to stress conditions (Guo et al., 2014). Especially, as an essential regulator of protein, the Hsp70 has the tendency to maintain internal cell stability, protecting the injured organisms by enhancing the stability of mRNA and translating preferentially under heat condition (McGarry and Lindquist, 1985). Previous studies showed that overexpression of Hsp70 gene was leading to increasing the tolerance of *Arabidopsis thaliana* (Shinya et al., 2014), *Capsicum annuum* (Guo et al., 2014; Usman et al., 2015), *Zea mays* (Ristic et al., 1991), and *O. sativa* (Wang et al., 2014) under heat and drought stress.

Although previous evidence indicated important roles of MeJA in regulating abiotic stress in plants, the function of MeJA in relation to heat tolerance remains unclear in perennial ryegrass. This study is aimed at investigating the effects of exogenous application of MeJA on osmotic adjustment, antioxidant defense, and the expression of JA-responsive genes after heat stress. The result would be useful to reveal possible MeJA-mediated mechanisms of heat tolerance and provides an option for alleviating heat damage in perennial ryegrass.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Perennial ryegrass cultivar "Esquire" seeds were provided by DLF SEED A/S Company in China office. "Esquire" seeds were germinated in plastic pots (20 cm length, 15 cm width, and 10 cm height) filled with quartz sand and distilled water in a growth chamber at 20/15°C (day/night). The humidity and illumination were set to 70% relative and 750 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, respectively. After 7-day germination, seedlings were planted in Hoagland's nutrient solution (Hope Bio-Technology Co., Ltd., Qingdao) for another 30 days. The pots' positions were rearranged daily in order to reduce the impact of the environment.

Treatments and Experimental Design

For exogenous MeJA concentration confirmation, a preliminary experiment was carried out with different MeJA concentrations (0, 20, 40, 60, 80, 100, 150, and 200 $\mu mol \cdot L^{-1}$) pretreatment on perennial ryegrass before heat stress. The results demonstrated that 100 $\mu mol \cdot L^{-1}$ MeJA had the most favorable effect on heat tolerance of perennial ryegrass under high-temperature stress, including relative water content (RWC), electrolyte leakage (EL), and total chlorophyll (Chl) content (**Supplemental Figure 1**). Accordingly, four treatments were designed for perennial ryegrass as follows: (1) Control check (labeled as CK), Hoagland's nutrient solution with 20/15°C (day/night) temperature for 21 days; (2) Only MeJA pretreatment (labeled as CK + MeJA), plants were first pretreated with Hoagland's nutrient solution containing 100 μmol/L MeJA with 20/15°C (day/night) for 7 days and then treated with Hoagland's nutrient solution with 20/15°C (day/night) for 14 days; (3) High-temperature treatment (labeled as H), Hoagland's nutrient solution with 20/15°C (day/night) temperature for 7 days, and then treated

with 38/30°C (day/night) high-temperature for 14 days; (4) MeJA pretreatment and high-temperature treatment (labeled as H + MeJA), plants were first pretreated with Hoagland's nutrient solution containing 100 μmol/L MeJA with 20/15°C (day/night) for 7 days and then treated with 38/30°C (day/night) high-temperature for 14 days. Each treatment had five biological replications. Samples were taken at 0 (labeled 0 day) and 14 (labeled 14 days) days after treatment with high temperature for all groups to analyze phenotypic and physiological variations. Samples from 0, 3, 6, 9, 12, and 24 h (labeled 0, 3, 6, 9, 12, and 24 h) after treatment with high temperature were used to analyze gene expression.

Measurement

For RWC, the 0.2-g fresh leaves were taken as fresh weight (FW). Then, leaves were placed in distilled water at 4°C for 24 h and weighed to record saturated weight (SW). Subsequently, dried samples at 105°C for 30 min, followed by drying at 75°C for 48 h, and weighed to get dry weight (DW). RWC was calculated by the formula: $\mathrm{RWC}\,(\%) = (\mathrm{FW} - \mathrm{DW}) / (\mathrm{SW} - \mathrm{DW}) \times 100\%$. The total Chl contents were extracted by incubating 0.1 g fresh leaves with a 10-ml solution of 80% acetone:95% methanol (1:1, V/V) in the dark until the leaves became colorless. The light absorption values of Chl a and Chl b were measured at 645 and 663 nm, respectively. The total Chl contents were calculated according to the following formula: $\mathrm{Chl}\,(\mathrm{a}+\mathrm{b})\ (\mathrm{mg/g}) = (20.2 \times \mathrm{OD645} + 8.02 \times \mathrm{OD663})/(\mathrm{DW} \times 1,000)$ (He and Gan, 2002). EL was determined by using a conductivity meter (Model 32, Yellow Springs Instrument Company). The method was clearly described in the study of Blum and Ebercon (1981).

For superoxide dismutase (SOD) activity, 1.5 ml of reaction solution [50 mM phosphate-buffered saline (PBS) containing 195 mM methionine, 60 μM riboflavin, and 1.125 mM nitro blue tetrazolium (NBT)] was mixed with 0.05 ml of enzyme extract, and then the reaction solution was placed under 800 μmol m^{-2} s^{-1} photosynthetically active radiation for 6 min. The absorbance was recorded at 560 nm (Giannopolitis and Ries, 1977). For peroxidase (POD) and catalase (CAT) activities, 0.05 ml of enzyme extract was added into 1.5 ml of reaction solution for POD determination or 1.5 ml of reaction solution for CAT assay (Chance and Maehly, 1955). For ascorbate peroxidase (APX) activity, the method was clearly described in the study of Nakano and Asada (1981). Changes in absorbance were monitored at 460, 240, or 290 nm every 10 s for 1 min for POD, CAT, or APX, respectively. To analyze malondialdehyde (MDA) content and antioxidant enzyme activities, 0.15 g of leaves was ground in 2 ml of 50 mM cold PBS (pH = 7.8). For MDA determination, 0.5 ml of supernatant was mixed with 1.0 ml of reaction solution and then incubated at 95°C for 15 min. The reaction solution was centrifuged at 8,000 *g* for 10 min, and the absorbance was measured at 532 and 600 nm (Dhindsa et al., 1981). The activity unit (U) of POD, CAT, and APX is defined as 0.01 changes within the first 1 min. Protein content was determined by using the method of Bradford (1976).

The plant JA ELISA Kit (MiBlo Inc., Shanghai, China) was used for the determination of JA and MeJA concentrations in plant tissue homogenates and other biological fluids. For both JA and MeJA content, measuring and setting the standard curve according to the kit procedures firstly, the linear regression equation of the calibration curve is calculated by using the concentration of the standard substance and the OD value. Here, 0.1 g of fresh leaves was ground with 0.01 $\mathrm{m{\cdot}ML^{-1}}$ cold PBS (pH = 7.2–7.4) to get the supernatant after being centrifuged at 2000–3000 $\mathrm{r{\cdot}min^{-1}}$ for 20 min at 4°C. Then, supernatant was treated following the manufacturer's instructions, and the absorbance was measured at 450 nm. The OD value of the sample is substituted into the equation to calculate the sample concentration, and then multiplied by the dilution factor, which is the actual concentration of the sample.

Identification of Related Genes in Perennial Ryegrass

In this study, four genes including *LpNAC022*, *LpNAC037*, *LpNAC045*, and *LpNAC054* from the NAC transcription factor families were selected, which play an important role in abiotic and biological stress response. Primer pairs for each gene were selected based on a previous published study (Nie et al., 2020). Another four genes from essential regulator of protein Hsp70 including *LpHsp70-009*, *LpHsp70-010*, *LpHsp70-015*, and *LpHsp70-020* were selected based on a previous study in our group, which is significant enhancing expression under heat stress condition. Furthermore, four genes in JA signal pathway including *LpLOX2*, *LpAOC*, *LpOPR3*, and *LpJMT* were identified, and the gene sequences were acquired from NCBI database of homologous genes including rice, wheat (*Triticum aestivum* L.), and corn. Alignment based on blast search with the conserved sequence and whole genome sequence of perennial ryegrass [downloaded from the Perennial Ryegrass Genome Sequencing Project[1], (Byrne et al., 2015)] was conducted, and primer pairs

[1] http://185.45.23.197:5080/ryegrassgenome

TABLE 1 | Primer information for all selected perennial ryegrass genes.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
LpAOC	CTACGAGGCCATCTACAGCA	AGGGGAAGACGATCTGGTTG
LpOPR3	AACCAAAGCAACCTTTCGCC	TTCAACCTGTTCCTGCGTCC
LpLOX2	GCACCATCGATGAGCGAAAC	ACCCCTGGCTCTGAAAATGG
LpJMT	CTTCGACCTCTCCCACCG	CTTCAACCTGTTCCTGCGTC
LpNAC022	ACGTTCCAAATAGGCAGTGG	TTCCCGTGCACCATGTATAA
LpNAC037	TCCTTTCGACGGAGCTTCTT	TCGTTATTAGCCTTGCACGC
LpNAC045	GCCGCCTCTACAACAAGAAG	TCGATGTCTGAGGAATCGTG
LpNAC054	TTGGGGAGAAGGAGTGGTTC	TTGCCGGAGTAGAAGACGAG
LpHSP70-009	GGTGTACGAGGGTGAGAGAG	CCTCCTGCACCATCTTCTCA
LpHSP70-010	CCTGCTGCTTGATGTCACTC	GGATGAGTACACCAGGCTGT
LpHSP70-015	GATCGTCGTCAAGCACAAGG	GCTGCGAGTCGTTGAAGTAG
LpHSP70-022	CATCATCAACGAGCCCACTG	ACCTCAAAGATGCCCTCCTC
eIF4A	AACTCAACTTGAAGTGTTG GAGTG	AGATCTGGTCCTGGAAAGA ATATG

for each gene were designed using the online tool Primer3[2]. All primer information was listed in **Table 1**.

Total RNA Isolation and Quantitative Real-Time PCR Expression Analysis

Direct-zol™ RNA MiniPrep Kit (Zymo Research Co.) was used for the extraction of total RNA according to the manufacturer's instruction manual. Genomic DNA was eliminated using DNase I (G Zymo Research Co.). A NanoDrop ND-2000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, United States) was used to determine RNA concentration, purity, and integrity, followed by 1% agarose gel electrophoresis. iScript™ cDNA (Bio-Rad Laboratories Inc.) was used for RNA reverse transcription following the manufacturer's instructions. The quantitative RT-PCR technique was used to validate the expression of genes. A 10-μl mixture contained 5 μl of abm® EvaGreen 2X qPCR Master Mix (Applied Biological Materials Inc., Canada), 1.5 μl of synthesized cDNA product, 0.3 μl of each primer, and 2.9 μl of ddH_2O. The following qRT-PCR reaction protocol was used: an enzyme activation step at 95°C for 10 min with one cycle, denaturation at 95°C for 15 s, and anneal/extension at 60°C for 60 s, for a total of 35 cycles. To verify the specificity of each primer, Tm and melting-curve analysis was obtained (65 to 95°C with fluorescence measured every 0.5°C increment). Technical samples and biological samples were used for all qRT-PCRs (Nie et al., 2020). The relative gene expression level was analyzed according to the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), and eIF4A was set as the reference gene to standardize the expression data (Yan et al., 2014). SPSS 19.0 (IBM, Armonk, NY, United States) was employed to the analysis of variance (ANOVA) at the 0.05 probability level. Data were transformed to meet normality and homogeneity of variance. Fisher's least significant difference (LSD) was used to determine differences between groups. Microsoft Excel 2007 (Microsoft, Redmond, WA, United States) was employed to generate the histograms used for data chart.

[2]http://primer3.ut.ee/

RESULTS

Effects of Exogenous Methyl Jasmonic Acid on Leaf Relative Water Content, Electrolyte Leakage, and Chlorophyll Content of Perennial Ryegrass After Heat Stress

The morphological appearance of perennial ryegrass was shown in **Figure 1A**. Leaves turned yellow and wilting after 14 days of heat stress in H and H + MeJA group, but MeJA-pretreated

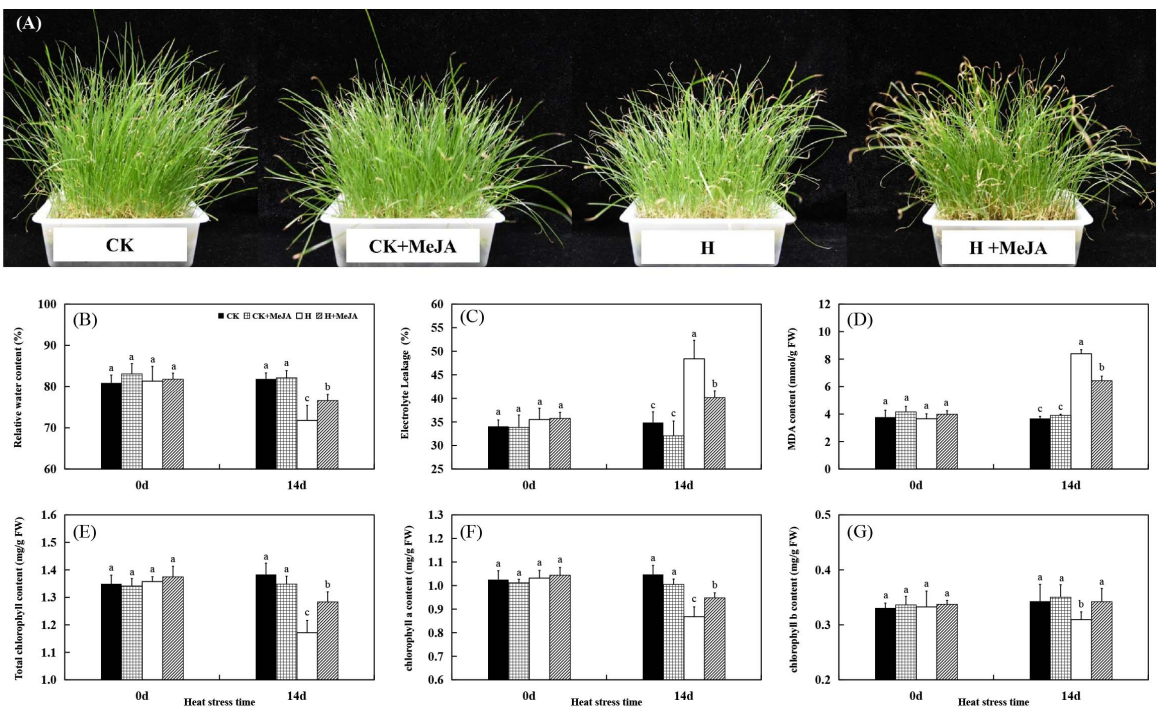

FIGURE 1 | Effects of exogenous methyl jasmonic acid (MeJA) on phenotypic changes **(A)**, relative water content (RWC) **(B)**, electrolyte leakage (EL) **(C)**, malondialdehyde (MDA) content **(D)**, total chlorophyll content **(E)**, chlorophyll a content **(F)**, and chlorophyll b content **(G)** of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean ($n = 5$). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$). CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

plants in the H + MeJA group were greener than untreated plants. Exogenous MeJA pretreatment had no significant effects on all detected physiological indicators under well-temperature conditions (heat stress at 0 day). After 14 days of heat treatment, leaf RWC decreased while MeJA-pretreated plants exhibited significantly higher RWC than untreated plants (**Figure 1B**). Also, after 14 days of heat stress, the EL of MeJA-pretreated plants in the H + MeJA group showed 7.81% lower than

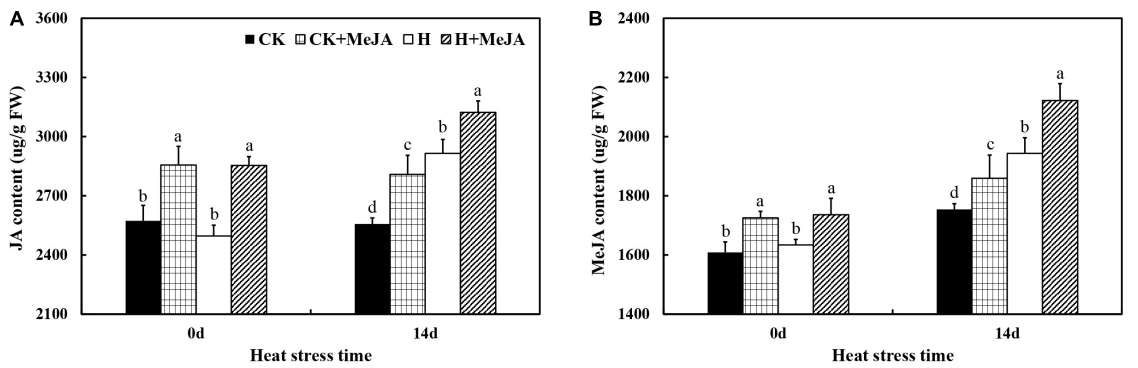

FIGURE 2 | Effects of exogenous methyl jasmonic acid (MeJA) on JA content **(A)** and MeJA content **(B)** in leaves of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean (n = 5). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$) on a given day. CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

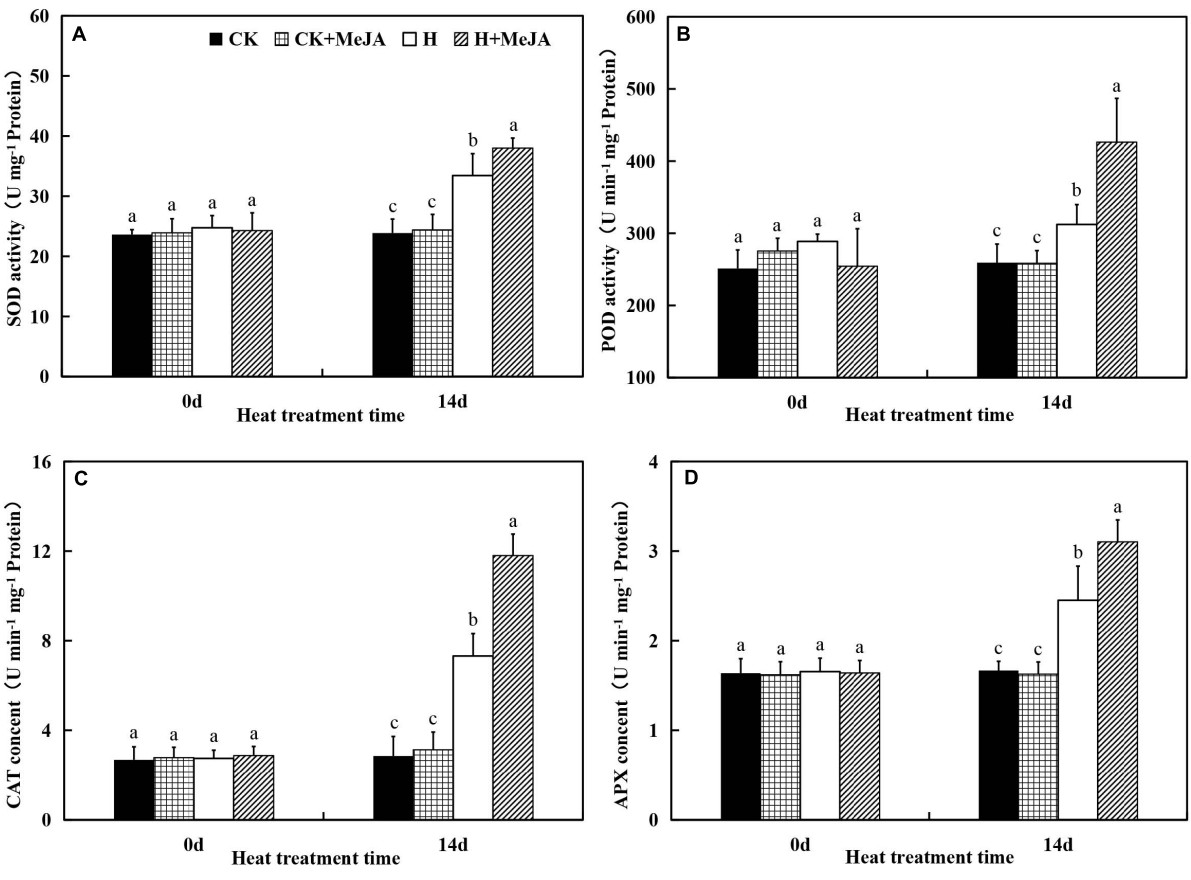

FIGURE 3 | Effects of exogenous methyl jasmonic acid (MeJA) on (**A**) superoxide dismutase (SOD), (**B**) catalase (CAT), (**C**) peroxide (POD), and (**D**) ascorbate peroxidase (APX) activities in leaves of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean (n = 5). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$) on a given day. CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

untreated plants in the H group (**Figure 1C**). The 14 days of heat stress treatment increased MDA content, but exogenous MeJA application maintained significantly lower MDA content in the H + MeJA group than untreated plants in the H group in response to high temperature (**Figure 1D**).

For Chl content, the CK group maintained no significant difference with plant in well-temperature condition in the CK + MeJA group, while heat induced significant degradation of total Chl, Chl a, and Chl b contents. However, in the H + MeJA group, MeJA-pretreated plants exhibited observably higher total Chl, Chl a, and Chl b contents than those in the H group at 14 days of heat stress (**Figures 1E–G**). Exogenous MeJA application had a much significant effect on total Chl b content in leaves under heat condition in the H + MeJA group.

Effects of Exogenous Methyl Jasmonic Acid on Endogenesis Jasmonic Acid and Methyl Jasmonic Acid of Perennial Ryegrass

After 7 days of exogenous MeJA application, it significantly increased the contents of endogenous JA and MeJA in leaves (**Figure 2**). After 14 days of high-temperature treatment, the content of endogenous JA in leaves increased about 13.99% in the H group compared with that in the CK group, while the highest endogenous content of JA was in the H + MeJA group after 14 days of heat stress. Also, the results showed that the endogenous JA content kept stable from 0 day to 14 days without heat stress treatment. For MeJA content, both heat stress and exogenous MeJA treatments could cause an accumulation of endogenous MeJA in leaves. Besides, compared with the MeJA content at 0 day, the endogenous MeJA content increased at 14 days in all groups, which was different with the JA biosyntheses and accumulation tendency.

Effects of Exogenous Methyl Jasmonic Acid on Antioxidant Defense of Perennial Ryegrass After Heat Stress

After 7 days of exogenous MeJA application, it did not have significant effects on SOD, CAT, POD, and APX activities. However, heat stress upregulated activities of SOD, CAT, POD, and APX in both H group and H + MeJA group after 14 days of high-temperature treatment (**Figure 3**). SOD activity

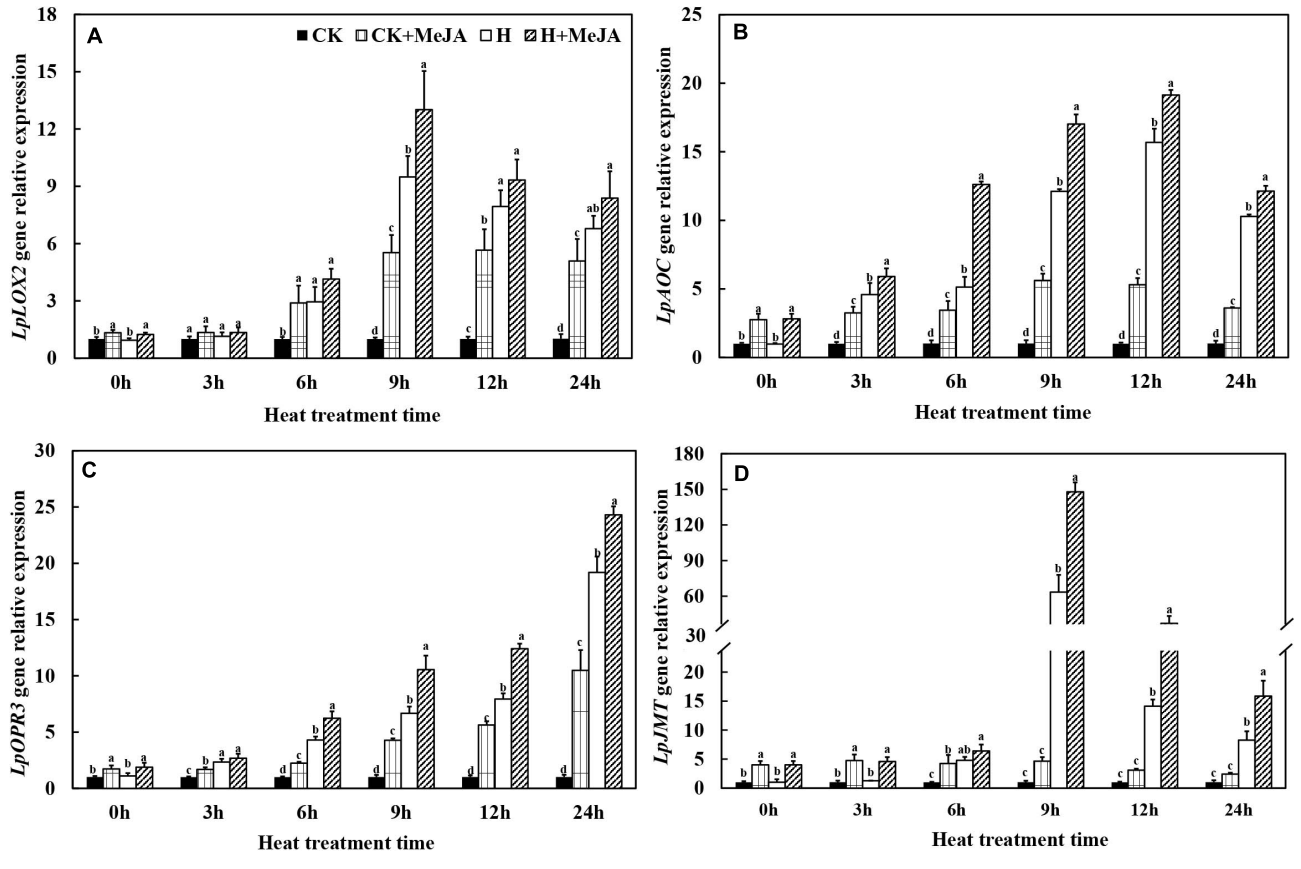

FIGURE 4 | Effects of exogenous methyl jasmonic acid (MeJA) on relative expression of JA biosynthesis-related genes *LpLOX2* **(A)**, *LpAOC* **(B)**, *LpOPR3* **(C)**, and *LpJMT* **(D)** in leaves of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean (n = 3). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$) on a given day. CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

significantly increased about 41.25% in the H group compared with the CK group after 14 days of heat stress, while plants in the H + MeJA group had significantly higher SOD activities than MeJA-untreated plants at 14 days after high-temperature treatment. Similar tendency was also found in POD, CAT, and APX activities. The results indicated that exogenous MeJA could enhance the antioxidant defense ability of plant leaves to resist the heat damage in perennial ryegrass.

The Expression of Jasmonic Acid Biosynthesis Pathway-Related Genes in Perennial Ryegrass

After 7 days of exogenous MeJA pretreatment, the expressions of *LpLOX2*, *LpAOC*, *LpOPR3*, and *LpJMT* genes were improved at 0 h (**Figure 4**). Heat stress significantly upregulated transcript levels of these four genes in leaves of the H and H + MeJA groups, and the expression peak values of *LpLOX2*, *LpAOC*, and *LpJMT* were at 9 or 12 h (**Figures 4A,B,D**). A different expression profile was found in *LpOPR3*, which showed a continuous increased gene expression within 24 h when exposed to high-temperature condition in the H and H + MeJA groups (**Figure 4C**). Furthermore, the expression of *LpJMT* gene increased about 62 times in the H group than CK and about 32 times in the H + MeJA group than the CK + MeJA group after 9 h of high-temperature treatment, indicating a heat-induced gene involved in MeJA biosynthesis and accumulation.

The Expression of NAC Transcription Factor and HSP-70 Protein Genes Responding to Heat and Methyl Jasmonic Acid Treatment

At 0 h, the exogenous MeJA had no significant effect on gene expression levels of *LpNAC037* and *LpNAC054*, while the expression of *LpNAC022* and *LpNAC045* increased after 7 days of exogenous MeJA pretreatment (**Figure 5**). Heat stress upregulated the gene expression of *LpNAC037*, *LpNAC045*, and *LpNAC054* in leaves in the H and H + MeJA groups, and the peak value appeared at 12 h. However, the expression profile of *LpNAC022* gene was very different from the other three genes, and either exogenous MeJA or heat treatment could enhance the expression from 3 h. Overall, there was no obviously superimposed effect on MeJA- or heat-induced expression

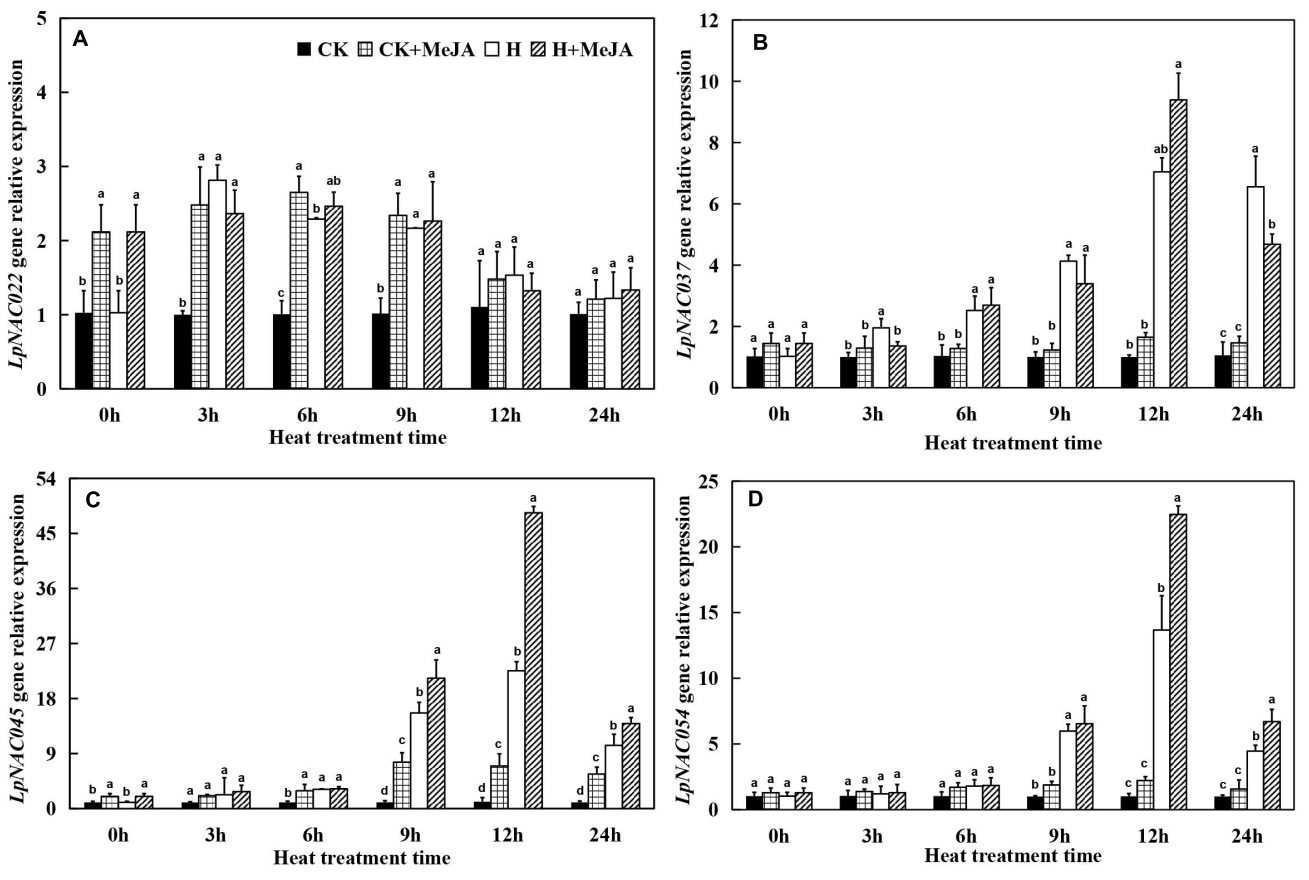

FIGURE 5 | Effects of exogenous methyl jasmonic acid (MeJA) on relative expression of *LpNAC022* **(A)**, *LpNAC037* **(B)**, *LpNAC045* **(C)**, and *LpNAC054* **(D)** in leaves of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean (n = 3). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$) on a given day. CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

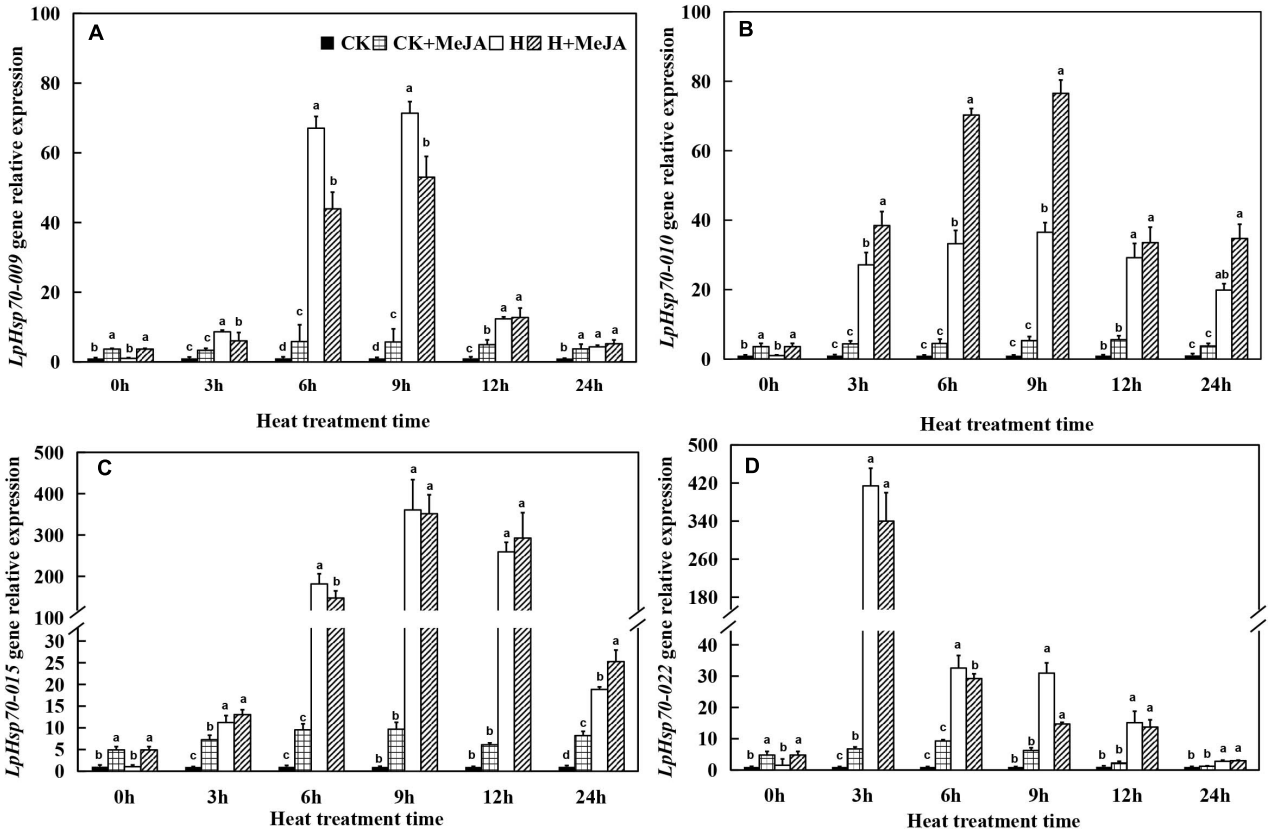

FIGURE 6 | Effects of exogenous methyl jasmonic acid (MeJA) on relative expression of *LpHsp70-009* **(A)**, *LpHsp70-010* **(B)**, *LpHsp70-015* **(C)**, and *LpHsp70-022* **(D)** in leaves of perennial ryegrass after heat stress. Vertical bars indicate ± SE of the mean (n = 3). No common letter above bar indicates a significant difference by least significant difference (LSD) ($P < 0.05$) on a given day. CK, control check; CK + MeJA, control check was pretreated with 100 μmol/L of MeJA; H, heat stress; H + MeJA, heat-stressed plants pretreated with 100 μmol/L of MeJA.

on *LpNAC022*, while *LpNAC037*, *LpNAC045*, and *LpNAC054* showed a significantly high expression in the H + MeJA group.

For *LpHsp70* genes, although exogenous MeJA pretreatment could enhance the expression of these four genes at 0 h, it was obviously induced by high-temperature treatment when heat stress was applied (**Figure 6**). Among them, *LpHsp70-009*, *LpHsp70-010*, and *LpHsp70-015* genes in both the H and H + MeJA groups showed significantly high expression at 6 or 9 h, while *LpHsp70-022* gene showed a rapid response to high temperature and maximum expression values at 3 h.

DISCUSSION

Plants have evolved multiple acclimation mechanisms to survive environmental stresses such as heat stress. The accumulation of JAs is one of the mechanisms of plant resistance to abiotic stress (Wasternack and Hause, 2013). Previous studies have found that exogenous MeJA could increase Chl content and improve the tolerance of cowpea (*Vigna sinensis*) and *Brassica napus* (Ahmadi et al., 2018) under salinity stress (Sadeghipour, 2017). Besides, it could also enhance the ability for water stress resistance in cauliflower (*Brassica oleracea* L.) (Wu et al., 2012) and strawberry (*Fragaria x ananassa*) (Jordi and Leon, 2016). Furthermore, MeJA improved drought tolerance of soybean plants as a potential growth regulator (Mohamed and Latif, 2017). In this study, exogenous MeJA significantly increased the RWC of perennial ryegrass, while it decreased the EL under heat stress, which indicated a positive effect on perennial ryegrass to resistant high-temperature stress. At the same time, the exogenous MeJA contributed to maintaining the Chl content stability of perennial ryegrass leaves under heat stress, which may have a potential relationship with leave senescence (He et al., 2005).

In general, under appropriate growth environment, the content of reactive oxygen species (ROS) in plant cells is in a state of dynamic balance. Stress could cause a large amount of ROS accumulation, membrane lipid peroxidation, and finally lead to metabolic disorders in plants (Choudhury et al., 2013). The active oxygen scavenging system in plants is mainly composed of antioxidant enzymes (SOD, POD, APX, etc.) and antioxidant substances (Baxter et al., 2014). SOD is responsible for catalyzing disproportionation of O^{2-} into H_2O_2, and CAT, POD, and APX are mainly involved in H_2O_2 elimination (Cheruth et al., 2009). A large number of findings supported that exogenous JAs could enhance SOD, CAT, POD, and APX activity or gene expression, thereby alleviating oxidative damage and stabilizing

cell membranes in plants under abiotic stress (Lehmann et al., 1995; Xin et al., 1997; Ghasempour et al., 1998). In this study, the activity of SOD, POD, CAT, and APX was further increased by exogenous MeJA treatment under heat condition and lower MDA content was accumulated, which was consistent with a previous study that the increased antioxidant enzyme activity favors the survival of plant to the heat stress and which induced the expression of related defense genes (Almeselmami et al., 2006).

Studies have shown that JA was a signaling molecule that regulates the expression of defense genes to various environmental stresses (Lehmann et al., 1995). Wasternack's study (Wasternack, 2007) showed that the genes related to the JA signal pathway are all induced by JA substances, and the JA biosynthesis is regulated by positive feedback. Previous results showed that exogenous MeJA treatment significantly induced the upregulation of *LOX2* (AT3G45140) in the JA biosynthesis pathway of *A. thaliana* seedlings (Mao et al., 2017). The bulbs of *Gladiolus hybridus* were treated with different concentrations of exogenous MeJA, which could induce the upregulated expression of *GhAOS*, *GhAOC*, and 12-oxo-phytodienoic acid reductase 3 (*GhOPR3*) genes in the JA signal pathway, and the expression level rose with the MeJA concentration increasing (Lian et al., 2013). Also, JA is catalyzed by JA carboxyl methyl transferase (JMT) to form MeJA, and JMT could perceive and respond to local and systemic signals generated by external stimuli, including exogenous MeJA itself (Seo et al., 2001). In this study, the result showed that exogenous MeJA treatment could enhance the expression of the key genes in the JA pathway, including *LpLOX2*, *LpAOC*, *LpOPR3*, and *LpJMT*. Furthermore, they were all increased at different treatment points under heat condition, indicating that the key genes of the JA signal pathway have a positive response to the heat stress. Several reports also showed that endogenous JAs rapidly and massively accumulate under stress conditions in plants (Xin et al., 1997), and with the upregulated expression of key genes in the JA pathway, the content of endogenous JA and MeJA increased gradually (Song et al., 2014; Nie et al., 2020). These results indicated that heat stress and exogenous MeJA may both play a positive feedback regulatory role in the signal pathway of JAs, thus enhancing the concentration of endogenous JAs in leaves. When plants are under stress, JA treatment may make plants produce anti-stress reactions, including synthesizing proteins with special functions, inducing or activating related enzymes and producing secondary active substances, so as to resist the harm of adversity (Cai et al., 2006). Moreover, when plants are stimulated by the stress, they could induce the synthesis and accumulation of JAs directly or not.

NAC genes are also involved in plants responding to biotic and abiotic stress (Margaret and Thomas, 2001; Hu et al., 2006, 2008; Yoo et al., 2007). Previous study demonstrated that stress-responsive NAC (SNAC) proteins had important roles in the control of abiotic stress tolerance (Kazuo et al., 2012) and the expression level would increase when treated with methyl-jasmonate (Zhou et al., 2013). In this study, the results showed that the expression of *LpNAC037*, *LpNAC45*, and *LpNAC54* were significantly induced by heat stress, while the expression profile of *LpNAC022* gene was different with them. Besides, previous study had found that several stress-responsive transcription factors encoded by *SNAC* also played an important role in stress tolerance when treated with exogenous MeJA in *A. thaliana* (Ooka et al., 2003). Hsp70s are also essential in regulating plant growth, development, and stress response in almost all cells (Miernyk et al., 1992; Su and Li, 2008, 2010). Also, the overexpression of mitochondrial *HSP70* could inhibit the apoptosis induced by high temperature and oxidation in rice (Qi et al., 2011). In this study, the results showed that heat stress enhanced the expression of *LpHsp009*, *LpHsp70-010*, *LpHsp015*, and *LpHsp022* genes, while we could not see the significant difference with exogenous MeJA treatment. However, exogenous MeJA prompted expressions of *LpHsp010* gene, showing that this gene may have a relationship with MeJA under heat conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GN and XZ conceived the project and designed the experiments. YS, YH, MT, JCa, and RW performed the experiments. YS and XD analyzed the data. YH, XD, and YS wrote the manuscript, and GN and XZ finalized at last. All authors discussed the results and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.664519/full#supplementary-material

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Regulation of Dual Activity of Ascorbate Peroxidase 1 From *Arabidopsis thaliana* by Conformational Changes and Posttranslational Modifications

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Ascorbate peroxidase (APX) is an important reactive oxygen species (ROS)-scavenging enzyme, which catalyzes the removal of hydrogen peroxide (H_2O_2) to prevent oxidative damage. The peroxidase activity of APX is regulated by posttranslational modifications (PTMs), such as S-nitrosylation, tyrosine nitration, and S-sulfhydration. In addition, it has been recently reported that APX functions as a molecular chaperone, protecting rice against heat stress. In this study, we attempted to identify the various functions of APX in *Arabidopsis* and the effects of PTMs on these functions. Cytosol type APX1 from *Arabidopsis thaliana* (AtAPX1) exists in multimeric forms ranging from dimeric to high-molecular-weight (HMW) complexes. Similar to the rice APX2, AtAPX1 plays a dual role behaving both as a regular peroxidase and a chaperone molecule. The dual activity of AtAPX1 was strongly related to its structural status. The main dimeric form of the AtAPX1 protein showed the highest peroxidase activity, whereas the HMW form exhibited the highest chaperone activity. Moreover, *in vivo* studies indicated that the structure of AtAPX1 was regulated by heat and salt stresses, with both involved in the association and dissociation of complexes, respectively. Additionally, we investigated the effects of S-nitrosylation, S-sulfhydration, and tyrosine nitration on the protein structure and functions using gel analysis and enzymatic activity assays. S-nitrosylation and S-sulfhydration positively regulated the peroxidase activity, whereas tyrosine nitration had a negative impact. However, no effects were observed on the chaperone function and the oligomeric status of AtAPX1. Our results will facilitate the understanding of the role and regulation of APX under abiotic stress and posttranslational modifications.

Keywords: abiotic stress, ascorbate peroxidase, chaperone, reactive oxygen and nitrogen species, posttranslational modifications

INTRODUCTION

Plants being sessile in nature are inevitably exposed to various abiotic and biotic stresses (Apel and Hirt, 2004; Mittler et al., 2004). Aerobic metabolism is related to various oxidation reactions that lead to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Stressful environments lead to the overproduction of reactive oxygen/nitrogen species (RONS), with the imbalance created between the production and scavenging causing detrimental effects. However, RONS play an important signaling role in plants and act as key regulators of various metabolic and physiological processes (Mittler et al., 2011; Baxter et al., 2014; Del Rio, 2015). Reactive oxygen species, such as superoxide radical, as well as reactive nitrogen species, such as nitric oxide, interact with each other to yield another type of RONS, peroxynitrite, indicating the cross talk between RONS (Halliwell, 2006; Radi, 2013; Khan et al., 2014). In particular, RONS lead to reversible modifications of various redox sensitive and functional catalytic groups of enzymes through oxidative and nitrosative modifications leading to RONS signaling. S-nitrosylation, S-sulfhydration, S-glutathionylation, and tyrosine nitration are few of the redox modifications caused by RONS. These modifications either reversibly or irreversibly alter the stability, structure, and function of proteins (Begara-Morales et al., 2013, 2014; Radi, 2013; Yang et al., 2015).

Organisms have a vast array of antioxidant systems to combat reactive oxygen species (Halliwell, 2006; Del Rio, 2015). Ascorbate peroxidase (APX) is one of the most important enzymes of the hydrogen peroxide (H_2O_2) detoxification system, which prevents the accumulation of toxic levels of ROS by reducing H_2O_2 into water using ascorbate as an electron donor (Asada, 1992; Shigeoka et al., 2002). More specifically, APX, which contains different isoenzymes which are present in different subcellular organelles, has been found in various higher plants. Based on their subcellular localization, APXs in *Arabidopsis* are classified as cytosolic (APX1, APX2, and APX6), peroxisomal (APX3, APX4, and APX5), and chloroplastic (stromal APX, thylakoid bound APX), while the intracellular location of another member remains unknown (Kubo et al., 1993; Santos et al., 1996; Panchuk et al., 2002; Chew et al., 2003; Narendra et al., 2006). The expression of the APX1 gene is induced in response to ozone, excessive light, high temperature, oxidative stress, and wounding (Kubo et al., 1995; Karpinski et al., 1997; Storozhenko et al., 1998; Shigeoka et al., 2002; Filiz et al., 2019). Plants overexpressing APX1 showed increased tolerance to salt and temperature stress (Gueta-Dahan et al., 1997; Badawi et al., 2004; Lu et al., 2007). Furthermore, APX1, despite being a cytosolic enzyme, is critical for the protection of chloroplasts from ROS and its absence has been shown to result in the compromise of the photosynthetic machinery (Davletova et al., 2005). In some other studies, *apx1* mutants, deficient in cytosolic *APX1,* were reported to show a sensitive phenotype in response to a combination of heat and drought stress and high-light intensities (Pnueli et al., 2003; Koussevitzky et al., 2008). Similarly, cytosolic APX1 from *Arabidopsis* (*AtAPX1*) was demonstrated to be crucial for tuning the regulation of H_2O_2, playing a key role in providing acclimation to a combination of heat and drought stress (Koussevitzky et al., 2008).

As mentioned, APX is classically known for its peroxidase function. However, a recent report on rice APX2 has revealed its additional function as a chaperone molecule. In addition, the conformational changes between the high molecular weight (HMW) and low molecular weight (LMW) forms of the protein have been linked to the dual activity of OsAPX2. For instance, it was shown that the protein showed a structural and functional transition in response to salt stress. The switch from HMW complexes to LMW forms were reported to lead to the dissociation of oligomers, which in turn increased the activity of APX under salt stress conditions (Hong et al., 2018). Previous studies on the peroxiredoxin protein reported the oxidative stress-induced regulation of 2-Cys Prx in human cells and yeast. In particular, 2-Cys Prx, in the oxidized form, undergoes a structural conversion from LMW form to HMW complexes leading to a functional switch from peroxidase to chaperone (König et al., 2002; Jang et al., 2004). Cytosolic ascorbate peroxidase possesses 2 substrate oxidation sites i.e., ascorbate and glutathione, and hence it exhibits dual activity in several plants. A recent report indicated that several plants were separated based on the amino acid composition for the glutathione binding site. Plants displaying an oxidizing ability for both AsA and GSH, such as *Oryza sativa*, were classified under group 1, whereas *Arabidopsis* with only the AsA oxidizing ability was placed in group 3 (Chin et al., 2019). This study focused on the functional and structural status of the dual nature of ascorbate peroxidase in *Arabidopsis thaliana.*

The research on NO and posttranslational modifications (PTMs) have brought in sight many target proteins modified by NO-derived molecules. Proteomics studies have suggested that APX is one of the targets of NO-derived molecules, such as SNO and $ONOO^-$. For instance, APX, like many other proteins, is regulated by various posttranslational modifications, such as S-nitrosylation (Begara-Morales et al., 2014; Yang et al., 2015), S-sulfhydration (Aroca et al., 2015), and tyrosine-nitration (Begara-Morales et al., 2016). S-nitrosylation regulates protein function either positively or negatively and promotes oligomerization or redox switch in proteins. S-nitrosylation of NPR1 facilitates oligomerization, which is important for the maintenance of protein homeostasis (Tada et al., 2008). S-nitrosylation of the insulin degrading enzyme (IDE) inhibits the enzymatic activity of IDE and promotes oligomerization (Ralat et al., 2009). Tyrosine nitration can cause lowering of pKa of the hydroxyl group, steric hindrance due to attachment of bulky group, or enhance the hydrophobicity of the proteins. It can result in either gain, loss, or no change of protein functions (Kolbert et al., 2017). In tobacco, tyrosine nitration leads to irreversible inhibition of two major H_2O_2 scavenging enzymes such as APX and catalase (Clark et al., 2000). Hydrogen sulfide (H_2S) is a messenger molecule that is highly reactive and toxic. It is known to modify proteins by reacting with cysteine residues to form persulfide, a process known as S-sulfhydration (Mustafa et al., 2009). S-sulfhydration is known to affect the protein function either positively or negatively,

for instance, in *Arabidopsis*, the enzyme activity of APX and glyceraldehyde-3-phosphate dehydrogenase is activated by S-sulfhydration (Aroca et al., 2015). The APX1 from the pea plant is reversibly regulated by S-nitrosylation and irreversibly by tyrosine nitration, creating antagonistic effects (Begara-Morales et al., 2014). Although the effects of PTMs on the peroxidase activity of APX have been studied in *Arabidopsis* and some other plant species, the conformational changes of APX due to PTMs remain unexplored.

In this study, we examined the functional and structural link of the APX1 protein in *Arabidopsis thaliana,* both *in vitro* and *in vivo*. As mentioned, the AtAPX1 protein functions both as peroxidase as well as a chaperone molecule, with this dual function being linked to its structural status. Abiotic stresses, such as heat and salt regulate this dual function and structural status of AtAPX1 through the association and dissociation of APX proteins, respectively. Furthermore, we confirmed the effect of various PTMs on the dual function and structural status of AtAPX1. This work provided a comprehensive study on the effects of abiotic stresses and posttranslational modifications on the dual activity of the APX protein.

MATERIALS AND METHODS

Cloning

The *AtAPX1* gene from *Arabidopsis thaliana* was cloned into the pJET1.2 vector (CloneJET, Thermo Fisher Scientific, Waltham, MA, United States) and further cloned into a pET28a (+) expression vector (Novagen, Madison, WI, United States). The coding region of *AtAPX1* was isolated from the genomic DNA of *Arabidopsis thaliana* by PCR with the gene specific primers having restriction enzyme sites, APX1-*Bam*HI-FP (5′-GGATCCATCCATGACGAAGAACTACCCAACCG-3′) and APX1-*Xho*I-RP (5′-CTCGAGTTAAGCATCAGCAAACCC AAGC-3′), using the *Pfu* DNA polymerase (Solgent, Daejeon, Korea). The amplified DNA fragment was cloned into the pJET1.2 cloning vector and transformed into the DH5α (Promega, Madison, WI, United States) bacterial strain. Then, the DNA fragments were cleaved using the respective restriction enzymes and cloned into the pET-28a vector and further transformed into the BL21 (DE3) (Invitrogen, Carlsbad, CA, United States) bacterial strain. Positive clones were confirmed through DNA sequencing and selected for protein expression.

Expression and Purification of Recombinant Proteins

The *AtAPX1* gene cloned in pET28a was induced using 0.3 mM isopropyl-β-D-thio-galactopyranoside (IPTG) (Promega, Madison, WI, United States) for 4 h at 37°C. The obtained His-tagged fusion protein was purified using a nickel-nitrilotriacetate-agarose (Ni-NTA) column (Peptron, Daejeon, Korea) following the manufacturer's instructions with phosphate-buffered saline supplemented with freshly prepared 1 mM ascorbate. The protein was eluted using 50 mM Tris–HCl (pH 7.5) with NaCl and thrombin for cleaving the His-tag at 4°C. Purified proteins were exchanged with 50 mM Tris–HCl (pH 7.5) using Amicon centrifugal columns (10 k NMWL) (Merck Millipore, Burlington, MA, United States) following the manufacturer's instructions, to be used for biochemical analyses.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed for further purification and determination of the molecular weight of recombinant AtAPX1 proteins. SEC was carried out using fast protein liquid chromatography (AKTA, Amersham Biosciences, Uppsala, Sweden) with a Superdex 200 10/300 GL gel-filtration column (Amersham Biosciences) following previously described methods, with minor modifications (An et al., 2011). Briefly, 50 mM Tris–HCl (pH 7.5) buffer was used to equilibrate the column and run the protein at a flow rate of 0.5 mL/min at 4°C. Absorbance was monitored at 280 nm. Fractions containing the desired protein were pooled and concentrated using Amicon centrifugal columns (10 k NMWL) (Merck Millipore). The column was calibrated using blue dextran (>2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa).

Ascorbate Peroxidase Activity Assay

The ascorbate peroxidase activity of purified protein was determined using ascorbate oxidation as previously described, with slight modifications (Nakano and Asada, 1981). The enzymatic activity of the total recombinant protein and fractions (F1-F3) separated by SEC was monitored by measuring the decrease in absorbance at 290 nm. The activity was measured immediately after adding 1 mM H_2O_2 to a 500 μL reaction mixture containing 0.5 mM ascorbate, 50 mM potassium phosphate buffer (pH 7.0), and AtAPX1 protein using a UV-Visible spectrophotometer (Evolution 300 UV-Vis Spectrophotometer; Thermo Scientific, Worcester, MA, United States) for 3 min. Potassium cyanide (KCN 8 mM) was used as an inhibitor of heme-containing protein.

Molecular Chaperone Activity Assay

The molecular chaperone activity assay was performed as previously described (Lee et al., 2015) by analyzing the ability of AtAPX1 in preventing the thermal aggregation of a heat-sensitive substance, malate dehydrogenase (MDH) (Lee et al., 1997; Moon et al., 2005). MDH was incubated in a 50 mM HEPES buffer (pH 8.0) at 43°C with various concentrations of AtAPX1 or its fractions separated by SEC. The thermal aggregation of the substrate was estimated by monitoring the change in turbidity at 340 nm for 15 min using an Evolution 300 Spectrophotometer (Thermo Scientific) equipped with a thermostatic cell holder.

Plant Growth Conditions and Stress Treatment

Arabidopsis thaliana (Columbia-0 ecotype) seeds were imbibed in sterile water for 30 min at 25°C and then surface sterilized with 4% sodium hypochlorite solution for 10 min, rinsed 5 times with sterile water, plated on one-half strength Murashige and

Skoog (1/2 MS) medium supplemented with 1% sucrose and 0.8% phytoagar, and then stratified at 4°C for 3 days. Plants were grown under a 16 h light/8 h dark cycle at 23°C. Then, 10 and 14 days old seedlings were used for abiotic stress treatments. For heat stress, 10 days old seedlings were transferred to a plant growth incubator set at 42°C for 1 h and then transferred to 23°C for recovery for 1 and 3 days. Likewise, 14 days old plants were transferred to 1/2 MS media supplemented with 200 mM NaCl for 6 h. After 6 h, half of the plants were transferred to 1/2 MS media for recovery and samples were collected after 1 day.

Extraction of Total Plant Protein and Western Blotting

Arabidopsis plants were immediately frozen upon harvesting and ground to a fine powder in liquid nitrogen using a pestle and mortar. A total of 0.5 mL of protein extraction buffer (50 mM Tris–HCl pH 7.5, 10% glycerol, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, and 1 × protease inhibitors) was added to each sample and incubated at 4°C for 1 h with constant rotating. The homogenate was centrifuged at least two times at 13,000 × *g* for 10 min and the supernatant was collected after each centrifugation. The protein content was measured using the Pierce BCA protein assay kit (Thermo-Scientific). Western blot analysis of total protein run on a 10% native-PAGE was performed to investigate the conformational changes in the AtAPX1 protein. Polyclonal mouse anti-AtAPX1 antibody was used to detect AtAPX1 protein complexes from total protein extracts. Native-PAGE was performed as previously described (Moon et al., 2005).

Treatment With GSNO, SIN-1, and NaHS

The recombinant AtAPX1 protein was incubated with various concentrations (0–1 mM) of S-nitrosoglutathione (GSNO) at 25°C for 30 min in the dark to achieve S-nitrosylation of protein. Excess of GSNO was removed using Amicon centrifugal filter units (10 k NMWL) (Merck Millipore). H_2S precursor sodium hydrogen sulfide (NaHS) was used to induce sulfhydration in the AtAPX1 recombinant protein. The AtAPX1 protein was incubated with various concentrations of freshly prepared NaHS (10 nM–100 μM) at 4°C for 30 min in the dark. Residual NaHS was removed using Amicon centrifugal filter units (10 k NMWL) (Merck Millipore). The S-nitrosylated and S-sulfhydrated proteins were detected with anti-biotin HRP linked antibody (Cell Signaling Technology, Seoul, Korea) using a biotin switch assay with slight modifications (Jaffrey and Snyder, 2001; Mustafa et al., 2009). The SIN-1 molecule (3-morpholinosydnonimine), a protein nitrating compound, was used as a peroxynitrite donor. Recombinant AtAPX1 protein was incubated with various concentrations (0–10 mM) of freshly prepared SIN-1 at 37°C for 1 h in the dark. Tyrosine nitrated proteins were detected with 3-nitrotyrosine antibody (Invitrogen, Rockford, IL, United States). Treated protein was used for the APX enzymatic activity assay, chaperone activity assay, SDS-PAGE, and native-PAGE analysis.

RESULTS

AtAPX1 Exists in Different Forms of Varying Sizes

In order to understand the structural status of the AtAPX1 protein, we used recombinant purified protein for analysis by SDS-PAGE, native-PAGE, and SEC. **Figure 1A** shows that recombinant AtAPX1 protein existed largely as a single band at a size of 27 kDa in 12% SDS-PAGE in the presence of DTT (reducing). Native-PAGE analysis revealed the presence of a dimeric form besides the low-molecular weight (LMW) and high-molecular weight (HMW) complexes of the AtAPX1 protein (**Figure 1B**). To further investigate the oligomeric status, we analyzed the AtAPX1 protein using size exclusion chromatography (SEC) (**Figure 1C**). SEC analysis showed three different peaks for the APX1 protein, which were represented by F1, having HMW complexes, the F2-LMW form, and the predominant F3 peak representing the major dimeric form. Each of the fractions corresponding to these three peaks were collected and reanalyzed using SEC to check their stability (**Figure 1E**). Collected fractions were subjected to reducing 12% SDS-PAGE, which demonstrated the presence of a single band at 27 kDa, representing the monomeric unit of AtAPX1, in all fractions. However, 10% native-PAGE analysis of collected fractions (**Figure 1D**) indicated that proteins and complexes in the F1 fraction (F1 > 2,000 kDa) stuck on the top of the 10% native-PAGE and were not able to travel through the gel because of their higher molecular weight. We found that the second SEC fraction (F2) showed a band of proteins between 158 and 440 kDa, whereas the F3 fraction corresponding to the dimeric form of AtAPX1 appeared below 158 kDa, representing the major peak in SEC analysis. The slight variation observed in the size of fractions in native-PAGE and SEC results might be due to the difference in underlying principle of both the separation techniques. These results indicated that AtAPX1 existed in multimeric forms, with the dimeric form being the dominant one.

Dual Function of APX as a Peroxidase and a Chaperone

Both OsAPX2 (Hong et al., 2018) and 2-Cys Prx (Jang et al., 2004), which exists in multimeric forms, are known to play a dual role, serving both as a peroxidase and a chaperone. The ascorbate-glutathione cycle is a pivotal antioxidant system involved in the regulation of H_2O_2 levels (Noctor and Foyer, 1998). Ascorbate peroxidase, being an important enzymatic antioxidant of this cycle, catalyzes the reduction of H_2O_2 to water using ascorbate as a specific electron donor. To investigate the role of the AtAPX1 recombinant protein as a typical ascorbate peroxidase, we performed an APX enzymatic activity assay by measuring the decrease in absorbance at 290 nm due to the oxidation of ascorbate. We accordingly observed that AtAPX1 showed an increase in enzymatic activity in a dose-dependent manner, as shown in **Figure 2A**.

To identify the potential function of AtAPX1 as a molecular chaperone, we investigated the chaperone activity of the

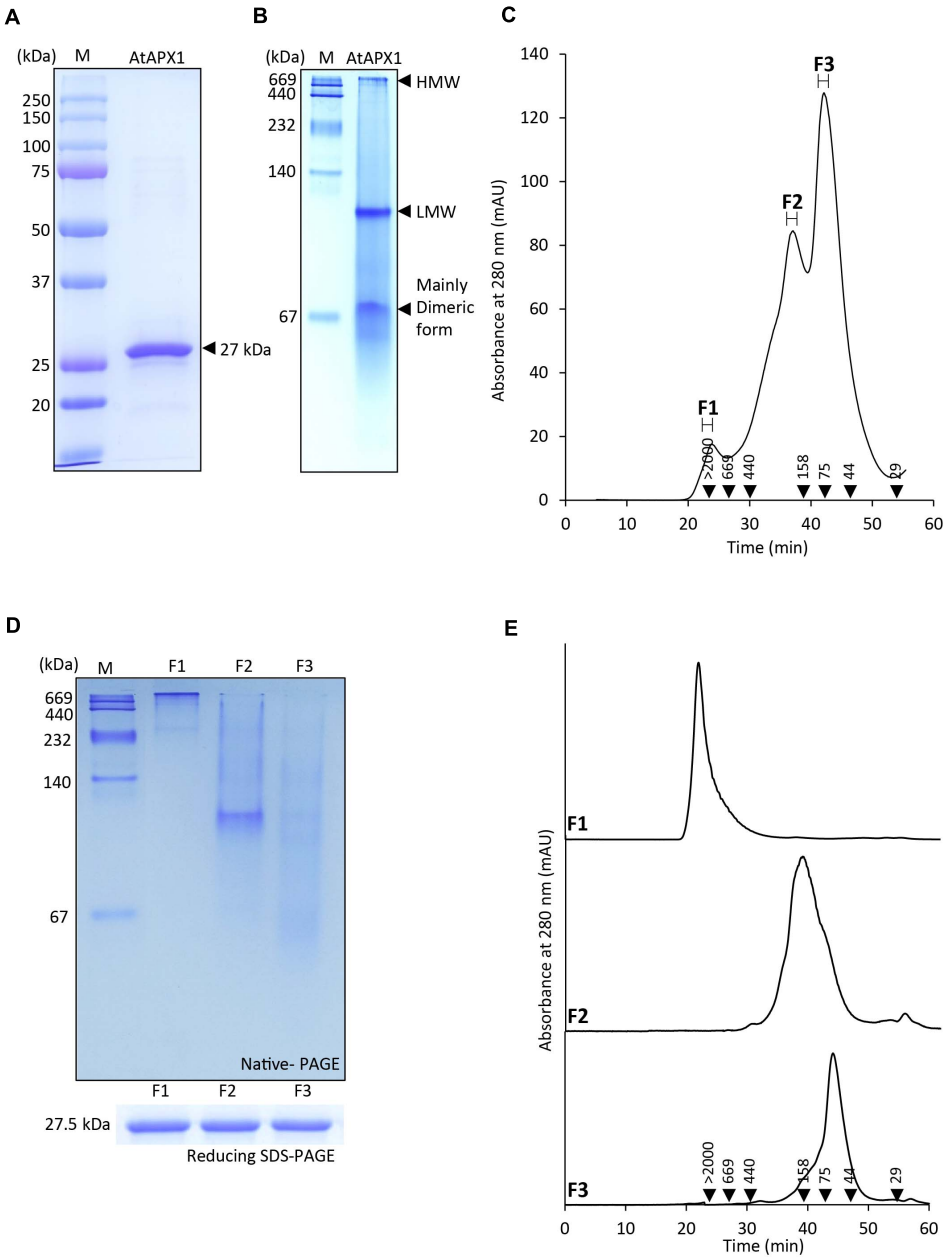

FIGURE 1 | Protein structure of AtAPX1 protein analyzed by **(A)** reducing SDS-PAGE, **(B)** native-PAGE, and **(C,E)** size exclusion chromatography (SEC). The values in the chromatogram represent the molecular weights of the standard proteins: blue dextran (>2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa). The three peaks (F1-F3) of separated proteins were collected and used for further analysis. **(D)** The fractions were separated by 10% native-PAGE and 12% reducing SDS-PAGE. **(E)** Each of the fractions was rechromatographed by SEC to confirm their stability.

recombinant protein by assessing its ability to inhibit the thermal aggregation of malate dehydrogenase (MDH), a heat-sensitive substrate. We found that the AtAPX1 protein showed a high chaperone activity as incubation of MDH with increasing amounts of AtAPX1 resulted in a concomitant decrease in the aggregation of MDH at 43°C (**Figure 2B**). We further noted that the aggregation of MDH was effectively suppressed at a subunit molar ratio of MDH to AtAPX1 of 1:2. Therefore, our results suggested that AtAPX1 exhibits both peroxidase and chaperone activities, similar to OsAPX2.

Functional and Structural Status of AtAPX1 Are Interconnected

As AtAPX1 appears in multimeric forms ranging from LMW to HMW form, with dual function, acting both as a peroxidase

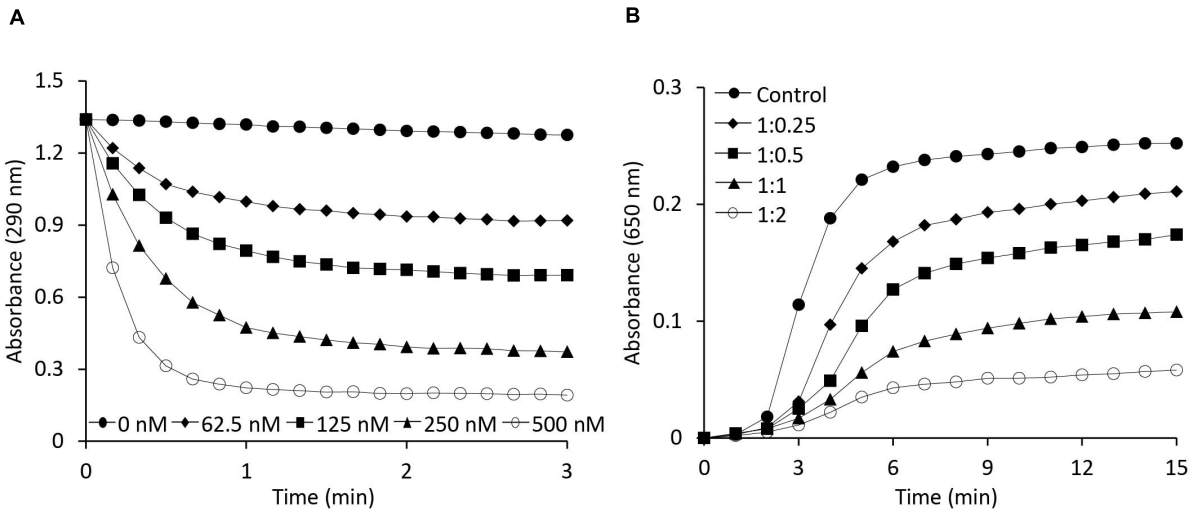

FIGURE 2 | Functional analysis of AtAPX1 protein as ascorbate peroxidase and molecular chaperone. **(A)** The APX activity was monitored by measuring the reduction in absorbance at 290 nm using (◆) 62.5 nM, (■) 125 nM, (▲) 250 nM, (○) 500 nM, and (●) 0 nM purified AtAPX1 protein. **(B)** Chaperone activity was measured at 650 nm using malate dehydrogenase (MDH) as a substrate. Thermal aggregation of 1 μM MDH was examined at 43°C for 15 min in the presence of purified AtAPX1 protein in molar ratios of MDH:AtAPX1, (◆) 1:0.25, (■) 1:0.5, (▲) 1:1, and (○) 1:2. (●) Control indicated the thermal aggregation of MDH in the absence of AtAPX1.

and a molecular chaperone, we investigated the connection between its structural conformations and functions. We collected and used the fractions (F1-F3) corresponding to the peaks of SEC for functional analysis. We found that the highest APX activity was exhibited by F3, the lowest molecular weight fraction, whereas the lowest APX activity was exhibited by F1, the highest molecular weight fraction. Furthermore, we observed that the APX activity of the F1 fraction was lower than that of the total protein (**Figure 3A**). In contrast, the chaperone activity was demonstrated to be the highest in the

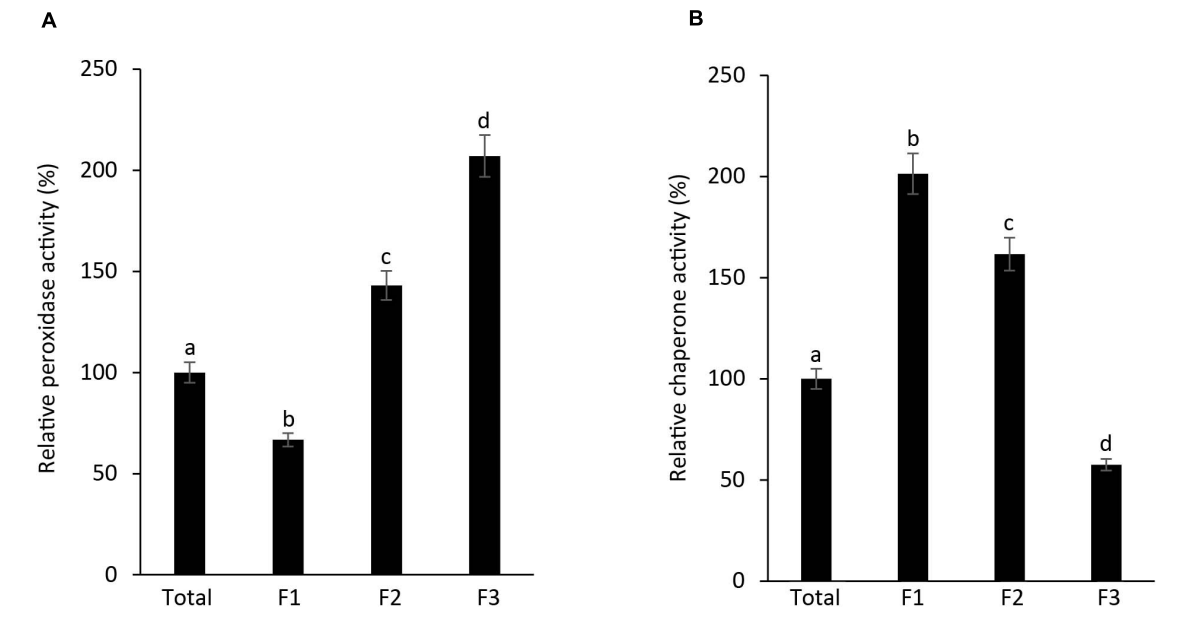

FIGURE 3 | AtAPX1 functions are related to differently sized protein structures. We separated the AtAPX1 protein into three fractions corresponding to the HMW complexes (F1), the LMW form (F2), and the dimeric form (F3). **(A)** Peroxidase activity of fractions was assayed by monitoring the reduction of ascorbate at 290 nm. **(B)** Chaperone activity was evaluated using a 1:1 molar ratio of MDH and AtAPX1. The activities of the three AtAPX1 protein fractions were compared with those of total protein which was set to 100%. Data are means (±) standard deviation of three independent experiments. Different letters indicate significant differences at $P < 0.05$ between the total protein and fractions by one-way ANOVA with Tukey's test.

F1 fraction, which represents high molecular weight complexes, whereas it was the lowest in the F3 fraction. These results suggested that multimerization of protein subunits promoted the molecular chaperone activity, whereas the LMW form has enhanced the peroxidase activity. Therefore, we concluded that the dual function of AtAPX1 was associated with its ability to form different protein complexes.

As is known, APX is a heme-containing enzyme and its activity is inhibited by potassium cyanide (KCN) and sodium azide, both of which are heme inhibitors (Nakano and Asada, 1981). Heme inhibitors bind to the heme moiety of the APX protein, inhibiting its peroxidase activity. In this study, we also investigated whether the function of APX as a molecular chaperone is being affected by heme inhibitor. To identify any relationship between the APX and chaperone activity of the protein, we performed a potassium cyanide inhibition test by incubating AtAPX1 proteins with various concentrations of KCN for 30 min (**Figure 4**). We respectively found that the chaperone activity of the AtAPX1 protein was not affected by KCN, whereas its APX activity was effectively suppressed. More specifically, the chaperone activity of KCN-treated AtAPX1 was demonstrated to be similar to that of non-treated protein, suggesting that the chaperone function of AtAPX1 does not depend on the heme moiety or its APX activity.

Abiotic Stress Modulates Structural Changes in AtAPX1 Protein

We found that the recombinant AtAPX1 protein showed oligomeric forms besides the major dimeric form and these different forms appear to play varying roles depending on the structural status of the protein. The yeast 2-Cys Prx was reported to shift from a LMW form to a HMW form after heat and oxidative stress, accompanied by a functional switching from peroxidase to molecular chaperone (Jang et al., 2004). In order to find out the effects of abiotic stress on the structural status of the AtAPX1 protein, we conducted a number of *in vivo* experiments. Col-0 plants were grown for 10 days and then transferred to a growth chamber set at 42°C for 1 h. For recovery, plants were transferred back to normal conditions and samples were collected after 1 and 3 days. Total protein was extracted and used for western blotting after native-PAGE. We noted that protein from control samples existed in different oligomeric forms similar to recombinant AtAPX1 proteins. Most of the protein was in HMW complexes, with the remaining existing in oligomeric and LMW complexes (**Figure 5A**). We also observed that after 1 h of heat stress, most of the protein formed HMW complexes; however, when plants were kept under recovery conditions for 3 d, the protein reverted back to its original form.

For the salt stress, 14 days old seedlings were transferred to 1/2 MS supplemented with 200 mM NaCl for 6 h. One half of the samples were collected, and the other half was transferred to 1/2 MS for recovery and samples were collected after 1 day. We found that the AtAPX1 protein from control samples detected using an anti-AtAPX1 antibody exhibited the same pattern of bands in native-PAGE, as shown in **Figure 5A**, whereas the majority of protein shifted to the LMW form after salt stress. As expected, this shift to the LMW form was reverted following the recovery of plants (**Figure 5B**).

We observed that the protein exhibited a transition from dimeric units to HMW complexes under heat stress, whereas the HMW complexes were dissociated under salt stress (**Figure 5C**). These results indicated that abiotic stress modulates the structural status of AtAPX1 *in vivo*.

Functional and Conformational Changes of AtAPX1 Induced by NO

S-nitrosylation, which binds a NO group to cysteine residues of proteins, thus modifying their function, is one of the most common posttranslational modifications (Astier et al., 2012; Begara-Morales et al., 2014). In particular, GSNO is known

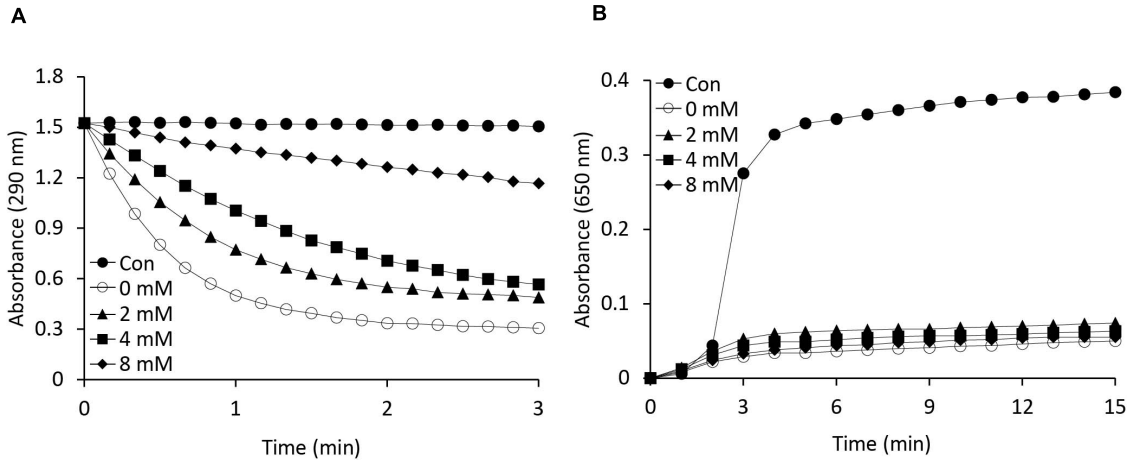

FIGURE 4 | Effects of a heme inhibitor (potassium cyanide) on the **(A)** peroxidase and **(B)** chaperone activities of AtAPX1 protein. To inhibit heme, we incubated AtAPX1 protein with (○) 0 mM, (▲) 2 mM, (■) 4 mM, and (◆) 8 mM potassium cyanide at 4°C for 15 min. (●) Control indicated measuring the activity without AtAPX1 protein.

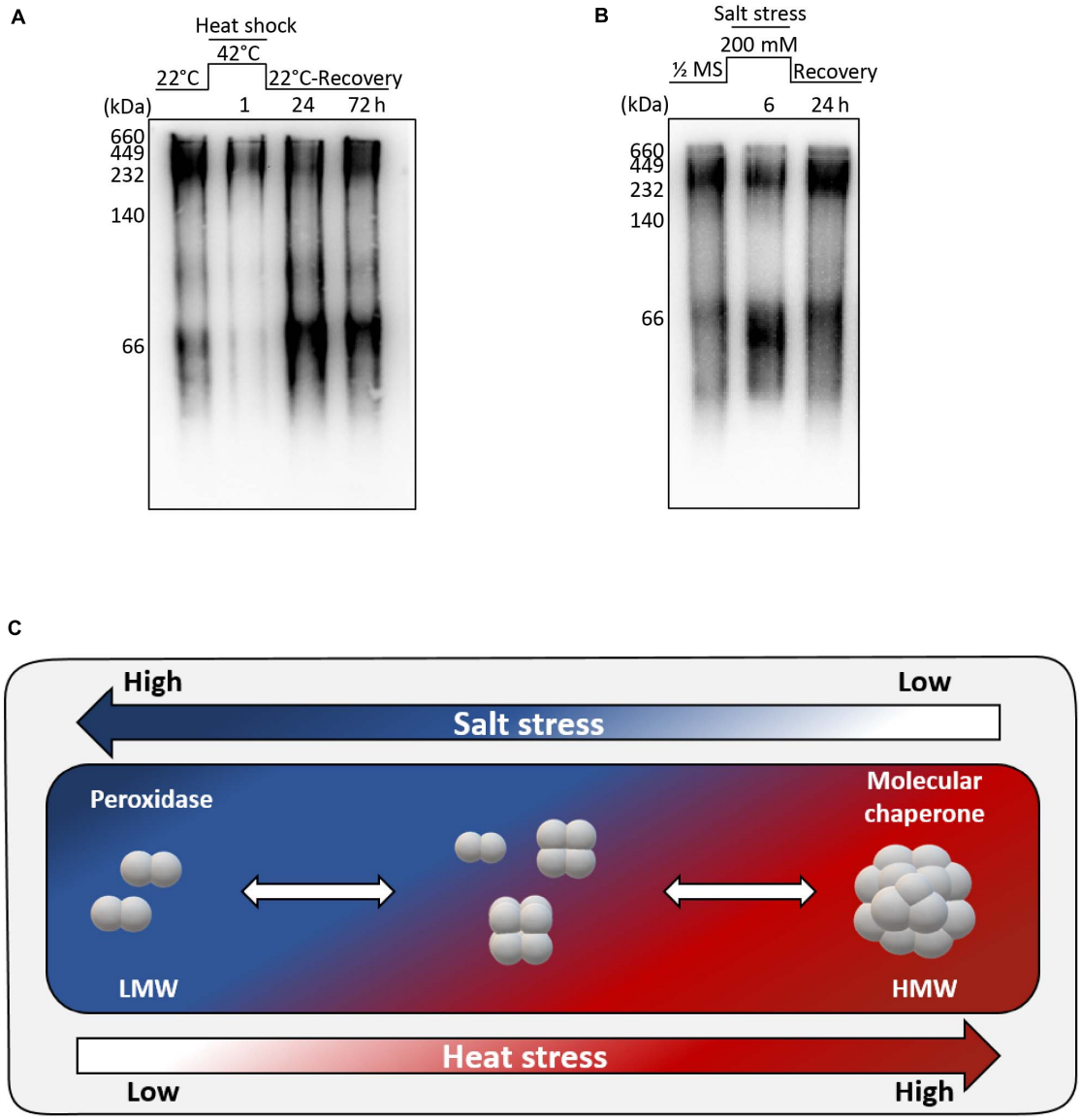

FIGURE 5 | Structural switch in AtAPX1 protein in response to abiotic stress. **(A)** For heat stress, 10 days old seedlings were subjected to heat stress at 42°C for 1 h, and then recovered in a growth chamber at 22°C for 24 h and 72 h. **(B)** For salt stress, 14 days old seedlings were transferred to 1/2 MS supplemented with 200 mM NaCl for 6 h. For recovery, *Arabidopsis* seedlings treated with salt stress were transferred to a new 1/2 MS containing no salt and grown for 24 h. **(A,B)** 20 μg of total protein was separated on 10% native-PAGE, followed by western blot analysis with an anti-AtAPX1 antibody (1:2,500 dilution). M, marker. **(C)** Model representation of molecular switching of AtAPX1 in response to abiotic stresses. APX1 in *Arabidopsis* exists in discretely sized multiple structures with a diverse range of molecular states, including dimeric, LMW, and HMW structures. AtAPX1 protein is converted to dimer or LMW forms under salt stress, whereas it is mainly present in HMW form under heat stress. This reversible switching is accompanied by the change of enzymatic functions of AtAPX1 between peroxidase and molecular chaperone.

to regulate the activity of APX1 either positively or negatively (De Pinto et al., 2013; Begara-Morales et al., 2014; Yang et al., 2015). We checked the effects of GSNO on the peroxidase and chaperone activity along with structural status of APX1. Our results were in accordance with previous reports indicating that S-nitrosylated APX1 showed substantial increase in the APX activity following treatment with GSNO (Yang et al., 2015); however, no effect was observed on its chaperone activity (**Figure 6A**). Moreover, we noted that native-PAGE analysis indicated that both treated and non-treated AtAPX1 protein samples showed similar structural behaviors (**Supplementary Figure 1**). In this study, we revealed for the first time that the GSNO-induced change in the activity of APX1 was not related to its oligomeric status.

We also pretreated purified recombinant APX1 protein with (10 nM–100 μM) NaHS for 30 min at 4°C to increase the concentration of *S*-sulfhydrated proteins. Then, we exchanged the buffer to eliminate residual NaHS before performing the

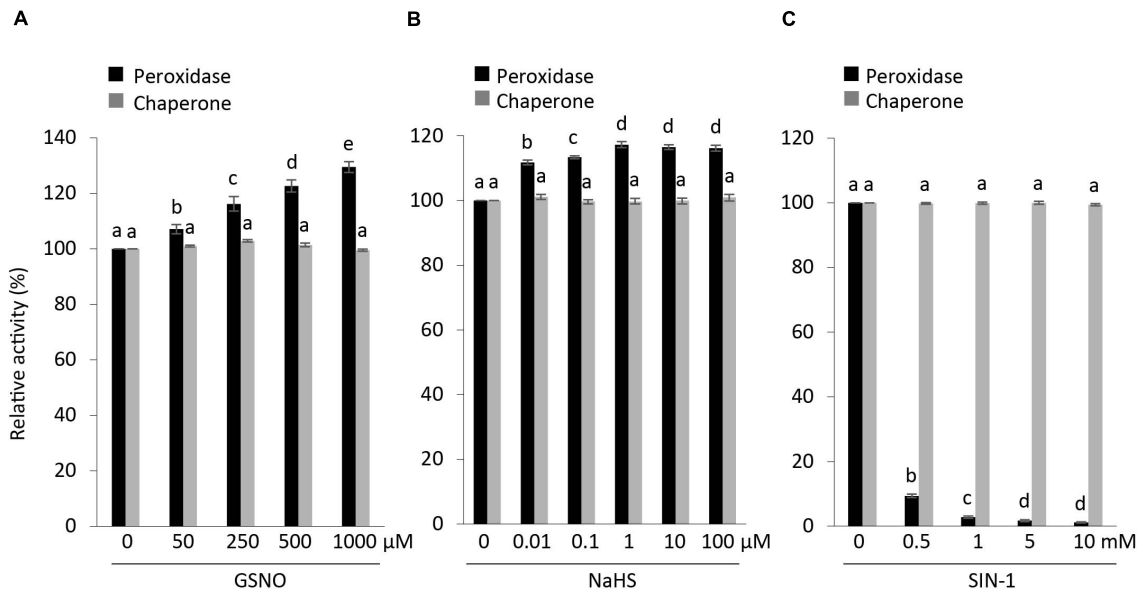

FIGURE 6 | Effects of posttranslational modifications on structural and functional status of AtAPX1. Comparison of peroxidase and chaperone activity of AtAPX1 protein treated with **(A)** GSNO (50–1,000 μM) for 30 min at 25°C in dark (S-nitrosylation), **(B)** NaHS (0.01–100 μM) for 30 min at 4°C in dark (S-sulfhydration), and **(C)** SIN-1 (0.5–10 mM) for 1 h at 37°C in dark (tyrosine nitration). The activities of treated protein were compared to non-treated protein whose activity was set to 100%. The black bars represent peroxidase activity and gray bars represent chaperone activity. Data are means (±) standard deviation of three independent experiments. Different letters indicate significant differences at $P < 0.05$ between non-treated and treated protein samples by one-way ANOVA with Tukey's test.

modified biotin switch assay and enzymatic activity assays. We accordingly observed an increase in peroxidase activity in NaHS-treated samples, whereas no effect was observed on chaperone activity and the oligomeric status of the AtAPX1 protein (**Figure 6B** and **Supplementary Figure 2**).

In order to evaluate the effect of SIN-1 (peroxynitrite donor) on the structure and function of AtAPX1, we incubated APX1 recombinant protein with 0.5–10 mM SIN-1. According to previous reports, SIN-1 inhibited the APX activity in pea plants (Begara-Morales et al., 2014). Our results corroborated with previous reports, as shown in **Figure 6C**, which depicts the inhibitory effects of the peroxynitrite donor. In contrast to the negative regulation of the peroxidase activity of the SIN-1-treated protein, we did not observe any effects on its chaperone activity. Treatment with SIN-1 did not cause any conformational changes either, as shown in **Supplementary Figure 3**, indicating that tyrosine nitration did not affect the structural status of AtAPX1.

Our analysis of the functional and structural status of AtAPX1 after posttranslational modifications revealed that PTMs affect the peroxidase activity of AtAPX1 both positively, as in the case of S-nitrosylation and S-sulfhydration, and negatively as in the case of tyrosine nitration. However, PTMs did not affect the chaperone activity in either case. In addition, almost no changes were observed in the structural status of AtAPX1 after PTMs.

DISCUSSION

Ascorbate peroxidase along with various enzymes and non-enzymatic antioxidants, such as ascorbate, and glutathione constitute the ascorbate-glutathione cycle. This cycle is important for maintaining and regulating the intracellular levels of hydrogen peroxide (Asada, 1992; Noctor and Foyer, 1998). In particular, APX being one of the most important enzymes in the defense system of plants, detoxifies H_2O_2 into water using ascorbate as an electron donor (Foyer and Noctor, 2005). The expression of *APX1,* which increases many folds during stress, can be detected in plant tissues even in the absence of stress (Pnueli et al., 2003). Despite being a cytosolic enzyme, it protects chloroplasts against ROS, indicating the importance of cytosolic enzymes (Davletova et al., 2005).

Recently, our group revealed a new function of the cytosolic OsAPX2 from rice, acting as a molecular chaperone besides its regular APX function (Hong et al., 2018). Therefore, to find out whether APX has a dual function not only in rice but also in other plants, we studied the role of the APX protein in *Arabidopsis thaliana*, a model plant. This study demonstrated that APX1 from *Arabidopsis* also possessed a dual function, acting both as a peroxidase and a molecular chaperone (**Figure 2**). In addition, we confirmed that APX1 existed in forms of various sizes, with these variations in protein conformation being related to its dual function (**Figures 1**–**3**). Our results from native-PAGE and SEC analysis (**Figure 1**) showed that AtAPX1 existed in homo-oligomeric forms, as the recombinant AtAPX1 protein was separated into three different fractions (F1, F2, and F3). We found that among them, F3, which contained mainly the dimeric form showed the highest APX activity, whereas the F1 fraction that contained HMW complexes showed the highest chaperone activity (**Figure 3**). Previous studies have shown that various proteins, such as OsAPX2 (Hong et al., 2018) and

2-Cys Prxs existing in bacteria (An et al., 2011), yeast (Jang et al., 2004), plant (König et al., 2002; Lee et al., 2015), and human (Moon et al., 2005) have a dual function, serving both as a molecular chaperone and a peroxidase. The functional switching of these proteins between peroxidase and molecular chaperone was correlated with the changes in the oligomeric status of the protein between its LMW and HMW form. These results corroborated with previous studies reporting on low molecular weight forms exhibiting high peroxidase activity, whereas oligomerization promoted a high chaperone activity. Therefore, these results indicated that the dual function of the APX1 protein was closely associated with the changes in its structural conformation.

As is known, APX is a heme-containing enzyme with protoporphyrin as a prosthetic group and its activity is inhibited by various inhibitors, such as sodium azide, potassium cyanide, and other thiol inhibitors (Shigeoka et al., 1980; Chen and Asada, 1990), which are potent inhibitors of heme-containing proteins. To find out whether the heme moiety is involved in the dual function of AtAPX, we measured both of its activities in potassium cyanide-treated AtAPX protein, where the heme moiety has been inactivated. We found that the peroxidase activity was decreased in a concentration-dependent manner, whereas the chaperone activity was not affected (**Figure 4**). These results excluded the involvement of the heme moiety in the chaperone activity of APX1, suggesting that the heme moiety is important for the peroxidase activity but not for the chaperone activity and conformational changes of APX1.

It has been reported that APX plays an important role in combating various abiotic stresses (Kubo et al., 1995; Karpinski et al., 1997; Koussevitzky et al., 2008). We thus monitored the *in vivo* structural status of AtAPX1 under abiotic stresses and found that it could be regulated by both heat and salt stress (**Figure 5**). The most significant finding was that the APX protein, which exists in various sizes *in vivo*, transitioned to HMW complexes under heat stress but reverted to various-sized forms during recovery (**Figure 5A**). Unlike heat stress, in plants subjected to salt stress the APX protein switched to an LMW form, but this switch was reverted when the salt stress was withdrawn (recovery) (**Figure 5B**). The switch to LMW forms indicated a high peroxidase activity, whereas the transition to HMW complexes represented a high chaperone activity. These *in vivo* results demonstrated the regulation of the dual function and structural status of the AtAPX1 protein by abiotic stresses. Hong et al. (2018) reported that the *in vivo* structural status of OsAPX2 in the IR-29 salt-sensitive rice cultivar was mainly represented by HMW complexes with very few LMW proteins. Therefore, they could not observe the structural change of OsAPX2 from LMW to HMW in response to heat stress *in vivo*. In the present study, we revealed that *Arabidopsis* AtAPX1 existed in various sizes from dimeric to HMW complexes, with their structure being regulated by salt and heat stresses.

It was recently reported that S-nitrosylation, tyrosine nitration, and S-sulfhydration are posttranslational modifications induced by reactive nitrogen species (RNS) and hydrogen sulfide (H_2S), an endogenous gaseous mediator that can regulate the peroxidase activity of the APX protein either negatively or positively, depending on the plant species (Begara-Morales et al., 2016; Aroca et al., 2018). To date, PTM studies on the APX protein have been limited only to its peroxidase activity. However, as this study identified and confirmed the dual function of APX, we examined the effects of PTMs on the structure and the dual function of APX using enzyme assays, as well as SDS- and native-PAGE. S-nitrosylation and S-sulfhydration are two PTMs targeting cysteine (Cys) residues in proteins. S-nitrosylation is the covalent attachment of a nitric oxide group (-NO) to the thiol group of a Cys residue, forming S-nitrosothiol (SNO), whereas S-sulfhydration involves the formation of a hydropersulfide moiety (-SSH) from Cys (Mustafa et al., 2009). The APX1 protein from *Arabidopsis* has 5 cysteine residues, of which 2 cysteine residues (Cys-32 and Cys-49) are S-nitrosylated (Yang et al., 2015). Cys-32 was also shown to be the target residue for S-sulfhydration when recombinant AtAPX1 protein was treated with NaHS and analyzed by LC-MS/MS (Aroca et al., 2015). Cys-32 is near the propionate side chain of the heme group and hence any modifications, such as S-nitrosylation and oxidation can cause local conformational changes around the heme group (Sharp et al., 2004; Yang et al., 2015). This situation could directly or indirectly regulate the binding affinity of APX1 with ascorbate, resulting in increased peroxidase activity. In contrast to the positive effects of S-nitrosylation and S-sulfhydration on the peroxidase activity of AtAPX1, the chaperone activity was not affected.

Tyrosine nitration is the addition of a nitro ($-NO_2$) group to the tyrosine residue of the target protein, which can promote conformational changes that can lead to gain, loss, or no change in the function of the target protein (Kolbert et al., 2017). Tyrosine nitration induces structural changes by enhancing the hydrophobicity of tyrosine residues (Souza et al., 2008). The pea APX1 was shown to have two of its tyrosines nitrated, of which Tyr235 has been suggested to be the most eligible candidate for inhibiting the activity of APX as it is present close to the pocket of the catalytic center and 3.6 Å away from the heme group (Patterson et al., 1995; Mandelman et al., 1998; Begara-Morales et al., 2014). Treatment of recombinant AtAPX1 with SIN-1 was demonstrated to lead to loss of peroxidase activity in a concentration-dependent manner. Loss of activity due to tyrosine nitration has been observed for most proteins (Corpas et al., 2013). These results concurred with previous reports on the loss of APX activity following tyrosine nitration of the APX1 protein in pea and tobacco (Clark et al., 2000; Begara-Morales et al., 2014). In contrast, the chaperone activity and the structural conformation were not affected by tyrosine nitration, indicating the direct effects of tyrosine nitration on the heme group.

In this work, we studied the link of multimeric forms of the APX1 protein from *Arabidopsis* with its dual function as a peroxidase and a molecular chaperone, and investigated its regulation *in vivo* under abiotic stresses. Moreover, this work was a comprehensive study on the effects of various PTMs on the dual function of AtAPX1, as well as their interconnection with the structural conformations of the protein.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SK and SSL conceived and designed the study. SK, PP, D-HB, SH, CC, and M-SC performed the experiments. SK, PP, D-HB, J-HK, SL, and SSL analyzed the data. J-HK, SL, H-WB, SYL, BC, and SSL contributed input and critically reviewed the manuscript. SK and SSL wrote the manuscript. SSL supervised the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.678111/full#supplementary-material

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Recent Advances in the Roles of HSFs and HSPs in Heat Stress Response in Woody Plants

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A continuous increase in ambient temperature caused by global warming has been considered a worldwide threat. As sessile organisms, plants have evolved sophisticated heat shock response (HSR) to respond to elevated temperatures and other abiotic stresses, thereby minimizing damage and ensuring the protection of cellular homeostasis. In particular, for perennial trees, HSR is crucial for their long life cycle and development. HSR is a cell stress response that increases the number of chaperones including heat shock proteins (HSPs) to counter the negative effects on proteins caused by heat and other stresses. There are a large number of HSPs in plants, and their expression is directly regulated by a series of heat shock transcription factors (HSFs). Therefore, understanding the detailed molecular mechanisms of woody plants in response to extreme temperature is critical for exploring how woody species will be affected by climate changes. In this review article, we summarize the latest findings of the role of HSFs and HSPs in the HSR of woody species and discuss their regulatory networks and cross talk in HSR. In addition, strategies and programs for future research studies on the functions of HSFs and HSPs in the HSR of woody species are also proposed.

Keywords: heat stress, woody plants, signaling network, molecular response, heat shock transcription factor, heat shock protein

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INTRODUCTION

As one of the most harmful abiotic stresses, heat stress poses a threat to plant life by impacting plant metabolism directly (Rennenberg et al., 2006). In order to successfully survive the occurrence of a long heatwave, plants have evolved a series of adaptive mechanisms to cope with the rising ambient temperature (Bäurle, 2016). Among those mechanisms, heat shock response (HSR) is a rapid response mechanism that protects the proteome against elevated temperature and other stresses (Mittler et al., 2012). It is wildly accepted that a class of molecular chaperones, heat shock proteins (HSPs), are rapidly induced under the drive of heat shock transcription factors (HSFs) when plants are exposed to extremely high temperatures (Hu et al., 2009). In the HSR regulatory network, HSFs are the central components of the effective defense systems (Xue et al., 2014), whereas HSPs are directly responsible for protein folding, assembly, translocation, and degradation (Molinier et al., 2006).

Perennial woody plants are greatly significant components of the global ecosystem and play a key role in limiting the emissions of carbon dioxide (CO_2) and other greenhouse gases.

In addition, woody plants are the main biomass resources for biofuels. Due to the perennial and long life cycle, woody plants experience more severe and extreme abiotic stresses during their lives compared to herbaceous plants. Woody plants may have evolved more complex stress-responsive mechanisms (Anderegg et al., 2012). The ubiquitous and conserved HSR has been extensively studied in herbaceous plants. However, similar research studies on forest trees remain limited (Mittler et al., 2012). In this study, we review the recent progress on the roles of HSFs and HSPs and their regulatory network in the HSR of woody plants. Unraveling this underlying interconnected mechanism will help to understand the complex regulatory networks of the fitness and adaptive advantage of higher plants.

HEAT SHOCK PROTEINS: MASTER PLAYERS OF HSR IN WOODY SPECIES

Heat shock proteins are found in all living organisms and are classified into at least six different types based on their molecular weight: sHSPs (small heat stress proteins), HSP40s, HSP60s, HSP70s, HSP90s, and HSP100s (Zhang et al., 2013, 2015a; Zandalinas et al., 2018). Massive production of HSPs in plants is a major characteristic response for the acquisition of thermotolerance.

Small Heat Stress Proteins

Small heat stress protein is a class of alpha-crystallin domain (ACD) chaperons with a molecular mass of 15–30 kDa. According to the sequence homology and subcellular localization, sHSPs are classified into 11 different classes. Class I–VI sHSPs are localized in the nucleus or cytoplasm, and the rest five classes of sHSPs are localized in mitochondria, chloroplasts, peroxisomes, or ER (Waters et al., 2008). The diversity of plant sHSPs reflects the molecular adaptability to various biotic and abiotic stresses (Hilton et al., 2012).

Until now, most studies have focused on the class I cytoplasmic sHSP and have divulged that sHSPs are involved in regulating thermotolerance in woody plants (**Table 1**), such as RcHSP17.8 in *Rosa chinensis* (*R. chinensis*) (Jiang et al., 2009, 2020), ThHSP18.3 in *Tamarix hispida* (*T. hispida*) (Gao et al., 2012), PtHSP17.8 in *Populus trichocarpa* (*P. trichocarpa*) (Li et al., 2016), CsHSP17.2 in *Camellia sinensis* (*C. sinensis*) (Wang et al., 2017a), and MsHSP16.9 in *Malus sieversii* (*M. sieversii*) (Yang et al., 2017). Among these genes, *RcHSP17.8* confers resistance to various stresses in *Escherichia coli* (*E. coli*), yeast, and *Arabidopsis* (Jiang et al., 2009). In addition, overexpression of *RcHSP17.8* in transgenic tobacco seedlings exhibits significant resistance to high temperatures and osmotic stresses, manifested by low electrolyte leakage and higher proline content under stress conditions (Jiang et al., 2020). Heterologous expression of *ThHSP18.3* protects yeast cells from salt, drought, heavy metals, and extreme temperatures (Gao et al., 2012). Overexpression of *PtHSP17.8* in *Arabidopsis* increases survival rate and root length under heat and salt stresses (Li et al., 2016). The *CsHSP17.2* in *C. sinensis* acts as a molecular chaperone to mediate heat tolerance by maintaining maximum photochemical efficiency and protein synthesis, enhancing the scavenging of reactive oxygen species (ROS), and inducing the expression of heat-responsive (HR) genes (Wang et al., 2017a). Overexpression of *M. sieversii MsHSP16.9* in *Arabidopsis* improves the tolerance of a plant to heat by alleviating the damages of ROS and regulating the expression levels of stress-related genes (Yang et al., 2017). Moreover, cytoplasmic class II and III sHSPs have also been reported to be involved in the HSR in woody species. For example, *SpHSP17.3*, a cytoplasmic class II *sHSP* gene from *Sorbus pohuashanensis* (*S. pohuashanensis*), responds to high temperature, salt, and drought stresses and has a certain effect on the adaptability of introduction and domestication (Zhang et al., 2020). Overexpression of a *Prunus mume* (*P. mume*) cytoplasmic class III sHSP gene (*PmHSP17.9*) improves the thermotolerance of transgenic *Arabidopsis* by enhancing superoxide dismutase (SOD) activity (Wan et al., 2016). Transient overexpression of a *Juglans regia* (*J. regia*) sHSP gene *JrsHSP17.3* in leaves enhances tolerance to cold, heat, and salt stresses by scavenging the accumulation of ROS and by accumulating osmotic adjustment substances (Zhai et al., 2016). In addition, the expressions of chloroplast small HSPs *CsHSP17.7*, *CsHSP18.1*, and *CsHSP21.8* (cytoplasmic classes I, II, and IV, respectively) from *C. sinensis* could be highly induced by heat and cold stresses. Overexpression of these small *CsHSPs* confers heat and cold tolerances in transgenic *Pichia pastoris (P. pastoris)* and *Arabidopsis* (Wang et al., 2017b). These studies indicate that sHSPs exhibit considerable influence in the adaptation of woody plants under heat and other abiotic stresses, which may be related to the long-term adaptive evolution of woody species.

In contrast, the organelle-localized sHSPs have been more studied in herbaceous plants. For example, the mitochondria-localized *GhHSP24.7* in cotton positively regulates seed germination through modulating the generation of ROS in a temperature-dependent manner (Ma et al., 2019). The chloroplast-localized *AsHSP26.8a* in creeping bentgrass plays a negative role in abiotic stresses through both abscisic acid (ABA)-dependent and ABA-independent signaling pathways and other stress signaling pathways (Sun et al., 2020). In *Arabidopsis*, an endoplasmic reticulum (ER)-localized sHSP, *sHSP22*, is involved in ABA and auxin signaling crosstalk (Li et al., 2018). However, the functional study of the organelle-localized sHSP in woody species has not been reported. In the future, more attention should be paid to these genes to reveal the response mechanisms of different organelle levels to high temperatures and other abiotic stresses in woody plants.

Other HSPs

Compared with the *sHSP* genes, members in other *HSP* gene families, such as *HSP40*, *HSP60*, *HSP70*, *HSP90*, and *HSP100*, have not been well-studied in woody species. There is a lack of results about individual gene function validated through genetic modification, although the genome-wide identification of such genes has been performed in several woody species. For example, Zhang et al. (2013, 2015a) have provided a comprehensive analysis of the gene organization and expression of *Populus HSF* and other *HSP* genes, such as *sHSP*, *HSP60*, *HSP70*,

TABLE 1 | Summary of the *HSP* and *HSF* genes involved in heat stress in woody plants.

Gene family	Gene symbol	Identified from species	Studied in species	Description	References
sHSP	*RcHSP17.8*	*Rosa chinensis*	*Escherichia coli*, *Yeast*, *Arabidopsis thaliana*, *Nicotiana tabacum*	Induced by heat and osmotic stresses; positive regulator of high temperature, drought, salt, and mannitol stresses	Jiang et al., 2009, 2020
	ThHSP18.3	*Tamarix hispida*	*Yeast*	Induced by heat and cold stresses; positive regulator of salt, drought, heavy metals, cold, and heat stresses	Gao et al., 2012
	PmHSP17.9	*Prunus mume*	*Arabidopsis thaliana*	Induced by ABA, heat, salt, drought, and oxidative stresses; positive regulator of heat stress	Wan et al., 2016
	PtHSP17.8	*Populus trichocarpa*	*Arabidopsis thaliana*	Induced by ABA, heat, cold, salt, PEG, and oxidative stresses; positive regulator of heat and salt stresses	Li et al., 2016
	JrsHSP17.3	*Juglans regia*	*Yeast*, *Juglans regia*	Induced by heat, cold, and salt stresses; positive regulator of salt, cold, and heat stresses	Zhai et al., 2016
	CsHSP17.2	*Camellia sinensis*	*Escherichia coli*, *Pichia pastoris*, *Arabidopsis thaliana*	Induced by heat, PEG, and salt stresses; positive regulator of heat stress	Wang et al., 2017a
	MsHSP16.9	*Malus sieversii*	*Arabidopsis thaliana*	Induced by heat stress; positive regulator of heat stress	Yang et al., 2017
	CsHSP17.7 *CsHSP18.1* *CsHSP21.8*	*Camellia sinensis*	*Yeast*, *Arabidopsis thaliana*	Induced by heat and cold stresses; positive regulator of cold and heat stresses	Wang et al., 2017b
	SpHSP17.3	*Sorbus pohuashanensis*	*Arabidopsis thaliana*	Induced by heat, salt, and drought stresses; positive regulator of salt stress	Zhang et al., 2020
HSF	*VpHSF1*	*Vitis pseudoreticulata*	*Nicotiana tabacum*	Induced by heat, drought, and pathogen *Erysiphe necator*; negative regulator of basal thermotolerance, osmotic stress, and pathogen; positive regulator of acquired thermotolerance	Peng et al., 2013

HSP90, and *HSP100*, under different abiotic stresses. A complex transcriptional regulatory network between *Populus HSFs* and *HSPs* has been generated based on their transcription patterns in poplar.

Several studies indicated that members in these HSP families play pivotal roles in regulating the thermotolerance in herbaceous species. For instance, knockdown of *CaHSP60-6* through virus-induced gene silencing in pepper [*Capsicum annuum* L. (*C. annuum* L.)] increases the heat sensitivity, which is manifested in the higher accumulation of ROS and lower membrane stability in silenced plants (Haq et al., 2019). Ectopic expression of a chrysanthemum *CgHSP70* in *Arabidopsis* enhances tolerance to heat, drought, or salinity, thereby protecting the plants from total damage (Song et al., 2014). Cytosolic *CaHSP70-1* from *C. annuum* is involved in the HSR through signal transduction pathways including calcium (Ca^{2+}), hydrogen peroxide (H_2O_2), and putrescine (Guo et al., 2014). In addition, overexpression of a soybean *GmHSP90A2* improves thermotolerance in *Arabidopsis* (Huang et al., 2019). However, the functional analysis of these high molecular weight HSPs in woody species is rarely reported. In order to explore the adaptability of woody species to elevated temperatures, more in-depth studies are needed.

HEAT SHOCK TRANSCRIPTION FACTORS ARE THE MAIN REGULATORS OF HSPs

The expression of the *HSP* genes is mainly regulated by HSFs. HSF/HSP transcriptional module-based HSR was recognized as an evolutionally conserved mechanism coping with heat stress (Scharf et al., 2012). Plants have evolved more complicated HSR compared to yeast and animals, for example, the HSF family of plants comprised of 18–52 members while yeast and *Drosophila* only have a single copy, and mammals have four HSFs (Andrási et al., 2020). Despite significant variability in

size and sequence of HSFs, their structures and functions are highly conserved across plant species (Lin et al., 2011). HSFs have a modular structure comprising a DNA-binding domain, an oligomerization domain, and a C-terminal activation domain (Scharf et al., 2012). Based on the variations in these three domains, especially the oligomerization domain, HSFs of plants can be divided into three classes (A, B, and C). Because of the vital regulatory responses to different stresses and developmental processes, the *HSF* gene family has been extensively characterized in the model plant *Arabidopsis*, as well as in several woody plants such as apple (*M. domestica*) (Giorno et al., 2012), poplar (*P. trichocarpa*) (Zhang et al., 2015a; Liu et al., 2019), desert poplar (*P. euphratica*) (Zhang et al., 2016), willow [*Salix suchowensis* (*S. suchowensis*)] (Zhang et al., 2015b), pear [*Pyrus bretschneideri* (*P. bretschneideri*)] (Qiao et al., 2015), tea (*C. sinensis*) (Liu et al., 2016), and grape [*Vitis vinifera* (*V. vinifera*)] (Liu et al., 2018). In *Arabidopsis*, rice, and tomato, *HSFA2* is strongly induced when plants are exposed to long-term heat stress or repeat-cycled heat stress and recovery (Scharf et al., 1998; Charng et al., 2007; Nishizawa-Yokoi et al., 2009), whereas *HSFA1a* seems to play a unique function as a master regulator of acquired thermotolerance and cannot be replaced by any other HSFs in tomato (Mishra et al., 2002). This implies that despite the existence of a certain conservative mechanism, HSF in different species may have functional specialization. Therefore, it is necessary to further study the gene function of HSF in various woody plants.

Transient overexpression of *Betula platyphylla* (*B. platyphylla*) *BpHSFA4* gene in leaves improves the salt stress tolerance by increasing the ability to scavenge ROS, thereby reducing cell damage or cell death and enhancing birch resistance (Liu et al., 2020). In contrast to the activity of the transcriptional activation of class A HSFs, the class B HSF proteins lack the activation domain and have a repression domain in the C-terminus. Peng et al. (2013) identified a novel class B2 *HSF* gene from Chinese wild *Vitis pseudoreticulata*, *VpHSF1*, which plays a key role in biotic and abiotic stress responses. Overexpressing *VpHSF1* in tobacco reduces the basal thermotolerance, improves

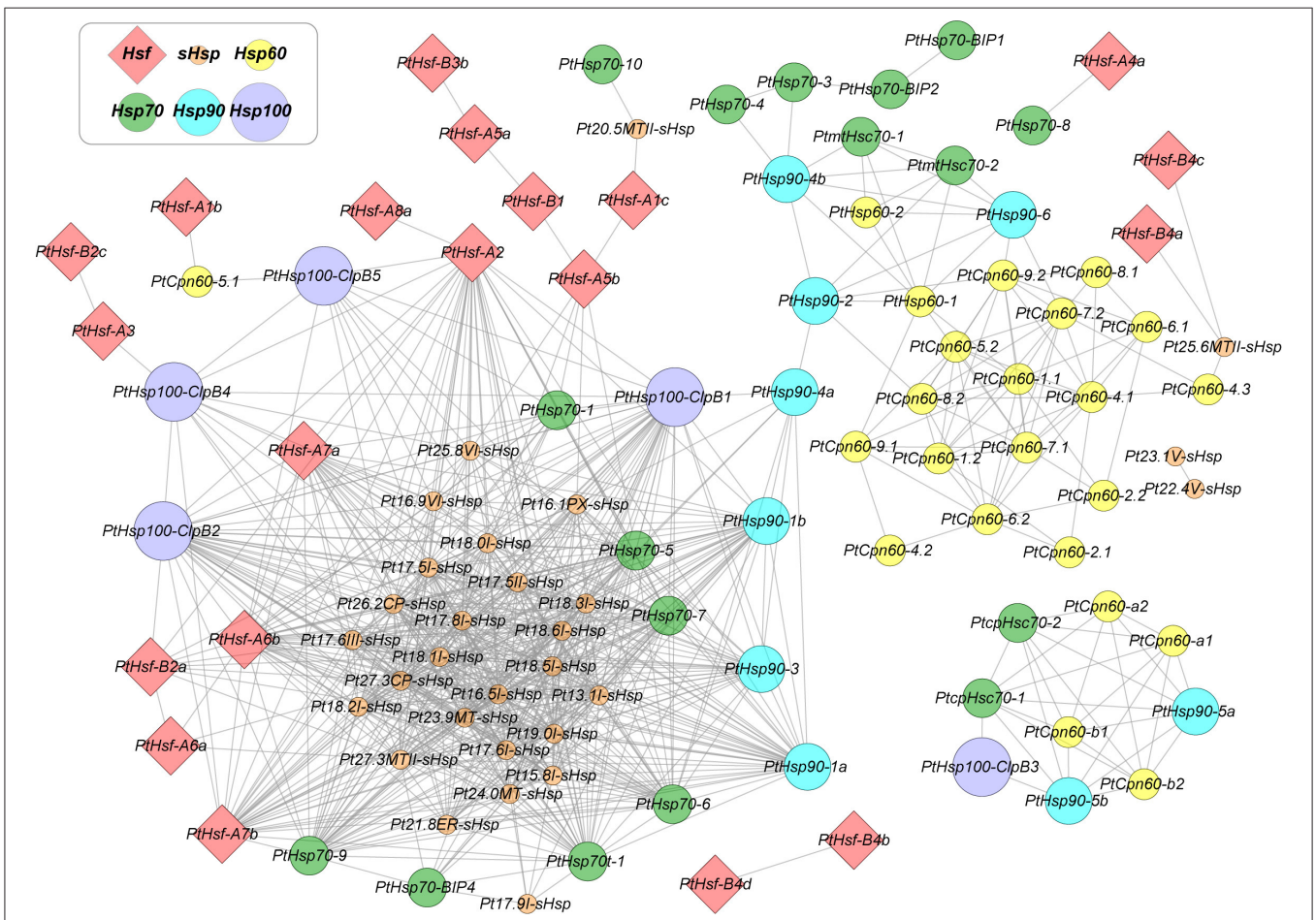

FIGURE 1 | Co-expression network of the *HSF* and *HSP* genes in *Populus*. Nodes represent *HSFs* and *HSPs* genes in *Populus*, and edges indicate pairwise correlation constructed by weighted gene co-expression network analysis (WGCNA). Node color codes represent different gene families. Red diamonds indicate HSFs, and solid circles with orange, yellow, green, cyan, and purple indicate *sHSPs*, *HSP60s*, *HSP70s*, *HSP90s*, and *HSP100s*, respectively. Lines represent the co-expression interaction. The network was created using Cytoscape.

the acquired thermotolerance, and enhances its susceptibility to osmotic stress and pathogen *Phytophthora parasitica* var. *nicotianae* Tucker (**Table 1**). Compared with herbaceous plants, the functional studies of HSFs in woody plants are still limited. *CpHSFB1* from *Carica papaya* (*C. papaya*) (Tarora et al., 2010) and *PsHSFB1* from *Paeonia suffruticosa* (*P. suffruticosa*) (Zhang et al., 2014) have been cloned from woody species, but their detailed biological functions have not been reported. Further unraveling the biological functions of each *HSF* can provide a better understanding of how woody plants can better cope with stresses during evolution.

HSFs AND HSPs FORM A COMPLEX REGULATORY NETWORK IN HSR

Plants possess a complex HSR regulatory network consisting of multiple *HSP* and *HSF* genes. HSFs contain a conserved DNA-binding domain at the N-terminus, which can recognize the DNA motif, 5′-nGAAnnTTCn-3′. This heat shock element (HSE) motif is commonly found in the promoters of HSF target genes (Andrási et al., 2020). Hierarchical transcriptional network among HSFs, HSPs, and other heat stress-responsive genes has been well-built in the model plant *Arabidopsis*. HSFA1s are the master regulators that respond quickly to heat stress, and other transcription factors, such as HSFA2 and DREB2A, were directly activated by HSFA1s (Liu et al., 2011; Yoshida et al., 2011). HSPs and ROS-scavenging enzymes are the main heat stress-induced proteins regulated by HSFs. They are required for protein quality control and oxidative homeostasis under heat–stress conditions (Ohama et al., 2017). Compared to *Arabidopsis*, woody species have very limited studies on HSFs. Salt-inducible PeHSF from *P. euphratica* directly binds to the HSE motifs in the *PeWRKY1* promoter and regulates its expression (Shen et al., 2015). HSFs regulate the expression of HSPs, and in turn, HSPs can physically interact with HSF proteins to affect the function of HSFs. In *Arabidopsis*, HSP90 and immunophilin ROF1 form a complex that co-imports HSFA2 into the nucleus and enhances its transcriptional activator activity under heat stress (Aviezer-Hagai et al., 2007; Meiri and Breiman, 2009). Another heat-inducible immunophilin ROF2, which is also a target of HSFA2, is recruited into the nucleus during accumulation and heterodimerizes with ROF1 in the complex, resulting in the inhibition of HSFA2. Therefore, the activity of HSFA2 in *Arabidopsis* is regulated by the two co-chaperones in both positive and negative manner by interacting with HSP90 (Meiri et al., 2010). In tomatoes, the constitutively expressed HSFA1a remains inactive in the complex formed with HSP70 and HSP90 under normal conditions. In contrast, the interaction of HSP90 with HSFB1 usually keeps the steady-state level of HSFB1 at a low level and targets HSFB1 to the degradation pathway of the 26S proteasome (Hahn et al., 2011). In addition, *V. vinifera VvGOLS1* has been heterologously expressed in *E. coli,* showing that it encodes a functional galactinol synthase. Transient expression assays showed that the heat stress factor VvHSFA2 transactivates the promoter of *VvGOLS1* in a heat stress-dependent manner (Pillet et al., 2012). However, the relationship between HSF and HSP, especially in woody species, remains unknown. With the rapid development of transcriptome-sequencing technology and the increase of big data in public databases, it is possible to use gene co-expression networks to explore the potential regulatory or interaction relationships between proteins. In this study, we use poplar as an example to construct an HSF-HSP co-expression network, due to the easy access to tremendous public expression data. As shown in **Figure 1**, we identified strong co-expressive relationships between *HSP90* (*PtHSP90-1a* and *PtHSP90-1b*) and *HSFA2* (*PtHSF-A2*), which is consistent with their protein–protein interactions in *Arabidopsis* and tomato (Meiri et al., 2010). This implies that HSR in woody species may share similar mechanisms across plant species. However, it is still an open question whether specific regulatory mechanisms or interaction relationships between HSFs and HSPs exist in woody plants.

FUTURE PERSPECTIVES

With ongoing greenhouse gas emissions, the ambient temperature is expected to rise over time. Woody plants have evolved more complex stress response mechanisms to cope with changing environments than herbaceous plants to guarantee a longer lifespan (Anderegg et al., 2012). However, the current knowledge of the functions of *HSF* and *HSP* genes during HSR in woody plants is still lacking. On the one hand, the transcriptional regulation or interaction between HSF and HSP in woody plants is unclear. Zhang et al. (2018) used the joint analysis of expression quantitative trait loci (eQTL) and co-expression network based on the *P. trichocarpa* natural variant populations to successfully identify the upstream regulatory transcription factor *WRKY* that controls the expression of the *HCT2* gene. This strategy provides a new method to identify the transcriptional regulatory relationship between genes. Therefore, the public gene expression database of woody plants can be used to preliminarily examine whether there is a potential regulatory or interaction relationship between HSFs and HSPs in these species. In addition, the continuous maturity of ChIP-Seq, CUT&Tag-Seq, and other technologies have effectively improved the ability to analyze the interaction between protein and DNA. The recently developed reverse-ChIP technology (Wen et al., 2020) can reversely mine the upstream regulators of specific target genes. In the future, the combined use of these methods can provide technical supports for the clarification of gene regulatory relationships in the HSR of woody plants. On the other hand, the traditional genetic transformation system is unstable and time-consuming for many woody species, which leads to insufficient research studies on the function of individual genes. Most of the studies just rely on the ectopic expression of *HSF* or *HSP* genes obtained from woody species in the model plant *Arabidopsis* or yeast. Therefore, fast and efficient gene function validation systems are urgently needed. The protoplast transient expression system (Zhang et al., 2018) can be used to quickly verify the relationship between HSFs and HSPs. And the recently

developed genome editing technology has been successfully applied in many woody species (Li et al., 2021). However, it is necessary to further improve the efficiency and accuracy of gene editing to overcome the limitation of its application in woody species. In addition, future research studies should also pay attention to the signals and cascade transduction pathways of woody plants after sensing high temperatures, how these signals further activate the HSR, and how the posttranscriptional and posttranslational regulatory pathways participate in the HSR of woody species. The combined use of these methods can provide a basis for further in-depth analysis of the function of HSFs and HSPs in HSR in woody plants.

AUTHOR CONTRIBUTIONS

JZ conceived the study. JZ, FT, and X-LH drafted the manuscript. TY, XY, J-GC, and M-ZL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Cyclic Nucleotide-Gated Ion Channel 6 Mediates Thermotolerance in *Arabidopsis* Seedlings by Regulating Hydrogen Peroxide Production *via* Cytosolic Calcium Ions

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We previously reported the involvement of cyclic nucleotide-gated ion channel 6 (CNGC6) and hydrogen peroxide (H_2O_2) in plant responses to heat shock (HS). To demonstrate their relationship with plant thermotolerance, we assessed the effect of HS on several groups of *Arabidopsis* (*Arabidopsis thaliana*) seedlings: wild-type, *cngc6* mutant, and its complementation line. Under exposure to HS, the level of H_2O_2 was lower in the *cngc6* mutant seedlings than in the wild-type (WT) seedlings but obviously increased in the complementation line. The treatment of *Arabidopsis* seeds with calcium ions (Ca^{2+}) increased the H_2O_2 levels in the seedlings under HS treatment, whereas treatment with a Ca^{2+} chelator (EGTA) inhibited it, indicating that CNGC6 may stimulate the accumulation of H_2O_2 in a manner dependent on an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$). This point was verified by phenotypic observations and thermotolerance testing with transgenic plants overexpressing *AtRbohB* and *AtRbohD* (two genes involved in HS-responsive H_2O_2 production), respectively, in a *cngc6* background. Real-time reverse transcription-polymerase chain reactions and Western blotting suggested that CNGC6 enhanced the gene transcription of HS factors (HSFs) and the accumulation of HS proteins (HSPs) *via* H_2O_2. These upon results indicate that H_2O_2 acts downstream of CNGC6 in the HS signaling pathway, increasing our understanding of the initiation of plants responses to high temperatures.

Keywords: heat shock, heat shock (stress) proteins, hydrogen peroxide, *Arabidopsis*, calcium ion

INTRODUCTION

Global warming is a serious environmental threat, and is an important limiting factor for normal plant growth and development. As fixed organisms, plants cannot escape from high temperature, but they have evolved methods and morphological variations to escape from its negative effects. As a countermeasure to heat shock (HS), plants can synthesize a series of

HS proteins (HSPs) in the responses of cell to HS conditions. They act as molecular chaperones, ubiquitin, and certain proteases to counteract protein denaturation, aggregation, and degradation, which protect the plant cells from heat-damage (Lawas et al., 2018). Thus, the synthesis of HSP is especially important for plant survival under HS conditions. In eukaryotes, HSP induction is dependent on HS factors (HSFs), which act as transcription factors to be bound in HS elements in the promoter regions of HSP genes (Akerfelt et al., 2010).

Several reactive oxygen species (ROS) are constantly generated as by-products of aerobic metabolism at multiple locations in plant cells, including the photosynthetic electron transport chain in chloroplasts, NADPH oxidase in the plasma membrane (PM), and peroxidase in the cell wall (Gechev and Hille, 2005). They are always greatly toxic and swiftly detoxified by different cellular enzymatic and nonenzymatic mechanisms. In other situation, plants purposefully release ROS as signal molecules to initial various biological processes including stress defense, programmed cell death, and stomatal behavior. Hydrogen peroxide (H_2O_2), as the major and most stable type of ROS, plays a key role in resistance reactions in plant cells, and it primarily originates from PM NADPH oxidase. In *Arabidopsis*, NADPH oxidase is encoded by 10 genes, from *AtRbohA* to *AtRbohJ*, which have distinct and shared biological features (Macpherson et al., 2008).

For example, H_2O_2 generated from AtRbohD and AtRbohF acts as a signaling molecule in ABA-induced stomatal closure and is crucial for jasmonic acid-induced expression of genes controlled by the MYC2 transcription factor (Maruta et al., 2011; Iwai et al., 2019), but regulates lateral root development negatively by altering the localization of superoxide in primary roots of *Arabidopsis* (Li et al., 2015). Under Cd stress, the differential regulation of H_2O_2 metabolism, redox homeostasis, and nutrient balance by AtRbohC, AtRbohD, and AtRbohF is of potential interest for biotechnology applications for the phytoremediation of polluted soils (Gupta et al., 2017). AtRbohF is considered a key modulator of defense-associated metabolism and a crucial factor in the interplay between intracellular oxidative stress and pathogenesis responses in *Arabidopsis* (Chaouch et al., 2012). In addition, the level of H_2O_2 has been reported to increase following exposure to high temperatures, resulting in elevated HSF activation and HSP accumulation (Banti et al., 2010), whereas peroxide scavengers and inhibitors of H_2O_2 generation inhibited HSP expression in HS-exposed plants (Königshofer et al., 2008), implicating the involvement of H_2O_2 in the HS signaling pathway. Mutations in *AtRbohB* and *AtRbohD*, two isoforms of NADPH oxidase which contribute to H_2O_2 production, were reported to show weaker defects under HS (Larkindale et al., 2005). Our work further indicated that AtRbohB and AtRbohD-dependent H_2O_2 production acts upstream of nitric oxide (NO) in the HS signaling pathway, involving variations in HSF DNA-binding activity and HSP expression (Wang et al., 2014).

Calcium ions (Ca^{2+}) mobilization is a core issue in various plant signaling pathways. Cyclic nucleotide-gated ion channels (CNGCs) are nonselective cation channels and the main entrances for Ca^{2+} influxes into cells (Jha et al., 2016). In *Arabidopsis* genome, there are 20 expressed CNGC genes, having both different and shared biological activities (Talke et al., 2003). For example, cyclic nucleotide-gated ion channel 6 (CNGC6), CNGC9, and CNGC14 fulfill part of redundant functions to generate and maintain tip focused Ca^{2+} oscillations, which are essential for proper root hair growth and polarity (Brost et al., 2019). CNGC2 and CNGC4-mediated Ca^{2+} entry is suggested to provide a vital link between the pattern-recognition receptor complex and Ca^{2+}-dependent immunity programs in PAMP-triggered immunity signal pathways in plants (Tian et al., 2019). The pollen-tube-specific CNGC7, CNGC8, and CNGC18 together with calmodulin (CaM) constitute a molecular switch that control the open or close of the calcium channel depending on cellular Ca^{2+} levels (Pan et al., 2019). CNGC9 is reported to mediate the elevation of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) to resist disease in rice (Wang et al., 2019). CNGCs are also believed to mediate Ca^{2+} signals in the HS pathway. We reported that CNGC6, a heat-responsive PM Ca^{2+}-permeable channel, is associated with the expression of HSP genes and the acquisition of thermotolerance in *Arabidopsis* (Gao et al., 2012). CNGC6 *via* Ca^{2+} signaling initiates plant resistant reactions to heat stress, but its precise regulatory mechanisms remain obscure. Further investigations into HS signaling will enrich our understanding of the initial heat stress signaling processes.

Calcium ions and H_2O_2 are well known as two universal intracellular secondary messengers. Studies of plants have shown a close relationship between their individual pathways; however, there is controversy regarding which one is upstream of the other. Lots of studies implicate a specific role of H_2O_2 in regulating Ca^{2+} signaling. For example, H_2O_2 production regulates the elevation of $[Ca^{2+}]_{cyt}$ in ABA signaling pathways in *Arabidopsis* guard cells (Jiang et al., 2013; Islam et al., 2019). On the contrary, some studies have pointed to the role of Ca^{2+} in influencing H_2O_2 signaling. For example, extracellular Ca^{2+} through H_2O_2 alleviates NaCl-induced stomatal openings in *Vicia* guard cells (Zhao et al., 2011). Also, crosstalk between Ca^{2+} signaling and H_2O_2 is required for some signaling networks, for example, their co-operation in the process of heavy metal stress resistance (Nazir et al., 2020). The relationship between Ca^{2+} and H_2O_2 is not yet fully understood in plants exposed to HS conditions.

In this investigation, we used the model plant *Arabidopsis* to explore the relationship between H_2O_2 and the Ca^{2+}-permeable channel CNGC6 under heat stress conditions. Our results demonstrate the involvement of H_2O_2 in CNGC6 signaling as a downstream factor in the HS signaling pathway, by stimulating *Hsf* transcription and HSP accumulation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type (WT) and mutant *Arabidopsis* were Col-0 ecotype. *atrbohB* and *atrbohD* mutant seeds were obtained from Dr. Miguel Angel Torress (University of North Carolina). The triple mutant *cngc6/rbohB/D* was obtained by crossing, while the transgenic lines *cngc6/35S::RbohB-1*, *cngc6/35S::RbohB-2*,

cngc6/*35S*::*RbohD-1*, and *cngc6*/*35S*::*RbohD-2* were obtained using the floral dip method.

The *Arabidopsis* seeds were surface sterilized in 2% (v/v) sodium hypochlorite for 1 min and then washed thoroughly with water. The sterilized seeds were placed on Murashige and Skoog (MS) medium containing 3% sucrose and 0.7% agar and kept at 4°C in the dark for 3 days. The plants were then transferred to a growth chamber set at 22°C and 120 μmol $m^{-2}s^{-1}$ on a 16-h daily light period.

For chemical treatment, 2 ml of H_2O_2 at various concentrations (0, 25, 50, 100, and 200 μM; Sigma-Aldrich, St. Louis, MO) were sprinkled onto the leaf surfaces of 8-day-old seedlings after filter sterilization. Sterilized water was used as a substitute for the control of seedlings. After 8 h of pre-treatment, the seedlings were subjected to HS conditions (Wang et al., 2014). In addition, 5 mM $CaCl_2$ or 2 mM EGTA (these reagents were prepared with sterilized water) was used to pre-treat the WT, *cngc6*, and COM12 seeds for 30 min before their being placed on MS medium in the fluorescence experiment, with sterilized water as the control.

Thermotolerance Testing

About 8-day-old seedlings, grown at 22°C, were incubated in sterilized 5 mM $CaCl_2$ at 37°C for 30 min, returned to 22°C for 2 h, then challenged at 45°C for 100 min, and then returned to 22°C for 5 days of recovery (Lewis et al., 2016). The seedlings that were still green and continuing to produce new leaves were registered as survivors. For Western blotting, 10-day-old seedlings were kept at 37°C for 2 h and collected for the analyses of HSP accumulation. All the experiments were repeated at least three times, and there were three independent biological replicates in each repeat (Peng et al., 2019).

Fluorescence Microscopy

Hydrogen peroxide was visualized using the specific fluorescent probe 5-(and-6)-chloromethyl-29,79-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen) as described previously (Wang et al., 2010) with some modifications. Wild-type and mutant seedlings were incubated in 1 ml of liquid MS medium (pH 5.8) with 10 μM CM-H_2DCFDA for 20 min. Thereafter, the roots were washed three times for 15 min each in liquid MS medium prior to visualization with a fluorescence microscope (Eclipse TE 200, Nikon, Tokyo, Japan). The signal intensities were calculated using MetaMorph (Molecular Devices, Sunnyvale, CA).

Vector Construction and the Generation of Transgenic Plants

To generate the *35S:6×Myc-RbohB* construct, the full-length *RbohB* coding sequence was amplified using the primers 5'-CGGGATC-CATGCGGGAGGAAGAAATG-3' and 5'-TCCA CAAGGAAAATTTCTAGCTGCAGTT-3'. To generate the *35S:6×Myc-RbohD* construct, the full-length *RbohD* coding sequence was amplified with the primers 5'-CGGGATCCATGA AAATGAGACGAGGCAA-3' and 5'-CCACAAAGAGAACTTCT AGCTGCAGTT-3'. The products were cloned in the *pCAMBIA1307-6×Myc* vectors using the BamHI and PstI sites.

The transformation of the constructs into *Arabidopsis* (*cngc6*) was performed according to the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* (strain GV3101). Transformants were screened on plates containing 15 mg l^{-1} of Basta. Homozygous T3 transgenic lines were selected for further analysis.

RT-qPCR Analysis

Total RNA (500 ng) was isolated from 10-day-old seedlings at 37°C for 1 h with a PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan) for first-stand cDNA synthesis, as per the manufacturer's instructions. The program was as follows: initial polymerase activation for 10 s at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The reactions were performed using an ABI Prism 7,000 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara Bio Inc.). Primer pairs were designed using Primer Express (Applied Biosystems). Detailed primer sequences are shown in **Supplementary Table 1**.

Western Blot Analysis

About 10-day-old seedlings were kept at 37°C for 2 h and then ground in liquid nitrogen. Total protein was extracted with an extraction buffer (10 mM HEPES, pH 7.9, containing 0.4 M NaCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride), and the extracts were purified by centrifugation at 14,000 × *g* for 20 min at 4°C. The supernatants were transferred to fresh tubes, and the protein content was measured according to the description of Bradford (1976). Total proteins (50 μg) were analyzed by Western blotting, as described previously (Wang et al., 2014).

Preparation of Protoplasts and Electrophysiology Analysis

Protoplasts were isolated as described previously (Demidchik and Tester, 2002) from 1 cm long of root tips of *Arabidopsis* seedlings cultivated vertically at 22°C for 8 days. Whole-cell voltage patch-clamping was carried out as described previously (Gao et al., 2012; Peng et al., 2019; Niu et al., 2020) with minor modification. Patch-clamp pipettes were pulled on a vertical electrode puller. The electrode was filled with pipette solution [0.5 mM $CaCl_2$, 2 mM Mg-ATP, 0.5 mM Tris-ATP, 4 mM $Ca(OH)_2$, 10 mM EGTA and 15 mM HEPES/Tris, pH 7.2, adjusted to an osmolality of 300 mOsm/kg with sorbitol; free Ca^{2+} concentration 100 nM]. The basal external solution comprised 10 mM $CaCl_2$ and 5 mM MES/Tris, pH 5.8, adjusted to an osmolality of 300 mOsm/Kg with sorbitol. The resistance of the electrode in the bath solution was approximately 20 MΩ. Seal resistances were up to 2 GΩ. After holding the whole-cell high seal resistances for 20 min, currents were recorded and data were sampled at 1 kHz and filtered at 200 Hz. Membrane potentials were corrected for liquid junction potentials and series resistance. An Axon 200B amplifier controlled by pCLAMP 9.0 software (Molecular Devices) was used to record the current signal. Basal currents were recorded at room temperature (20–22°C). HS treatment (37 ± 1°C) was performed using continuous bath perfusion.

RESULTS

Effects of HS on H_2O_2 Production in the Wild-Type, *cngc6*, and a Complemented Line COM12 Seedlings

In this work, we presented evidence for the involvement of H_2O_2 in Ca^{2+} signaling in plant thermotolerance. CNGC6, activated by HS and mediated Ca^{2+} influxes, functioned as a signal in the induction of H_2O_2 generation to stimulate the transcription of *Hsfs* and HSPs accumulation. Thus, CNGC6 was found to promote heat tolerance in *Arabidopsis* seedlings.

Hydrogen peroxide is a plant signaling molecule that plays a vital role in many environmental stress responses. Lots of studies suggest a key role for CNGCs in controlling H_2O_2 production (Walker and Berkowitz, 2013; Cui et al., 2020). To elucidate the relationship between H_2O_2 and CNGC6 in thermotolerance, we first determined the transcription levels of *AtRbohB* and *AtRbohD* at the seedling stage using the wild-type plants, a T-DNA insertion mutant (*cngc6*; SALK_042207), and a complementation line (COM12; *cngc6* + *CNGC6*; Gao et al., 2012). The result showed that no clear difference existed between the expression levels in these seedlings under normal conditions; however, both of their expression levels were stimulated by high temperatures and varied depending on the expression level of *CNGC6* (**Supplementary Figure 1**), implying that it had a role in the generation of H_2O_2. Thus, we examined endogenous H_2O_2 accumulations in these seedlings using the special fluorescent probe CM-H_2DCFDA. This probe can be transported into cells, where its acetate groups are passively cleaved by intracellular esterases, producing the fluorescent compound dichlorodihydrofluorescein (DCF; Chozinski et al., 2016).

Fluorescence analysis indicated that under normal conditions (22°C), no clear difference in the abundance of H_2O_2 was observed among the seedlings. After HS treatment at 45°C for 30 min (Wang et al., 2014), the H_2O_2 level increased by 208% in the wild-type seedlings, higher than the increase observed in *cngc6* (108%); however, it was nearly rescued in COM12 seedlings (187%; **Figures 1A,B**). We also found that not all the production of H_2O_2 responsive to HS was inhibited in *ncgc6* mutant. Thus, these results suggest that the production of H_2O_2 observed after HS treatment was partially due to the activation of CNGC6.

Effect of Ca^{2+} on the H_2O_2 Accumulation in the Wild-Type Seedlings

Cyclic nucleotide-gated ion channel 6 is a heat-responsive Ca^{2+}-permeable channel in the PM of plant cells (Gao et al., 2012). Ca^{2+} is one of the most multifunctional ions existed in eukaryotes, and it has been confirmed to coordinate with H_2O_2 in many physiological processes (Ferreira et al., 2003). Thus, it is reasonable to consider that CNGC6 elevates the H_2O_2 level through Ca^{2+} to induce thermotolerance.

To test this hypothesis, the H_2O_2 levels were examined in the wild-type, *cngc6*, and COM12 seedlings pre-treated with 5 mM $CaCl_2$ or 2 mM EGTA (a Ca^{2+} chelator) before germination as described previously (Liu et al., 2005; Peng et al., 2019). Fluorescence analysis showed that under normal growth conditions, the H_2O_2 levels in wild-type, *cngc6*, and COM12 seedlings were rather stable. However, under HS conditions, 5 mM Ca^{2+} treatment elevated the H_2O_2 level to 411, 303, and 389% of their individual controls in the wild-type, *cngc6*, and COM12 seedlings, respectively. Whereas 2 mM EGTA reduced the increase in H_2O_2 to 245 and 213% of the wild-type and COM12 controls, respectively, but there was no clear effect on the *cngc6* mutant (**Figures 1C–H**).

Effects of H_2O_2 on the Thermotolerance of *cngc6* Seedlings

Subsequently, a solution containing a series of concentrations of H_2O_2 was used to pre-treat the wild-type and *cngc6* seedlings. Under HS conditions, the internal H_2O_2 level was higher in the wild-type seedlings than in the *cngc6* seedlings. Exogenous application of H_2O_2 stimulated the internal H_2O_2 level in these seedlings depending on the H_2O_2 concentration, reaching a maximum value at 100 μM and decreasing slightly at 200 μM (**Figures 2A,B**). The survival ratios of the wild-type and *cngc6* seedlings changed in the same manner as their internal H_2O_2 levels, reaching the maximum at 100 μM (**Figures 2C,D**).

Taken together, these results (**Figures 1, 2**) showed that heat-responsive Ca^{2+} channel CNGC6 regulated H_2O_2 production; however, an increased internal H_2O_2 level rescued the impaired thermotolerance of the CNGC6-deficient mutant, indicating H_2O_2 involvement in CNGC6 signaling as a downstream factor.

AtRbohB and *AtRbohD* Overexpression in a *cngc6* Background Increases Thermotolerance

We even reported that H_2O_2 acts as a signal in heat tolerance using the mutants *rbohB* and *rbohD*, which show poor thermotolerance due to a deficiency in H_2O_2 (Wang et al., 2014). To further investigate the effect of CNGC6 on H_2O_2 signaling under HS conditions, we obtained two *AtRbohB*-overexpressing transgenic lines, *cngc6/35S::RbohB-1* and *cngc6/35S::RbohB-2*, and two *AtRbohD*-overexpressing transgenic lines, *cngc6/35S::RbohD-1* and *cngc6/35S::RbohD-2*, and examined the influences of excess internal H_2O_2 on *CNGC6*-deficient mutants under HS conditions. The increased expression of *AtRbohB* and *AtRbohD* was confirmed according to real-time quantitative PCR (RT-qPCR; **Figures 3A, 4A**).

Dichlorodihydrofluorescein fluorescence analysis indicated that *AtRbohB* and *AtRbohD* overexpression enhanced the internal H_2O_2 levels in these transgenic plants under normal and HS conditions (**Figures 3, 4**). Under normal conditions, no clear phenotypic difference was observed between *cngc6* mutant and these transgenic lines. However, under high temperature conditions, *AtRbohB* or *AtRbohD* overexpression greatly improved the survival ratio of the transgenic lines in comparison with their background *cngc6* according to their individual transcriptional levels (**Figures 3, 4**).

These results showed that the overexpression of *AtRbohB* or *AtRbohD* restored heat tolerance in a *CNGC6*-deficient

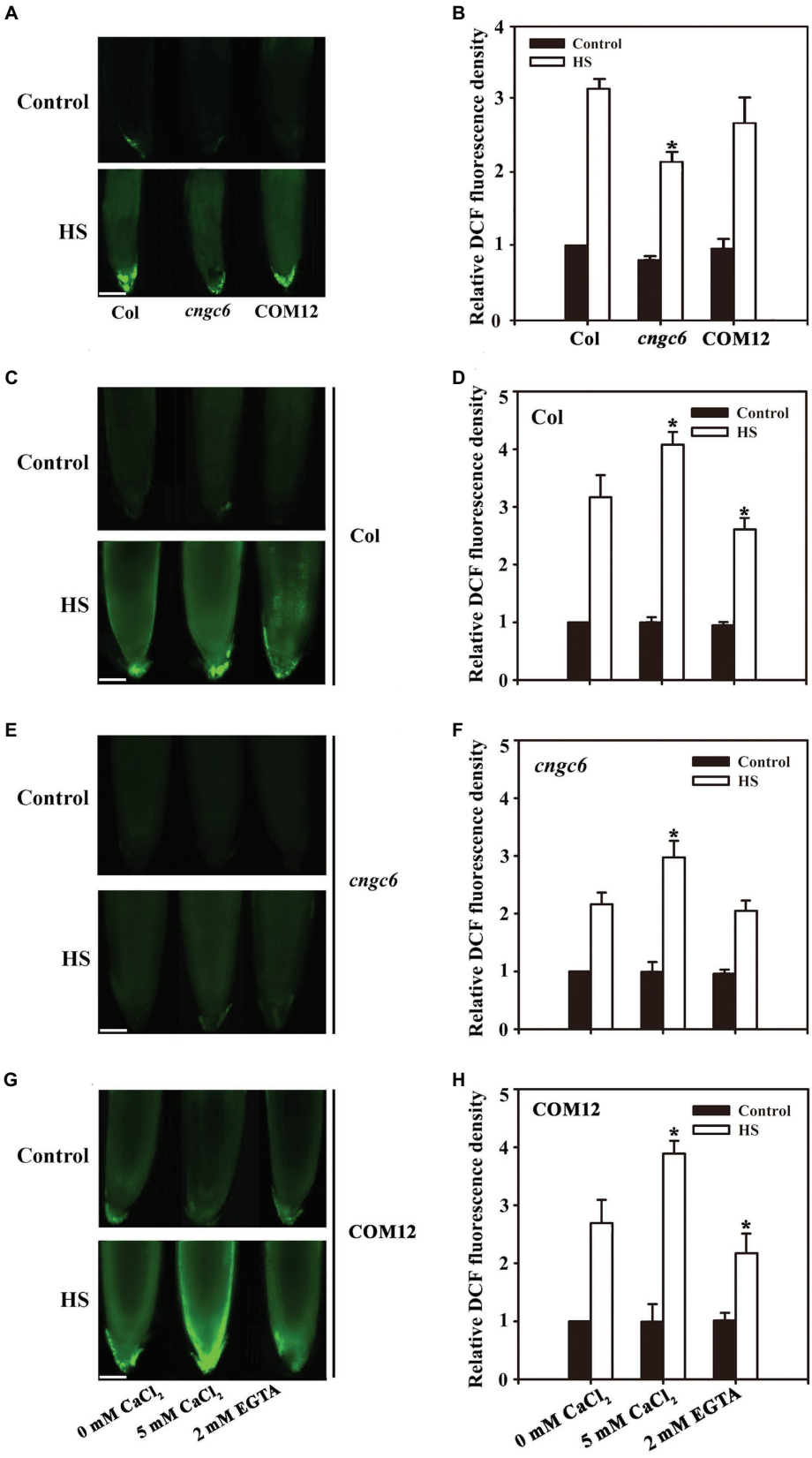

FIGURE 1 | (Continued)

FIGURE 1 | Effects of calcium ions (Ca^{2+}) on hydrogen peroxide (H_2O_2) accumulation in *Arabidopsis* seedlings. **(A)** About 8-day-old wild-type (WT), *cngc6*, and COM12 seedlings grown at 22°C were exposed to 45°C (heat shock, HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels in the seedlings were then examined by fluorescence microscopy using roots dyed with 5-(and-6)-chloromethyl-29,79-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Bar = 100 μm. **(B)** Relative dichlorodihydrofluorescein (DCF) fluorescence densities in the roots. The data presented are the means ± SE of measurements taken from five independent experiments with at least 10 roots for each treatment. *$p < 0.05$ vs. Col (Student's *t*-test). **(C,E,G)** About 8-day-old seedlings of wild-type **(C)**, *cngc6* **(E)**, and COM2 **(G)** were exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels in the plants were then examined by fluorescence microscopy using roots stained with CM-H_2DCFDA. Bar = 100 μm. **(D,F,H)** The relative DCF fluorescence densities in the roots of wild-type **(D)**, *cngc6* **(F)**, and COM2 **(H)**. The data presented are the means ± SE of measurements taken from five independent experiments with at least 10 roots for each treatment. *$p < 0.05$ vs. 0 mM $CaCl_2$ (Student's *t*-test).

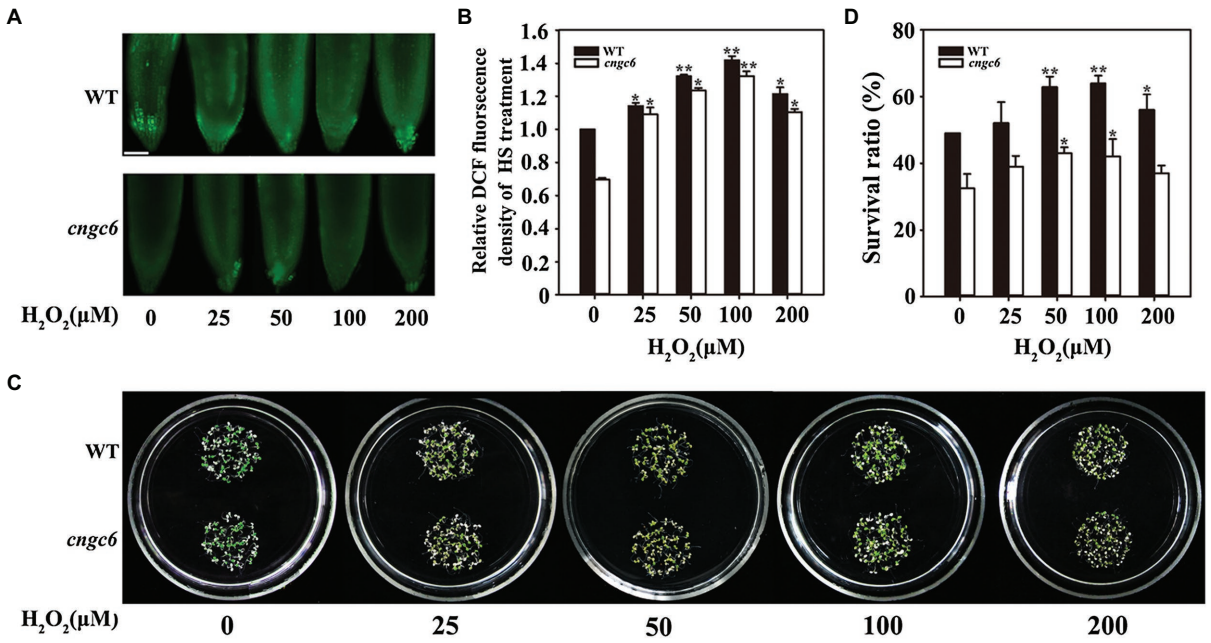

FIGURE 2 | Effects of H_2O_2 on the thermotolerance of WT and *cngc6* seedlings. **(A)** About 8-day-old WT and *cngc6* seedlings grown at 22°C were pre-treated with 2 ml of 0, 25, 50, 100, or 200 mM H_2O_2 for 8 h and then exposed to 45°C (HS) for 30 min. The H_2O_2 levels were then assessed by fluorescence microscopy in roots stained with CM-H2DCFDA. Bar = 100 mm. **(B)** Relative DCF fluorescence densities in the roots. The data presented are means ± SE of measurements taken from at least 10 roots for each treatment. *$p < 0.05$ and **$p < 0.01$ vs. 0 mM H_2O_2 (Student's *t*-test). **(C)** Seedlings were exposed to 45°C for 100 min, then returned to 22°C and photographed 5 days later. **(D)** Survival ratios of the seedlings after HS treatment. The data presented are means ± SE of at least five independent experiments with 50 seedlings per experiment. *$p < 0.05$ vs. 0 mM H_2O_2 (Student's *t*-test).

mutant, providing genetic proof for the relationship between CNGC6 and H_2O_2 in HS signaling.

Effects of HS on the Thermotolerance of the *cngc6/rbohB/D* Triple-Mutant Seedlings

To further examine the roles of CNGC6 and H_2O_2 in plant thermotolerance, we obtained the *cngc6/rbohB/D* triple mutant by crossing, which was deficient in *CNGC6*, *RbohB*, and *RbohD* transcription according to RT-qPCR analysis (**Figure 5A**). Under normal and HS conditions, the H_2O_2 level in the *cngc6/rbohB/D* seedlings was similar to that in the *rbohB/D* seedlings (**Figures 5B,C**), revealing that the deficiency of *CNGC6* did not remarkably reduce H_2O_2 accumulation in the *rbohB/D* seedlings. Under normal conditions, *cngc6/rbohB/D* seedlings showed similar phenotypes with other seedlings (**Figure 5D**, Control). Under HS conditions, the survival ratio of the *cngc6/rbohB/D* seedlings was near to that of the *rbohB/D* seedlings (**Figures 5D,E**), showing that the deficiency of *CNGC6* did not obviously aggravate the heat susceptibility of *rbohB/D*.

Effects of H_2O_2 on the Activity of Ca^{2+}-Permeable Channel

The results provided evidence of the function of CNGC6 on the H_2O_2-mediated acquisition of heat tolerance. In *Arabidopsis*, a specific role for H_2O_2 in regulating Ca^{2+} mobilization has also been found (Islam et al., 2019).

To confirm whether H_2O_2 influences the action of heat-responsive Ca^{2+}-permeable channels, we determined the effects of internal H_2O_2 on the function of CNCG6 in the PM of root protoplasts of *Arabidopsis* with the whole-cell patch-clamp technique (Gao et al., 2012; Peng et al., 2019). Under normal conditions at 22°C, the Ca^{2+} current in *cngc6* (−136 pA) was lower than in the wild-type (−178 pA) at −200 mV. Under HS at 37°C, the inward Ca^{2+} current was swiftly

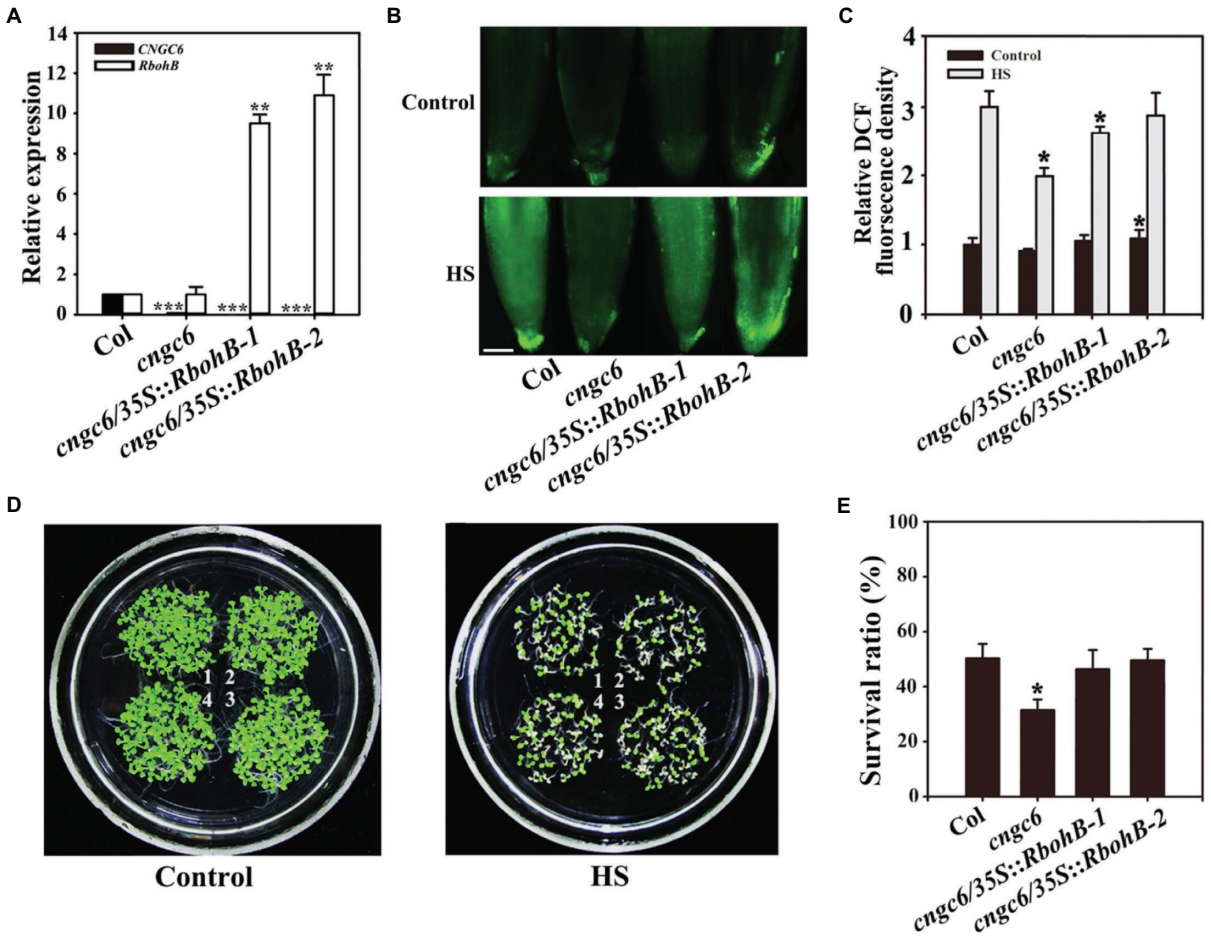

FIGURE 3 | Improved thermotolerance through *AtRbohB* overexpression in a *cngc6* background. **(A)** Real-time quantitative PCR (RT-qPCR) analysis of *AtCNGC6* and *AtRbohB* transcription in wild-type, *cngc6*, *cngc6/35S::RbohB-1*, and *cngc6/35S::RbohB-2* plants. The experiments were repeated three times with similar results. Each data point represents the mean ± SD (n = 3). Asterisks indicate a significant difference relative to Col (Student's t-test: $^{**}p < 0.01$ and $^{***}p < 0.001$). **(B)** About 8-day-old wild-type, *cngc6*, *cngc6/35S::RbohB-1*, and *cngc6/35S::RbohB-2* seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels in the plants were then examined by fluorescence microscopy using roots stained with CM-H_2DCFDA. Bar = 100 μm. **(C)** The relative DCF fluorescence densities in the roots. The data presented are the means ± SE of measurements taken from five independent experiments with at least 10 roots for each treatment. $^{*}p < 0.05$ vs. Col. **(D)** Seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 100 min, then returned to 22°C and photographed 5 days later. The clusters are as follows: 1, wild-type; 2, *cngc6*; 3, *cngc6/35S::RbohB-1*; and 4, *cngc6/35S::RbohB-2*. **(E)** Survival ratios of the seedlings after HS treatment. The data presented are the means ± SE of at least five independent experiments with 50 seedlings per experiment. $^{*}p < 0.05$ vs. Col (Student's t-test).

elevated to −375 pA in the wild-type within 1 min. However, only a slight increase (to −171 pA) was observed in *cngc6* (**Figures 6A,B**), in accordance with our previous reports (Gao et al., 2012; Peng et al., 2019; Niu et al., 2020). In the *rbohB/D* double mutant with low internal H_2O_2 levels, the Ca^{2+} currents exhibited similar changing trends to those in the wild-type under both of normal and HS conditions (**Figure 6C**). However, in the *cngc6/rbohB/D* triple mutant, the Ca^{2+} currents showed no clear difference with those in *cngc6* under normal and HS conditions (**Figure 6D**). In two transgenic lines with high endogenous H_2O_2 levels, *cngc6/35S::RbohB-1* and *cngc6/35S::RbohD-1*, the Ca^{2+} currents were similar to those of *cngc6* (non-transgenic background; **Figures 6E,F**), indicating that H_2O_2 had no obvious affection on the activity of Ca^{2+} channel.

Effect of CNGC6 on the Transcription of *Hsf* and the Expression of AtHSP21 and AtHSP17.7 Through H_2O_2

To investigate the underlying mechanisms of CNGC6- and H_2O_2-induced plant thermotolerance, the mRNA level of *Hsf* in the wild-type, *cngc6*, *rbohB/D*, and *cngc6/rbohB/D* seedlings as well as in the two individual *RbohB*- and *RbohD*-overexpressing transgenic lines (*cngc6/35S::RbohB-1* and *cngc6/35S::RbohD-1*) was analyzed using RT-qPCR. Under normal conditions, there was no clear difference among the levels in these seedlings (**Figure 7**, Control). After the HS treatment, *Hsf* (*Hsf2A*, *HsfA7a*, and *HsfB2b*) mRNA levels were dramatically elevated. However, in *cngc6*, *rbohB/D*, and *cngc6/rbohB/D* seedlings, they were lower than in the wild-type seedlings (and lowest for *cngc6/rbohB/D*) but they were significantly

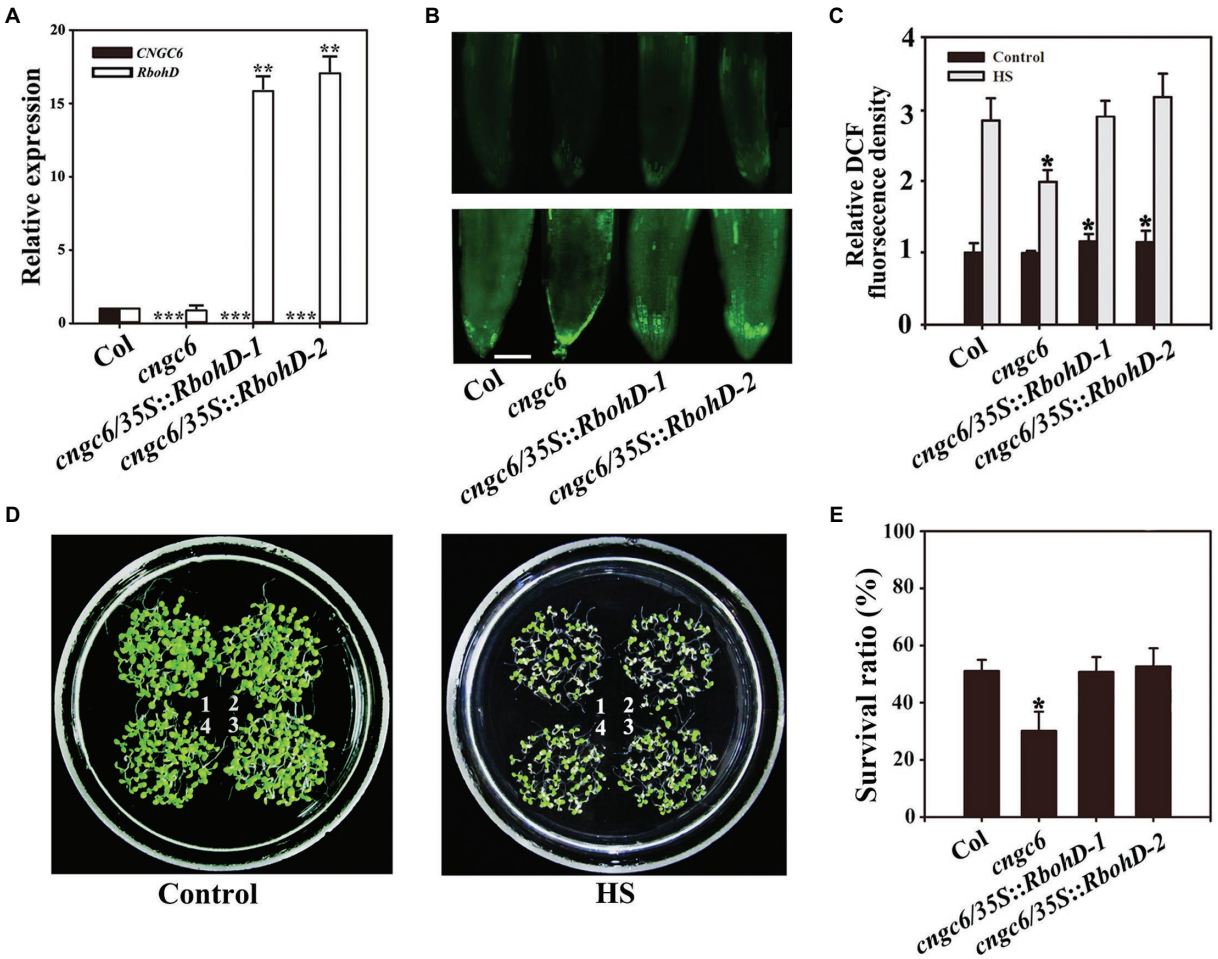

FIGURE 4 | Improved thermotolerance through *AtRbohD* overexpression in a *cngc6* background. **(A)** RT-qPCR analysis of *AtCNGC6* and *AtRbohD* transcription in wild-type, *cngc6*, *cngc6/35S::RbohD-1*, and *cngc6/35S::RbohD-2* plants. The experiments were repeated three times with similar results. Each data point represents the mean ± SD ($n = 3$). Asterisks indicate a significant difference relative to Col (Student's *t*-test: $^{**}p < 0.01$ and $^{***}p < 0.001$). **(B)** About 8-day-old wild-type, *cngc6*, *cngc6/35S::RbohD-1*, and *cngc6/35S::RbohD-2* seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels in the plants were then examined by fluorescence microscopy using roots stained with CM-H_2DCFDA. Bar = 100 μm. **(C)** The relative DCF fluorescence densities in the roots. The data presented are the means ± SE of measurements taken from five independent experiments with at least 10 roots for each treatment. $^{*}p < 0.05$ vs. Col. **(D)** Seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 100 min, then returned to 22°C and photographed 5 days later. The clusters are as follows: 1, wild-type; 2, *cngc6*; 3, *cngc6/35S::RbohD-1*; and 4, *cngc6/35S::RbohD-2*. **(E)** Survival ratios of the seedlings after HS treatment. The data presented are the means ± SE of at least five independent experiments with 50 seedlings per experiment. $^{*}p < 0.05$ vs. Col (Student's *t*-test).

stimulated by 50 μM H_2O_2 and were activated in the two transgenic lines compared with their background *cngc6* (**Figure 7**, HS).

Heat shock proteins, as molecular chaperones, are crucial for all organisms to survive under severe stress through the maintenance of proteostasis (Akerfelt et al., 2010). Thus, we subsequently determined the influences of CNGC6 and H_2O_2 on the expression of AtHSP17.7 and AtHSP21 in these plants using Western blotting analysis. Neither AtHSP17.7 nor AtHSP21 was observed at 22°C; however, both of them accumulated at 37°C (**Figure 8**). The level of protein expression was lower in the mutants than in the wild-type (and lowest for *cngc6/rbohB/D*), and it was greatly elevated by 50 μM H_2O_2 in the *cngc6* mutant. In addition, its accumulation was increased in the *cngc6/35S::RbohB-1* and *cngc6/35S::RbohD-1* plants in comparison with the *cngc6* mutant (non-transformed background; **Figure 8**). In all these experiments, tubulin was adopted to ensure equal sample loading.

These results revealed that the application of H_2O_2 and the overexpression of *AtRbohB* or *AtRbohD* prompted HSP expression in a *cngc6* mutant, providing further evidence that CNGC6 acts upstream of H_2O_2 in the HS pathway.

DISCUSSION

The Relationships Among Ca^{2+}, CNGC6, and H_2O_2 Accumulation in Plant Thermotolerance in *Arabidopsis* Seedlings

High external temperatures always result in elevated $[Ca^{2+}]_{cyt}$ and the accumulation of H_2O_2 in plant cells, as they play

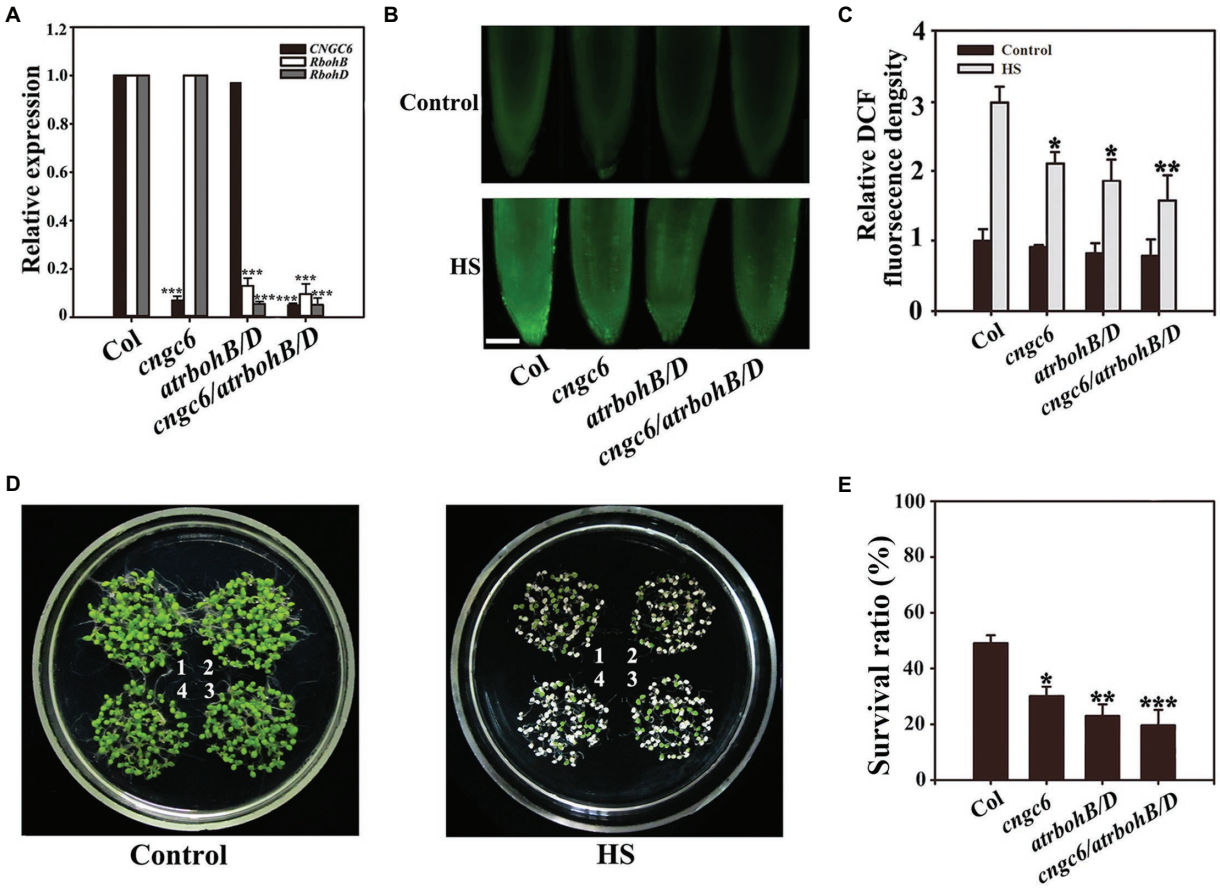

FIGURE 5 | Survival status of the *cngc6/rbohB/D* triple mutant. **(A)** RT-qPCR analysis of cyclic nucleotide-gated ion channel 6 (*CNGC6*), *RbohB* and *RbohD* transcription in wild-type, *cngc6*, *rbohB/D*, and *cngc6/rbohB/D* seedlings. The experiments were repeated three times with similar results. Each data point represents the mean ± SD (n = 3). Asterisks indicate a significant difference relative to Col; ***$p < 0.001$ (Student's t-test). **(B)** About 8-day-old wild-type, *cngc6*, *rbohB/D*, and *cngc6/rbohB/D* seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 30 min. The H_2O_2 levels in the seedlings were then examined by fluorescence microscopy using roots stained with CM-H_2DCFDA. Bar = 100 μm. **(C)** Relative DCF fluorescence densities in the roots. The data presented are the means ± SE of measurements taken from five independent experiments with at least 10 roots for each treatment. *$p < 0.05$, and **$p < 0.01$ vs. Col (Student's t-test). **(D)** About 8-day-old seedlings grown at 22°C were exposed to 45°C (HS) or maintained at 22°C (Control) for 100 min, then returned to 22°C and photographed 5 days later. The clusters are as follows: 1, wild-type; 2, *cngc6*; 3, *rbohB/D*; and 4, *cngc6/rbohB/D*. **(E)** Survival ratios of the seedlings after HS treatment. The data presented are the means ± SE of at least five independent experiments with 50 seedlings per experiment. *$p < 0.05$ and **$p < 0.01$ vs. Col (Student's t-test).

crucial roles in the response of plant to HS (Liu et al., 2005; Sun and Guo, 2016). However, the relationship between H_2O_2 and Ca^{2+} signaling pathways in thermotolerance is unclear. Herein, our work showed that CNGC6, a heat-activated Ca^{2+}-permeable channel, stimulates H_2O_2 accumulation to regulate the gene expression of *Hsfs* and HSPs accumulation to promote plant heat tolerance.

Hydrogen peroxide, an essential second messenger in a wide variety of biological processes, is stimulated by various factors to counteract exogenous stresses in plants. We previously reported that H_2O_2 acts as a signal in the induction of heat tolerance through NO (Wang et al., 2014). NO was even found to be associated with elevating intracellular levels of free Ca^{2+} under HS conditions (Peng et al., 2019). Recently, several studies have focused on the function of Ca^{2+} in initiating H_2O_2 accumulation in plants (Ferreira et al., 2003; Zhao et al., 2011). Therefore, we speculated that there should be a close relationship between Ca^{2+} and H_2O_2 in HS signaling pathway.

In plants, the CNGC proteins are expressed differentially in numerous tissues (Zelman et al., 2012). Molecular genetic studies have revealed that CNGCs frequently function in numerous biological processes, including plant growth and development, adaptations to increased Ca^{2+} concentration, and plant responses to abiotic and biotic stresses (Gao et al., 2016; Jha et al., 2016). Our prior work has demonstrated that AtCNGC6 is a heat-activated PM Ca^{2+}-permeable channel that conducts Ca^{2+} into the cytoplasm to help regulate HS responses. A T-DNA insertion mutant *cncg6* was used for those investigations due to its lower Ca^{2+} current than the wild-type, which is nearly totally restored in the transgenic line COM12 plants after HS treatment (Gao et al., 2012; Peng et al., 2019), indicating that CNGC6 regulates the influx of Ca^{2+} into plant cells.

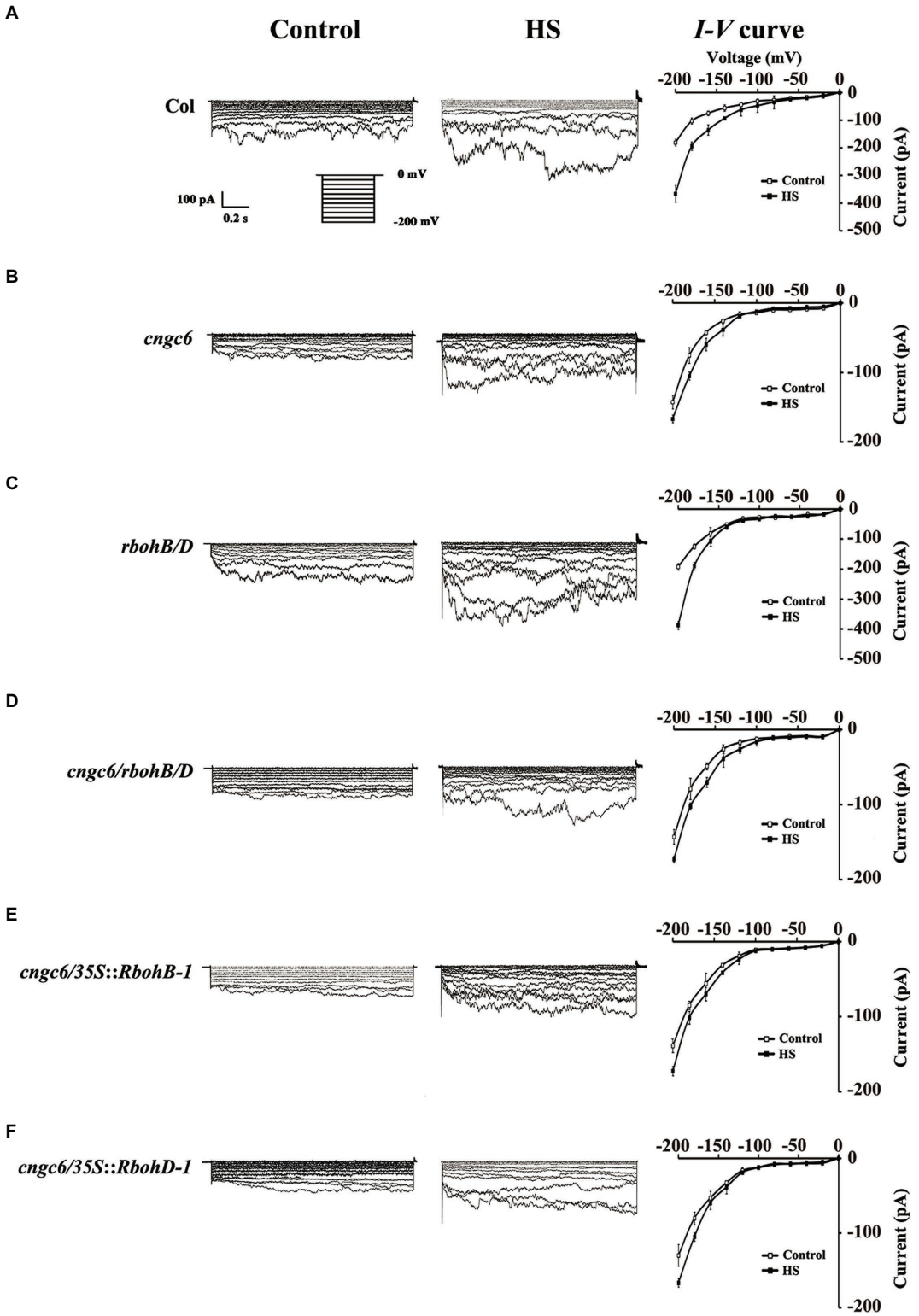

FIGURE 6 | Patch-clamp analysis of Ca^{2+}-permeable channels in wild-type, *cngc6*, *rbohB/D*, *cngc6/rbohB/D*, *cngc6/35S::RbohB-1*, and *cngc6/35S::RbohD-1* seedlings. The Ca^{2+} current before HS (at 22°C, control) and after HS (at 37°C, HS) was compared in the root cell protoplasts of 8-day-old wild-type **(A)**, *cngc6* **(B)**, *rbohB/D* **(C)**, *cngc6/rbohB/D* **(D)**, *cngc6/35S::RbohB-1* **(E)**, and *cngc6/35S::RbohD-1* **(F)** plants. The Ca^{2+} current was recorded by step voltage clamp. Each trace is a representative current from six protoplasts. Currents in the protoplasts are shown in the left and middle columns, respectively. The *I–V* curve is shown in the right column (mean ± SD, $n = 6$).

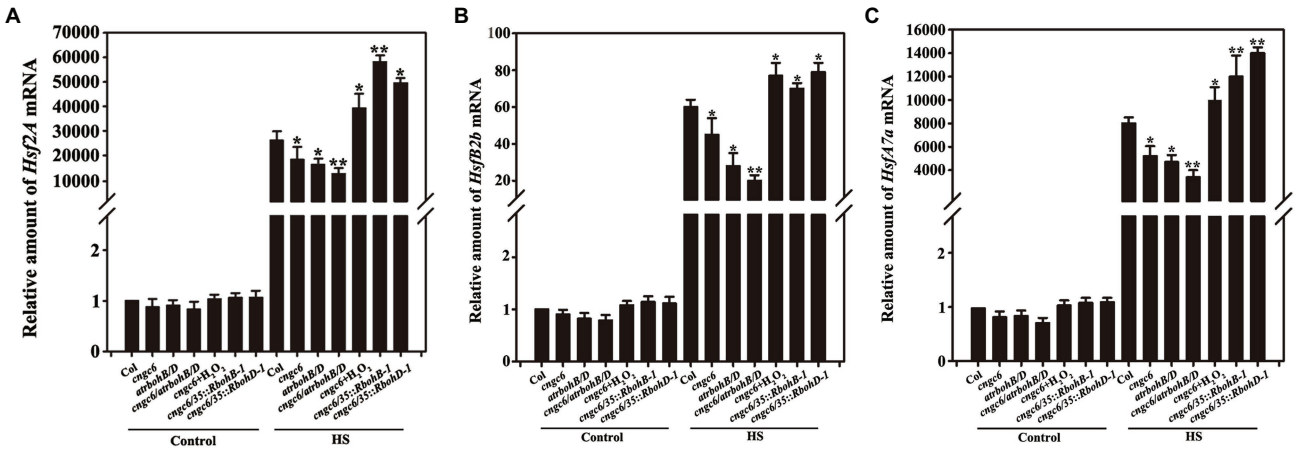

FIGURE 7 | Analysis of the effects of CNGC6 on H_2O_2-induced *Hsfs* by RT-qPCR. About 10-day-old seedlings grown at 22°C was exposed to 37°C (HS) or maintained at 22°C (Control) for 60 min, then used for analysis of *Hsf* (**A**, *Hsf2A*; **B**, *HsfA7a*; and **C**, *HsfB2b*) mRNA expression. The data are the mean ± SE of at least five independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ vs. Col (Student's *t*-test).

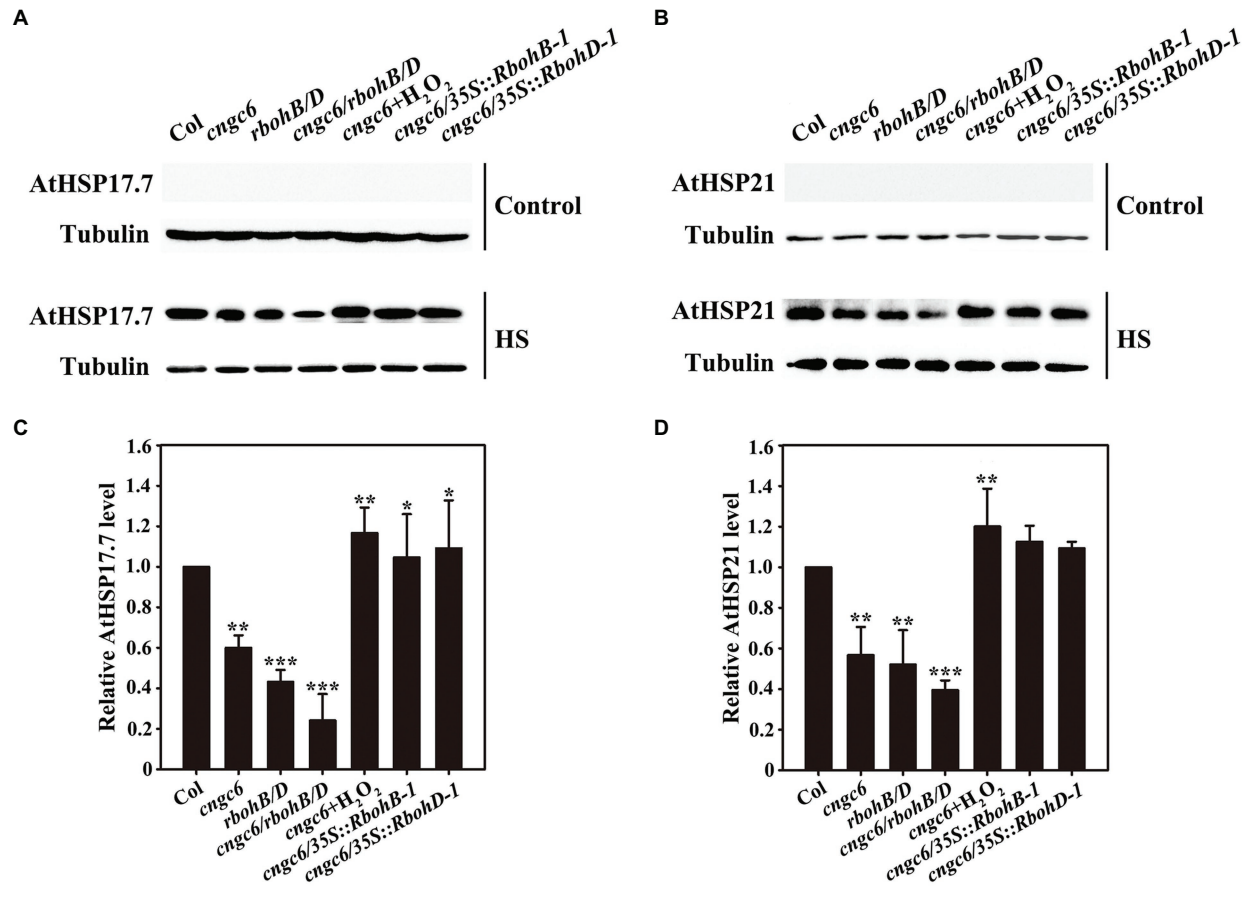

FIGURE 8 | Effects of CNGC6 *via* H_2O_2 on AtHSP17.7 and AtHSP21 expression. **(A,B)** About 10-day-old seedlings grown at 22°C were exposed to 37°C (HS) or maintained at 22°C (Control) for 2 h. Total protein was then extracted, separated by SDS-PAGE, and analyzed by Western blotting. Tubulin was used as an internal control. Three independent experiments were carried out; the results indicate similar trends in protein accumulation. **(C,D)** The relative HSP17.7 **(C)** and HSP21 **(D)** level after HS treatment. The data presented are the means ± SE of measurements taken from three independent experiments and represents the relative intensity of each signal. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. Col (Student's *t*-test).

Thus, we used the *cngc6* mutant and the COM12 plants to examine the relationship between H_2O_2 and CNGC6 in plant thermotolerance.

The mRNA level of *AtRbohB/D* is stimulated by HS depending on CNGC6 expression levels (**Supplementary Figure 1**), indicating that CNGC6 regulates H_2O_2 accumulation under HS conditions. Thus, we first examined H_2O_2 levels using the fluorescent probe CM-H_2DCFDA. The results showed that high temperatures stimulated H_2O_2 accumulation according to their *CNGC6* expression levels in the seedlings (**Figures 1A,B**), indicating an important role of CNGC6 in the regulation of H_2O_2 production in the HS pathway.

Because of the role of CNCG6 in conducting Ca^{2+} into the cytoplasm in HS-treated plants, we determined the effects of Ca^{2+} on H_2O_2 accumulations in the wild-type, *cngc6*, and COM12 seedlings. The results showed that Ca^{2+} increased H_2O_2 accumulation in the seedlings under high temperature, whereas the Ca^{2+} chelator EGTA clearly reduced H_2O_2 accumulations in the wild-type and COM12 seedlings (**Figures 1C–H**), indicating that CNGC6-mediated free Ca^{2+} is a crucial factor in promoting H_2O_2 signaling. Thus, we propose that CNGC6 participates in stimulating internal H_2O_2 levels *via* free Ca^{2+} in the HS pathway. However, EGTA had no clear effect on the H_2O_2 level in *cngc6* seedlings, which might be due to the smaller increase in free Ca^{2+} under HS exposure (**Figures 1E,F**).

Effects of CNGC6 and H_2O_2 on Heat Tolerance in *Arabidopsis* Seedlings

To interpret the effects of CNGC6 and H_2O_2 on thermotolerance, we determined the effects of H_2O_2 on the survival of wild-type and *cngc6* seedlings exposed to HS conditions. Exogenous applications of H_2O_2 enhanced the internal H_2O_2 levels and the survival ratios of both of HS-treated wild-type and *cngc6* seedlings (**Figure 2**). The overexpression of two HS-responsive H_2O_2 synthesis-related enzymes, *RbohB* and *RbohD*, simultaneously elevated the internal H_2O_2 levels and the survival ratios of these transgenic lines, in comparison with their non-transgenic background *cngc6* under HS conditions (**Figures 3, 4**), respectively, indicating that an increase in internal H_2O_2 restored the heat sensitivity of the mutant plants because of the absence of *CNGC6*. We also identified a strange phenomenon in that a high H_2O_2 concentration (200 μM) could not produce a high internal H_2O_2 level under HS conditions (**Figures 2A,B**), which is likely due to plant self-protection against oxidative damage as discussed previously (Wang et al., 2014; Wu et al., 2015).

Next, we obtained the triple mutant *cngc6/rbohB/D*, which showed a phenotype similar to that of the *rbohB/D* double mutant under normal and HS conditions (**Figure 5**), revealing that deficiencies in *CNGC6* and *RbohB/D* do not aggravate the heat susceptibility due to a deficiency in *RbohB/D*.

Collectively, the upon results provide physiological and genetic proof for the existence of a novel HS signaling pathway in which CNGC6 is activated by high temperatures to mediate H_2O_2 accumulation to confer plant thermotolerance.

Effects of H_2O_2 on Ca^{2+} Fluxes in the Responses of *Arabidopsis* Seedlings to HS Stress

Hydrogen peroxide is the especially stable one of ROS and regulates plant growth, development, and stress adaptations. It acts through increasing $[Ca^{2+}]_{cyt}$ as a second messenger, by the activation of the PM Ca^{2+}-permeable influx channels as a primary part of this process (Ordoñez et al., 2014; Richards et al., 2014; Shabala, 2019). However, only few studies have drawn the opposite conclusion that Ca^{2+} influx influences H_2O_2 generation. For example, the silencing of two tomato CNGC genes, *SICNGC1* and *SICNGC14*, was reported to strikingly promote both pathogen-induced and flg22-elicited H_2O_2, revealing that two *SICNGCs* inhibit ROS production and attenuate non-host resistance and PAMP-triggered immunity (Zhang et al., 2018). Accordingly, we wondered whether H_2O_2 stimulates Ca^{2+} influxes to confer thermotolerance.

A marked elevation in Ca^{2+} current was presented in the response to a swift temperature increase from 22 to 37°C in the wild-type. However, the current was clearly inhibited in *cngc6*, *cngc6/rbohB/D*, *cngc6/35S::RbohB-1*, and *cngc6/35S::RbohD-1* plants but not obviously varied in *rbohB/D* plants (**Figure 6**), showing no great effect of H_2O_2 on the activity of Ca^{2+}-permeable channel. These results, in combination with those shown in **Figures 2–5**, proposed that the HS-induced alteration in Ca^{2+} unidirectionally stimulates H_2O_2 signaling in plants. A plausible interpretation for these data is that supplementation with H_2O_2, a downstream signal molecule, rescued the heat-susceptible phenotype of the *CNGC6*-deficient seedlings (**Figures 2–5**) but could not elevate the heat-responsive activity of CNGC6 (**Figure 6**).

The Mechanism Underlying the Effects of CNGC6 *via* H_2O_2 on Thermotolerance

To examine the mechanisms by which CNGC6 influences heat tolerance *via* H_2O_2, we determined the effects of CNGC6 and H_2O_2 on *Hsf* transcript and HSP expression under HS conditions.

Heat shock factors are known as downstream elements in the HS signaling pathway to regulate heat tolerance by deciding the expression of HSPs as the response to phosphorylation (Kotak et al., 2007). Our current data indicated that a reduction in the level of CNGC6 prohibits the transcript levels of *Hsfs*, whereas applications of H_2O_2 and overexpression of *RbohB* and *RbohD* elevates them in *cngc6* plants (**Figure 7**). Therefore, H_2O_2 appears to restore the CNGC6 effects, thereby influencing the *Hsfs* transcription and inducing to thermotolerance.

Heat shock protein genes, stimulated by HSFs linking to promoter elements, are categorized depending on their molecular masses, for example, HSP110, HSP100, HSP90, HSP70, and small HSPs, which are the most important ones among them due to their irreplaceable role in plant tolerance against high temperatures (Carre et al., 2019). To interpret the relationship between CNGC6 and H_2O_2 in the HS signaling pathway, we used HSP21 and HSP17.7, two small HSPs, to examine how CNGC6 mediates thermotolerance through H_2O_2. Western-blot analysis revealed that the reduced *CNGC6* level in *cngc6* mutant decreased

HSP21 and HSP17.7 expression under HS conditions, whereas application of H_2O_2 and the overexpression of *RbohB* or *RbohD* in *cngc6* plants increased the accumulation of HSP21 and HSP17.7 (**Figure 8**), indicating that CNGC6 activated HSP expression *via* H_2O_2. Taken together, the mechanism through which CNGC6 influences thermotolerance *via* H_2O_2 involves variations in HSP gene expression.

These upon results suggest that CNGC6, the HS-responsive Ca^{2+}-permeable channel, takes part in the initiation of HS signaling transduction through H_2O_2. We previously suggested a model for the HS signaling pathway in which the HS signal was received by an unknown receptor, resulting in an elevated H_2O_2 level and then stimulating NO production and AtCaM3 expression to initiate plant resistance against high temperatures (Xuan et al., 2010; Wang et al., 2014). Additionally, feedback inhibition existed between NO and H_2O_2 in the HS signaling pathway in *Arabidopsis* (Wu et al., 2015). AtCaM3 also inhibited excess NO accumulation and enhanced plant thermotolerance through stimulating *S*-nitrosoglutathione reductase by direct binding (Zhang et al., 2020). Recently, we found that CNGC6 through free Ca^{2+} acts upstream of NO in plant response to HS (Peng et al., 2019). In this work, CNGC6 was also proposed to act upstream of H_2O_2 through free Ca^{2+} in the HS pathway. Ca^{2+} and AtCaM3 are associated with HSP gene expression in *Arabidopsis* (Zhang et al., 2009). CaM, upon binding to Ca^{2+}, attaches to specific targets, increasing their functions as part of a HS-responsive Ca^{2+} signaling pathway, for instance, CaM-binding protein kinase 3 (Liu et al., 2008) and PP7 (Liu et al., 2007). Thus, these findings suggest that interactions exist among Ca^{2+} channels, H_2O_2, NO, and the Ca^{2+}/CaM-dependent target proteins to participate in regulating HSP expression in the HS pathway.

ACCESSION NUMBERS

Sequence data from this article can be found in GenBank/EMBL under the following accession numbers: *AtRbohB* (At1G09090), *AtRbohD* (AT5G47910), *CNGC6* (At2g23980), and *Actin2* (At3g18780).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BL and LZo conceived the project and designed the research. WW and JZ carried out the phenotypic observation, RT-qPCR analysis, *Arabidopsis* transgenic experiments, and Western blot analysis. WW and LA carried out the whole-cell voltage patch-clamping. LZn and DW participated in the data analysis. LZo wrote the article with contributions from all authors and revised and proofread the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.708672/full#supplementary-material

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Identification of Genes and MicroRNAs Affecting Pre-harvest Sprouting in Rice (*Oryza sativa* L.) by Transcriptome and Small RNAome Analyses

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Pre-harvest sprouting (PHS) is one of the primary problems associated with seed dormancy in rice (*Oryza sativa* L.). It causes yield loss and reduces grain quality under unpredictable humid conditions at the ripening stage, thus affecting the economic value of the rice crop. To resolve this issue, understanding the molecular mechanism underlying seed dormancy in rice is important. Recent studies have shown that seed dormancy is affected by a large number of genes associated with plant hormone regulation. However, understanding regarding the effect of heat stress on seed dormancy and plant hormones is limited. This study compared the transcriptome and small RNAome of the seed embryo and endosperm of two contrasting *japonica* rice accessions, PHS susceptible (with low seed dormancy) and PHS resistant (with high seed dormancy), at three different maturation stages. We found that 9,068 genes and 35 microRNAs (miRNAs) were differentially expressed in the embryo, whereas 360 genes were differentially expressed in the endosperm. Furthermore, we identified and verified the candidate genes associated with seed dormancy and heat stress-related responses in rice using quantitative real-time PCR. We newly discovered eight hormone-related genes, four heat shock protein-related genes, and two miRNAs potentially involved in PHS. These findings provide a strong foundation for understanding the dynamics of transcriptome and small RNAome of hormone- and heat stress-related genes, which affect PHS during seed maturation.

Keywords: rice (*Oryza sativa* L.), pre-harvest sprouting (PHS), dormancy, stress responses, transcriptome, small RNAome

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the essential crops and the staple food for approximately 50% of the global population (Wei et al., 2013). In the rice field, pre-harvest sprouting (PHS) is crucial issue as it not only reduces the rice grain yield but also significantly affects the grain quality, resulting in a significant economic loss. Under normal conditions, rice seeds can be germinated

by controlling their maturation. However, under excess moisture conditions during the period between seed maturation and harvest, the proper seed dormancy is broken, which causes PHS, an important issue that must be overcome to avoid the reduction in grain quality and yield (Gubler et al., 2005). Therefore, understanding the molecular basis of seed dormancy and maturation is crucial for controlling PHS.

Abscisic acid (ABA) plays an important role in the induction and maintenance of seed dormancy (Gubler et al., 2005; Finkelstein et al., 2008; Graeber et al., 2012; Shu et al., 2016). Abscisic aldehyde oxidase 3 (AAO3) catalyzes the final step of ABA biosynthesis. *Arabidopsis thaliana* mutants *aao3-2* and *aao3-3* and the rice mutant *osao3* exhibit lower seed dormancy and ABA levels than the corresponding wild type (González-Guzmán et al., 2004; Shi et al., 2021). In rice, the basic helix-loop-helix (bHLH) transcription factor gene *OsbHLH035* mediates seed germination. The *Osbhlh035* mutants showed delayed seed germination and up-regulated *OsAAO3* expression (Chen et al., 2018). ABA response and signaling are also involved in seed dormancy. GEM, a member of the GRAM (Glycosyltransferases, Rab-like GTPase Activators, Myotubularins) domain family proteins, is an ABA-responsive protein, and the *gem-1* mutant shows increased seed dormancy (Mauri et al., 2016). In rice, the overexpression of the F-box gene, *OsFbx352*, improves the germination rate of seeds when treated with ABA, and decreases the expression of ABA biosynthesis-related genes (*OsNced2* and *OsNced3*) (Frey et al., 2012; Song et al., 2012).

Ethylene, another plant hormone, promotes seed germination by counteracting ABA signaling (Arc et al., 2013; Corbineau et al., 2014). The metabolic precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), produced by ACC synthase (ACS), is oxidized by ACC oxidase (ACO) to form ethylene (Zarembinski and Theologis, 1994; Wang et al., 2002). Transgenic *Arabidopsis* lines overexpressing the bHLH transcription factor gene *PIF5* exhibit up-regulated *ACS4* expression and increased ethylene accumulation (Khanna et al., 2007). In addition, *aco2* mutants exhibit reduced seed germination via the ACC-mediated reversion of ABA (Linkies et al., 2009). In tomato (*Solanum lycopersicum*), overexpression of the *Ethylene Response Factor 2* (*SlERF2*) gene results in premature seed germination (Pirrello et al., 2006). On the other hand, the *Arabidopsis* mutant *aterf7* exhibits delayed seed germination and increased ABA sensitivity during seed germination (Song et al., 2005).

In addition to plant hormones, heat shock proteins (HSPs) affect seed dormancy and temperature. Plants synthesize HSPs under high temperature conditions (Kotak et al., 2007; Xue et al., 2014; Muthusamy et al., 2017). Moreover, plants overexpressing *HSPs* exhibit enhanced heat stress tolerance (Chauhan et al., 2012; Mu et al., 2013; Kaur et al., 2015). In *Arabidopsis* and tomato, the overexpression of *GhHSP24.7*, which encodes a small mitochondrial HSP, accelerates seed germination via temperature-dependent generation of reactive oxygen species (Ma et al., 2019). In *Arabidopsis*, mutation of the *AtDjA3* gene (which encodes the J protein, also referred to as HSP40 or DnaJ protein) reduces the germination of seeds when treated with ABA and increases the expression of *ABA-INSENSITIVE 3* (*ABI3*), which is a negative regulator of germination (Parcy et al., 1994; Salas-Muñoz et al., 2016). In rice, overexpression of *OsHSP18.2* improves seed germination, and a motif responsible for ABA-inducible expression is present in its promoter (Kaur et al., 2015).

MicroRNAs (miRNAs) regulate the expression of target genes by binding to and cleaving their complementary sequences or inhibiting their translation (Vaucheret, 2006; Axtell, 2013). Furthermore, the biosynthesis and function of miRNAs are regulated by plant hormones and abiotic stress in plants (Reyes and Chua, 2007; Khraiwesh et al., 2012; Sanan-Mishra et al., 2013). However, little is known about miRNAs that regulate PHS, depending on the seed maturation stage.

This study aimed to identify candidate genes and miRNAs that determine the PHS rate in rice.

MATERIALS AND METHODS

Plant Materials and PHS Assay

Two rice (*Oryza sativa* L. ssp. *japonica*) accessions, Gopum (PHS susceptible) and Jowoon (PHS resistant), were used in this study. Both accessions were bred in an experimental field of Seoul National University, Suwon, Republic of Korea. Seeds and panicles of Gopum and Jowoon were harvested at 30, 45, and 60 days after heading (DAH). Seeds were sampled by dividing them into embryo and endosperm. To perform the PHS assay, three freshly harvested panicles were incubated at 25°C under 100% relative humidity for 7 days. The number of germinated seeds in each panicle was recorded and expressed as a percentage of the total number of seeds per panicle (Lee et al., 2017). Three replicates were used at each time point. Statistical analyses were performed using the Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Total RNA Extraction and Construction of mRNA and Small RNA Libraries

In total, 10 embryos and endosperms were sampled from one panicle of each plant. Total RNA was extracted from 100 embryos and endosperms, each obtained from 10 plants at three time points (30, 45, and 60 DAH). Briefly, each sample was homogenized in liquid nitrogen using a pestle and mortar and total RNA was then extracted using TRIzol Reagent (Invitrogen, United States), as described previously (Wang et al., 2012). The mRNA libraries were constructed from 2 μg of total RNA using SENSE mRNA-Seq Library Prep Kit V2 for Illumina platforms (LEXOGEN, Austria), based on the manufacturer's instructions. Small RNAs (18–30 nt) were isolated from 20 μg of total RNA using Urea-PAGE gel, and then small RNA libraries were constructed using Small RNA-Seq Library Prep Kit for Illumina Platforms (LEXOGEN, Austria), based on the manufacturer's instructions. Four replicates of the mRNA and small RNA samples were prepared.

RNA-Seq Analysis

RNA-seq was performed using the Illumina HiSeq 2500 platform to generate 101-bp paired-end reads. Raw sequences were filtered

and trimmed using Trimmomatic v0.3.6. The clean reads were mapped onto the International Rice Genome Sequencing Project (IRGSP) 1.0 reference genome using HISAT2 v2.1.0 with default parameters. The mapped reads were transformed into BAM files using samtools v1.8. The number of mapped reads was quantified using the featureCounts function in Rsubread v2.4.3. To estimate gene expression levels, the mapped read counts were transformed into counts per million (CPM). Subsequently, the CPM values were normalized using the trimmed mean of M-values. The log-transformed CPM values were then used to generate heatmaps with the pheatmap package v1.0.12 in R. Differentially expressed genes (DEGs) with fold-change (FC) ≥ 2 and false discovery rate (FDR) ≤ 0.05 were identified using the edgeR package v3.32.1 by comparing the two rice accessions at each time point.

Gene Ontology Enrichment Analysis

Gene ontology (GO) enrichment analysis was performed to determine the biological roles of the selected genes (Moon et al., 2020). Significantly enriched GO terms were selected with fold enrichment > 2 and FDR < 0.05 using the GO Resource web server.[1]

MapMan Analysis

The MapMan software v3.6.0 was used to map the transcriptome data and define functional categories for identifying significantly overrepresented functional genes (Zhang et al., 2020). A dataset containing the IDs of DEGs was constructed with two overviews: regulation and cellular response.

Validation of RNA-Seq Data by Quantitative Real-Time PCR

To validate the RNA-seq data, cDNA was synthesized from each RNA sample using SuperScript III Reverse Transcriptase (Invitrogen, United States), based on the manufacturer's instructions. Then, quantitative real-time PCR (qRT-PCR) was performed using Light Cycler 480 SYBR Green I Master (Roche, United States) with SYBR Green detection and gene-specific primers. The Ct values for genes were obtained using Os03g0718100 (*OsACT1*) as a control, and relative expression values were calculated using the $\Delta\Delta$Ct method. Gene-specific primers were designed using NCBI primer BLAST.[2] The primer sequences of the candidate genes are listed in **Supplementary Table 15**. Statistical analyses were performed using the Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Small RNA-Seq Data Processing and Differential Expression Analysis of miRNAs

The removal of adaptor sequences and selection of 15–26 nt small RNAs were performed using Cutadapt (v3.4). To obtain high-quality small RNA reads, reads with Phred quality score < 25 were removed using Trimmomatic. The clean reads were then aligned on RNACentral (v.17.0). *Oryza* sp.-specific non-coding RNA sequences (mostly structural RNAs, including rRNAs, tRNAs, snRNAs, and snoRNAs) were filtered, and the remaining reads were mapped onto the IRGSP-1.0 rice reference genome sequence using bowtie (v1.2.2). To annotate mature rice miRNAs, the IRGSP-mapped small RNA reads were aligned against the mature rice miRNA sequences registered in miRBase (v22) and PmiREN. The filtered read counts were visualized by generating a heatmap using the pheatmap package v1.0.12 in R. Differential expression analysis of miRNAs was performed using edgeR, and miRNAs with $\log_2$FC > 1 and FDR < 0.05 were selected as differentially expressed miRNAs (DEMs).

RESULTS

Difference in PHS Rates Between Gopum and Jowoon

We defined three seed maturation stages in this study: early (30 DAH), mid (45 DAH), and late (60 DAH) stages. The PHS rate of Gopum (PHS susceptible accession) was higher than that of Jowoon (PHS resistant accession) at every stage (**Figure 1A**). In particular, the PHS rate of Gopum was significantly higher than that of Jowoon at 45 DAH by approximately 35-fold (**Figures 1B,C**). Therefore, we performed RNA-seq and RNAome analyses to identify genes and miRNAs responsible for the difference in the PHS rate between Gopum and Jowoon.

Identification of DEGs in the Embryo and Endosperm of Gopum and Jowoon

To understand the transcriptional changes in PHS-related candidate genes at different seed maturation stages in Gopum and Jowoon, we performed RNA-seq analysis of the embryo and endosperm at 30, 45, and 60 DAH. After read filtering and trimming, the clean reads were mapped onto the IRGSP 1.0 rice reference genome sequence. The results showed that 16,897 and 14,049 genes were expressed in the embryo and endosperm samples, respectively. The log-transformed CPM values of each gene are depicted as a heatmap in **Figures 2A,B**.

To identify PHS-related candidate genes, gene expression was compared between Gopum and Jowoon samples at 30, 45, and 60 DAH (hereafter referred to as GJ30, GJ45, and GJ60, respectively). In the embryo samples, 2,980, 3,636, and 5,958 DEGs were identified in the GJ30, GJ45, and GJ60, respectively (**Figure 2C**). In the endosperm samples, no DEG was identified in GJ30, whereas 226 and 215 DEGs were identified in GJ45 and GJ60, respectively (**Figure 2D**). Genes that were up- or down-regulated in Jowoon compared with those in Gopum in GJ30, GJ45, and GJ60 of embryo and endosperm are shown in **Figures 2E,F**. Interestingly, the endosperm had significantly less DEGs compared with the embryo. Thus, it is likely that DEGs in the embryo cause the difference in PHS rate between Gopum and Jowoon.

Functional Annotation of DEGs

We performed GO enrichment analysis to predict the biological functions of DEGs in the embryo and endosperm. GO terms

[1] http://geneontology.org

[2] http://www.ncbi.nlm.nih.gov/tools/primer-blast/

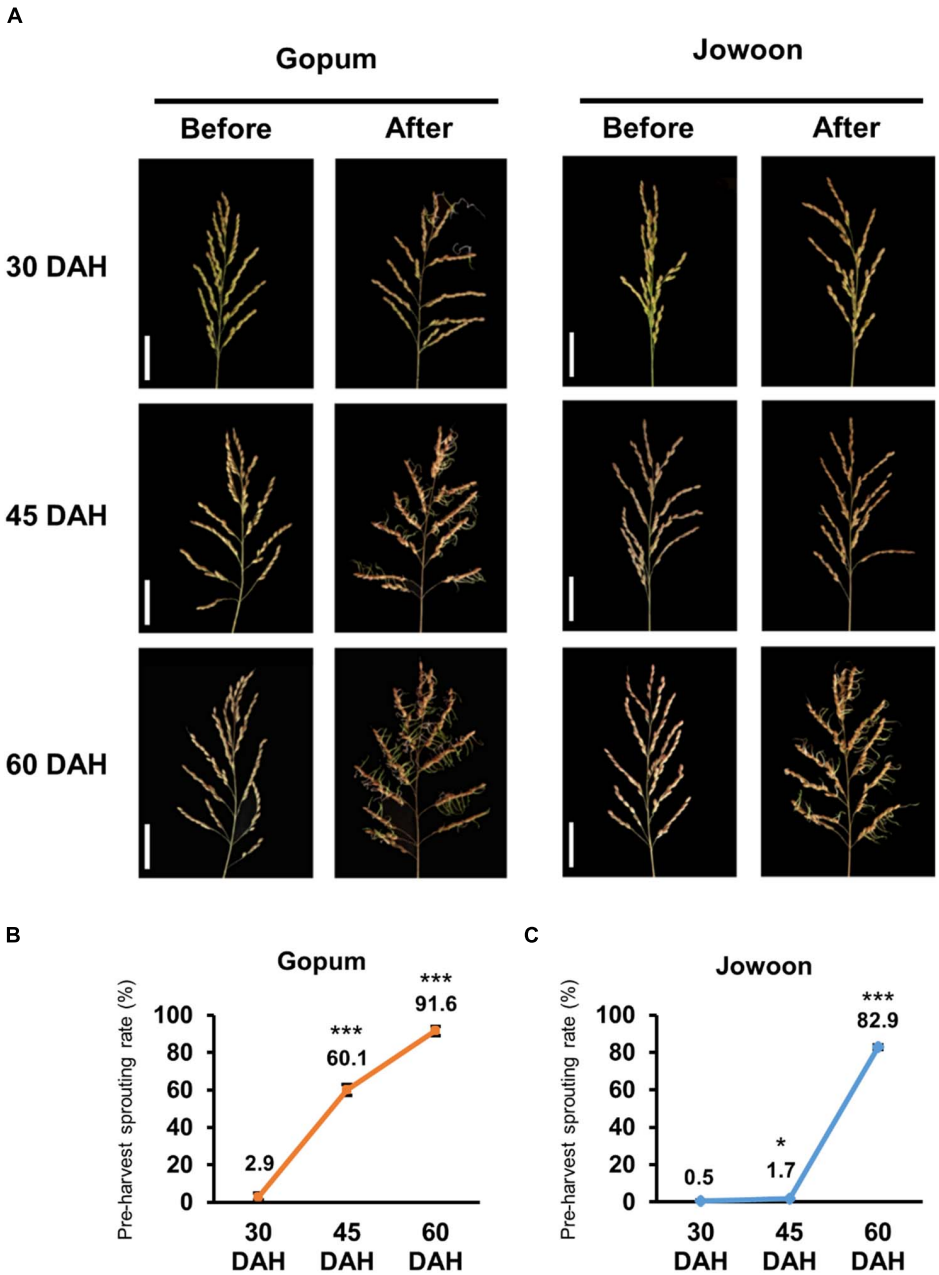

FIGURE 1 | Pre-harvest sprouting (PHS) assay in Gopum and Jowoon. **(A)** PHS phenotypes of Gopum and Jowoon at 30, 45, and 60 days after heading (DAH). The phenotypes before PHS assay (before) and after PHS assay (after) are shown. Scale bar = 5 cm. **(B,C)** PHS rate of Gopum **(B)** and Jowoon **(C)** at 30, 45, and 60 DAH ($N = 3$). Significance is determined by Student's *t*-test, $^{*}P < 0.05$ and $^{***}P < 0.001$.

in the biological process category with fold enrichment > 2 and FDR < 0.05 were identified. In the embryo samples, DEGs identified in the GJ30, GJ45, and GJ60 were enriched with hormone-related GO terms, such as "response to abscisic acid," and abiotic stress-related GO terms, such as "response to heat" (**Figure 3**). In endosperm samples, no significant GO term related to hormones or abiotic stress was identified (**Supplementary Tables 7–9**). GO term was not identified in the genes down-regulated in Jowoon, compared with those in Gopum of endosperm GJ60. All GO terms in the biological process category are listed in **Supplementary Tables 1–9**.

Identification of Hormone- and Abiotic Stress-Related DEGs in the Embryo

High-throughput transcriptome data can be visualized in diverse overviews using the MapMan software, based on multiple omics data of plants (Usadel et al., 2005; Jung and An, 2012). In this

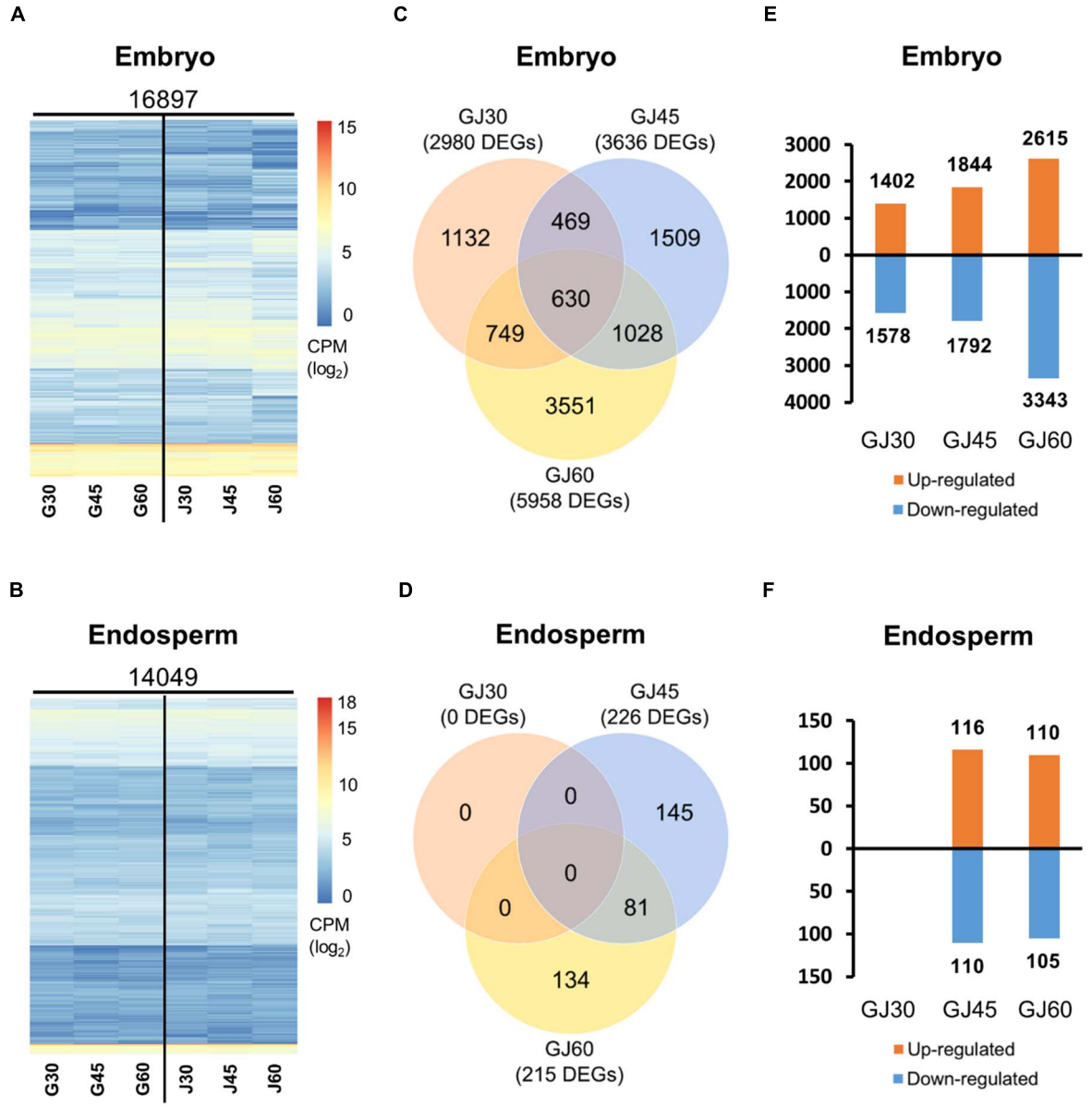

FIGURE 2 | Identification of differentially expressed genes (DEGs) based on the RNA-seq data of the embryo and endosperm of Gopum and Jowoon. **(A,B)** Heatmaps of expressed genes in the embryo **(A)** and endosperm **(B)** of Gopum and Jowoon, respectively. Expressed genes, mapped onto the International Rice Genome Sequencing Project (IRGSP) rice reference genome sequence, are shown as log-transformed counts per million (CPM) values. G30, G45, G60, J30, J45, and J60 indicate Gopum and Jowoon samples at 30, 45, and 60 DAH, respectively. **(C,D)** Comparison of Gopum and Jowoon based on the number of DEGs in the embryo **(C)** and endosperm **(D)**. GJ30, GJ45, and GJ60 indicate the comparisons between Gopum and Jowoon at 30, 45, and 60 DAH, respectively. **(E,F)** Numbers of genes up- or down-regulated in Jowoon compared with those in Gopum in the embryo **(E)** and endosperm **(F)** of GJ30, GJ45, and GJ60.

study, we used the MapMan software to visualize the function and expression profiles of DEGs at each seed maturation stage in the embryo and endosperm samples of Gopum and Jowoon. The DEGs identified in the embryo (GJ30, GJ45, and GJ60) and endosperm (GJ45 and GJ60) were analyzed using the regulation and cellular response overviews. The hormone-related DEGs identified in the regulation overview, showed the highest changes in indole-3-acetic acid (IAA)-, ABA-, and ethylene-related genes in the embryo of GJ30, GJ45, and GJ60. In addition, in the abiotic stress of the cellular response overview, heat stress-related genes showed the highest expression change in the embryo of GJ30, GJ45, and GJ60 (**Figures 4A–C**). In the endosperm, no significant changes in hormone− and abiotic stress-related genes were observed in the both GJ45 and GJ60 (**Figures 4D,E**). These results suggest that IAA−, ABA−, ethylene−, and heat stress-related genes with differential expression between the embryos of Gopum and Jowoon are responsible for the difference in the PHS rate between the two accessions. The regulation and cellular

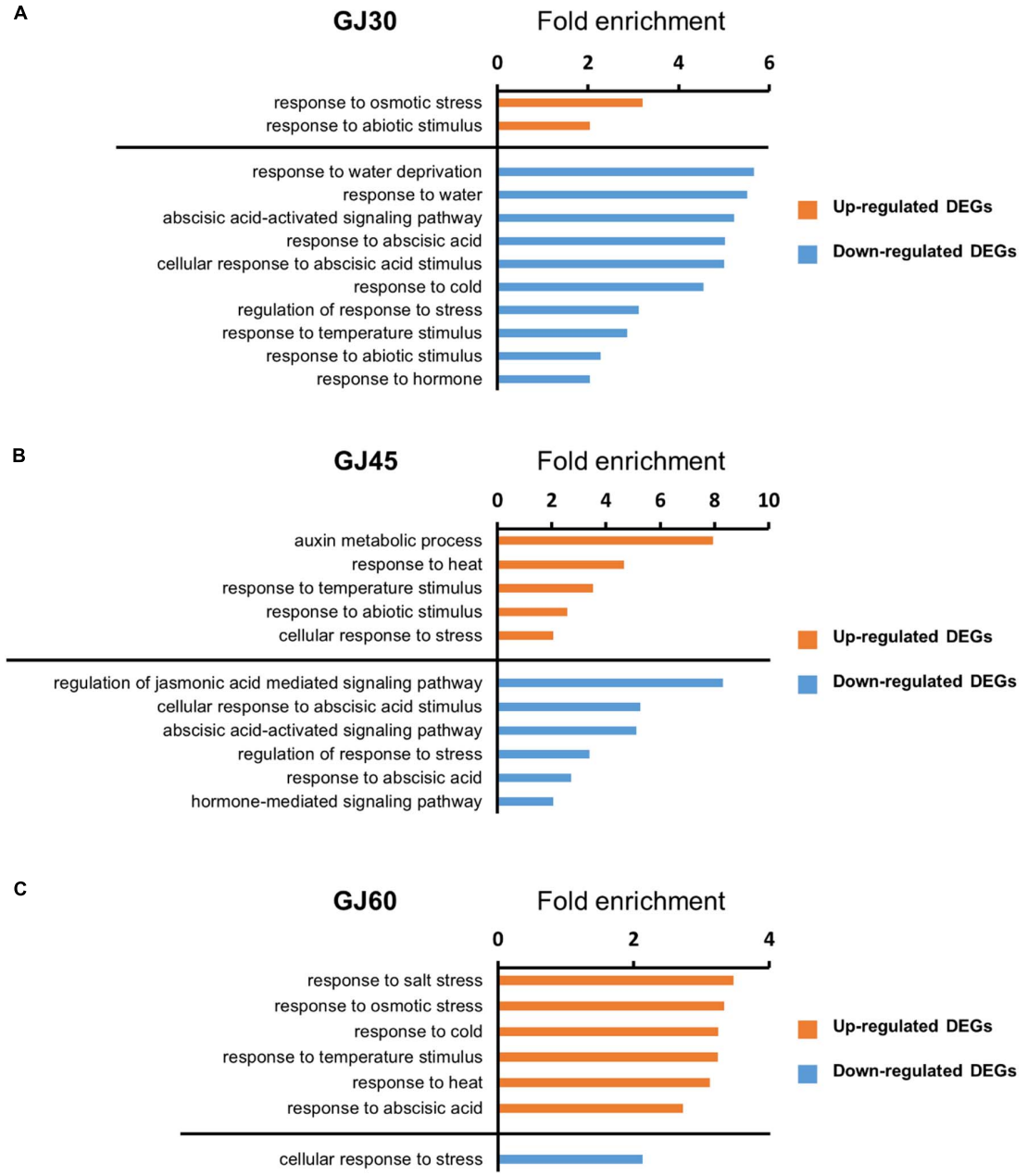

FIGURE 3 | Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) identified in the embryo in the biological process category. **(A–C)** GO terms (fold enrichment > 2 and false discovery rate < 0.05) of DEGs, which were up- or down-regulated in Jowoon compared with those in Gopum, related to hormones and abiotic stress in the embryo of GJ30 **(A)**, GJ45 **(B)**, and GJ60 **(C)**.

response overviews of all GJ30, GJ45, and GJ60 of embryo and endosperm are shown in **Supplementary Figures 1–4**.

Genes Potentially Responsible for the Difference in the PHS Rate Between Gopum and Jowoon

We found that several genes, including *AAO*, *AO*, *GRAM* domain containing gene, *bHLH*, *ACO*, *ERF*, and *HSP*, were differentially expressed between the embryos of Gopum and Jowoon at 30, 45, and 60 DAH. The review of literature, Rice Annotation Project Database (RAP-DB) and MSU Rice Genome Annotation Project, and related searches revealed several hormone and abiotic stress-related genes with known or expected functions in the embryo (**Supplementary Tables 10–12**). In addition, we selected PHS-related candidate genes, which have not yet been published. The expression of these genes was validated by qRT-PCR (**Figure 5**).

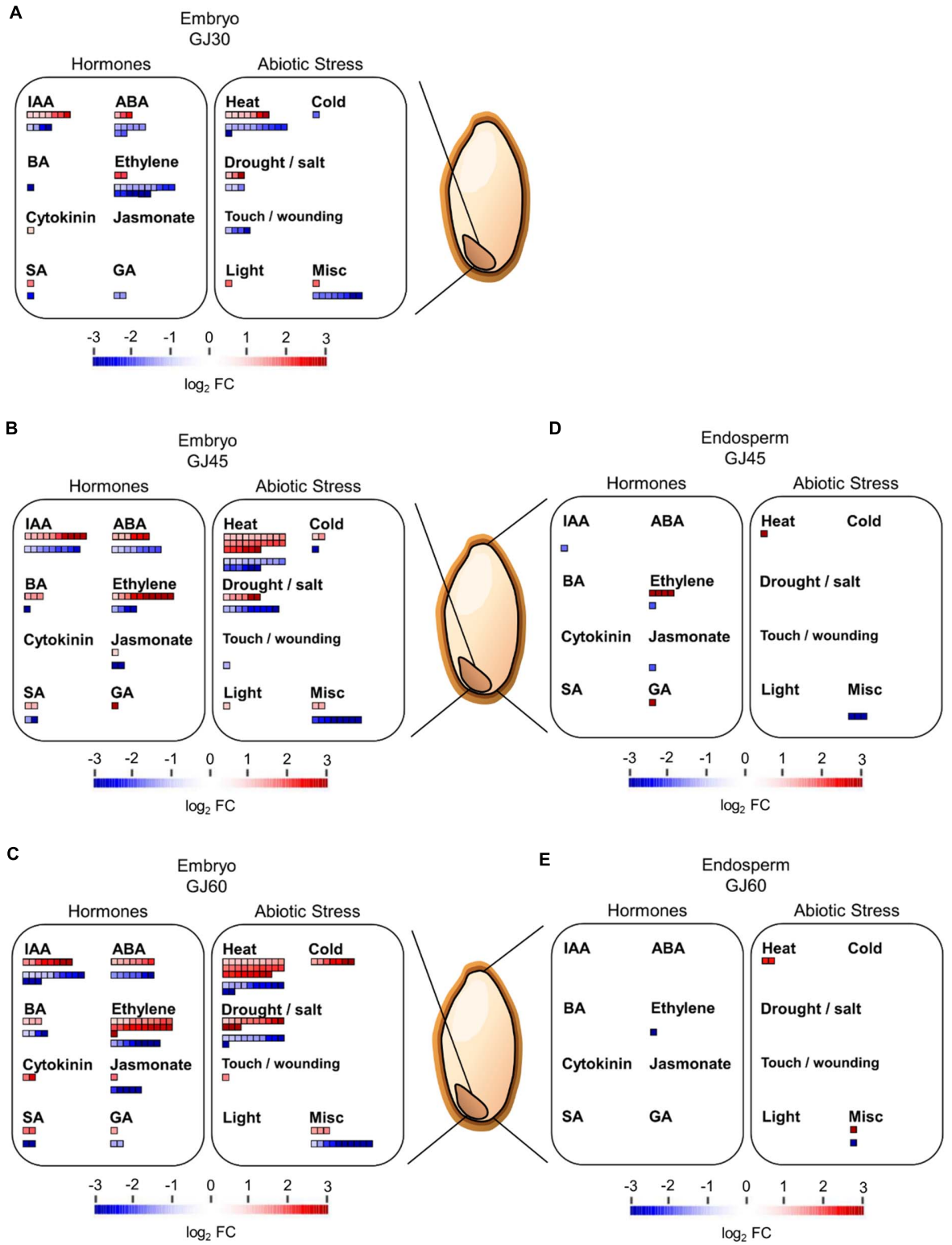

FIGURE 4 | MapMan analysis of differentially expressed genes (DEGs), identified in the embryo and endosperm of Gopum and Jowoon. **(A–E)** Identification of hormone- and abiotic stress-related DEGs in the embryo of GJ30 **(A)**, GJ45 **(B)**, and GJ60 **(C)** and in endosperm of GJ45 **(D)** and GJ 60 **(E)**. The regulation overview and cellular response overview were applied, and the figures were modified from **Supplementary Figures 1–4** for DEGs related to hormones and abiotic stress.

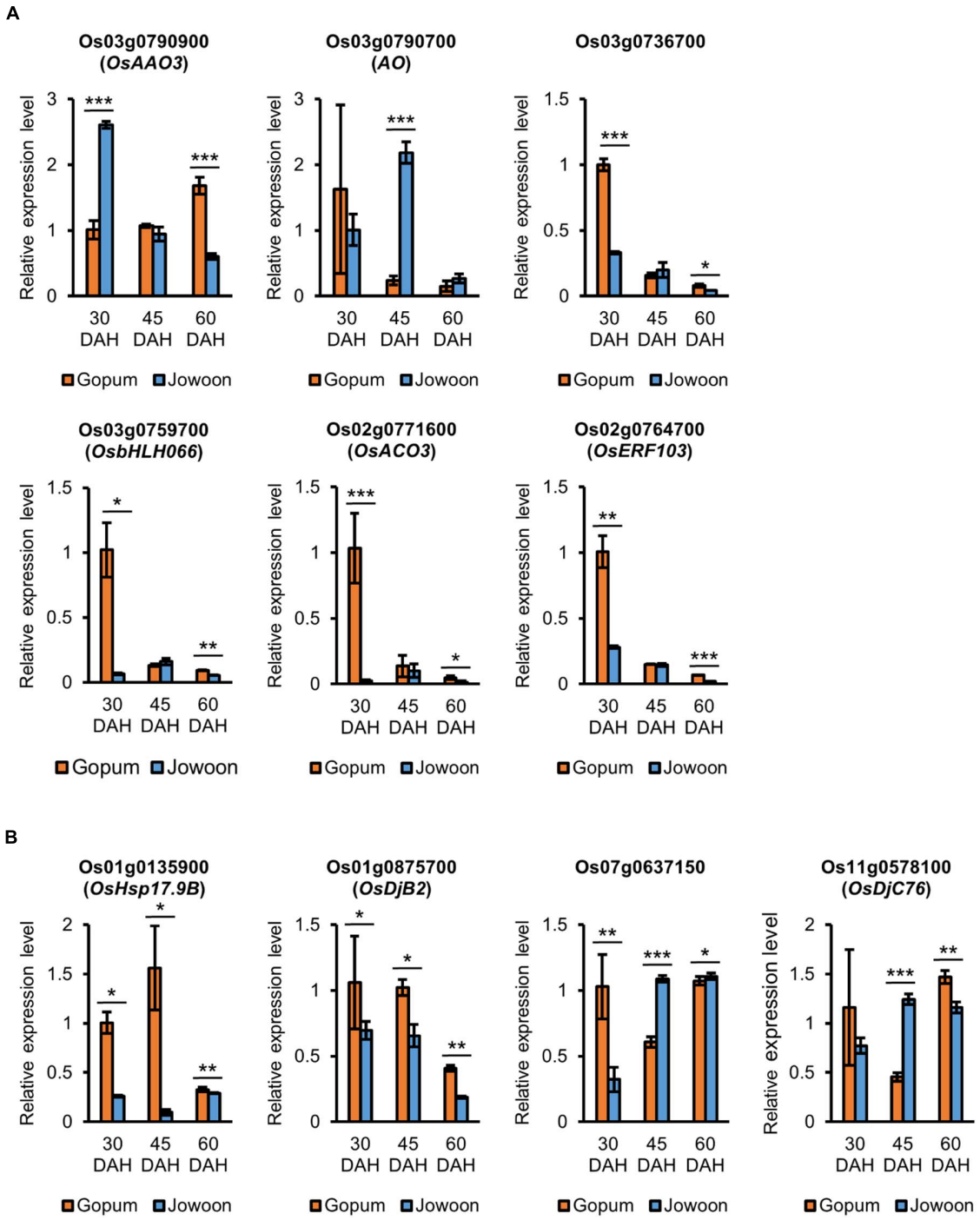

FIGURE 5 | Relative expression levels of pre-harvest sprouting (PHS)-related candidate genes by quantitative real-time PCR (qRT-PCR). **(A)** Relative expression levels of abscisic acid (ABA)- and ethylene-related genes. ABA-related genes (top) and ethylene-related genes (bottom) are shown. **(B)** Relative expression levels of *heat shock protein* (*HSP*) genes. Data represent mean ± standard error of mean (SEM; $N = 3$). Significance is determined by Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Os03g0790900 (*OsAAO3*), Os03g0790700 (*AO*), and Os03g0736700 (which contains the GRAM domain) were selected as ABA-related genes, and Os03g0759700 (*OsbHLH066*), Os02g0771600 (*OsACO3*), and Os02g0764700 (*OsERF103*) were selected as ethylene-related genes. Among these genes, Os03g0790900 (*OsAAO3*) and Os03g0790700 (*AO*) were up-regulated in Jowoon compared with those in Gopum at 30 and 45 DAH, respectively (**Figure 5A**). Our results suggest

that Os03g0790900 (*OsAAO3*) and Os03g0790700 (*AO*) are associated with ABA and affect the low PHS rate of Jowoon at 30 and 45 DAH, respectively. In addition, Os03g0736700 (which contains the GRAM domain), Os03g0759700 (*OsbHLH066*), Os02g0771600 (*OsACO3*), and Os02g0764700 (*OsERF103*) were up-regulated in Gopum compared with those in Jowoon at 30 DAH (**Figure 5A**). These results suggest that the abovementioned genes are associated with ethylene and affect the higher PHS rate of Gopum compared with that of Jowoon at 45 DAH.

The selected *HSP* genes including Os01g0135900 (*OsHsp17.9B*), Os01g0875700 (*OsDjB2*), Os07g0637150 (which is similar to *DnaJ*), and Os11g0578100 (*OsDjC76*) were up-regulated in Gopum compared with those in Jowoon at 30 DAH (**Figure 5B**). These results suggest that the abovementioned *HSP* genes are associated with the higher PHS rate of Gopum compared with that of Jowoon at 45 DAH. In addition, the high expression of Os07g0637150 and Os11g0578100 (*OsDjC76*) at 45 DAH in Jowoon is associated with the PHS of Jowoon seeds at 60 DAH (**Figure 5B**).

Details of hormone and abiotic stress-related genes in GJ30, GJ45, and GJ60 of the embryo and endosperm, respectively, are summarized in **Supplementary Tables 10–14**. All primers used for qRT-PCR are listed in **Supplementary Table 15**.

Identification of PHS-Related Candidate miRNAs and Corresponding Target Genes

On the basis of our transcriptome data, we speculated that the embryo plays a greater role in PHS than the endosperm. Furthermore, we predicted that miRNAs associated with PHS would be expressed in the embryo. To identify miRNAs and their corresponding target genes affecting the difference in PHS rate between Gopum and Jowoon, we performed small RNA-seq analysis of Gopum and Jowoon embryos at 30, 45, and 60 DAH. After read filtering and trimming, the clean small RNA reads were mapped onto the IRGSP 1.0 rice reference genome sequence. The IRGSP-mapped small RNA reads were aligned against the mature rice miRNA sequences in miRBase and PmiREN. Consequently, 2,835 miRNAs were identified in the embryo samples. The heatmap of filtered read counts of miRNAs is shown in **Figure 6A**. Next, we performed DEM analysis ($\log_2$FC > 1; FDR < 0.05) at each stage to identify PHS-related candidate miRNAs. In total 7, 10, and 26 DEMs were identified in the GJ30, GJ45, and GJ60, respectively (**Figure 6B**). The up- and down-regulated miRNAs in Jowoon compared with those in Gopum in GJ30, GJ45, and GJ60 are shown in **Figure 6C**.

The target genes of DEMs were identified using miRNA target gene prediction. We considered the anti-correlation in expression level between DEMs and the corresponding target genes and validated the expression of target genes by qRT-PCR. Subsequently, two miRNAs and two target genes, expected to be associated with PHS, were selected (**Figures 6D,E**). The osa-miR5827 and osa-miR1862h were expressed at higher levels in Jowoon than in Gopum at all stages, whereas the expression of the corresponding target genes, Os03g0728900 (*OsbHLH084*) and Os11g0209600 (*OsFbox594*), respectively, was lower in Jowoon than in Gopum at 30 DAH (**Figures 6D,E**). These results suggest that Os03g0728900 (*OsbHLH084*) and Os11g0209600 (*OsFbox594*) are associated with the higher PHS rate of Gopum compared with that of Jowoon at 45 DAH. In addition, the high expression of osa-miR5827 and osa-miR1862h in Jowoon might be associated with the low PHS rate of Jowoon, especially the case with osa-miR1862h.

The DEMs and their corresponding target genes are summarized in **Supplementary Tables 16–18**.

DISCUSSION

Rice is one of the most important crops in the world. However, the reduction in rice grain quality and yield due to PHS results in a significant economic loss (Gubler et al., 2005; Wei et al., 2013). Therefore, to overcome PHS, it is necessary to understand the molecular mechanisms underlying seed dormancy and maturation, as well as genes and miRNAs involved in PHS. In this study, we performed RNA-seq and small RNA-seq analyses of the embryo and endosperm tissues of the PHS susceptible accession Gopum and PHS resistant accession Jowoon at three seed maturation stages: 30, 45, and 60 DAH.

Our RNA-seq data revealed the expression of *OsSdr4*, *OsPYL/RCAR5*, and *OsDOG1L-3*, which are well known to negatively control seed germination (Sugimoto et al., 2010; Graeber et al., 2012; Kim et al., 2012; Wang et al., 2020). The expression of Os07g0585700 (*OsSdr4*) was higher in Jowoon than in Gopum at 60 DAH. In addition, the expression of Os05g0213500 (*OsPYL/RCAR5*) and Os01g0306400 (*OsDOG1L-3*) was overall higher in Jowoon than in Gopum; however, no significant expression changes were noted between Gopum and Jowoon based on the seed maturation stages (**Supplementary Figure 5**).

The results of the GO enrichment and MapMan analyses revealed that hormone- and heat stress-related GO terms and gene expression differences between Gopum and Jowoon were significantly observed in the embryo and rarely in the endosperm (**Figures 3, 4** and **Supplementary Tables 7–9**). These results suggest that the expression of hormone- and heat stress-related genes in embryo plays a crucial role in PHS. Thus, using the embryo data, we identified genes that are expected to affect PHS. We identified three ABA- and three ethylene-related genes. *AAO* is involved in the last step of ABA biosynthesis. Previously, *aao3* and *ao3* mutants showed reduced seed dormancy and ABA levels (González-Guzmán et al., 2004; Shi et al., 2021). The expression of Os03g0790900 (*OsAAO3*) and Os03g0790700 (*AO*) was higher in Jowoon than in Gopum at 30 and 45 DAH, respectively (**Figure 5A**). It is possible that the high expression level of Os03g0790900 (*OsAAO3*) and Os03g0790700 (*AO*) is associated with high ABA levels in Jowoon seeds at 30 and 45 DAH, respectively, resulting in low PHS rate (**Figure 7**). GEM, which contains a GRAM domain, is involved in ABA signaling and is activated by ABA, and the *Arabidopsis gem-1* mutant shows increased seed dormancy (Mauri et al., 2016). We identified Os03g0736700, which contains a GRAM domain, as a DEG whose expression level was higher in Gopum than in

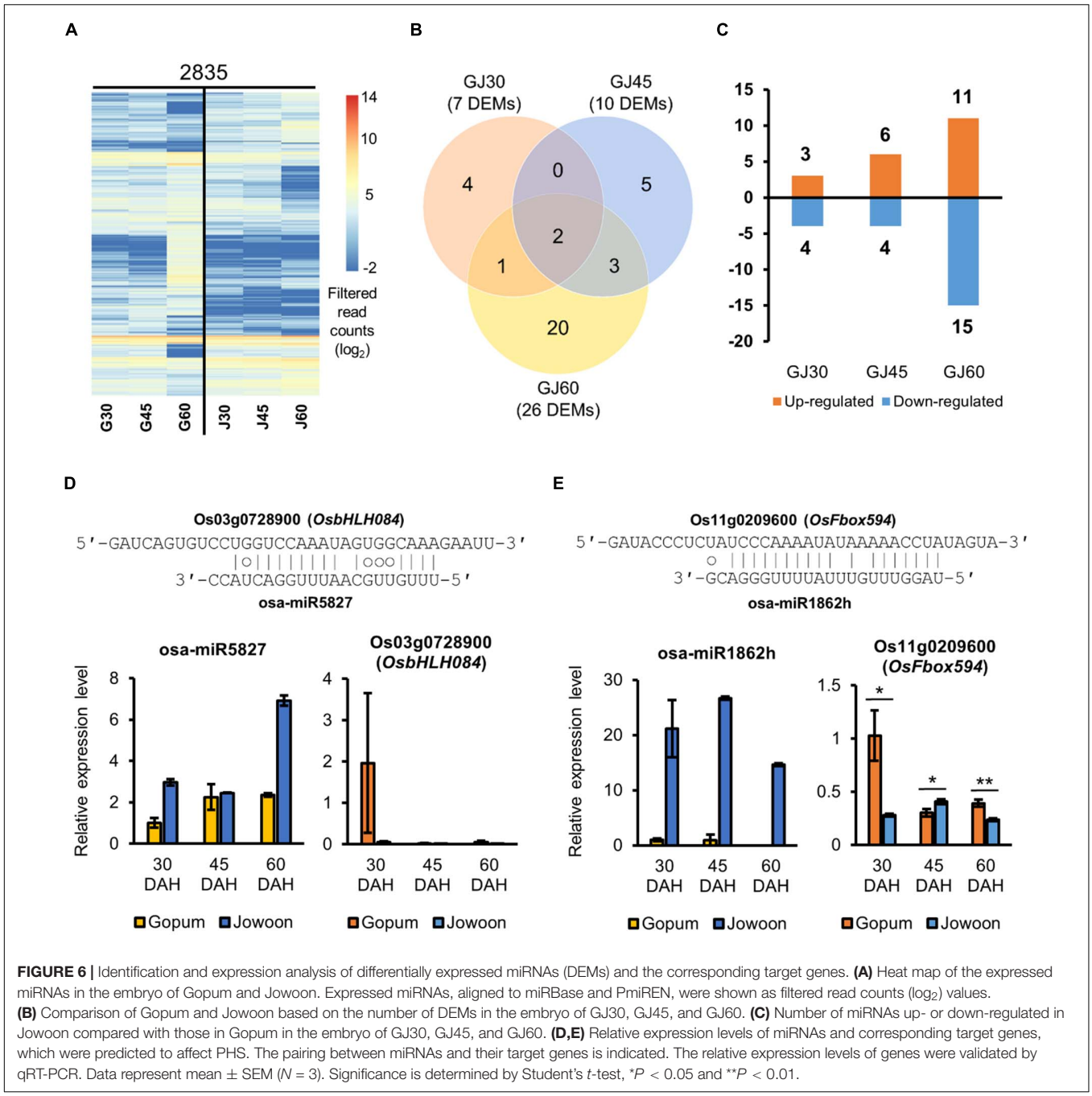

FIGURE 6 | Identification and expression analysis of differentially expressed miRNAs (DEMs) and the corresponding target genes. **(A)** Heat map of the expressed miRNAs in the embryo of Gopum and Jowoon. Expressed miRNAs, aligned to miRBase and PmiREN, were shown as filtered read counts (log_2) values. **(B)** Comparison of Gopum and Jowoon based on the number of DEMs in the embryo of GJ30, GJ45, and GJ60. **(C)** Number of miRNAs up- or down-regulated in Jowoon compared with those in Gopum in the embryo of GJ30, GJ45, and GJ60. **(D,E)** Relative expression levels of miRNAs and corresponding target genes, which were predicted to affect PHS. The pairing between miRNAs and their target genes is indicated. The relative expression levels of genes were validated by qRT-PCR. Data represent mean ± SEM ($N = 3$). Significance is determined by Student's *t*-test, $^*P < 0.05$ and $^{**}P < 0.01$.

Jowoon at 30 DAH (**Figure 5A**). We predict that Os03g0736700 responds to ABA and affects the high PHS rate of Gopum after 30 DAH (**Figure 7**).

Ethylene promotes seed germination (Arc et al., 2013; Corbineau et al., 2014). ACS synthesizes the metabolic precursor of ethylene, ACC, which is oxidized by ACO to form ethylene (Zarembinski and Theologis, 1994; Wang et al., 2002). In *Arabidopsis*, *PIF5* overexpression lines showed high *ACS4* expression and increased ethylene level (Khanna et al., 2007). In addition, the *aco2* mutant showed reduced seed germination (Linkies et al., 2009). The *aterf7* mutant showed delayed seed germination, whereas tomato *SlERF2* overexpression lines showed early seed germination than the wild type (Song et al., 2005; Pirrello et al., 2006). Os03g0759700 (*OsbHLH066*, which encodes a bHLH transcription factor), Os02g0771600 (*OsACO3*), and Os02g0764700 (*OsERF103*) showed higher expression in Gopum than in Jowoon at 30 DAH (**Figure 5A**). Therefore, we speculate that these three genes, involved in ethylene biosynthesis and response, contribute to the high PHS rate of Gopum at 45 DAH (**Figure 7**).

HSPs, associated with ABA, control seed germination. In *Arabidopsis* and tomato, the overexpression of *GhHSP24.7*

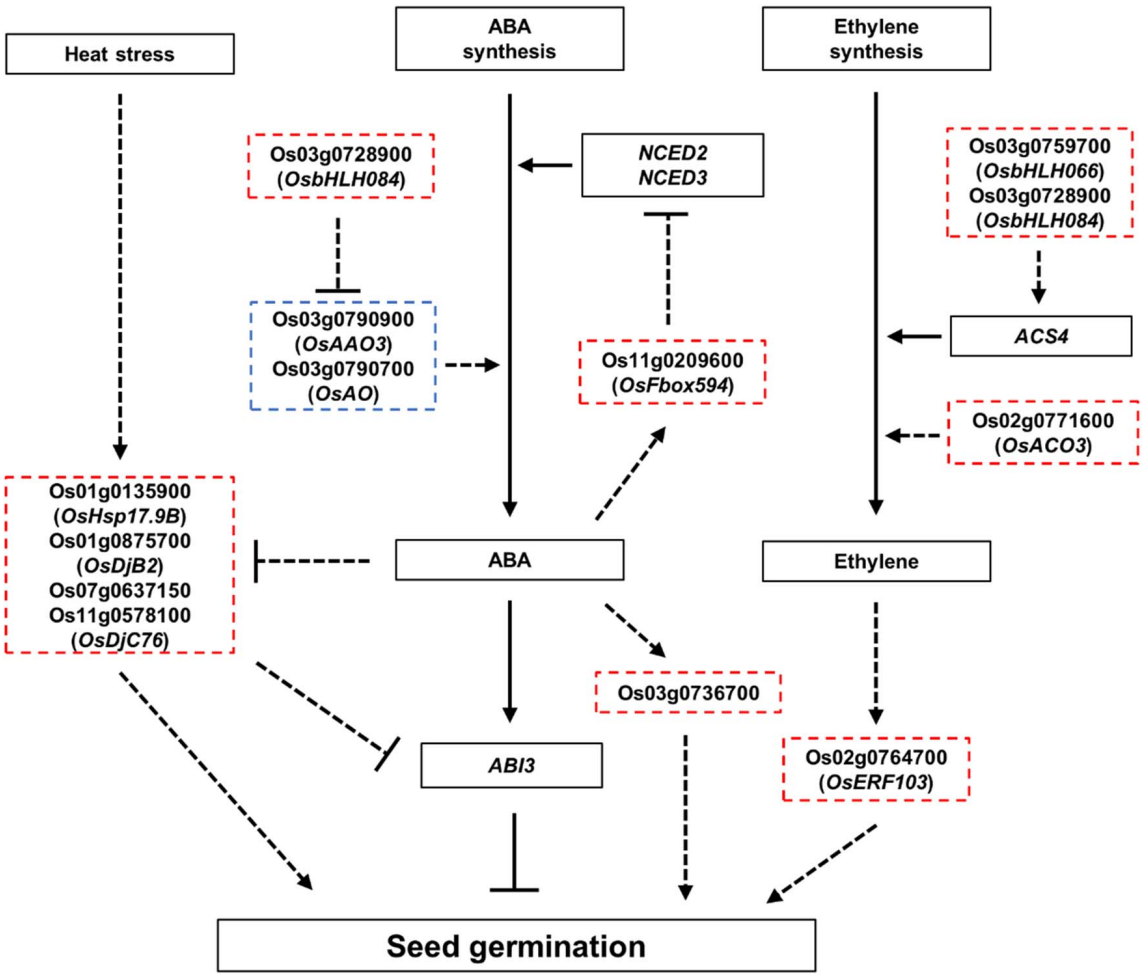

FIGURE 7 | Proposed model showing the role of ABA, ethylene, and HSPs in seed germination. Red and blue dashed boxes indicate genes expected to positively and negatively regulate the seed germination, respectively. Arrows indicate positive regulation, and bars indicate negative regulation. Bold lines indicate gene functions supported by strong evidence, and dashed lines indicate gene function with limited evidence in seed germination, which requires further investigation.

accelerated seed germination (Ma et al., 2019). In addition, the *Atdja3* mutant showed reduced seed germination, when treated with ABA, and higher expression of *ABI3* than the wild type (Salas-Muñoz et al., 2016). In rice, an ABA-inducible motif was identified in the *OsHSP18.2* promoter region, and *OsHSP18.2* overexpression lines showed enhanced seed germination (Kaur et al., 2015). In the present study, Os01g0135900 (*OsHsp17.9B*) and Os01g0875700 (*OsDjB2*) showed higher expression in Gopum than in Jowoon at all stages and are, therefore, expected to contribute to the overall high PHS rate of Gopum (**Figure 5B**). In addition, Os07g0637150 (similar to *DnaJ*) showed higher expression in Gopum at 30 DAH, whereas Os11g0578100 (*OsDjC76*) showed higher expression in Jowoon at 45 DAH (**Figure 5B**). We speculate that these genes are associated with the high PHS rate of Gopum at 45 DAH and that of Jowoon at 60 DAH. On the basis of previous studies (Kaur et al., 2015; Salas-Muñoz et al., 2016), the *HSP* genes, which showed low expression in Jowoon, may be affected by ABA synthesized from the high expression of Os03g0790900 (*AAO3*) and Os03g0790700 (*AO*). These *HSP* genes may affect the low PHS rate in Jowoon by enhancing the expression of *ABI3*, which is a negative regulator of seed germination (**Figures 5**, **7**).

We speculate that the difference in the PHS rates of Gopum and Jowoon is caused by the difference in gene expression in the embryo. Little is known about the role of miRNAs in PHS. Therefore, we performed small RNA-seq analysis of the embryo. Using target prediction, we identified F-box and bHLH-related genes as the targets of DEMs from our small RNAome data (**Supplementary Tables 16, 17**). We selected osa-miR5827, osa-miR1862h, Os03g0728900 (*OsbHLH084*), and Os11g0209600 (*OsFbox594*), considering the anti-correlation between miRNA and the corresponding target genes (**Figure 6**). We speculate that the higher expression of osa-miR5827 and osa-miR1862h in Jowoon and that of Os03g0728900 (*OsbHLH084*) and Os11g0209600 (*OsFbox594*) in Gopum at 30 DAH affects the PHS phenotype of the two accessions. In previous studies, *PIF5* and the *OsbHLH035* were reported to be associated with ethylene biosynthesis (by affecting *ACS4* expression) and

ABA biosynthesis (by affecting *AAO3* expression), respectively (Khanna et al., 2007; Chen et al., 2018). *OsbHLH084*, which was identified in this study, is expected to be involved in ethylene biosynthesis by affecting the expression of *ACS4* or in ABA biosynthesis by lowering the expression of Os03g0790900 (*OsAAO3*) and Os03g0790700 (*AO*) in Gopum compared with their expression in Jowoon at 30 and 45 DAH, respectively, leading to the high PHS rate of Gopum after 30 DAH (**Figure 7**). In addition, on the basis of a previous study (Song et al., 2012), it can be expected that Os11g0209600 (*OsFbox594*) represses the ABA biosynthesis gene, NCED, and affects the high PHS rate of Gopum after 30 DAH (**Figure 7**).

CONCLUSION

In conclusion, by comparing the PHS susceptible rice Gopum with the PHS resistant rice Jowoon, we identified eight hormone-related genes, four HSP-related genes, and two miRNAs as the potential regulators of the difference in PHS between Gopum and Jowoon. In addition, we demonstrated a bioinformatics pipeline that can be used to identify candidate genes and miRNAs involved in PHS; this involves RNA-seq and small RNA-seq analyses and the MapMan analysis of the transcriptome data. A comprehensive review of the previous studies and the results of the present study suggest that interaction between ABA and ABA-related genes and between ABA and HSPs contributes to seed dormancy during seed maturation. On the basis of these results, we propose a model in which seed germination is affected by ABA, ethylene, and HSPs (**Figure 7**). Additional studies, such as the mutagenesis of hormone-related and *HSP* genes identified in this study and the measurement of ABA and ethylene contents, are needed to elucidate the interaction between ABA and ABA-related genes, ethylene and ethylene-related genes, and ABA and HSPs. Overall, this study provides big data on the expression dynamics of hormone- and abiotic stress-related genes and miRNAs, which can be utilized in various ways to manipulate seed maturation and PHS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The datasets generated and analyzed for this study can be found in the NCBI SRA repository, https://www.ncbi.nlm.nih.gov/sra/, with the GEO accession no. GSE174017 and no. GSE175561.

AUTHOR CONTRIBUTIONS

CS conceived the research and designed the experiments. MP, DL, J-SJ, and CS planned and performed the field experiments, sampling, and PHS assay. WC, S-YS, HM, Y-SG, and K-HJ performed computational analysis. CS and MP prepared the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.727302/full#supplementary-material

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Heat Shock Signaling in Land Plants: From Plasma Membrane Sensing to the Transcription of Small Heat Shock Proteins

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Heat stress events are major factors limiting crop productivity. During summer days, land plants must anticipate in a timely manner upcoming mild and severe temperature. They respond by accumulating protective heat-shock proteins (HSPs), conferring acquired thermotolerance. All organisms synthetize HSPs; many of which are members of the conserved chaperones families. This review describes recent advances in plant temperature sensing, signaling, and response. We highlight the pathway from heat perception by the plasma membrane through calcium channels, such as cyclic nucleotide-gated channels, to the activation of the heat-shock transcription factors (HSFs). An unclear cellular signal activates HSFs, which act as essential regulators. In particular, the HSFA subfamily can bind heat shock elements in HSP promoters and could mediate the dissociation of bound histones, leading to HSPs transcription. Although plants can modulate their transcriptome, proteome, and metabolome to protect the cellular machinery, HSP chaperones prevent, use, and revert the formation of misfolded proteins, thereby avoiding heat-induced cell death. Remarkably, the HSP20 family is mostly tightly repressed at low temperature, suggesting that a costly mechanism can become detrimental under unnecessary conditions. Here, the role of HSP20s in response to HS and their possible deleterious expression at non-HS temperatures is discussed.

Keywords: heat shock transcription factor, heat shock response, acquired thermotolerance, cyclic nucleotide-gated channels, calmodulins, small heat-shock proteins, heat stress, global warming

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INTRODUCTION

During summer days, mild or severe heat stress (HS) typically occurs at midday and lasts until late afternoon in terrestrial systems (Dong et al., 2017). To survive, a plant must sense, early in the morning, a minor temperature increment to establish a suitable genetic program. The heat shock response (HSR) contains molecular defenses, including heat shock proteins (HSPs), that

Abbreviations: ARP6, actin-related protein 6; AT, acquired thermotolerance; CaM, calmodulin; CaMBD, calmodulin-binding domain; CBK3, calmodulin-binding protein kinase 3; CML, calmodulin-like protein; CNBD, cyclic nucleotide-binding domain; CNGC, cyclic nucleotide-gated channels; ER, endoplasmic reticulum; IQ, isoleucine-glutamine; HS, heat stress; HSE, heat shock element; HSF1, heat shock transcription factor; HSP, heat shock protein; HSR, heat shock response; miRNAs, microRNAs; ncRNA, non-encoding RNA; ROS, reactive oxygen species; siRNAs, small interfering RNAs.

must accumulate rapidly under rising temperatures to minimize foreseeable damage (Song et al., 2012; Serrano et al., 2019). Acquired thermotolerance (AT) refers to the plant adaptive capacity to survive noxious HS when exposed to sublethal temperatures, requiring the accumulation of HSPs. Under HS, both transcriptome and proteome-based studies have indicated regulatory responses of HSPs (Qin et al., 2008; Finka et al., 2011; Mangelsen et al., 2011; Xin et al., 2016; Guihur et al., 2020; Zhao et al., 2021). A conserved subfamily called "heat-induced molecular chaperones" contains the HSP100s, HSP90s, HSP70s, HSP60s, HSP40s, and HSP20s (Al-Whaibi, 2011; Jee, 2016). They are 20 times more likely to be heat-induced compared to non-chaperone proteins (Wang et al., 2004; Finka et al., 2011, 2015; Guihur et al., 2020). HSP chaperones prevent and repair protein misfolding and aggregation, reducing cell damage (Ben-Zvi and Goloubinoff, 2001; Wang et al., 2004; Zeng et al., 2004; Liberek et al., 2008; Mogk and Bukau, 2017). In particular, HSP20s are the most heat-responsive in plants due to their dramatic induction (Vierling, 2003; Guihur et al., 2020). They also prevent the aggregation of heat-labile proteins and could stabilize lipids at the plasma membrane (Haslbeck and Vierling, 2015). At low temperature, HSP20s are tightly repressed, suggesting that their inappropriate expression could be deleterious for plants (Sun et al., 2016). Moreover, HS generates stress granules that contain molecular chaperones, such as HSP20s, HSP101, untranslated mRNAs, elongation initiation factors, RNA-binding proteins and transcription factors (McLoughlin et al., 2016, 2019; Chantarachot and Bailey-Serres, 2018; Kosmacz et al., 2019). Theses cytoplasmic and chloroplastic bodies seem to have an important role in protein translation during and after HS (Merret et al., 2017; Chodasiewicz et al., 2020).

In most eukaryotes, including land plants, HSP accumulation depends on a signal that arises at the plasma membrane and results in the activation of heat shock transcription factor (HSF) families (Nover et al., 2001; Mishra et al., 2002; Hayashida et al., 2011; Liu et al., 2011; Scharf et al., 2012; Fragkostefanakis et al., 2015; Kijima et al., 2018). Plant cells can sense a wide temperature range through changes in the plasma membrane fluidity. Calcium channels, such as cyclic nucleotide-gated channels (CNGCs), can mediate calcium entry during HS as shown in *Arabidopsis thaliana* and *Physcomitrium patens* (Gong et al., 1998; Saidi et al., 2009; Finka et al., 2012; Gao et al., 2012; Tunc-Ozdemir et al., 2013). Yet, a fraction of HSFA1 is associated with the HSP70-HSP90 complex, and a large inactivated fraction might remain unbound under non-stressful conditions (Kyle Hadden et al., 2006; Westerheide et al., 2006; Saidi et al., 2009; Hahn et al., 2011). Following the activation of CNGCs, and a still unclear signaling pathway, HSFA1 is translocated into the nucleus and can bind specific DNA motifs present in the promoter of HSP genes, called "heat shock element" (HSE) (Santoro et al., 1998; Liu and Charng, 2012). HSFA1 could also trigger regulatory responses, including DNA methylation, histone modification, and chromatin remodeling (Zhao et al., 2021). In particular, bound histones to HSP genes, such as H2A.Z, must be evicted to allow for RNA polymerase II docking for the transcription of HSP (Franklin, 2010; Kumar and Wigge, 2010; Probst and Mittelsten Scheid, 2015). Yet, the mechanisms of heat sensing, particularly the components between the CNGCs sensors and the activation of the main regulator HSFA1 are not elucidated (Larkindale et al., 2005; Mittler et al., 2012). This review addresses the heat perception and signaling pathway in land plants, with a particular emphasis on the activation of HSFA1 at the plasma membrane, leading to the accumulation of HSP chaperones. In addition, the role of HSP20s at non-HS temperatures and their putative effect in plant cells are discussed. Understanding these critical processes would facilitate the production and selection of thermotolerant cultivars to face global warming.

HEAT SENSING AND SIGNALING IN LAND PLANTS

Calcium Entry Across the Plasma Membrane Triggers the Heat Shock Signaling

Plant cells developed an efficient mechanism for sensing the increase in temperature, as well as a signaling cascade for a rapid adaptive response. The nucleus, endoplasmic reticulum, cytosol, mitochondria, and chloroplast may also contain heat sensors (Bussell et al., 2010; Franklin, 2010; Schwarzländer and Finkemeier, 2012; Hentze et al., 2016; Sun and Guo, 2016; Chang et al., 2017; Lin K. F. et al., 2018). However, various observations have indicated that the primary heat sensing might occur at the plasma membrane. For instance, electrophysiology measurements in *P. patens* protoplasts, expressing the calcium-sensitive fluorescent protein aequorin reporter, have demonstrated a saturated accumulation of cytosolic Ca^{2+} within the first 10 min at 38°C (Saidi et al., 2009). Artificially preventing the entry of periplasmic Ca^{2+} in *A. thaliana* and *P. patens* protoplast showed a lack of HSP expression. A defective HSR has also been described in the presence of ionomycin and thapsigargin, which are ionophores known to release Ca^{2+} from internal stores (Saidi et al., 2009; Finka et al., 2012). Similar observations were reported for tobacco, maize, and rice (Gong et al., 1998; Li et al., 2004; Wu and Jinn, 2010; Wu et al., 2012). Yet, this phenotype has not been observed in *Chlamydomonas reinhardtii*, suggesting another mechanism of heat perception in green algae (Schmollinger et al., 2013). Thus, the HSR seems to depend on Ca^{2+} entry across the plasma membrane in land plants (**Figure 1**; Demidchik et al., 2018).

Embedded Cyclic Nucleotide-Gated Channels Act as Plasma Membrane Thermosensors

In both plants and animals, plasma membrane contains CNGCs, which are tetrameric cation channels and contain six transmembrane domains. They modulate Ca^{2+} entry from the apoplast and other ions, such as Mg^{2+}, K^{+}, Na^{+}, or Pb^{+} (Véry and Sentenac, 2002). CNGCs can be assembled as homotetrameric or heterotetrameric complexes, allowing for the formation of a large array of sensors capable of responding to different intensities of environmental cues (Clough et al., 2000; Tan et al., 2020). The cytosolic C-terminus harbors a

cyclic nucleotide-binding domain (CNBD) and a calmodulin-binding domain (CaMBD), in which a calmodulin (CaM) binding isoleucine-glutamine (IQ) motif is embedded (Kaplan et al., 2007; Jarratt-Barnham et al., 2021). *A. thaliana* AtCNGC2 and four acts as a heat sensor since its lack of expression leads to a hyper thermosensitive phenotype, resulting in a higher accumulation of HSPs at lower temperature. Similar results were observed in the orthologs CNGCb and CNGCd of *P. patens* (A Finka et al., 2012; Finka and Goloubinoff, 2014). In addition, the lack of AtCNGC6 in *A. thaliana* leads to a fewer transcript levels of HSP18.2, HSP25.3, and HSP70 compared to control plants at 37°C, ultimately impacting the HSR (Gao et al., 2012). In rice, reduced thermotolerance was observed in *Oscngc14* and *Oscngc16* mutants, resulting in a higher accumulation of hydrogen peroxide, leading to the cell death (Cui et al., 2020). These results strongly suggest that CNGCs act as thermosensors in land plants. Other calcium channel families have been also implicated in heat response. For example, the lack of synaptotagmin A activity led to decreased HSPs synthesis in *A. thaliana* at 45°C (Yan et al., 2017). ANNEXIN calcium channels can modulate cytosolic calcium signature under oxidative and heat stresses (Liao et al., 2017). Glutamate receptor-like channels have been suggested to participate in Ca^{2+} signaling since exogenous glutamate improves basal thermotolerance in maize (Li et al., 2019). Therefore, the heat sensing by the plasma membrane of plants contains calcium channels, including CNGCs, that can respond to incremental temperatures, mediating Ca^{2+} entry and triggering the signaling pathway for the accumulation of HSPs.

Calmodulins Response to Heat

The signaling molecules located in the cytosol and required to activate HSFA1s are not yet uncovered (**Figure 1**). Yet, both CNBD and CaMBD present on the cytosolic part of CNGCs suggest that cyclic nucleotide monophosphate and CaMs can mediate the heat signaling (Gao et al., 2012). CaMs are made of calcium-binding loops, called "E" and "F" that can each bind two Ca^{2+} ions and can respond to biotic and abiotic stress in plants (Rhoads and Friedberg, 1997; Mccormack et al., 2005; Fischer et al., 2013; Virdi et al., 2015). *A. thaliana* contains nine CaMs; among them are the first seven, which are highly conserved. In addition, 50 members of calmodulin-like proteins (CMLs) have been described as important players in stress perception and plant development (Aldon and Galaud, 2006; Vadassery et al., 2012). Interestingly, AtCaM2, AtCaM4, AtCaM6, AtCaM7, and AtCML8 were found to bind the C-terminal of several CNGC families (Fischer et al., 2017). At 37°C, AtCNGC6 was negatively regulated by AtCaM2/3/5 and AtCaM7, which interact with the IQ motif of AtCNGC6 and impact Ca^{2+} entry (Niu et al., 2020). A potentially important role in the heat signaling has been demonstrated for *AtCaM3* where the knockout mutant has shown reduced levels of HSP18.2 and HSP25.3 transcripts at 37°C, negatively impacting basal thermotolerance. The overexpression of AtCaM3 leads to a significant increase in the HSPs level and improves the resistance against noxious temperatures (Zhang et al., 2009). AtCaM3 has been also suggested to activate several components of the heat shock signaling pathway, such as mitogen-activated protein kinase 6 and calmodulin-binding protein kinase 3 (CBK3) (**Figure 1**; Liu et al., 2005; Yan et al., 2017). Moreover, AtCBK3 promotes HSFA1 activation by phosphorylation. Under HS, the lack of AtCKB3 dramatically reduced HSP18.2 and HSP25.3 levels, resulting in defective basal thermotolerance, whereas the *Atcbk3* overexpression line rescued the hypersensitivity phenotype (Liu et al., 2008; Yip Delormel and Boudsocq, 2019). In other plant species, several CaMs have been described to mediate the heat signal. In rice, OsCaM1-1 was shown to positively regulate Ca^{2+} signals, resulting in HSP accumulation (Wu and Jinn, 2012; Wu et al., 2012). In wheat, CaM1-2 has been found to act upstream of HSP26 and HSP70 at 37°C (Liu et al., 2003). Therefore, CaMs have been mentioned to play a critical role in the heat signaling of land plants and responding to other environmental stimuli (Virdi et al., 2015). Yet, other components of the heat signaling pathway between CNGC sensors and HSFA1s remain to be identified (**Figure 1**).

Heat Shock Transcription Factor A1 Acts as a Key Regulator of the Heat Shock Signaling Pathway

HSFs are essential regulators of the heat signaling pathway in many organisms (Gallo et al., 1993; Mishra et al., 2002; Nicholls et al., 2009; Anckar and Sistonen, 2011). In contrast to vertebrates, which contain fewer members (six for humans), plant HSF families have more members that reflect their strategy for a sessile adaptation in changing environment (von Koskull-Doring et al., 2007; Huang et al., 2016; Gomez-Pastor et al., 2018). For example, *A. thaliana* contains 21 HSFs, 24 for tomato, 52 for soybean, or 56 for wheat (Scharf et al., 2012; Xue et al., 2014; Fragkostefanakis et al., 2015). Plant HSFs are classified into three classes: HSFA, B, and C. All HSFs have a DNA binding, oligomerization, and nuclear localization domains. Yet, HSFAs differ in the presence of an activator region at the C-terminal, which binds HSEs, whereas HSFBs contain an inhibitor region. Under HS, several subfamilies of HSFA are required for the accumulation of HSPs. HSFB contains subfamilies, which can act as coactivators of HSPs transcription and, also, as antagonist repressors, competing for HSFAs at the end of HS (Czarnecka-Verner et al., 1997; Santoro et al., 1998; Mishra et al., 2002; Mitsuda and Ohme-Takagi, 2009; Ikeda et al., 2011; Scharf et al., 2012; Liu and Charng, 2013; Fragkostefanakis et al., 2015; Guo et al., 2016). In *A. thaliana,* the *hsfA1a* mutant was shown to be ineffective in accumulating several HSP transcripts at 37°C, resulting in the absence of AT (Liu et al., 2011). Similar observations were previously made in tomato where HSFA1 has been shown to be a master regulator for AT (Mishra et al., 2002). Studies in several monocotyledonous species, such as wheat and rice, have also demonstrated the important role of HSFA in mediating the heat signal (Yokotani et al., 2008; Zhang et al., 2013; Guo et al., 2016). Furthermore, the role of HSFA1 is not only limited to the transcription of HSPs, but it also activates several transcription factors, such as HSFA2, HSFA3, HSFA7a, multiprotein bridging factor 1C, and dehydration-responsive element-binding protein 2A, which are

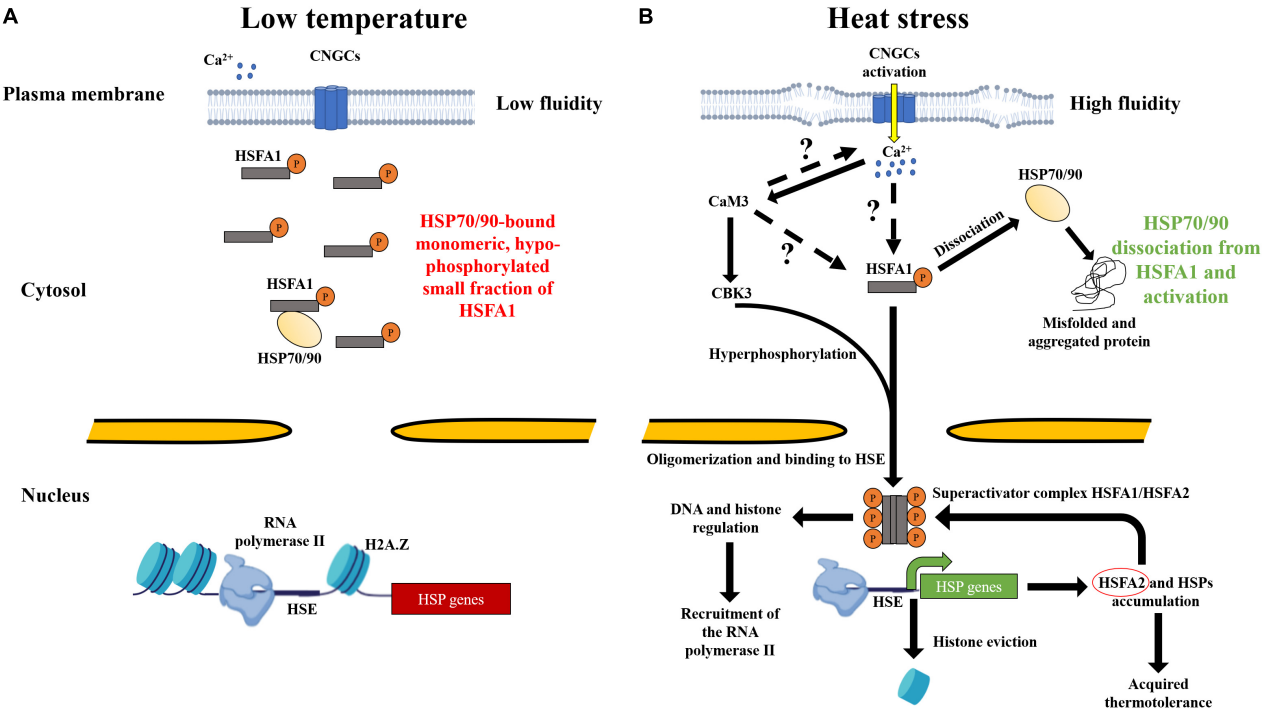

FIGURE 1 | Heat perception by the plasma membrane and mechanisms leading to the onset of acquired thermotolerance in land plants. **(A)** At low temperature, the rigidity of the plasma membrane prevents the activation of hypophosphorylated key regulator HSFA1, while a small fraction might still bind the HSP70-HSP90 complexes in the cytosol. Histones, such as H2A.Z, associate and condense the DNA and prevent the RNA polymerase from accessing HSP genes for active transcription. **(B)** The increase in temperature increases the fluidity of the plasma membrane, resulting in the activation of CNGCs heat sensors. Transient periplasmic Ca^{2+} entry into the cytosol trigger is an unknown signaling cascade that activates HSFA1. Excessive temperatures are expected to denature heat-labile proteins in the cytosol. Misfolded and aggregated proteins are assumed to recruit the HSP90 and HSP70 being in part bound to HSFA1. The dissociation of HSP70-90 is a key step that leads hyperphosphorylation of HSFA1 by CBK3 and leads to its translocation into the nucleus to bind HSEs. HSFA1 might mediate a signal to the chromosome remodeling machinery to remove bound histones from HSP genes. HSFA1 can also activate histone and DNA modifications required for the regulatory responses of HSPs. RNA polymerase II is then recruited for the transcription of HSFA2 (red circle), forming a superactivator complex with HSFA1 and leading to accumulation of HSPs, ultimately conferring acquired thermotolerance in land plants.

required for HSP synthesis and thermotolerance in *A. thaliana* (Suzuki et al., 2011; Yoshida et al., 2011; Liu and Charng, 2013; Ohama et al., 2017). When accumulated, HSFA2 can form a heterodimer with HSFA1 and thereby forming a superactivator complex for sustaining HSPs expression under HS (Chan-Schaminet et al., 2009). Thus, the family of HSFAs has been identified as a major regulator required for the onset of AT in land plants (Mishra et al., 2002; Hahn et al., 2011; Yoshida et al., 2011).

At low temperature, inactive cytosolic HSFA1s are hypophosphorylated and bound to the complex HSP70-HSP90 (**Figure 1**; Hahn et al., 2011; Morimoto, 2012). The traditional model suggests that, upon HS, HSP70-HSP90 complex is hijacked by the increased cytosolic levels of unfolded or misfolded thermolabile proteins, leaving HSFA1 free to trigger the HSR (**Figure 1**; Zou et al., 1998; Kim and Schöffl, 2002; Yamada et al., 2007; Hahn et al., 2011). Although thermolabile proteins become denatured and recruit molecular chaperones upon heat exposure, the prevention of Ca^{2+} entry through the plasma membrane led to an absence of HSR (Saidi et al., 2009). In addition, treatment with HSP90 inhibitors triggers a minor HSR at low temperature at rest, whereas a full-blown HSR can be obtained at higher temperatures (Kyle Hadden et al., 2006; Westerheide et al., 2006; Saidi et al., 2009). Therefore, even if all HSP90s are dissociated from HSFA1s, a large fraction of HSFA1s is required to be activated independently from a non-elucidated signal, which strictly depends on the calcium entry across the plasma membrane (**Figure 1**).

Histones and DNA Regulation Lead to the Transcription of HSPs

The expression of HSPs in plants is regulated by epigenetics, such as DNA methylation, histone modification, and chromatin remodeling (Boyko et al., 2010; Gao et al., 2014; Gallego-Bartolomé, 2020; Zhao et al., 2021). HSPs gene must be unwrapped from histones to become actively transcribed when HSF1As are bound to HSEs (**Figure 1**). Following HS, a global rearrangement of the chromatin has been observed in rice, rye, and *A. thaliana* (Santos et al., 2011; Tomás et al., 2013; Probst and Mittelsten Scheid, 2015). These results indicate that, at low temperature, HSPs genes are compacted by histones, and chemical modifications are required for HSPs expression to be allowed under HS. Interestingly, in *A. thaliana*, actin-related protein 6 (ARP6) has been reported to be an essential

component of the chromatin remodeling complex required for H2A.Z incorporation (**Figure 1**; March-Díaz and Reyes, 2009). The *Atarp6* mutant exhibited a higher accumulation of HSP70 at 12, 22, and 27°C (Kumar and Wigge, 2010; Cortijo et al., 2017). Histone chemical modifications, such as methylation and acetylation, have been also indicated to be important for the regulation of HSPs in plants (Lämke et al., 2016; Yamaguchi et al., 2021). Regarding non-coding RNA (ncRNA), such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), its involvement in the regulation of the HSR has been suggested in several land plant species (Khraiwesh et al., 2012; Li et al., 2014; Zhao et al., 2016; Liu et al., 2017; Lin J. S. et al., 2018; Zhao et al., 2021). Thus, following a short period of HS, epigenetic processes have also been implicated in developing a transcriptional heat memory (Bäurle and Trindade, 2020).

THE ROLE OF SMALL HEAT SHOCK PROTEINS UNDER HEAT STRESS

Among HSP chaperones, the HSP20 (sHSP) family is the most responsive to heat, whereas, at low temperatures, it is mainly repressed (Waters et al., 1996; Sun et al., 2002; Vierling, 2003; Guihur et al., 2020). HSP20s proteins are composed of subunits between 12 and 43 kDa and have an alpha-crystalline domain suggested to bind denatured proteins (Haslbeck et al., 2005; Basha et al., 2012; Waters and Vierling, 2020). *A. thaliana* contains 19 HSP20 (sHSPs) divided into six classes according to their localization (Sun et al., 2002; Waters and Vierling, 2020). Following HS, heat-labile proteins can be bound by HSP20s, in an ATP-independent manner, and folded into their native state by chaperone machinery, thereby preventing further heat-caused denaturation (Waters et al., 1996; Glover and Lindquist, 1998; Veinger et al., 1998; Goloubinoff et al., 1999; Swindell et al., 2007; Haslbeck and Vierling, 2015; Mogk et al., 2015; Mogk and Bukau, 2017). As shown initially in *E. coli*, the small HSP IbpB has been revealed to interact with HSP40, HSP60, and HSP70 chaperone complexes and assist in protein refolding (Veinger et al., 1998). Similar observations have been made in *Pisum sativum* and *Synechocystis sp.* (Mogk et al., 2003). In addition, HSP20s stabilize lipid bilayers and thereby protect the plasma membrane from high fluidity under excessive temperatures (Horváth et al., 2008; Haslbeck and Vierling, 2015).

HSP20s accumulation is essential for basal thermotolerance and the onset of AT in plants. In *A. thaliana*, an *AtHSP17.6II* mutant was unable to establish the AT, whereas the overexpression of LimHSP16.45 from *Lilium davidii* rescued the sensibility to HS (Yang et al., 2020). Supporting these observations, *A. thaliana* RNAi lines of six cytosolic HSP20s showed higher thermosensitivity, whereas the HSP20s overexpression lines restored the phenotype (McLoughlin et al., 2016). In wheat, chloroplastic HSP26 was shown to be required for seed maturation, germination, and development of HS tolerance (Chauhan et al., 2012). Similar observations have been described in other plant species, such as in tobacco and rice (Lee et al., 2000; Zhang et al., 2016). Besides providing protection against noxious temperatures, HSP20s also confer resistance to salt, drought, and cold stresses (Sun et al., 2002;

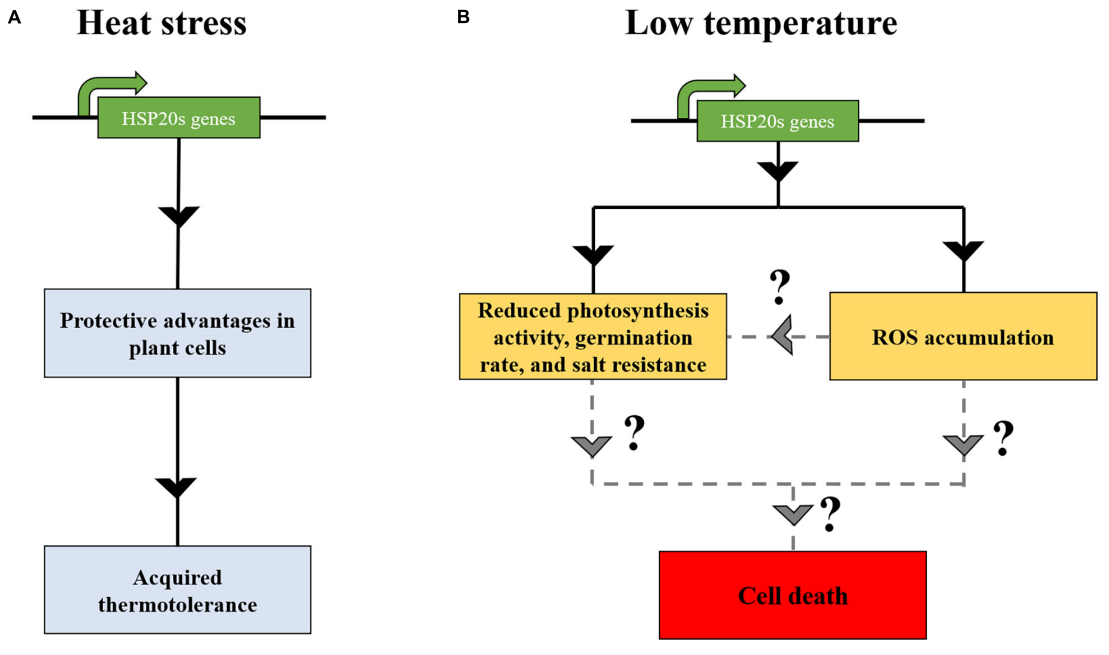

FIGURE 2 | The expression of HSP20s at low temperature might induce deleterious effects in land plants. **(A)** Under heat stress, HSP20s are accumulated, conferring protective advantages in plant cells, leading to the onset of acquired thermotolerance. **(B)** The overexpression of HSP20s at low temperatures might induce deleterious effect on plant growth and development (Sun et al., 2016), such as ROS accumulation (Ma et al., 2019), eventually resulting in the cell death (Sun et al., 2016; Ma et al., 2019). Solid lines indicate the consequences of HSP20s expression at low temperature, whereas dashed lines indicate potential cell death effect on plant cells.

Sarkar et al., 2009; Song and Ahn, 2010; Yang et al., 2014). HSP20s were also described to play key roles in somatic embryogenesis, pollen development, and seed germination (Sun et al., 2002; Volkov et al., 2005; Chauhan et al., 2012).

In several plant species, transcriptome and proteome-based analyses have demonstrated a nearly total absence of HSP20s at non-HS temperatures (Hernandez and Vierling, 1993; Simões-Araújo et al., 2003; Finka et al., 2012; Guihur et al., 2020). In contrast, other HSP chaperones families might have a substantial constitutive expression (Finka et al., 2012; Guihur et al., 2020). This raises a question of why plants tightly suppress HSP20s synthesis at non-HS temperature (low temperature) The complete HSP20 repression suggests that its constitutive expression would be problematic (**Figure 2**). To date, one study has reported a deleterious effect of one HSP20 in *A. thaliana*. *A. stolonifera* HSP17 overexpression in *A. thaliana* led to a reduction in leaf chlorophyll content and photosynthesis activity at both 22 and 40°C. The mutant showed hypersensitive response to exogenous abscisic acid and salinity during germination and during post-germinative growth (Sun et al., 2016). AtHSP24.7 has been described as a central activator of temperature-dependent seed germination (Ma et al., 2019). AtHSP24.7 overexpression accelerated seed germination and caused the accumulation of reactive oxygen species (ROS). In the study of Ma et al. (2019), an absence of negative physiology impact on plants was observed. Yet, it remains to demonstrate that other HSP20 family members behave similarly to HSP24.7, which could increase ROS content and, thereby, inducing apoptosis when achieving a critical threshold. Other related studies have indicated that the overaccumulation of HSP molecular chaperones might be deleterious for plants. For instance, although the overexpression of HSP70-1 improved basal thermotolerance in *A. thaliana*, it resulted in a dwarf phenotype, altering root growth (Sung and Guy, 2015). Furthermore, overexpression lines of HSP90.2, HSP90.5, and HSP90.7 reduced the resistance to salt and drought stress and produced a lower germination rate and lower fresh weight (Song et al., 2009). Thus, plants seem to have established a sophisticated mechanism to tightly regulate the expression of HSP chaperones, presumably to not affect plant fitness (**Figure 2**).

CONCLUSION

In recent years, the threat of global warming and the wide-reaching implications of the adverse effects on plant growth and crop yields have called for more studies about HS. This review has described some aspects of the heat perception and molecules involved in the signaling, ultimately triggering the accumulation of protective HSPs. There is strong evidence in literature showing that the plasma membrane, embedded with CNGCs, acts as a central hub for the perception of incremental temperature. Yet, an unidentified signal, potentially involving calmodulins and kinases, triggers the translocation of HSFA1s into the nucleus to activate essential regulatory responses, such as histone and DNA regulation (**Figure 1**). To further investigate the heat shock signaling pathway, several questions remain unanswered; among them are the following:

- What are the missing partners involved in the heat signal transduction between CNGCs and the activation of HSFA1 upon HS?
- How are CNGCs subunits assembled to sense and respond to a wide temperature scale?
- Does the overexpression of HSP20s at low temperature induce deleterious phenotypes in land plants?

All these issues need further research to address a comprehensive picture of heat sensing and AT.

AUTHOR CONTRIBUTIONS

BB made the figures and AG has updated **Figure 1**. Both the authors conceived the central ideas of the manuscript, interpreted data from literature, contributed to writing, reviewed, edited, and approved its final version of the manuscript.

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Mitochondria-Targeted SmsHSP24.1 Overexpression Stimulates Early Seedling Vigor and Stress Tolerance by Multi-Pathway Transcriptome-Reprogramming

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Among the diverse array of heat shock proteins across the three domains of life, mitochondria-targeted small heat shock proteins (sHSPs) are evolved in the plant lineage. However, they remained mysterious and understudied. In this study, we reported a systematic study of a novel mitochondria-targeted nuclear sHSP from eggplant (*Solanum melongena* L.; SmsHSP24.1). Differential expression of *SmsHSP24.1* indicated its positive role exerted during stress conditions. *Escherichia coli*-BL21 cell line overexpressing the *SmsHSP24.1* showed excellent thermo-tolerance ability, tolerating up to 52°C. Spectrometry and electron microscopy revealed a multimeric structure of the protein which acted as a molecular chaperone at high temperatures. Overexpression of *SmsHSP24.1* significantly enhanced resistance against heat, drought, and salt stresses and showed rapid germination in constitutively overexpressed eggplant lines. RNA-seq analysis reveals an apparent upregulation of a set of reactive oxygen species (ROS) scavenging enzymes of the glutathione (GHS) pathway and mitochondrial electron transport chain (ETC). Significant upregulation was also observed in auxin biosynthesis and cell-wall remodeling transcripts in overexpressed lines. qPCR, biochemical and physiological analysis further aligned with the finding of transcriptome analysis and suggested an essential role of *SmsHSP24.1* under various stress responses and positive physiological influence on the growth of eggplants. Therefore, this gene has immense potential in engineering stress-resilient crop plants.

Keywords: eggplant, mitochondria, chaperon, abiotic stress, heat tolerance, small heat shock protein

INTRODUCTION

Environmental factors continuously influence the performance of agronomic traits. Heat stress, among others, is one of the significant regulating factors that influence the metabolic pathways, growth, and development of crop plants and ultimately reduces the yield. Heat stress also imbalance the delicate cellular homeostasis, which turns down total protein synthesis, stability, and activity (Mayer and Bukau, 2005). This deregulation of cellular homeostasis activates a chain of events, such as instability of cell membranes and altered osmosis, a sudden increase in the concentration of reactive oxygen species (ROS), etc., which leads to organelle malfunction, imbalance in phytohormones production and signaling, and reprogramming of transcriptomic and metabolic pathways, thereby limiting the growth and productivity of plants (Hasanuzzaman et al., 2013). With increasing global temperature, heat stress poses severe threats for crop production, including eggplant (*Solanum melongena*) in the Indian subcontinent. It is estimated that across South Asia, the temperature would rise by approximately 2.2°C annually, which will cause significant damage to the performance of the eggplant in the coming years (Hoegh-Guldberg et al., 2018). Therefore, studying the impact of high temperature (HT) on the growth and development of crop plants is vital to maximizing agricultural production and ensure food security, especially for the resource-poor farmers in the South Asian region whose livelihoods directly depend on agriculture. Thus, it is important to understand the mechanism behind heat stress tolerance in eggplants.

Throughout the evolution, plants developed several stress tolerance mechanisms as they continuously expose to different stresses including heat (Suzuki, 2016). To counter this, plants have evolved diverse mechanisms executed at varying levels of metabolomics or proteomics pathways. Small heat shock proteins (sHSP; 12–42 kD molecular weight) are considered one such important protective mechanism, which is active in all living organisms, including plants. There are a total of 12 diversified subfamilies of sHSP present ubiquitously in three domains of life (Sun et al., 2020). Among all the subfamilies of sHSPs, the mitochondria localized sHSPs (Mito-sHSP) are evolved exclusively within the plant lineage (Waters and Vierling, 2020) with rare exceptions in *Drosophila* HSP22 (Morrow et al., 2000). Also, previous studies showed that plants tend to accumulate more mitochondrial small heat shock protein compared with HSP60 and HSP70 under heat stress conditions (Vierling, 1991; Lenne and Douce, 1994).

It is well known that mitochondria are the source of ATP production and also lead to ROS formation under adverse environmental conditions (Zhang et al., 2009; Huang et al., 2016). However, cellular ROS has both positive and negative effects on growth and development based on the concentration gradient (Bailly et al., 2008). Previous studies have demonstrated the relation of cellular ROS gradient and availability of small HSPs during stress conditions by protecting ROS scavenging enzymes (Kong et al., 2014). Higher seed vigor, longevity, and seedling establishment were achieved by overexpressing *OsHSP18.2* in *Arabidopsis* (Kaur et al., 2015). Higher rates of germination and elongated hypocotyls were observed in *CsHSP17.2* overexpressed *Arabidopsis* (Wang et al., 2017). Similarly, transcriptional reprogramming due to overexpression of small HSPs involved in the different biological pathways has also been reported in creeping bentgrass (Sun et al., 2016). However, a similar role of Mito-sHSP is still emerging in plants compared with other cytosolic sHSPs. It has been well documented that *Drosophila* Mito-sHSP22 is involved in protecting mitochondrial protein homeostasis and plays a crucial role in aging regulation and is essential for longevity (Morrow et al., 2004; Morrow and Tanguay, 2015). This shows the importance of mitochondria localized sHSP proteins under both normal and stressed conditions. A recent study on cotton shows that under heat stress, Mito-sHSP24.7 promotes rapid germination by blocking electron transport at cytochrome c, leading to enhanced ROS, which facilitate seed testa rupture and early germination (Ma et al., 2019). Another study on apple has demonstrated that mitochondrial sHSP protects the first protein complex of the electron transport chain NADH (ubiquinone oxidoreductase) during heat stress (Downs and Heckathorn, 1998). The study of Sanmiya et al. (2004) revealed that overexpression of tomato mitochondrial sHSP23.8 protein in transgenic tobacco lines shows higher thermotolerance, whereas tobacco plants that carry antisense of sHSP23.8 mRNA were susceptible to heat. Similarly, overexpression of sHSP23.8 in transgenic tomatoes shows increased thermotolerance in both T0 and T1 progenies under heat shock conditions (Nautiyal et al., 2005).

All these pieces of evidence suggest a crucial role of mitochondria localized nuclear small heat shock protein in protecting plants from various stresses. To date, compared with other subfamilies of heat shock proteins, only a few Mito-sHSPs are studied to understand their physiological or stress-protective role, such as pea (Lenne et al., 1995; Avelange-Macherel et al., 2020), tomato (Banzet et al., 1998; Liu and Shono, 1999), maize (Lund et al., 1998, 2001), rice (Mani et al., 2015), Cotton (Ma et al., 2019), and *Arabidopsis* (Scharf et al., 2001; Hooper et al., 2017; Escobar et al., 2021). Therefore, much of the function of mitochondria localized sHSPs and their potential role in plant growth and development remain largely unknown. In this study, we had investigated the physiological functions of a mitochondria-targeted small HSP (Mito-*SmsHSP24.1*) along with its protective role under stress conditions from a novel source which was the eggplant. In order to understand the impact of Mito-*SmsHSP24.1* under normal and stressed conditions, we had developed transgenic eggplants that constitutively overexpressed Mito-*SmsHSP24.1*. We had systematically studied its effect on growth and development under normal and stressful conditions. Transcriptome profiling indicated global reprogramming in crucial paths which led to significant alteration of crucial agronomic traits, specifically in early germination and seedling vigor in transgenic lines. Our results revealed a novel source of mitochondria localized sHSP protein, which could be beneficial for engineering climate-resilient crops in the future.

MATERIALS AND METHODS

Plant Material and Growth Conditions for Stress Treatments

We used eggplant (*S. melongena*) variety BARI Begun-4 which was developed by the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh. Specifically, 3-week old seedlings were grown hydroponically in Yoshida nutrient solution (YS; HIMEDIA, Mumbai, India) and subjected to different stresses like heat (45°C), salt (200 mM NaCl), and drought (150 mM) mannit to understand the response of *SmsHSP24.1* expression in leaf tissues. Plants irrigated with water served as treatment controls (CT). Eggplant leaves were collected at 0, 1, 2, 4, 6, 12, and 24 h after stress treatment and used for RNA isolation followed by subsequent gene expression analysis.

For physiological analysis of *SmsHSP24.1* overexpressing transgenic eggplant lines, three different southern positive lines (OE1, OE3, and OE7) were used under both normal and stress conditions. Specifically, 2-week old seedlings of OE1, OE3, and OE7 lines along with wild-type plants (WT) shifted in soil pots were used for heat and drought stress experiments, and seedlings transferred to Yoshida nutrient solutions were used for salt stress. After 1 week of adaptation, seedlings were exposed to heat shock (45°C) for up to 36 h. Yoshida nutrient solution was supplemented with 200 mM NaCl for salt treatment for 12 h and as drought treatment, water was withheld for 10 consecutive days to study the growth parameters of transgenic lines (OE) compared with the control (WT). Similarly, after combined heat (45°C) and dt stress by withdrawal water for 25 days at ICGEB net house field, leave tissues were collected. These samples were used to study oxidative damaged, biochemical and enzymatic assays as well as expression profiling of the target transcripts, i.e., *NADH-ubiquinone oxidoreductase*, q*uinol-cytochrome c-oxidoreductase*, *aminopeptidase* (*PepA*), *auxin-responsive gene* (*SAUR*), *superoxide dismutase* (*SOD1, SOD2*), *catalase* (*CAT*), and *ascorbate peroxidase* (*APX*) transcript by qRT-PCR.

Extraction of Plant Genomic DNA, RNA, and qRT-PCR Analysis

Genomic DNA was extracted from leaves of 21-days-old eggplant seedlings using the PureLink Genomic Plant DNA Purification Kit (Invitrogen[1], United States) according to the instructions of the manufacturer. Total RNA was extracted from eggplant tissues using the PureLink Plant RNA Reagent kit (Invitrogen; see text footnote 1). RNA was treated with RNase-free DNase (NEB[2], Ipswich, MA, United States) and used for first-strand cDNAs synthesis according to Verso cDNA Synthesis Kit (Thermo Fisher Scientific, United States; see text footnote 1). Real-time qRT-PCR was performed with SYBR Green PCR Master Mix and Applied Biosystems in 7500 Real-Time PCR Systems (Thermo Fisher Scientific, United States; see text footnote 1). Eggplant 18S rRNA-specific primer pair was used as an internal reference gene. All primer pairs used for RT-PCR and qRT-PCR are mentioned in **Supplementary Table 2**.

Sequence Identification, Isolation of cDNA, Phylogenetic Analysis, and Homology Modeling of Novel SmsHSP24.1 Protein

The putative eggplant mitochondria localized nuclear *SmsHSP24.1* gene was identified using NCBI expressed sequence tags (ESTs) database (**Supplementary Figure 1A**). The full-length *SmsHSP24.1* cDNA was amplified by nested PCR using a high-fidelity DNA polymerase (KOD plus, Toyobo, Japan) with primer pairs of *SmsHSP24.1*_F1/R1 and F2/R2 (**Supplementary Table 2**) and cloned into PCR-4-TOPO vector (Invitrogen, United States). Sequence confirmation was done using universal M13 forward and reverse primers through the Macrogen sequencing platform (Seoul, Republic of Korea) (macrogen.com/ko). MODELLER 9 version 11 (Sali et al., 1995) program was used for homology modeling of SmsHSP24.1 against Mito_AtHsp21 (Protein databank ID P31170) (**Supplementary Figure 1B**). Evaluation tools ProCheck (Laskowski, 1993) and Verify3D (Eisenberg et al., 1997) were applied to assess the predicted three-dimensional model of SmsHSP24.1 protein. The Muscle (Edgar, 2004) program was used for multiple sequence alignment, and a phylogenetic tree was constructed using MEGAX (Kumar et al., 2018) according to the neighbor-joining method with bootstrap value 1,000 (**Supplementary Figure 1C**).

Subcellular Localization of mGFP-Fused SmsHSP24.1 Protein in Tobacco Leaves and Eggplant Cell Culture

Bioinformatics-based prediction tools TargetP[3] (Denmark) and MitoFates (Fukasawa et al., 2015) were used to check the SmsHSP24.1 protein targeting signal (**Supplementary Figure 2**). To further *in vivo* confirmation, *SmsHSP24.1* protein-coding sequence without stop codon was cloned into mGFP based pCAMBIA1302 expression vector with hygromycin selection. *Bgl*II and *Spe*I restriction enzymes were used for the cloning of this sequence (**Supplementary Figures 3A-C**). Primers used for cloning confirmation are enlisted in **Supplementary Table 2**. *Agrobacterium* strain EHA105 was used for transient expression of the final expression cassette in tobacco leaf epidermal cells by following the method described by Kokkirala et al. (2010). After 72 h of inoculation, the leaf tissues were observed under a confocal fluorescence scanning microscope (Zeiss LSM510, Germany).

Similarly, stably integrated eggplant cell suspension culture with the same construct was used to further confirmation by co-localization with mitochondria specific dye MitoTracker^{TM} Red FM (Cat no: M22425; Invitrogen, United States) following a method similar to the one developed in the crop improvement lab of Reddy (data unpublished, ICGEB). Samples were prepared by adding 10 μl of suspended cells on a slide, and photographs were taken by laser scanning confocal microscopy (Zeiss

[1]https://www.thermofisher.com

[2]http://www.neb.com

[3]http://www.cbs.dtu.dk/services/TargetP/

LSM510, Germany). The fluorescence signal was collected using an emission filter of a 500–535 nm bandpass for GFP and 581–644 nm bandpass for MitoTracker™ Red FM. All fluorescence experiments were independently repeated at least three times.

Construction of Binary Expression Vectors and Generation of Overexpression Lines

The *SmsHSP24.1* coding region (*Nde*I + *Not*I) was first cloned into Gateway compatible entry vector (pL12R34-Ap) under constitutive cauliflower mosaic virus promoter (CaMV 35S) (*Kpn*I + *Nde*I) and nopaline synthase terminator (Nos) (*Not*I + *Sac*I) and then transferred into the plant transformation vector pMDC100 (Invitrogen, United States) containing *nptII* (kanamycin-resistant) gene as a plant selection marker (**Supplementary Figures 3D-G**). *Agrobacterium* strain, EHA105 was used for plant transformation, and 21-days-old cotyledonary leaves were used as starting material. Kanamycin (100 mg/l) was used at a 10-day interval to select transformed lines. The putative transgenic lines were screened by PCR using *nptII* and *SmsHSP24.1* gene-specific primers. For Southern blot analysis of putative transgenic lines, approximately 20 μg genomic DNA was digested with the restriction enzyme, *Nde*I. DNA fragments were separated on a 0.8% agarose gel and blotted onto the Hybond™ N^+ nylon membrane (GE Healthcare Limited, United Kingdom). Subsequently transferred N^+ nylon membrane was hybridized with a 500 bp of *SmsHSP24.1* gene-specific DIG-labeled probe (PCR DIG Probe Synthesis Kit, Roche, Germany). The blot was then washed and detected according to the instructions of the manufacturer (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche, Germany).

Recombinant Protein Production, Thermal Stability, and Protein Solubility Assay

The protein-coding sequence *SmsHSP24.1* was cloned between *Nde*I and *Not*I restriction sites of pET28a vector (**Supplementary Figures 3H-K**; Primers enlisted in **Supplementary Table 2**) for recombinant protein production in *E. coli* BL21 (DE3) cells. 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction at different temperatures (37, 28, and 18°C) and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE).

For studying the thermotolerance capacity of SmsHSP24.1 protein, we followed a method similar to the one described by Wang et al. (2017). *Escherichia coli* harboring pET28a:SmsHSP24.1 and pET28a blank plasmid were used for the assay at 37, 42, 48, 52, 55, and 58°C, respectively. Cell viability was estimated by counting the number of colony-forming units. Parallelly total soluble protein was also analyzed from supernatants and pellets in SDS–PAGE, visualized by Coomassie Blue staining.

Chaperone Like Activity Assay of SmsHSP24.1

For the chaperone-like activity assay, thermal-induced aggregation of alcohol dehydrogenase (ADH) (50°C) and citrate synthase (CS) (45°C) were measured in the presence or absence of SmsHSP24.1 protein at various molar ratios, according to Ihara et al. (1999). Protein aggregation was monitored by measuring light scattering at 360 nm in three replicates for each treatment. The measurements were performed using Helios Gamma Spectrophotometer (Thermo Spectronic, Cambridge, United Kingdom). For further investigation, electron microscopic analysis was performed with purified SmsHSP24.1 protein and the model substrate, citrate synthase (CS) before and after thermal-induced aggregation according to Zhang et al. (2015). Electron micrographs were recorded with a JEOL 1400 transmission electron microscope operated at 100 kV in ICGEB, India facility.

Seed Germination Assay

Seed germination assay of WT and OE lines was done under normal and abiotic stress conditions. MS growth medium was supplemented with 200 mM NaCl for salt stress and 150 mM mannitol for drought stress. For heat stress, seeds were first kept in an incubator at 45°C heat for 2 and 4 h then placed in full strength MS growth medium. A growth room condition of 16 h photoperiod at 25 ± 2°C temperature was provided. Germination rates were calculated each day, up to 7-day incubation, and representative seedlings were photographed.

Measurements of Chlorophyll Content and Physiological Parameters

Total chlorophyll content of OE and WT seedlings under untreated and stressed conditions were measured using a spectrophotometric method described by Lichtenthaler and Wellburn (1976) with slight modification. Approximately 150 mg of leaf tissue was used for chlorophyll measurement, with each reaction was performed in three replicates. The absorbance was measured at 441, 646, 652, and 663 nm using a Helios Gamma Spectrophotometer (Thermo Spectronic, Cambridge, United Kingdom). Physiological parameters, namely plant height (cm), shoot length, root length, fresh weight, and dry weight of transgenic and WT lines were measured under normal and abiotic stress conditions.

Biochemical and Enzymatic Activity Assays of SmsHSP24.1 Protein Overexpressed Lines

Transgenic lines and WT plants from untreated and stressed conditions were harvested for assay of antioxidant enzyme activity. Approximately, 200 mg of fresh leaves were finely ground in ice-cold 0.2 M phosphate buffer (pH 8) and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was kept on ice. The superoxide dismutase (SOD) activity was performed as previously described by Beyer and Fridovich (1987). Absorbance was measured at 560 nm Ascorbate peroxidase (APX) activity was determined by following dihydrogen dioxide (H_2O_2) dependent

oxidation of ascorbic acid (ASC) at 290 nm. The assay for catalase (CAT) activity was performed according to the protocol of Beaumont et al. (1990), which monitored the dismutation of H_2O_2 at 240 nm. Electrolyte leakage was measured as previously described (Sairam et al., 2002). Measurement of proline was also performed according to the protocol of Bates (1973).

Oxidative Damage and Dead Cell Detection Assay

Leaf discs from both WT and transgenic plants of similar age were treated with 10 μM methyl viologen (MV; paraquat) for oxidative damage, 200 mM NaCl as salt stress, and 150 mM mannitol as drought stress. For MV stress, leaf discs were incubated for 6 h at 28°C dark in a shaker incubator and then exposed to light. Salt and drought-imposed leaf discs were observed after 72 h of incubation under the light. Generation of H_2O_2 was detected with 3, 3-diaminobenzidine (DAB) as previously reported by Thordal-Christensen et al. (1997). *In vivo* generation of O^{-2} in tissues after applying the stresses mentioned was detected by staining with 1% nitro blue tetrazolium (NBT) described by Fryer et al. (2002). Similarly, cell death was also detected in treated samples after staining with lactophenol trypan blue as described by Keogh et al. (1980).

RNA-Seq and Transcriptome Analysis

Total RNA was extracted from the leaves of 3-week-old WT and T2 generation transgenic eggplants (OE7) under either normal or heat stress (45°C) conditions using the RNAeasy Plant Mini Kit (Qiagen[4], Germany) according to the instructions of the manufacturer. The quality and quantity of RNA were confirmed by RNase-free agarose gel electrophoresis and a Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). Library construction and RNA sequencing were carried out by Agri-Genome Labs, Kerala, India. Only samples with RNA integrity of N7.0 were used for RNA-seq analysis. Illumina HiSeq 2500 platform (Illumina Inc., CA, United States) was used for RNA sequencing. All the RNA-seq data were then successfully submitted to the NCBI SRA database (PRJNA750594). The pre-processed and rRNA removed reads were aligned to the tomato reference genome, and the gene model downloaded from Sol Genomics Network[5]. After aligning the reads with the reference genome, the aligned reads were used to estimate genes and the expression of transcripts using the cufflinks program (version 2.2.1, United States). Differential expression analyses of all control and treated samples were performed using the cufflinks program. Levels of gene expression were measured by the fragments per kilobase of transcript per million fragments mapped (FPKM) plot. Log2 fold change cut-off 2 and *p*-value cut-offs.01 and.05 were used separately as cut-offs for up and down-regulated genes and isoforms. Clustered heat maps of up- and down-regulated genes of sample comparison with a *p*-value cut-off of.05 were generated. Gene ontology (GO) enrichment analysis was performed using the GOSeq tool (default parameters; United States). GOseq functions in calculating the significance of over-representation of each GO category amongst differentially expressed genes (DEGs). The KEGG pathway analysis was carried out for the available differentially expressed genes using a bio-conductor tool, path view with the tomato reference genome.

[4]https://www.qiagen.com

[5]ftp://ftp.solgenomics.net/tomato_genome/Heinz1706/wgs/assembly/build_3.00/S_lycopersicum_chromosomes.3.00.fa.tar.gz

Statistical Analysis

Statistical analyses were performed by one-way ANOVA and the differences between means were compared with Tukey HSD.5 values obtained for the particular dataset.

RESULTS

Identification, Cloning, and Sequence Analysis of *SmsHSP24.1* Gene From Eggplant

The putative 1032 bp gene was retrieved by NCBI ESTs blast using the tomato Mito-*slHSP23* (BAA32547.1) full-length cDNA as a query. *In silico* sequence analysis using eggplant draft genome database (Hirakawa et al., 2014), we found one copy of mitochondria-targeted *small HSP* gene at Sme2.5_00899.1_g00005.1 contig with two exons and an open reading frame (ORF) of 636 bp. We amplified the *SmsHSP24.1*-ORF with nested PCR primers that encode a protein with an apparent molecular weight of 24.1 kDa and an isoelectric point of (pI) 4.84. Blastp homology search and multiple sequence alignment and homology modeling indicated that SmsHSP24.1 contained a conserved α-crystalline domain (ACD) of 81-amino-acid at the C-terminus (positions + 115 to + 196). Phylogenetic analysis with the deduced amino acid sequences from a diverse range of species revealed that this protein was closely related to tomato (SlsHSP23.5) and *Arabidopsis* (AtsHSP26.4). Therefore, we designated our protein as SmsHSP24.1 and submitted it to the NCBI gene bank (AXS76128.1).

SmsHSP24.1 Response to Multiple Environmental Stresses

We had investigated *SmsHSP24.1* transcript abundance to understand its physiological role under abiotic stress conditions. The level of *SmsHSP24.1* transcript in leaves under heat (45°C), salt (200 mM NaCl), and osmotic stress (150 mM mannitol) were compared with untreated seedlings as relative fold-change (**Figures 1A-C**). The relative fold change of *SmsHSP24.1* under heat stress was found to be the highest among all the treatments. An elevated transcript level was observed within 1 h after the heat treatment which reached a maximum of 9-fold change at 2 h time point and then gradually declined (**Figure 1A**). In the case of salt stress, the expression dynamics of the *SmsHSP24.1* transcript were found to be different from the heat stress. An approximately 5-fold increase of *SmsHSP24.1* transcript was observed at the 4 h time point of salt stress after which it reduced gradually until 12 h and then a significant upregulation was observed again at the 24 h timepoint (**Figure 1B**). A quick expression of *SmsHSP24.1* was

witnessed when treated with mannitol reaching a peak as soon as this osmotic agent was added to the plant. However, after 2 h the level of *SmsHSP24.1* transcript started to decline steadily up to 12 h and at 24 h, it was 1.5-fold lower than the level transcript observed at 1 h of drought stress (**Figure 1C**).

SmsHSP24.1 Is a New Member of Mitochondria Localized Nuclear-Encoded Protein

In silico analysis using TargetP (see text footnote 3) and MitoFates (Fukasawa et al., 2015) revealed that the *SmsHSP24.1* protein was localized in mitochondria with a significantly high score of 0.792 and 0.997, respectively. Similarly, *in vivo*, transient expression assay of Agrobacterium harboring *SmsHSP24.1* fused with mGFP on tobacco leaf (3-week-old seedlings) also indicated discreet localization other than the nucleus (**Figures 1D,E**). Furthermore, co-localization analysis with mitochondria specific dye (MitoTracker^{TM}, United States) FM (Cat no: M22425; Invitrogen) in eggplant cell suspension-culture conclusively confirmed that this novel SmsHSP24.1 was targeted to the mitochondria (**Figure 1F**).

Heterologous Overexpression of SmsHSP24.1 Protein Acts as a Molecular Chaperon and Enhances Thermo-Tolerance of *E. coli* Cells Under Severe Heat Stress

We performed a series of experiments to analyze whether the novel SmsHSP24.1 protein can function as a chaperone and enhance survivability under severe stress conditions. First, we had expressed the coding sequence of SmsHSP24.1 protein in the BL21(DE3) strain of *E. coli* using the expression vector pET28a. Western blot analysis with an anti-His-tag antibody demonstrated the presence of the recombinant protein in the *E. coli* cells (**Figures 2A-C**). A Thermotolerance assay was conducted with a pET28a vector carrying *SmsHSP24.1* and blank pET28a as an experimental control (**Figure 2D**). No growth difference was observed under normal growth conditions, but 65% of *E. coli* culture carrying pET28a-SmsHSP24.1 construct found to be effectively tolerated temperature up to 55°C for 1 h, whereas the control cells failed to survive (**Figure 2E**). Only 10% of the cells carrying the blank pET28a at 50°C survived 1 h compared with 50% of *E. coli* carrying the pET28a-SmsHSP24.1 construct. SDS–PAGE analysis of the soluble fractions further revealed a significant increase in total soluble proteins in the pET28a-SmsHSP24.1 carrying BL21 cells compared with pET28a-blank at different temperatures (**Figure 2F**).

To further understand, we conducted a transmission electron microscopic thermal-aggregation assay of two commonly used heat-sensitive model substrate proteins, ADH, and CS, at different molar ratios of SmsHSP24.1. Thermal inactivation and aggregation of CS and ADH were detected by light scattering at 45°C and 50°C, respectively (**Figures 2G,H**). SmsHSP24.1 delayed aggregation of CS at a molar ratio of 1:1 (SmsHSP24.1:CS), and at a 2:1 molar ratio, it fully protected CS from aggregation. Similarly, the aggregation of ADH was also found to be entirely prevented at a 2:1 (SmsHSP24.1:ADH) molar ratio. Transmission electron microscopic (TEM) analysis further showed CS is in its native state at 4°C, whereas it was completely denatured and aggregated at 45°C without the presence of SmsHSP24.1 (**Figure 2I**), On the other hand, while incubated with SmsHSP24.1, the formation of polyhedron assembly under TEM was observed which correlates with the thermal protection of CS at 45°C up to 60 min (**Figure 2J**).

Generation of Constitutively Overexpressed SmsHSP24.1 Eggplant

To further evaluate the physiological role of SmsHSP24.1, we developed independent transgenic eggplant lines constitutively overexpressing SmsHSP24.1 protein (**Supplementary Figure 4A**). PCR screening with two sets of primers (*NptII* primer sets ∼0.45 kb and SmsHSP24.1 + NosT terminator junction primer set ∼0.9 kb) found 17 out of 20 putative T0 plants to be positive (**Supplementary Figure 4B**). Homozygous T2 OE lines were selected under 100 mg/L kanamycin and reconfirmed by PCR (**Supplementary Figures 4C,D**). Same lines were further used for Southern blot analysis to confirm the copy number of transgene integration using the *SmsHSP24.1* gene-specific probe. We obtained two single, two double, one triple, and one multi-copy (containing five copies of insertions) transgenic events with morphological parameters similar to WT eggplants (**Supplementary Figure 4E**). Similarly, the copy number of Mito-sHSP (*SmsHSP24.1*) in the WT eggplants genome was also confirmed by Southern blot analysis (**Supplementary Figure 4F**). qRT-PCR analysis showed a higher level of transgene expression compared with the WT (**Supplementary Figure 4G**). Henceforth, we chose single copy integrated transgenic lines (OE1, OE3, and OE7) with high transgene expression from the T2 generation for subsequent physiological analysis.

SmsHSP24.1 Overexpression Exerts Improved Tolerance to Abiotic Stresses

Transgenic (OE1, OE3, and OE7) and WT plants were tested under normal and stress conditions. Both grew well and produced new leaves in the untreated condition. However, upon heat stress (45°C) for 36 h, OE lines exhibited marked thermotolerance compared with WT (**Figure 3A**) and wholly recovered when the stress was withdrawn. During drought stress induced by withholding water for 10 days, WT plants exhibited severe wilting, and some died, while only minor signs of dehydration were observed in OE lines. After 1 week of re-watering, 80% of the OE lines revived compared with 30% WT plants (**Supplementary Figure 5**). Also, when seedlings were treated with 200 mM NaCl for 3 days, WT plants either exhibited significant growth inhibition or died compared with OE lines. It was also observed that seedlings of OE lines were less prone to chlorosis compared with WT plants (**Figure 3B**). Furthermore, the OE seedlings exhibited significantly greater fresh weight, dry weight, root length, shoot length, and chlorophyll content under heat, salt, and

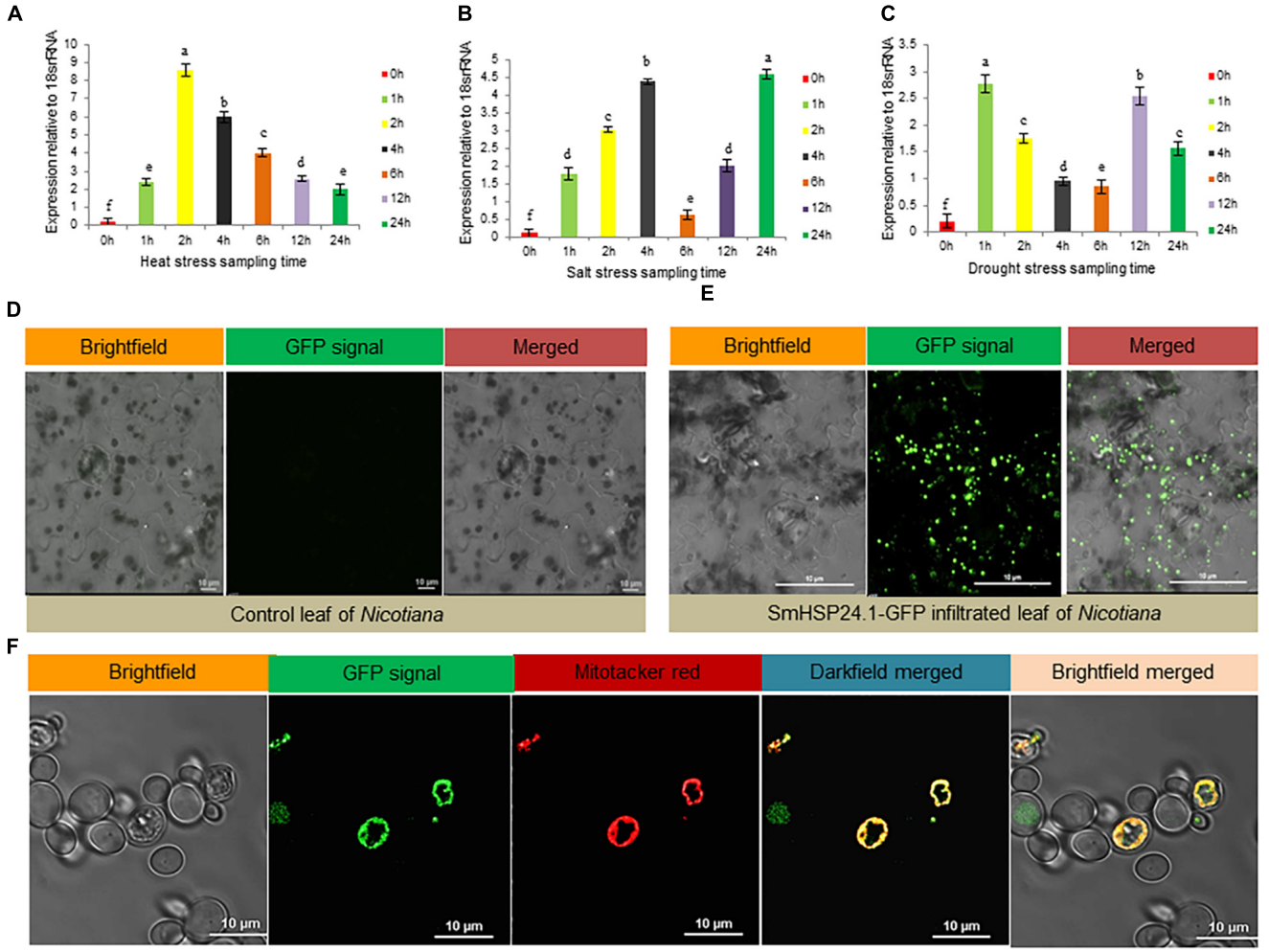

FIGURE 1 | Stress-responsive expression analysis and subcellular localization of SmsHSP24.1 protein. **(A)** Time-dependent relative quantitative RT-PCR analysis of 3-week-old eggplant seedlings grown in soil pot at 45°C heat. **(B,C)** Relative RT-PCR analysis of seedlings grown hydroponically in Yoshida nutrient solution containing 200 mM NaCl salt and 150 mM mannitol. RT-qPCR data are $M \pm SD$ ($n = 3$). The letters a, b, c, and others indicate significant differences between samples ($P < 0.05$). **(D)** Fluorescence scanning confocal microscopy of the Agro-infiltrated control tobacco epidermal cells appeared with no mGFP fluorescent signal. **(E)** Agro-infiltrated tobacco epidermal cells with pCAMBIA:CAMV35SP:SmsHSP24.1-mGFP:NosT construct revealed punctate structures in leaf epidermal cells which appeared to be localized in the mitochondria. **(F)** Stably transformed eggplant suspension cells with pCAMBIA:CAM35SP:SmsHSP24.1-mGFP:NosT construct were directly observed under a confocal microscope with co-localization of mitochondria specific dye MitoTracker™ FM Red. The bar represents 10 µm.

drought stress compared with the WT plants (**Figures 3C-E** and **Supplementary Figures 5A-C**).

A field study of combined heat and drought stress was also conducted at the ICGEB net house facility, New Delhi. In here, two and half-month-old seedlings of both OE lines and WT plants were subjected to 25 days of water withdrawal and an average of 43°C air and 50°C soil temperature at the reproductive stage. Under such conditions, most WT seedlings were found to be severely injured, whereas OE lines remained green and healthy (**Figures 4A-C**). OE lines (OE1, OE3, and OE7) recovered quickly without any effect on flowering and fruit setting after withdrawal of stress, whereas the WT plants were completely retarded (**Figure 4D**). Physiological parameters also showed higher fresh and dry weight in all OE lines compared with WT under control and stress conditions (**Figure 4E**). Besides, semi-quantitative RT-PCR analysis showed increased accumulation of *SmsHSP24.1* transcript in all OE lines both in control and combined stressed plots compared with WT (**Figure 4F**). Enzymatic activity of ROS-scavenging enzymes, such as SOD, APX, and CAT was also monitored in both OE and WT lines from the combined stress plot. OE lines were found to maintain a high level of all the ROS-scavenging enzymes compared with WT plants (**Figure 4G**). Similarly, proline content showed an increase in overexpressed lines, whereas MDA content had decreased significantly after stress treatment in the OE lines compared with WT plants (**Figures 4H,I**). This data was found to correlate with RNA-seq, and semi-quantitative RT-PCR analysis carried out on 3-week-old heat stress OE lines.

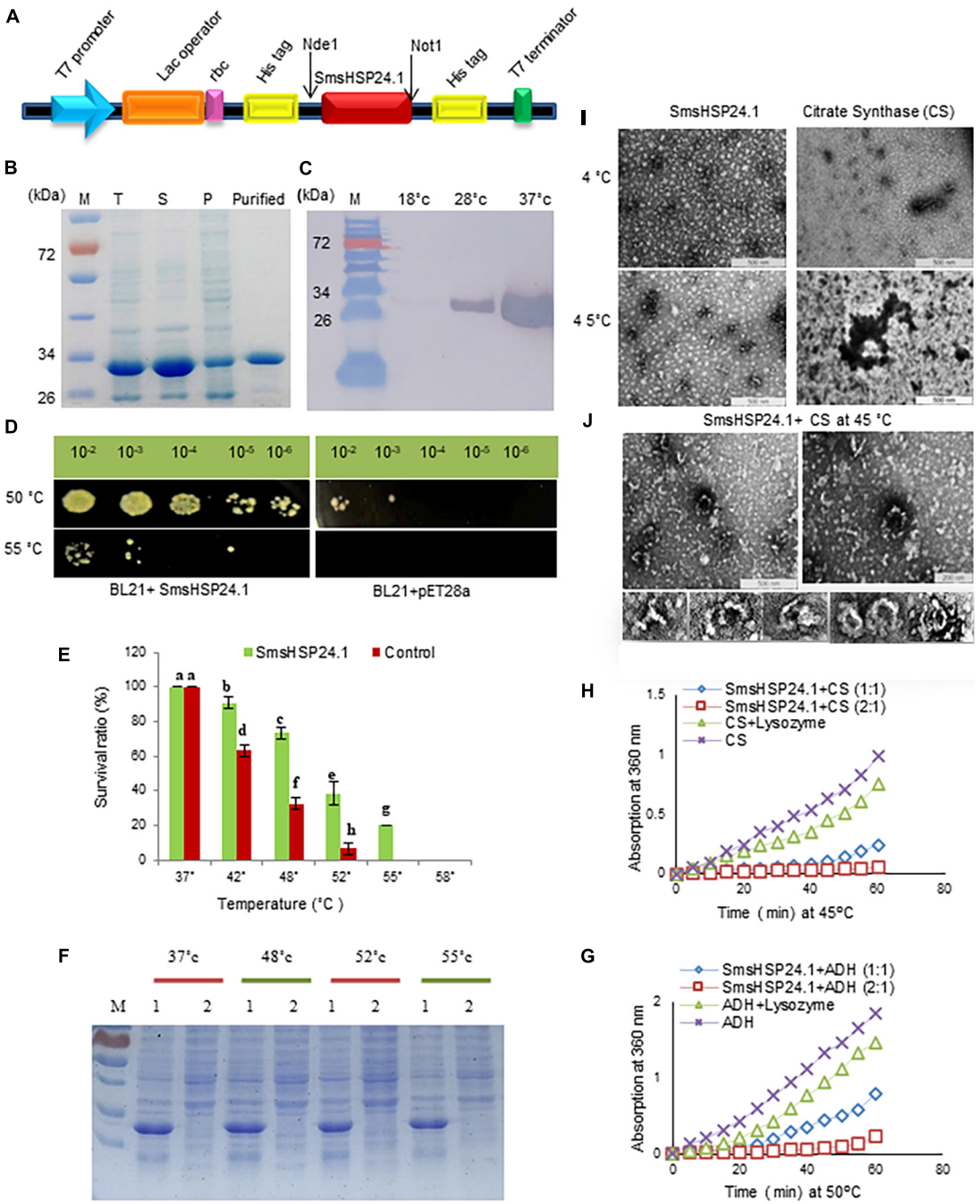

FIGURE 2 | Recombinant SmsHSP24.1 protein expression and chaperone activity assay. **(A)** Schematic diagram of SmsHSP24.1 protein expression cassette. **(B)** SDS–PAGE analysis of the proteins present in the total lysates (T), soluble (S), insoluble pellet (P), and purified SmsHSP24.1 protein. **(C)** Western blot of SmsHSP24.1 protein with Anti-His tag antibody. **(D)** pET28a:SmsHSP24.1 and control pET21a plasmid harboring BL21 cells grown in LB medium after being heat-shocked for an hour at 50°C and 55°C. **(E)** Colony-forming units counted from heat-treated and non-treated cells at different temperatures. **(F)** Proteins solubility was analyzed in SDS–PAGE at different temperature periods; 1: BL21a:SmsHSP24.1, 2: Blank BL21a. **(G,H)** Thermal aggregation of CS and ADH detected by light scattering at 360 nm. **(I)** Transmission electron microscopy images of SmsHSP24.1 and citrate synthase (CS) protein at 4°C and 45°C, respectively, Scale bars represent 500 nm. **(J)** Electron microscopy images clearly show SmsHSP24.1 protein prevents model substrate CS when incubated at 45°C for 60 min. Scale bars represent 500 nm (Left panel) and 200 nm (Right panel), SmsHSP24.1protein forming different polyhedron assemblies (Lower panel).

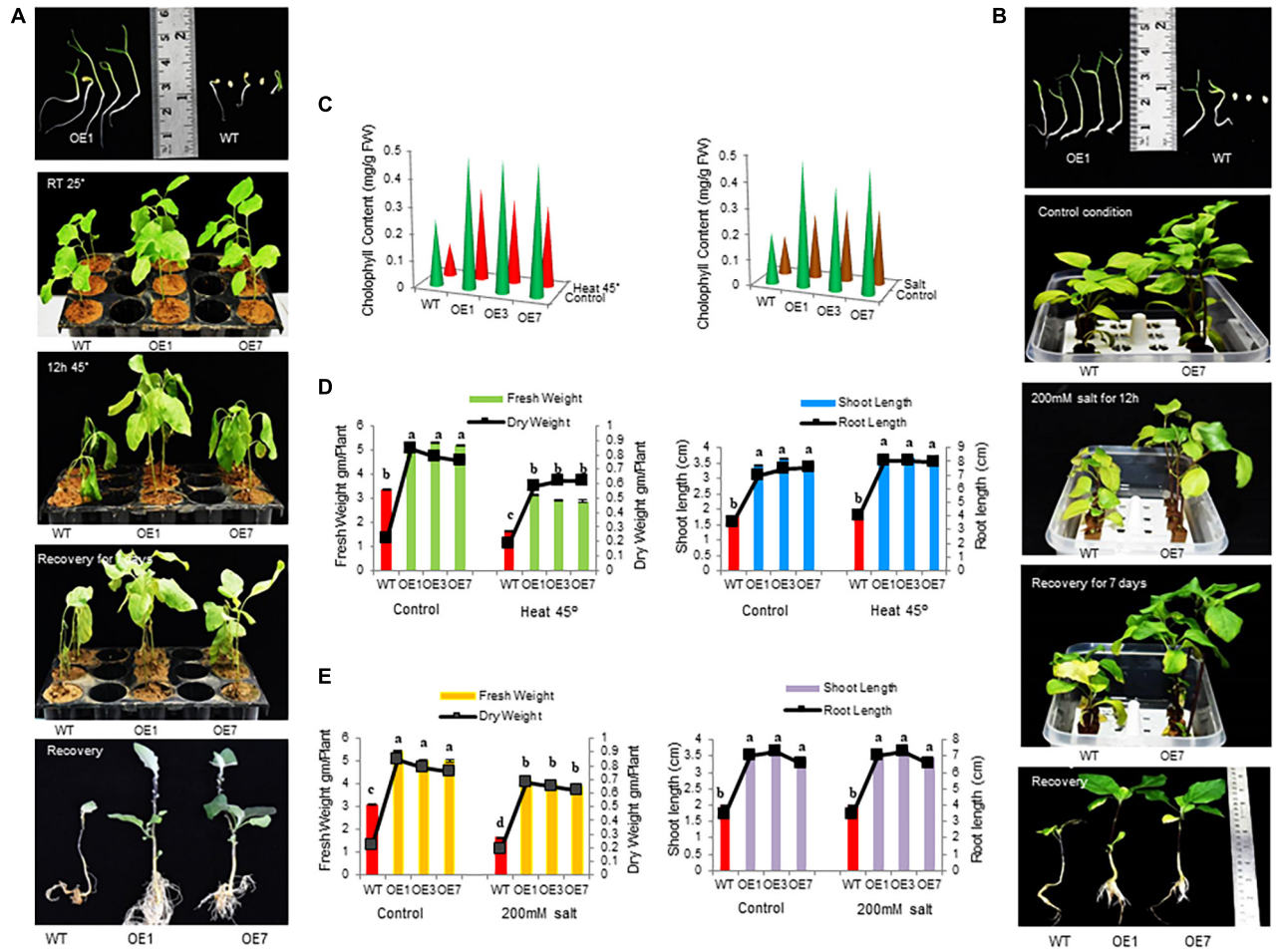

FIGURE 3 | Growth parameters of transgenic eggplant lines overexpressing SmsHSP24.1 protein under heat and salt-stressed conditions. **(A)** Growth parameters of transgenic (OE) as well as WT eggplant lines at 45°C. **(B)** Growth parameters under 200 mM NaCl salt stress condition of the transgenic (OE) and WT eggplant lines. **(C–E)** Total fresh weight, dry weight, root length, shoot length, and chlorophyll contents were significantly higher in all transgenic seedlings compared with the WT line. Data are M ± SD (n = 3). The letters a, b, c, and others indicate significant differences between different lines ($P < 0.05$).

SmsHSP24.1 Overexpressing Plant Maintains an Elevated Level of Cellular Reactive Oxygen Species Under Both Normal and Stress Conditions

To evaluate the impact of overexpressed *SmsHSP24.1* in mitigating ROS damage, we conducted leaf disc assay separately for heat (45°C heat for 36 h), osmotic stress (150 mM Mannitol), salt (200 mM NaCl), and MV (10 μM) to investigate the H_2O_2, O^{2-} contents and cell death after stress (**Figure 5** and **Supplementary Figure 6**). Reduced and slow chlorosis was observed for SmsHSP24.1 overexpressing event compared with WT plants after stress treatments. The OE lines overexpressing *SmsHSP24.1* showed a significantly lower level of ROS production and cell death compared with WT plants after MV (**Supplementary Figure 6A**) and heat treatment as were evident by DAB, NBT, and Trypan blue staining (**Figure 5A**). We also noticed that the H_2O_2 levels in WT plants were approximately 3- to 4-fold higher than OE lines after the heat, salt, mannitol, and MV treatment (**Figure 5B**). However, it was interesting to note that under normal conditions, the OE lines maintained a slightly higher cellular H_2O_2 level compared with WT plants. However, we have observed that this slight increment of cellular ROS in OE lines did not hamper the growth under normal environmental conditions, while no lethal increment in the level of H_2O_2 was observed in OE lines compared with WT plants upon stress treatment (**Figure 5B**). This finding was also supported by a comparative analysis of chlorophyll to be retained and ion leakage between OE lines and WT plants. OE lines were found to prevent chlorophyll damaged and reduced ion leakage probably due to the protection from oxidative damage through decreased ROS production under stress conditions (**Figures 5C,D**). As described in the previous data (**Figure 4C**), OE lines are able to maintain a higher level of ROS scavenging enzymes compared with WT plants due to which ROS production could not be increased up to lethal level.

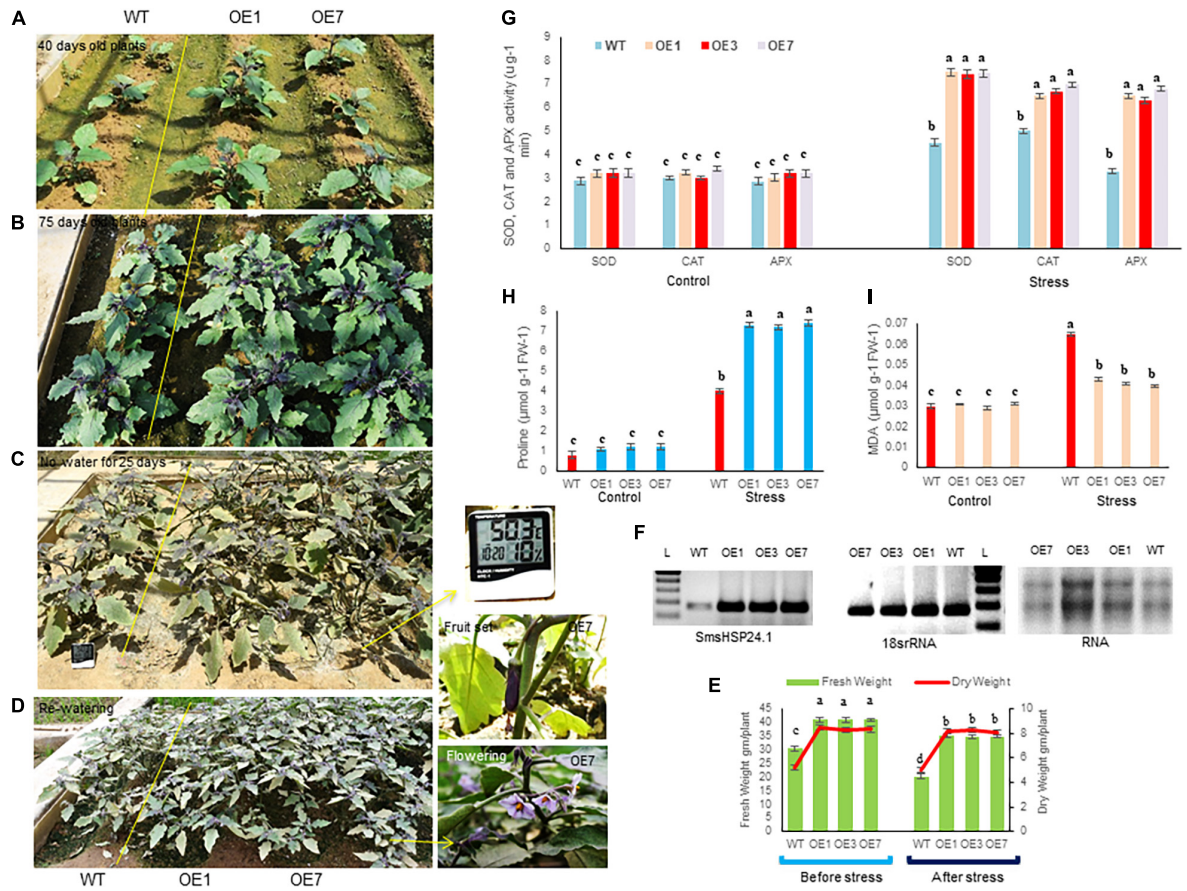

FIGURE 4 | Transgenic plants overexpressing *SmsHSP24.1* maintained physiological balance at the field level under combined (heat and drought) stress. **(A,B)** Mature transgenic as well as WT plants grown in normal field conditions. **(C)** Phenotypes of 75 days old eggplant seedlings imposed by water withdrawal for 25 days with 45°C average heat from nature. **(D)** Phenotypes of transgenic and WT seedlings after re-watering for 15 days. Initiation of flower and fruit sets after recovery from stresses. **(E)** Total fresh weight and dry weight were significantly higher in all transgenic seedlings under control and stress conditions. Data are M ± SD (n = 3). The letters a, b, c, and others indicate significant differences between different lines ($P < 0.05$). **(F)** Semi-quantitative RT-PCR analysis of *SmsHSP24.1* from field-grown OE and WT lines show increased accumulation of SmsHSP24.1 transcript in all OE lines both under control and stress conditions. **(G–I)** Different biochemical parameters such as proline, MDA, and ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were determined in transgenic as well as WT lines. Data are $M \pm SD$ (n = 3). The letters a, b, c, and others indicate significant differences between samples from different OE lines ($P < 0.05$).

SmsHSP24.1 Overexpressed Lines Exhibit Early Seed Germination and Seedling Vigor

We observed a significant rate of early seed germination and seedling vigor, both *in vitro* and field condition in OE lines (OE1, OE3, and OE7) compared with WT (**Supplementary Figure 7A**). Furthermore, a 95% germination rate was observed within 3 to 4 days in OE lines (OE1, OE3, and OE7) in the growth medium, while 6 days were required to attain an 80% germination rate in WT (**Supplementary Figure 7A**). A similar result was observed when seeds were sown in the field under normal conditions. Physiological parameters obtained at 2 weeks after germination revealed significantly higher total fresh weight, dry weight, root length, shoot length, and chlorophyll content in all OE seedlings compared with WT seedlings (**Supplementary Figures 7B-D**).

We further conducted transcriptome analysis to understand the possible molecular mechanisms underlying the effect of early seed germination and seedling vigor. The comprehensive transcriptome data have been presented separately; however, changes in transcription were noticed in three key regulatory pathways; ETC and GSH, as well as auxin biosynthesis and transport (**Figures 6A,B** and **Supplementary Figure 8**). In ETC, *NADH-ubiquinone oxidoreductase* transcript was found to be significantly upregulated while *quinol-cytochrome c-oxidoreductase* was slightly down-regulated in OE lines compared with WT lines under both normal and stressed conditions. qPCR cross-validation also revealed similar results (**Figure 6C**). *PepA* (*aminopeptidase*) gene from the GSH pathway was found to be highly upregulated in OE lines. It was approximately 5-fold higher in OE lines compared with WT (**Figure 6C**). Similarly, significant upregulation was also observed for auxin carrier proteins like auxin-responsive *SAUR-like* genes. Likewise,

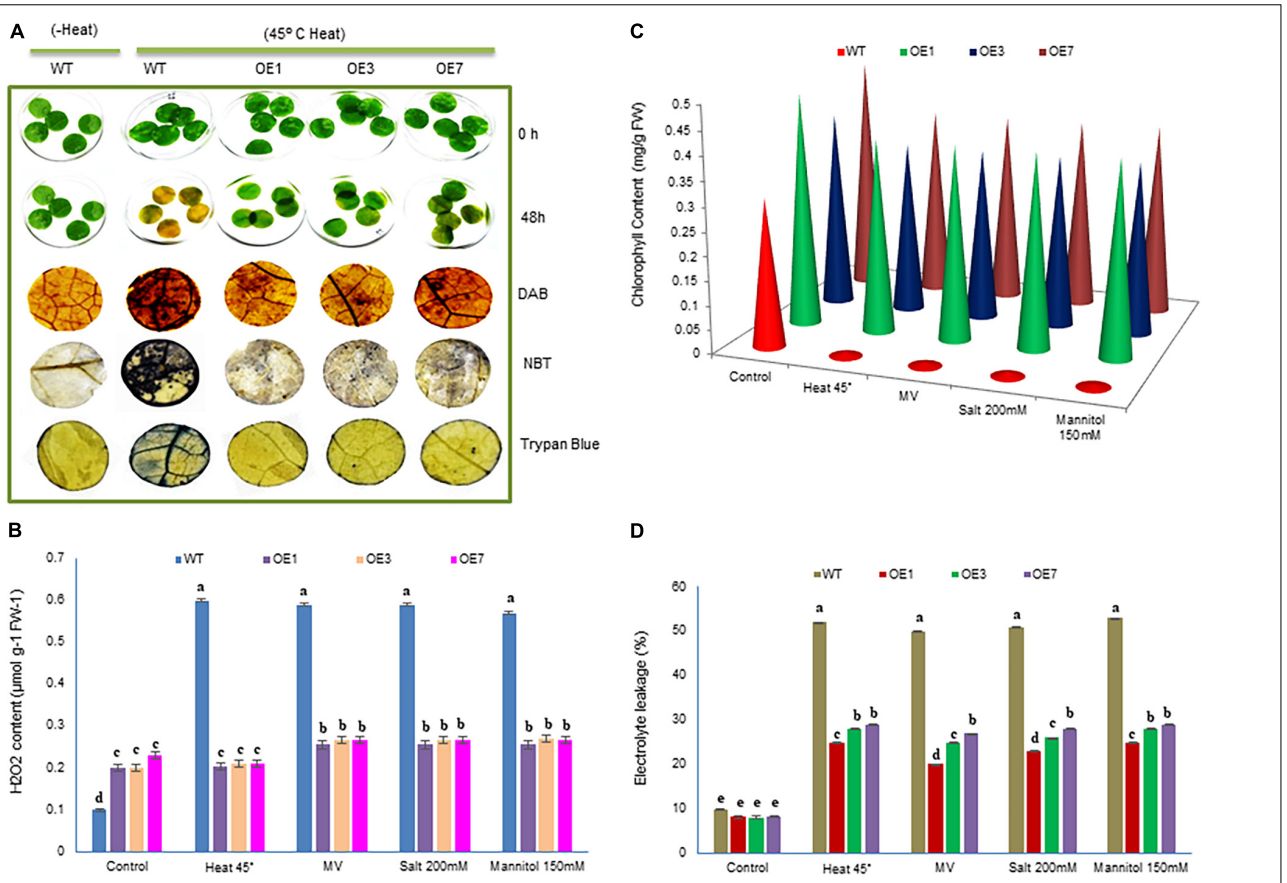

FIGURE 5 | Analysis of cellular damage in response to heat, salinity, and drought stresses. **(A)** Leaf strips of WT and three transgenic eggplants lines (OE) under 45°C heat stress for 48 h. Untreated (without heat) WT leaves were used as a control. Relative H_2O_2 and O^{2-} accumulation and dead cells in the leaf strips of transgenic and WT lines were detected by histochemical staining. **(B–D)** H_2O_2, chlorophyll content, and electrolytic leakage were measured in response to heat, salinity, MV (methyl viologen), and osmotic stresses. Data are $M \pm SD$ ($n = 3$). The letters a, b, c, and others indicate significant differences between different lines ($P < 0.05$).

RNA-seq values of differentially expressed transcripts of *SOD1*, *SOD2*, *CAT*, and *APX* in OE lines, further confirmed by qPCR (**Figure 6C**).

SmsHSP24.1 Overexpression Leads to Global Transcriptomic Reprogramming

Transcriptome profiling of OE lines compared with WT plants further provide evidence of a significant impact on global transcriptome due to the overexpression of *SmsHSP24.1*. During this experiment, an average of 56.1 and 83.5 million raw reads (2 × 100 bp) were generated from transgenic (OERT) and WT (WTRT) lines, respectively, under controlled environmental conditions. Similarly, an average of 73.5 and 82.5 million raw reads (2 × 100 bp) were generated under the heat-stressed conditions at 45°C for 2 h from transgenic (OE2hH) and WT (WT2hH) line respectively.

After adaptor trimming and base quality check, it was found that more than 95% of total reads were of high quality (HQ) except for the WT2hH plant, in which after the removal of rRNA, the value was 87%. Mapping of cleaned reads with tomato reference genome and gene model downloaded from Sol Genomics Network (Fernandez-pozo et al., 2015) found 65.6% of OERT, 52.8% of WTRT, 56.88% of OE2hH, and 57.76% of WT2hH reads to be mapped (**Supplementary Table 3**). Differential gene expression analysis revealed significant transcription alteration in some of the physiologically important pathways (**Figures 6A,B** and **Table 1**). Heat map illustration with cluster dendrogram based on FPKM plot and GO classification of DEGs in WT and OE also revealed the up- and down-expression of important genes (**Figures 7**, **8** and **Supplementary Figures 9–11**). Among the several DEGs, *Photosystem II reaction center protein M* (*Solyc09g064580*), *Chloroplastic matK* (*Solyc09g061390*), genes involved in electron transport chain (*Solyc09g074540*, *Solyc11g044636*) were significantly upregulated in OE_vs_WT samples at RT (**Figure 7** and **Table 1**), while *SOD* (*Solyc01g067740*), ABC transporter that regulates lipid transporter (*Solyc03g113070*), *GLB1*(nitrogen regulatory P-II-like protein) regulated fatty acid biosynthesis (*Solyc07g008240*) (Duan et al., 2017), in OE_vs._WT samples after heat treatment, respectively (**Supplementary Figure 9**). Similarly, significant down-regulation was also observed in transmembrane transporters like *F-box domain-containing protein* (*Solyc07g044920*), *iron-nicotianamine*

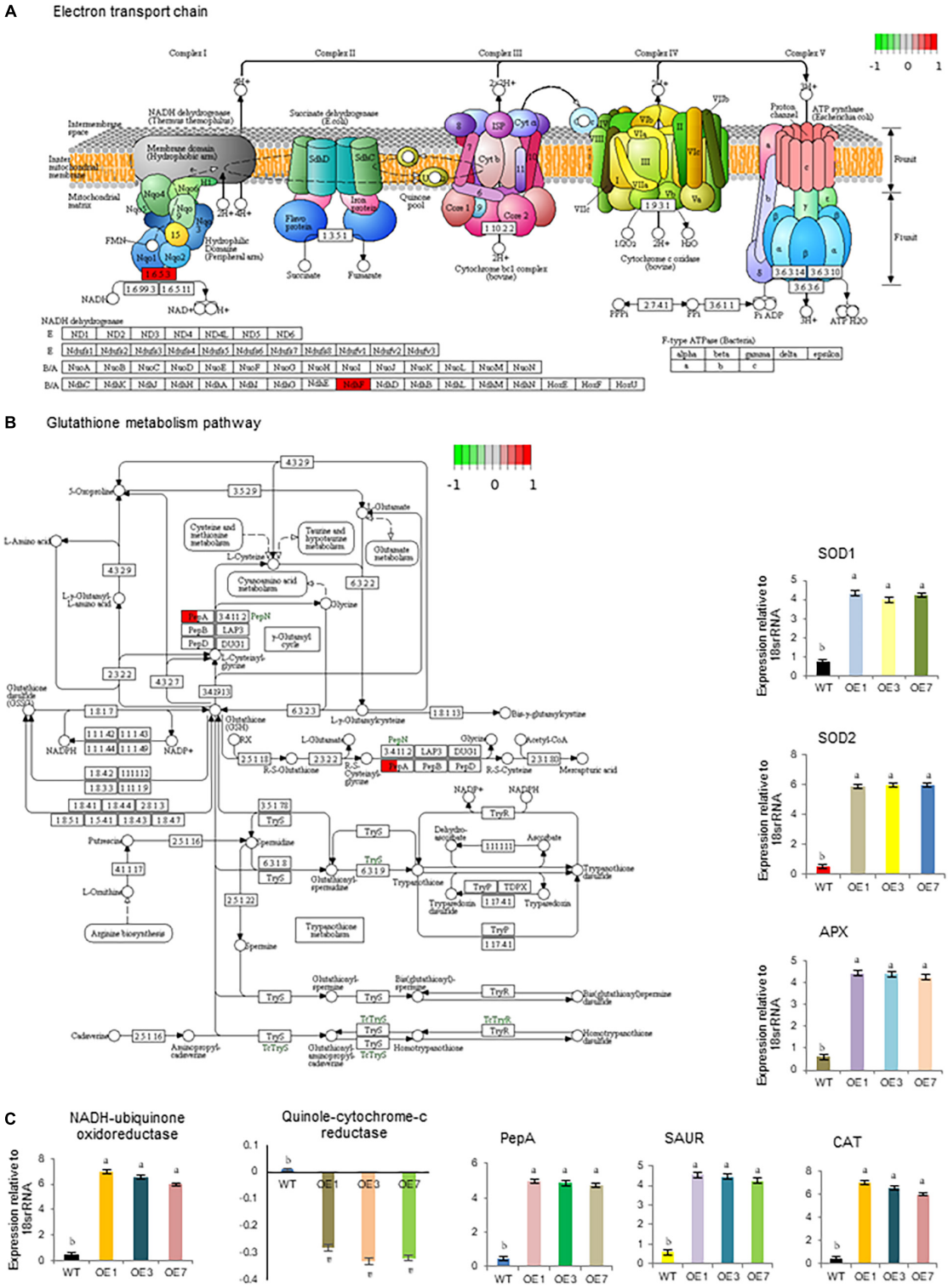

FIGURE 6 | Important biological pathways significantly affected SmHSP24.1 overexpressed transgenic (OE) plants as compared with wild-type (WT). **(A)** First protein complex NADH-ubiquinone oxidoreductase significantly affected the electron transport chain (ETC). **(B)** PepA (aminopeptidase) positively affected the Glutathione metabolism pathway. **(C)** Time-dependent relative quantitative RT-PCR validation of some selected genes (*NADH-ubiquinone oxidoreductase*, *Quinol-cytochrome c-oxidoreductase*, *PepA*, *SOD1*, *SOD2*, *CAT*, *APX,* and *SAUR*) highly affected in these pathways. mRNA level was normalized with respect to housekeeping gene *18srRNA* and fold change in expression was calculated as compared with WT sample. RT-qPCR data are $M \pm SD$ ($n = 3$). The letters a, b, c, and others indicate significant differences between different lines ($P < 0.05$).

TABLE 1 | Upregulated DEGs in overexpressed OE lines compared with the wild-type (WT) eggplant line.

Gene symbol	log2(fold change)	Protein name
DGEs related to auxin-response and transport		
Solyc01g110900.1	2.22	auxin-induced protein 15A-like
Solyc01g110940.3	2.07	Auxin-induced protein 15A; auxin-induced protein 15A-like
Solyc03g033590.1	2.59	Auxin-induced SAUR-like protein; auxin-induced protein 15A
Solyc03g082510.1	2.21	Auxin-responsive protein SAUR32
Solyc03g123410.1	4.40	auxin-binding protein ABP19a-like
Solyc05g008060.3	2.26	Auxin efflux carrier component
Solyc06g053840.3	4.58	Auxin-responsive protein
Solyc07g041720.1	3.24	Auxin-binding protein ABP19a; auxin-binding protein ABP19a-like
Solyc09g007810.3	3.36	Auxin response factor
Solyc09g083280.3	2.02	Auxin-responsive protein
Solyc10g080880.2	2.39	Auxin efflux carrier component
Solyc11g069190.2	2.31	Auxin response factor
Solyc11g011660.2	2.73	Uncharacterized protein
Solyc06g053260.1	3.18	Small auxin-up protein 58
DGEs related to cell wall reorganization		
Solyc02g087060.3	2.28	WAT1-related protein
Solyc02g091920.3	4.04	Xyloglucan endotransglucosylase/hydrolase
Solyc02g092840.1	3.44	xyloglucan galactosyltransferase XLT2-like
Solyc03g071570.3	2.73	Pectate lyase
Solyc05g014000.3	3.49	Pectate lyase
Solyc05g055840.3	2.64	Putative UDP-glycosyltransferase 86A1-like
Solyc05g055845.1	2.47	Putative UDP-glycosyltransferase 86A1-like
Solyc06g009190.3	2.59	Pectinesterase
Solyc06g062580.3	2.07	Beta-galactosidase
Solyc06g083580.3	2.49	Pectate lyase
Solyc07g017600.3	3.14	Pectinesterase
Solyc07g049610.1	3.23	xyloglucan galactosyltransferase XLT2-like
Solyc07g052690.3	2.38	Beta-amylase
Solyc07g052695.1	2.46	Beta-amylase
Solyc07g052980.3	2.64	Xyloglucan endotransglucosylase/hydrolase
Solyc08g075020.3	3.49	Pectin acetylesterase
Solyc08g079040.1	2.14	Putative xyloglucan galactosyltransferase gt19
Solyc09g008320.3	2.08	Xyloglucan endotransglucosylase/hydrolase
Solyc09g091430.3	4.28	Pectate lyase
Solyc11g005770.2	3.38	Pectinesterase
Solyc02g063140.3	3.92	3-ketoacyl-CoA synthase
DGEs related to lipid biosynthesis		
Solyc02g069490.3	2.52	Sterol side chain reductase
Solyc02g085870.3	3.24	3-ketoacyl-CoA synthase
Solyc03g025320.3	4.43	Alcohol acyl transferase
Solyc04g009380.2	2.41	S-acyltransferase; Palmitoyltransferase
Solyc05g009270.3	3.88	3-ketoacyl-CoA synthase
Solyc05g012790.3	2.68	S-acyltransferase; Palmitoyltransferase
Solyc05g013207.1	3.79	3-ketoacyl-CoA synthase
Solyc06g074390.3	2.88	Fatty acyl-CoA reductase
Solyc08g067260.3	2.26	3-ketoacyl-CoA synthase
Solyc09g083050.3	3.07	3-ketoacyl-CoA synthase
Solyc10g011820.3	2.55	Putative delta (8)-fatty-acid desaturase-like
Solyc11g072990.2	2.50	3-ketoacyl-CoA synthase
Solyc12g006820.2	2.23	3-ketoacyl-CoA synthase
DGEs related to cellular redox homeostatsis		
Solyc01g006290.3	3.63	Peroxidase
Solyc01g006300.3	3.19	Peroxidase
Solyc02g087190.1	2.12	Peroxidase
Solyc03g093180.1	3.24	Peroxisomal membrane protein 11-4
Solyc02g087850.1	2.30	Putative ovule protein
Solyc05g053300.3	2.37	Dihydrolipoyl dehydrogenase
Solyc08g062970.1	2.48	Putative glutaredoxin-C6-like
Solyc08g082590.3	2.01	Uncharacterized protein
Solyc10g006970.3	2.20	Uncharacterized protein
Solyc10g007110.3	2.26	Tyrosine aminotransferase 1
Solyc11g066390.2	3.93	Superoxide dismutase [Cu-Zn]
DGEs related to histone modification		
Solyc01g110150.2	2.04	Putative histone-lysine N-methyltransferase SETD1B-like
Solyc02g077480.1	2.63	Histone H3.2; Histone H3.1
Solyc06g005420.1	2.08	Histone H4; CaH4
Solyc06g084020.3	3.64	Histone H1
Solyc10g008910.1	2.80	Histone H3.2; Histone H3.1
Solyc11g073250.2	2.55	Histone H2A
DGEs related to chlorophyll biosynthetic process		
Solyc01g105030.3	2.19	Chlorophyll a-b binding protein CP24 10A, chloroplastic; CAB-10A; LHCP
Solyc01g105050.3	2.51	Chlorophyll a-b binding protein CP24 10B, chloroplastic; CAB-10B; LHCP
Solyc06g063360.3	2.10	Chlorophyll a-b binding protein, chloroplastic
Solyc10g006230.3	2.06	Chlorophyll a-b binding protein 7, chloroplastic; LHCI type II CAB-7
Solyc10g018580.1	2.27	Protein TIC 214; Translocon at the inner envelope membrane of chloroplasts 214
Solyc11g021290.2	2.03	Protein TIC 214; Translocon at the inner envelope membrane of chloroplasts 214
Solyc11g021300.1	2.41	Protein TIC 214; Translocon at the inner envelope membrane of chloroplasts 214; AtTIC214
Solyc12g035550.1	2.95	Protein TIC 214; Translocon at the inner envelope membrane of chloroplasts 214; AtTIC214
Solyc10g018300.2	2.22	Transketolase, chloroplastic; TK
Solyc01g094750.3	4.14	cytochrome P450 86A8-like
Solyc01g107730.3	3.30	CycD3
Solyc02g089160.3	2.47	Cytochrome P450 85A1; C6-oxidase; Dwarf protein 1.10.2.2
Solyc03g111950.3	3.27	cytochrome P450 71A3-like
Solyc04g054260.3	2.32	cytochrome P450 CYP736A12-like
Solyc05g055400.3	5.046	CYP77A19
Solyc08g081220.1	2.68	cytochrome P450 86A22
Solyc11g007540.2	2.67	CYP77A20
Solyc09g064500.3	2.26	Photosystem II reaction center Psb28 protein
Solyc06g060340.3	2.66	Photosystem II 22 kDa protein, chloroplastic; CP22
Solyc01g109260.3	2.44	Putative plastid division protein PDV2-like
DGEs related to transcription factors		
Solyc04g054910.3	3.07	ethylene-responsive transcription factor RAP2-13-like
Solyc05g052030.1	2.73	Ethylene response factor 4
Solyc08g008305.1	2.33	ethylene-responsive transcription factor ERF061
Solyc01g110310.3	3.56	GATA transcription factor
Solyc02g067340.3	2.15	Transcription factor
Solyc02g077710.1	3.87	GATA zinc finger domain-containing protein 14-like
Solyc03g121240.1	2.50	transcription factor bHLH87 isoform X2
Solyc06g070900.3	3.21	TCP transcription factor 17
Solyc10g055410.2	3.29	Transcription factor
DGEs related to ATP synthesis coupled electron transport		
Solyc01g017110.1	3.73	NAD(P)H-quinone oxidoreductase subunit 5, chloroplastic; NADH-plastoquinone oxidoreductase subunit 5
Solyc01g017333.1	4.48	Uncharacterized protein
Solyc01g065620.1	2.10	NAD(P)H-quinone oxidoreductase chain 4, chloroplastic; NAD(P)H dehydrogenase, chain 4; NADH-plastoquinone oxidoreductase chain 4
Solyc03g043610.2	3.17	ATP synthase subunit a; F-ATPase protein 6
Solyc11g044636.1	2.12	NAD(P)H-quinone oxidoreductase subunit 5, chloroplastic; NAD(P)H dehydrogenase subunit 5; NADH-plastoquinone oxidoreductase subunit 5.

transporter (*Solyc03g082620*), *calcium-binding protein CML44* (*Solyc04g058170*), and *LOB1* (*Solyc06g064540*) under normal conditions, while *ADF-H actin-binding protein* (*Solyc02g063450*), cell division cycle protein- *CDC27B* (*Solyc03g061590*), cell number control protein- *PLAC* (*Solyc02g079390*), and FA_desaturase domain-containing protein (*Solyc10g011810*) in WT eggplants compared with OE lines upon heat treatment (**Supplementary Figure 10** and **Supplementary Table 1**). GO term-based gene classification for DEGs in WT, and OE samples also revealed a large number of genes affected significantly. Twelve major pathway-related genes were found to have distinctive differential expression in OE treated lines compared with WT (**Figure 8** and **Table 1**). Similarly, a total of 185 genes are down-regulated in the same event (**Figure 8** and **Supplementary Table 1**). Among the genes for transcription factors, BZIP transcription factor (*Solyc01g109880.3*), *ethylene-responsive factor 2*; ethylene-binding protein (*Solyc09g075420.3*), and NAC4 domain protein (*Solyc11g017470.2*) were found to be down-regulated whereas a total of 18 different transcription factors (Tfs), most of which were auxin-responsive/ethylene-responsive, DREB, GATA, and TCP family transcripts were found to be upregulated in OE lines.

Well-known ROS-scavenging genes such as *SOD*, *catalase*, *ascorbate-peroxidase* were significantly upregulated in OE lines under both normal and treated conditions. An extensive set of genes involved in fatty acid biosynthesis (28 in number, **Table 1**), chlorophyll biosynthesis, genes involved in the developmental pathway (32 in number), and mitochondrial integral membrane protein have identified upregulated. Among phytohormones, auxin biosynthesis/carrier-related transcripts upregulated in OE lines (**Table 1**). A total of 14 auxin pathway genes were significantly upregulated in OE lines compared with WT lines. Transcript for auxin-induced *SAUR-like* protein showed 4.3-fold upregulation. Significant upregulation was also observed for cytokinin oxidoreductase and cytokine-responsive transcripts in OE lines. This considerable alteration in transcription might have significant effects on OE lines, and our RNA-Seq data reveal substantial transcriptional reprogramming in several physiologically essential pathways of the OE lines (**Table 1**). DEGs involved in other essential pathways were also found to be down-regulated (**Supplementary Table 1**), such as 8 DEGs involved in hydrolase activity [GO:0016787], 6 DEGs with ubiquitin-protein ligase activity [GO:0061630], 5 DEGs of cell wall organization or biogenesis pathway [GO:0071554] and 2 each of auxin catabolic process [GO:0009852] and chlorophyll catabolic process [GO:0015996].

DISCUSSION

Unlike other organisms, plants have diverse and abundant low-molecular-weight (12-42 kD) sHSP (Sun et al., 2016; Waters and Vierling, 2020). However, until its chaperone activity, the other roles of organelle targeted sHSPs have not been well studied. It is worth mentioning that Mito- and ER-localized sHSPs have evolved explicitly in plant lineage and are not found in other eukaryotes (Waters and Vierling, 2020) except for the presence of HSP22 in *Drosophila*. Interestingly, it is now well documented that apart from the chaperone activity, HSP22 has been essential for the longevity of *Drosophila* (Morrow and Tanguay, 2015). Therefore, it was crucial to understanding the evolutionary advantage that a plant acquires from the Mitochondria-targeted sHSPs.

We had identified and characterized a novel sHSP "SmsHSP24.1" localized in the mitochondria of eggplants. *In silico* analysis revealed the presence of a conserved mitochondrial signal aligned with the previously reported Mito-sHSP from different plants. Agroinfiltration on tobacco-leaf and eggplant cell suspension culture using MitoTracker^{TM} FM conclusively proved that this novel SmsHSP24.1 is mitochondria localized protein. We used eggplant cell suspension to determine host protein localization. According to an earlier report, phylogenetic distances are expected to have an impact on protein trafficking and localization in different host systems (Kirienko et al., 2012). Also, *in silico* prediction of only one copy of *Mito-sHSP* (*SmsHSP24.1*) in the eggplant genome was confirmed by Southern blot analysis (**Supplementary Figure 4F**). Therefore, we further studied to understand the role of SmsHSP24.1 under both normal and stressed conditions.

Expression analysis in WT plants clearly showed that *SmsHSP24.1* is sensitive to different environmental stresses, especially to heat, salt, and mannitol stresses. A rapid increase of its transcript level indicates an early response of *SmsHSP24.1* against stress conditions. This is important in the context of its localization. The mitochondrion is the powerhouse of the cell and cell metabolism (Morrow and Tanguay, 2015) and also a source of cellular ROS production during stress (Huang et al., 2016). Thus, maintenance of mitochondrial protein homeostasis was crucial for survival. We had discussed the possible role of SmsHSP24.1 in the later part of the discussion. To further understand its physiological role, we developed overexpressing eggplant lines and have studied the molecular activity of SmsHSP24.1 both *in vitro* and *in vivo*.

Unlike other chaperons, the affinity of sHSPs to selectively bind with aggregation-prone proteins in an ATP-independent way is high (Kuang et al., 2017). A thermal survivability test revealed the ability of SmHSP24.1 protein protection against aggregation of *E. coli* cells up to 55°C for 1 h. Our results complement similar findings from previously reported thermotolerance of RcHSP17.8 in *E. coli* (Jiang et al., 2009); CsHSP17.2 in *E. coli* and *Pichia pastoris* cells (Zhang et al., 2015; Wang et al., 2017). It was conclusively understood from our study that *E. coli* cells were able to survive under heat stress due to the heterologous overexpression of SmsHSP24.1. The mechanisms by which SmsHSP24.1 performs the chaperoning activity are difficult to define due to the limited structural information (Rutsdottir et al., 2017). Our Electron microscopy study suggested the formation of the polyhedral structure by SmsHSP24.1 which could bind with its client protein even at a higher temperature of 50°C. Our results which were in agreement with previous findings (Zhang et al., 2015) suggested a possible mechanism of ATP- independent chaperon activity of sHSPs induced by the formation of multimeric topological structures.

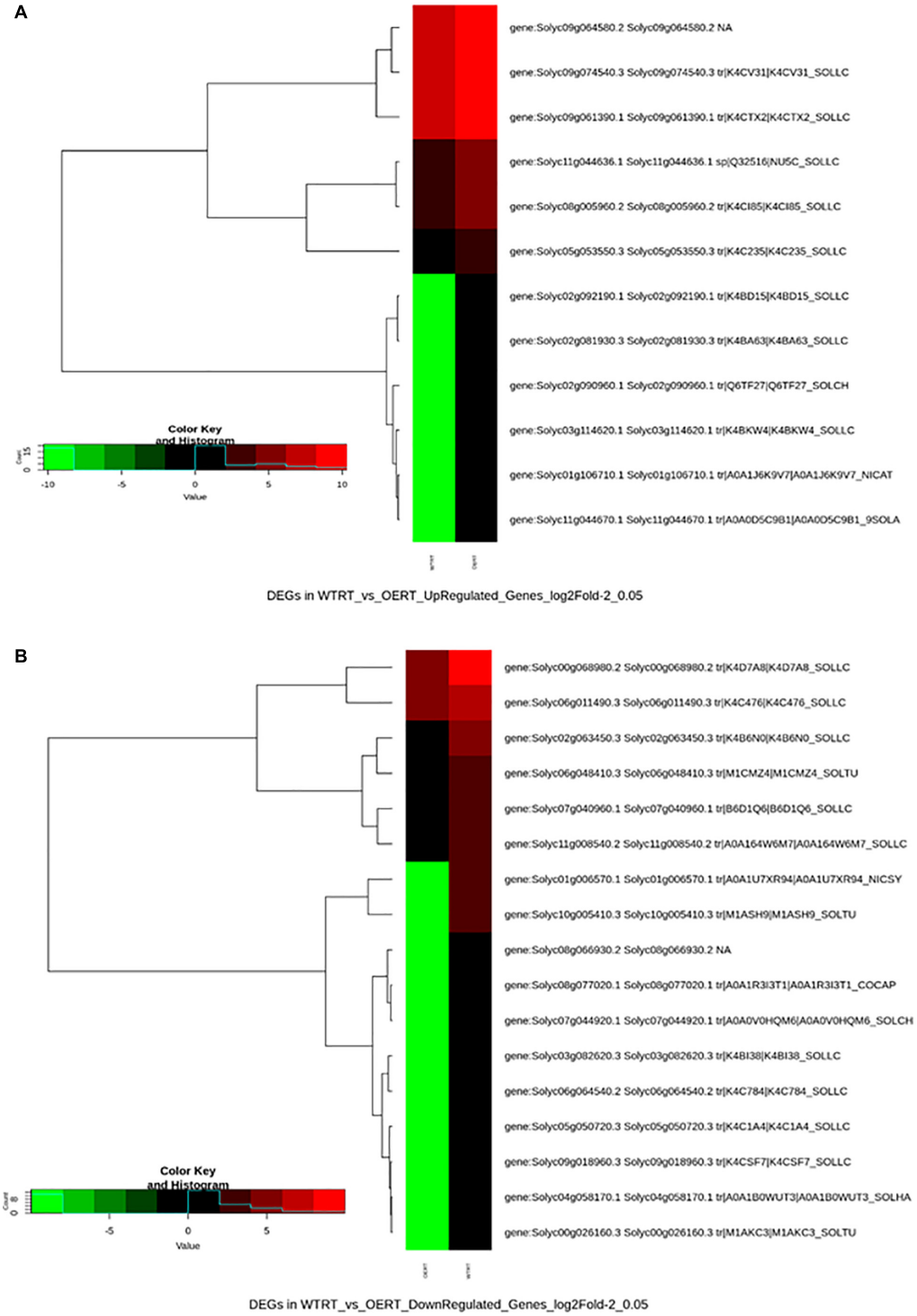

FIGURE 7 | Heat map of differentially expressed genes (DEGs) in SmHSP24.1 overexpressed transgenic (OE) and wild type (WT) lines under control conditions**. (A)** Heatmap generated from differentially expressed upregulated genes in OERT vs. WTRT lines. **(B)** Heatmap generated from differentially expressed down-regulated genes in OERT vs. WTRT lines.

Apart from chaperon activity, seeds from OE lines showed a quicker germination rate compared with the WT plants, along with rapid seedling growth and vigor irrespective of the integration site. Altered phenotypes such as pollen growth and development, seed and root development, response to pathogen and UV damage, *etc.* have also been reported in previous

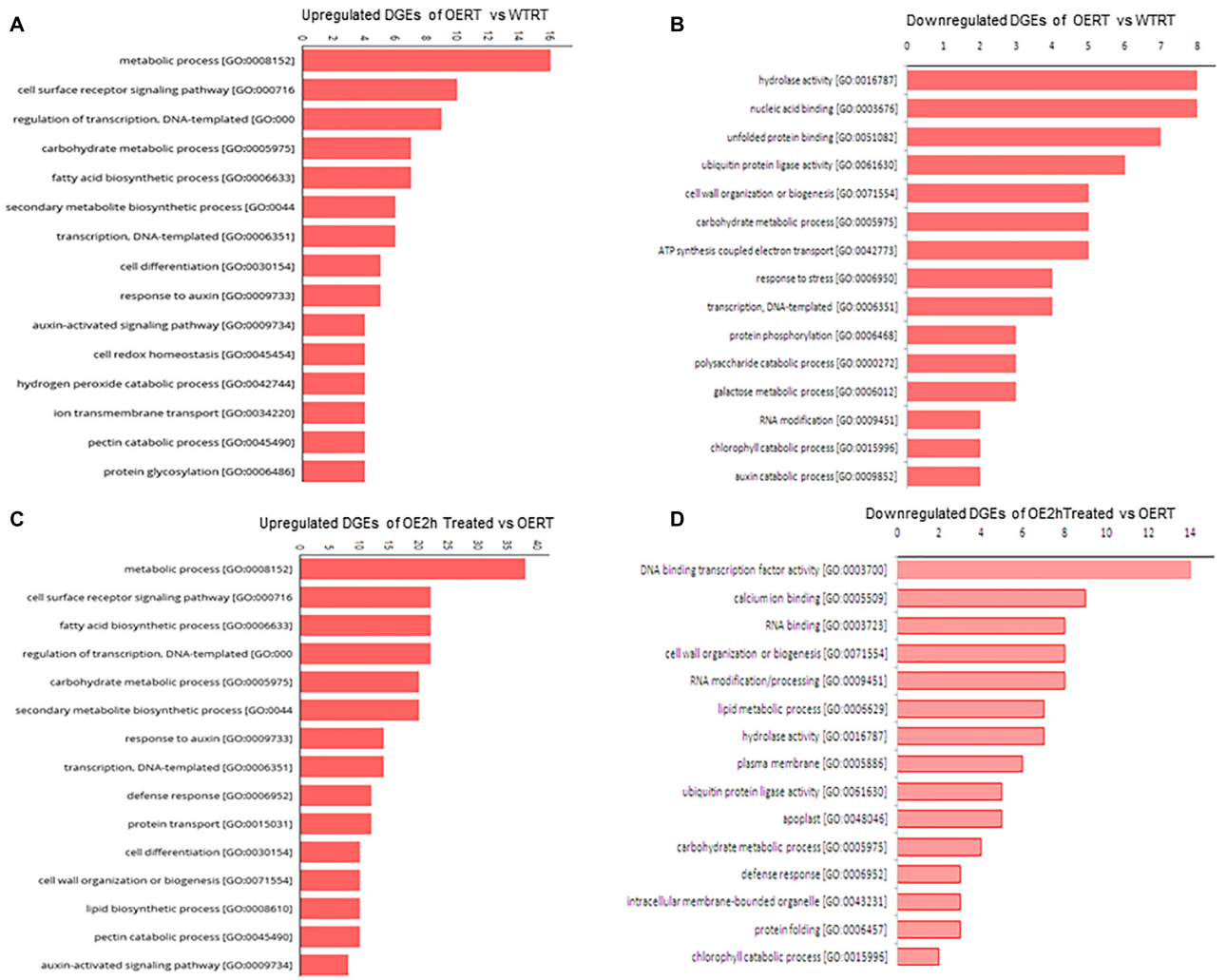

FIGURE 8 | Gene ontology (GO) classification of DEGs in SmHSP24.1 overexpressed OE and WT lines under control and stress conditions. **(A,B)** GO for both up- and down-regulated differentially expressed genes in SmHSP24.1 overexpressed OERT vs. WTRT lines under control environmental growth condition. **(C,D)** GO for both up- and down-regulated differentially expressed genes in SmHSP24.1overexpressed transgenic line under heat stress conditions. The y-axis and x-axis indicate the names of clusters and the gene number of each cluster respectively. Only the biological processes were used for GO term analysis. OERT: SmHSP24.1overexpressed line in room temperature, WTRT: WT line in room temperature, OE2h: SmHSP24.1overexpressed line under 2 h heat (45°C) stress condition.

studies of sHSPs (Sun et al., 2002; Murakami et al., 2004; Zou et al., 2009). Agrobacterium-mediated transformation can cause insertional inactivation of functional genes which can alter phenotype, but all our lines with different copy numbers show an even rate of germination. This indicates to be an effect of SmsHSP24.1 overexpression, opening up opportunities for exploiting this feature for the development of rapid germination, quick seedling growth, and vigor. Such traits are essential in commercial cultivation for high yield and efficient resource utilization (Finch-Savage and Bassel, 2016).

It has progressively emerged that cellular ROS homeostasis is crucial for seed germination and has evolved as an "oxidative window of seed germination" regulation (Bailly et al., 2008; El-Maarouf-Bouteau and Bailly, 2008). Mitochondria are the central regulators of ROS production (Noctor et al., 2007). It has been well accepted that NADH:ubiquinone oxidoreductase (complexes I) and ubiquinol:cytochrome c oxidoreductase (complexes III) are the main producers of superoxide production in higher eukaryotes (Dröse and Brandt, 2008; Larosa and Remacle, 2018). Our transcriptome data revealed interesting results that hypothesize the possible role of SmsHSP24.1 in rapid seed germination. The transcript level was lower for quinol-cytochrome c- oxidoreductase (EC 1.10.2.2), whereas it was higher for NADH-ubiquinone oxidoreductase (EC 1.6.5.3) in OE lines under both stressed and normal conditions compared with WT plants. This differential expression of electron acceptor and donor, especially for NADH-ubiquinone oxidoreductase, the first electron receptor, and quinol-cytochrome c- oxidoreductase, the third complex in the electron transport chain might have creates an imbalance in the electron gradient which during

normal growth conditions leads to an elevated level of cellular ROS. Besides, biochemical analysis of ROS production also suggests a slightly higher ROS level in OE lines than WT plants under normal conditions. This supports our hypothesis that overexpression of SmsHSP24.1 increases germination by altering the cellular ROS gradient. In contrast to the elevated cellular ROS, no negative growth effect was observed in SmsHSP24.1 OE lines. The probable reason is the simultaneous elevation of several necessary ROS-scavenging transcripts as observed in RNA-Seq analysis, such as Mito-*superoxide dismutase* (*Solyc06g048410.3, Solyc06g048420.2*), *catalase* (*CAT*) (*Solyc04g082460.3*), *APX* (*Solyc09g007270.3*) in OE lines compared with WT. Some key cell redox homeostasis transcripts, e.g., *PepA glutaredoxin*, *proline dehydrogenase*, *glutamate synthase* genes have also been found to be upregulated in OE lines. qPCR expression crosscheck along with enzymatic analysis supports these findings. These indicate *SmsHSP24.1* overexpression leads to an elevation of cellular ROS at normal growth conditions without being lethal to the host cell; however, this caused a reprogramming of global transcripts in OE lines. Rapid seedling growth is determined by hypocotyl elongation occurring through cell expansion which is centrally controlled by the auxin hormone. Previous studies have demonstrated that overexpression of *AtSAUR36* (Stamm and Kumar, 2013), *AtSAUR41* (Kong et al., 2013), *AtSAUR19* (Spartz et al., 2012), and *AtSAUR63* (Chae et al., 2012) promote hypocotyl elongation as a result of increased cell expansion. Similar to these findings, our RNA-seq data suggest overexpression of a set of genes related to auxin-biosynthesis and carrier pathway. Overexpression of selected eggplant-specific auxin-responsive protein *SAUR32* gene was validated by their expression analyses in the OE lines. These findings suggested that OE lines maintain a significantly higher *SAUR32* transcript level and other auxin biosynthesis and carrier transcripts that positively regulate hypocotyl growth, hinting at a complex gene regulatory network of SmsHSP24.1 protein on plant growth and development.

Ribonucleic acid-seq data also provide insight into the molecular mechanism of increased heat tolerance of OE plants. Results suggest that cellular transcript reprogramming not only maintains redox homeostasis but also alters several key physiological pathways. Field analysis of OE lines under combined severe heat and drought conditions demonstrate better crop performance over WT eggplants. RNA-seq analyses also

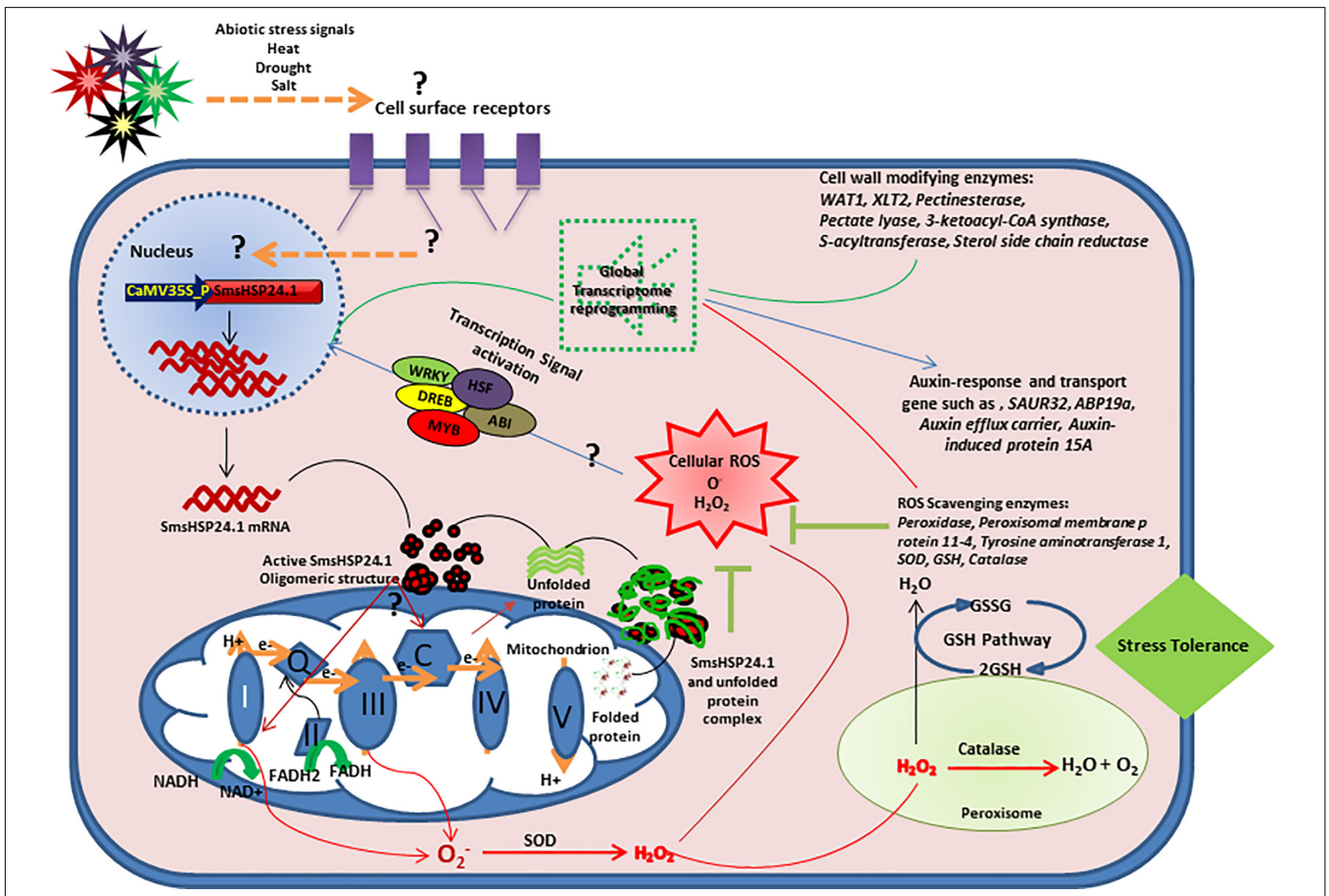

FIGURE 9 | Hypothetical model for understanding the molecular mechanism of SmsHSP24.1-mediated plant abiotic stress response. Overexpression of SmsHSP24.1 might interact with mitochondrial electron transport chain (ETC) which leads to increased ROS. A set of reactive oxygen species (ROS) scavenging enzymes of the glutathione (GHS) pathway also upregulated and maintains ROS below lethal dose which might act as a transcriptional activation signal of several stress-induced transcription factors and ultimately lead to change in global transcription.

suggest altered cell wall biogenesis in OE lines (**Table 1**). It has been well documented through previous studies that the modification of the biophysical properties of a cell wall was a crucial response toward environmental stimuli such as heat, necessary to maintain the overall function and growth (Lima et al., 2013; Wu et al., 2018).

Other significant altered transcripts in our study were found to be related to lipid metabolism, chloroplast (including photosystem II receptor protein) and mitochondrial membrane proteins, histone and histone modification, and transcription factor genes. Profound transcriptional reprogramming during heat stress has been reported in several plants affecting several traits (Zou et al., 2011; Barah et al., 2013). Our results also showed a similar effect on the global transcriptome in OE lines. A dynamic change in lipid composition in the cell membrane is crucial during stress signaling and response (Hou et al., 2016). Among 13 DEGs, fatty acyl Co-A reductase (FAR), a key enzyme in the biosynthesis of long-chain fatty alcohols such as cuticular wax (Rowland et al., 2006), works as an interface between plants and their biotic and abiotic environments, significantly restricting non-stomatal water loss and serves as the first line of defense (Chai et al., 2018).

Similarly, a total of 20 chloroplast-related DEGs have been found in OE lines. Chlorophyll is one of the major targets of heat stress damage, crucial for plant growth and development. Significantly higher upregulation of these genes is vital for chlorophyll development and photosynthesis during normal and stress conditions. Chromatin re-modeling is another way of regulating expression. Histone modification and alteration of DNA methylation patterns are crucial ways to counter environmental changes. This is often coordinated with dynamic changes in stress-responsive genes (Kim et al., 2015). Similarly, Tfs are also involved in normal growth and development and abiotic stress responses. Tfs, such as ethylene-responsive elements, have also been observed. Multiple transcriptions reprogramming due to an overexpression of SmsHSP24.1 confers abiotic stress tolerance and improved growth and development under normal environmental conditions.

Finally, we had identified a novel mitochondrion localized SmsHSP24.1 protein in eggplant and characterized the same under both normal and stressed conditions. While overexpression of *SmsHSP24.1* enhanced abiotic stress tolerance, especially in temperatures up to 45°C in the field condition, rapid germination and seedling vigor were also observed. Thus, based on our findings, we had proposed a hypothesis for the possible mechanism of overexpression of SmsHSP24.1 and its impacts on ROS regulation and reprogramming of the global transcriptome in transgenic eggplant lines compared with WT (**Figure 9**). Further, it is interesting to study the targeted cytosolic or mitochondrial protein profiling in both normal and stress conditions. A recent study by Escobar et al. (2021), has shown that Mito-sHSP regulates cellular homeostasis by coordinating between different subcellular compartments especially between plastids, cytosol, and mitochondrion in *Arabidopsis*. Thus, in the future lines of work, comparative studies of protein profiling in OE lines against WT plants corresponding to the finding of our transcriptome analysis are useful to understand the regulatory mechanism of Mitochondria localized small HSPs in plant growth and development. Findings from the present study also suggested that mitochondria-targeted sHSPs provided an evolutionary advantage to plants that could be harnessed for the development of stress-resilient crop plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA750594.

AUTHOR CONTRIBUTIONS

MR, MS, IA, BB, and MK designed the experiments. MK, BB, and VP performed experiments and data analysis. MR and MS wrote the manuscript. MR, MS, IA, HK, SH, and CK supervised the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.741898/full#supplementary-material

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REVIEW
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Adaptation Strategies to Improve the Resistance of Oilseed Crops to Heat Stress Under a Changing Climate: An Overview

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Temperature is one of the decisive environmental factors that is projected to increase by 1. 5°C over the next two decades due to climate change that may affect various agronomic characteristics, such as biomass production, phenology and physiology, and yield-contributing traits in oilseed crops. Oilseed crops such as soybean, sunflower, canola, peanut, cottonseed, coconut, palm oil, sesame, safflower, olive etc., are widely grown. Specific importance is the vulnerability of oil synthesis in these crops against the rise in climatic temperature, threatening the stability of yield and quality. The natural defense system in these crops cannot withstand the harmful impacts of heat stress, thus causing a considerable loss in seed and oil yield. Therefore, a proper understanding of underlying mechanisms of genotype-environment interactions that could affect oil synthesis pathways is a prime requirement in developing stable cultivars. Heat stress tolerance is a complex quantitative trait controlled by many genes and is challenging to study and characterize. However, heat tolerance studies to date have pointed to several sophisticated mechanisms to deal with the stress of high temperatures, including hormonal signaling pathways for sensing heat stimuli and acquiring tolerance to heat stress, maintaining membrane integrity, production of heat shock proteins (HSPs), removal of reactive oxygen species (ROS), assembly of antioxidants, accumulation of compatible solutes, modified gene expression to enable changes, intelligent agricultural technologies, and several other agronomic techniques for thriving and surviving. Manipulation of multiple genes responsible for thermo-tolerance and exploring their high expressions greatly impacts their potential application using

CRISPR/Cas genome editing and OMICS technology. This review highlights the latest outcomes on the response and tolerance to heat stress at the cellular, organelle, and whole plant levels describing numerous approaches applied to enhance thermos-tolerance in oilseed crops. We are attempting to critically analyze the scattered existing approaches to temperature tolerance used in oilseeds as a whole, work toward extending studies into the field, and provide researchers and related parties with useful information to streamline their breeding programs so that they can seek new avenues and develop guidelines that will greatly enhance ongoing efforts to establish heat stress tolerance in oilseeds.

Keywords: antioxidants, CRISPR/Cas9 technology, heat stress, oilseeds, omics technology, signaling, smart technologies, tolerance

INTRODUCTION

Oilseeds are ranked fourth in important food commodities after cereals, vegetables and melons, and fruits and nuts, and they occupy about 213 Mha of the world's arable land (OECD-FAO, 2020). However, the utilization and demand of oil crops continuously increases due to high population pressure, vagaries in dietary choices, cumulative global affluence, and the need for more renewable bio-products (Villanueva-Mejia and Alvarez, 2017). Vegetable oil is used as a biofuel, so it has a great future as an essential energy source (Lu et al., 2011). Factually, the primary sources of vegetable oils are oilseed crops, including rapeseed, soybean, cotton, peanut, palm oil, and sunflower (Abiodun, 2017), which are used in human diets as salad dressings, oil, margarine, frying oil, and numerous other products. Due to their specific chemical and physical properties, vegetable oil is an important feedstock used to produce multiple industrial materials, including promising applications such as biofuel and constituting an alternative to petroleum derivatives (Lu et al., 2011). Oilseed crops are a significant source of animal (Ponnampalam et al., 2019) and human nutrition (Rahman et al., 2018a) and industrial products (Liu et al., 2018a), and biodiesel production (Mohammad et al., 2018) has been increasing day by day. The quality and consumption of oilseed crops have been improved through different genetic engineering techniques (Tan et al., 2011).

Numerous environmental stresses affecting plant growth and development have induced grave anxiety in the context of potential climate change. Across the globe, contemporary agriculture is facing unprecedented environmental pressure and stress due to climatic variability (Argosubekti, 2020). Plants' growth in open environments faces several challenges, including heat, drought, cold, waterlogging, and salinity (Ashraf et al., 2018). Elevated temperature is one of the major concerns for the world as different models have predicted the rise of carbon dioxide (CO_2), causing an increase in the ambient temperature leading to global warming (NOAA, 2017), which would have severe consequences on agriculture production systems across the globe. The Intergovernmental Panel on Climate Change (IPCC) estimates that the global ambient temperature will increase by 1.5°C from 2030 to 2052 (IPCC, 2018). Temperature-induced heat stress is articulated as the shift in air temperature exceeding the threshold level for an extended period that could cause injuries or irreversible damage to crop plants in general (Teixeira et al., 2013). Therefore, heat stress has proven to be a great menace and ever-looming threat to fruitful crop production around the globe (Hatfield and Dold, 2018; Tariq et al., 2018). The consequences of global climate change and spatial, temporal, and regional patterns are of considerable concern in agriculture production (Porter and Moot, 1998). Heat stress speeding up crop growth and not allowing the proper completion of crop growth stages results in immature development (Rahman et al., 2018a), perturbing carbon assimilation. This is an urgent matter, given that the geographical distribution of plant species depends to a large extent on their adaptation to different temperature zones (Keller and Seehausen, 2012).

Additionally, the world population is expected to reach 9 billion by 2050. Agriculture production needs to be enhanced up to 70% regardless of climate change and its impacts on agriculture (Rahman et al., 2018b). However, all the growth stages in plants are affected adversely by heat stress right from germination to growth and development, reproductive phase, seed yield (Hasanuzzaman et al., 2013; Ahmad et al., 2016), and seed quality in oilseed crops (Ahmad et al., 2021a). The rise in global temperature will ultimately damage the ecosystem comprehensively (Kanojia and Dijkwel, 2018). Specifically, heat stress is a severe threat to oilseed crops as it impairs the production and quality of the yield; for example, the seed yield decreased up to 39% in camelina and 38% in canola under elevated temperature scenarios (Jumrani and Bhatia, 2018; Ahmad et al., 2021b).

The temperature fluctuations have made it imperative to develop climate-resilient varieties that display better adaptability for growth under varied environmental conditions (Bhat et al., 2021). However, achieving this objective will be complicated by the fact that the performance of oilseeds may be hampered by environmental impacts related to climate change and the associated increase in pests and diseases, which are likely to become more challenging in the near future (Jaradat, 2016; Rahman et al., 2019). Therefore, hypothetically, several options can be used to achieve improvements in seed yield and related traits (either alone or in combination), increase seed oil content, or reduce seed yield losses due to abiotic stresses, including high temperature at the sensitive crop stage (Valantin-Morison

and Meynard, 2008). The resilience of oilseed crops under heat stress is led by conventional breeding techniques, including hybridization, artificial selection, and induced mutagenesis; though, these methods are complicated due to the polyploid nature of oil crops and require extensive time and labor investments to accomplish (Yang et al., 2017). In the coming decades, the growing demand for oilseeds can be achieved by using advanced molecular breeding techniques such as complementary breeding tools, which would be very useful to accelerate all crop improvement programs to produce climate-resilient crops. While transgenic approaches have so far been successfully used in oilseeds to improve a wide range of traits (Meesapyodsuk et al., 2018; Na et al., 2018; Shah et al., 2018; Kim et al., 2019; Wang et al., 2019), only a small number of these devices have made it to the market due to poor public perception as well as the disproportionately high cost and length of existing regulatory processes (Mall et al., 2018). Therefore, in this review, we aim to analyze recent results on the response and tolerance to heat stress at the cell, organelle, and whole plant level and describe the numerous approaches used to increase heat tolerance in oilseed crops.

HEAT STRESS AND ITS THRESHOLD IN OILSEEDS

In general, the threshold level is defined as a point after which some irreversible changes might occur. Therefore, the threshold level of heat stress is the moment after which plants lose their membrane stability. The scorching impact of high-temperature stress can be defined by the duration of exposure, the intensity of focus, and the degree of elevated temperature. Temperature limits of 35°C are considered heat stressors in tropics and subtropics (Bita and Gerats, 2013; Awais et al., 2017a; Ahmad et al., 2021a; Waraich et al., 2021a); however, temperatures above 25°C are thought to be stressors in rabi (winter) crops (Wahid et al., 2007; Abbas et al., 2017). The impact of high-temperature stress and the threshold temperatures of important oilseed crops at different growth stages is presented in **Table 1**.

HEAT STRESS SENSING AND SIGNALING

A healthy plant needs a compact and robust network of interconnected systems that responds rapidly to stimuli, initiates metabolic responses, and exhibits unique plasticity to adapt to adverse conditions. Heat stress can affect plant functioning in various ways by destabilizing membrane fluidity, multiple proteins, transport systems, enzyme efficiency, RNA stability, and de-polymerization of the cytoskeleton (Hasanuzzaman et al., 2013). The adaptation process to stress is complex and occurs mechanistically through genes, metabolites, and proteins that are collectively involved in many regulatory pathways. The initial step of stress perception involves molecular or structural changes through which a signaling cascade is established, leading to membrane fluidity responses, adaptive changes in proteins, and alteration of DNA and RNA sequences (Lohani et al., 2020). The initial site of stress sensing is mostly the plasma membrane that stimulates the activation of Ca^{+2} channels in the plasma membrane resulting in oscillations of the cytosolic Ca^{+2} level. Ca^{+2} acts as a secondary messenger, and signals rely on Ca^{+2} sensors and others such as calcineurin B-like proteins (*CBLs*), calmodulin (*CaMs*), calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs/CPKs), G protein-coupled receptors (GPCR), mitogen-activated protein kinase (MAPKs), pyrabactin resistance 1-like (PYR/PYL) protein, matrix metalloproteinases (MMPs), and other enzymes. For the most part, this mechanism of calcium detection has been elucidated in several models and also in oilseed plants.

Calmodulin and Calmodulin-Like Proteins

CaM and CML-containing helix-loop-helix EF-hand domains are a family of Ca^{2+} sensors in plants and control downstream targets based on Ca^{2+} fluctuations (Lohani et al., 2020). Eighteen CAMTAs have been identified in *B. napus*, the maximum of any plant species reported to date (Rahman et al., 2016). Diversified expression of these BnaCaM/CML genes indicated significant roles in different tissues in response to stress conditions, including heat stress. It was critical in the upregulation of heat stress tolerance (He et al., 2020). These proteins played essential roles in 13 metabolic processes and cellular responses, including protein biosynthesis, carbohydrate metabolism, protein folding, signal transduction, carbon assimilation and assembly, cell cycle, energy pathway, cell defense and rescue, nitrogen metabolism, lipid metabolism, transcription regulation, amino acid metabolism, and secondary metabolite biosynthesis (Wang et al., 2012).

Calcineurin B-Like Proteins

In contrast to calmodulin, which regulates several proteins, calcineurin B-like proteins are apparently linked to calcineurin B-like protein kinases (CIPK) or SNF1-related protein kinases (SnRK3) (Chen et al., 2012). The structural composition of calcineurin B-like interacting protein kinases contains an N-terminal kinase catalytic domain. This junction domain links it to the highly variable C-terminal regulatory part (Chaves-Sanjuan et al., 2014). The C-terminal regulatory environment consists of the FISL motif with a unique 24 amino acid stretch, essential for the CBLCIPK binding (Albrecht et al., 2001). Yuan et al. (2014) stated the description of CBL and CIPK genes in *B. napus* and revealed the presence of 23 CIPKs and 7 CBLs. Interaction studies of BnCBL1-BnCIPK6 protein were established by bimolecular fluorescence complementation (BiFC) and its regulation under stressed conditions in *B. napus* (Chen et al., 2012).

Calcium-Dependent Protein Kinase

Calcium-dependent protein kinases act as a third component of the Ca^{2+} sensing apparatus in plants, functioning as a responder to various sensors with the ability to self-modify authorization through the action of various enzymes (Chen et al., 2012), making calcium-dependent protein kinases very important in their dual function of detecting Ca^{2+} and responding through phosphorylation events in opposition to high-temperature signals. There are multiple calcium-dependent protein kinase essentials to react to specific stress stimuli under

TABLE 1 | Effect of heat stress in different oilseed crops at different growth stages.

Oilseed	Heat stress/duration	Impact on plant	Growth stage	References
Soybean (*Glycine max* L.)	42/34°C	Length between nodes and internodes decreased.	Seed filling	Allen Jr et al., 2018
	45°C/6 days	Chlorophyll content and yield	Reproductive phase	Khan et al., 2020
	38°C/8 hours	Decreased seed production	The appearance of the first flower	Cohen et al., 2021
	40°C/14 days	Reduced seed production and yield	Seed fill	Djanaguiraman et al., 2011
	42/28°C	Leaf weight, stomatal density, photosynthesis, and chlorophyll fluorescence	Reproductive phase	Jumrani et al., 2017
Sunflower (*Helianthus annuus* L.)	25°C/7 days after first anthesis to physiological maturity	Decreased the size of the embryo	Reproductive stage	Chimenti et al., 2001
	38°C/3 weeks	Increased lipid peroxidation and hydrogen peroxide content	Reproductive stage	Razik et al., 2021
	35°C/7 days	Decreased the seed weight per plant, decreased oil content	Seed fill stage/reproductive stage	Rondanini et al., 2003
	33°C/6 weeks	Decreased the leaf growth in sunflower	Vegetative	De la Haba et al., 2014
Canola (*Brassica napus* L.)	35°C/14 days	Reduction in gas exchange and water relations	Reproductive stage	Ahmad et al., 2021c
	37°C/2 days	Seed photosynthesis machinery, impairment of carbohydrates incorporation	Reproductive stage	Huang et al., 2019a
	35°C/7 days	Abnormal vegetative growth	Reproductive stage	Chen et al., 2021a
	32°C/7 days	Female reproductive organs are more sensitive than male reproductive organs	Reproductive stage	Chen et al., 2021b
	28°C/10 days	Reduced water relation and seed yield	Reproductive stage	Waraich et al., 2021b
Groundnut (*Arachis hypogaea* L.)	34°C/6 days	Reduction in number of pegs and pods	Reproductive	Prasad et al., 2000
	40°C/6 days	90% reduction in pod formation	Micro-sporogenesis	Prasad et al., 2001
	40°C	The photochemical efficiency of PSII decreased	Vegetative	Yang et al., 2013
	41°C/18 days	Fatty acid profile	Flowering	Lwe et al., 2020
Cotton (*Gossypium hirsutum* L.)	40°C/6 h	Reduction of photosynthetic material, total soluble sugars, and proline content	Reproductive stage	Mohamed and Abdel-Hamid, 2013
	45°C	Reduction in photosynthesis and cell membrane stability	Reproductive stage	Saleem et al., 2021
	38 and 45°C/1 week	Increased lipid membrane damage through increased malondialdehyde (MDA)	Reproductive stage	Sarwar et al., 2019
Castor bean (*Ricinus communis* L.)	35°C/13 days	Effect biomass production	Germination stage	Ribeiro et al., 2014
	35°C/7days	Heat shock proteins	Germination stage	Ribeiro et al., 2015
Linseed (*Linum usitatissimum* L.)	>30°C/7 days	Pollen viability	Reproductive stage	Gusta et al., 1997
	42°C/ 1 day	Gene expression	Reproductive stage	Saha et al., 2021
Camelina (*Camelina sativa* Crantz)	25–35°C/3 h in a day	Reduced photosynthetic rate	Reproductive stage	Carmo-Silva and Salvucci, 2012
	35°C/14 days	Reduction in gas exchange and water relations	Reproductive stage	Ahmad et al., 2021a
	35°C/10 days	Oxidative damage	Reproductive stage	Ahmad et al., 2021b
	35°C/14 days	Photosynthetic rate and water status decreased	Reproductive stage	Ahmad et al., 2021c
	32°C/12 days	Reduced growth rate and gas exchange	Reproductive stage	Waraich et al., 2021a

high-temperature stress. Wang et al. (2018a) also studied the interaction partners of BnCPK2 using bimolecular fluorescence complementation and the split ubiquitin-based pairing system (mbSUS) and revealed a role for BnCPK2 in regulating cell death and modulating ABA signaling and ROS homeostasis, and obtained probable interactions with the NADPH oxidase-like respiratory burst oxidase homolog D (RbohD) (Asano et al., 2012; Wang et al., 2018b). Under heat stress, GmTCTP and GmCDPKSK5 were reported in soybean, and their interaction works in response to heat stress in developing soybean seed (Wang et al., 2017). The burst of cytosolic Ca^{2+} or CDPK stimulates respiratory burst oxidase homolog D (RBOHD),

another plasma membrane-located protein with a role in the hydrogen peroxide generation through NADPH oxidase phosphorylation. The downstream signal path of RBOHD is involved in heat shock responses which consist of specific mitogen-activated protein kinases (MAPKs), HSFs, and MBF1c.

G Protein-Coupled Receptors

G protein-coupled receptors act as plasma membrane-localized receptors in plants that perceive different stress signals and play an essential role in the response of plants under abiotic stresses (Choudhury et al., 2011). These receptors bind to other ligands to sense and transmit the information related to extracellular stress stimuli. Ligand binding to G protein-coupled receptors causes conformational deviations and facilitates the exchange of GTP for GDP, leading to the activation of heterotrimeric guanine nucleotide-binding proteins (G proteins). The association of GPCRs and ligand-bound G proteins activates small Ras-related GTP-binding proteins in canola, which subsequently sets in motion a Ca^{2+} inositol triphosphate (IP3)-mediated signaling pathway under abiotic stress (Shokri-Gharelo and Noparvar, 2018; Nongpiur et al., 2019). Gao et al. (2010) examined *B. napus* and revealed the role of BnGB1 in signal pathways and could also improve the defense system of plants under environmental stresses (Gao et al., 2010).

Mitogen-Activated Protein Kinase Signaling Cascade

The mitogen-activated protein kinase signaling cascade assimilates and channels signal transduction to express the stress-responsive genes facilitated through phosphorylation and acts as and is involved in converging points in the mechanism of abiotic stress tolerance (Chinnusamy et al., 2004). Mitogen-activated protein kinase signaling cascades are comprised of MAPK kinases (MAP2Ks, MAPKKs, MEKs, and MKKs), MAPKK kinases (MAP3K, MAPKKKs, and MEKK), and MAPKs (MPKs). Mitogen-activated protein kinases function as on-off signaling switches aiming at downstream targets through phosphorylation. Consecutive phosphorylation and de-phosphorylation of threonine or serine residues by MAPKKKs command the activation of MKKs and then tyrosine and threonine residues to activate MPKs (Sun et al., 2014). Then, the activated terminal MAPKs ensue forward with the signal transduction by phosphorylation-arbitrated control of transcription enzymes or factors. However, Liang et al. (2013) identified 12 MPK and 7 MKK members. Sun et al. (2014) identified 66 MAPKKK genes in B. napus. The expressed BnMAPKKK genes were regulated by high-temperature stress and hormone-induced stress stimuli by transmitting external signals to the nucleus via sequential phosphorylation. Mitogen-activated protein kinases act as a signaling molecule sensing and modulating terminal heat stress, which subsequently controls the plant response to heat stress (Krysan and Colcombet, 2018).

The Pyrabactin Resistance 1-Like Protein

The pyrabactin resistance 1-like protein (BnPYL1-2, BnPYR1-3, and BnPYL7-2) is an essential regulatory constituent of abscisic acid signaling networks in *B. napus* (Di et al., 2018). Abscisic acid is sensed by the ABA receptor (pyrabactin resistance 1-like) in the ABA core signal transduction pathway (Ma et al., 2009; Miyazono et al., 2009). When PYR/PYL is bound by ABA, they inhibit the enzymatic activity of protein phosphatase 2C (PP2C), leading to the release of serine/threonine-protein kinase SRK2 (SnRK2) (Ma et al., 2009). Serine/threonine-protein kinase SRK2 is activated through the activation of loop auto-phosphorylation (Soon et al., 2012), and started by phosphorylate transcription factors, like the abscisic acid-responsive element binding factor (ABF), which is essential to activate ABFs (Kobayashi et al., 2005). These activated abscisic acid-responsive element binding factors enter the nucleus to upregulate the expression of downstream abscisic acid-induced stress-associated genes.

Matrix Metalloproteinases

Matrix metalloproteinases were found in humans and are a family of zinc-dependent endopeptidases, but a number of matrix metalloproteinases are also located in plants. Speculated results showed that plant matrix metalloproteinases played a role in the growth and development of plants and their response to different stresses. Still, there is a dire need to explore their biological functions (Ratnaparkhe et al., 2009). Pak et al. (1997) revealed the first high plant matrix metalloproteinases (*Gm1-MMP*) which were found to play a significant role in the expansion of soybean leaf. Heat stress-responsive matrix metalloproteinases (*Gm2-MMP*) confer heat stress tolerance by regulating the growth and development of plants which may help researchers to understand the biological functions of the matrix metalloproteinases family in plants (Liu et al., 2017, 2018b).

Phytochrome A and Phytochrome B

Phytochromes A and B are the most abundant phytochromes in de-etiolated and dark-grown seedlings. PhyB is present in two alternative isoforms: the active Pfr, with a maximum absorbance in the far-red region, and the inactive Pr, which absorbs maximally at the red region (Sakamoto and Kimura, 2018). Phytochrome B mediates signaling pathways to improve plant resistance to environmental stresses by reducing transpiration rate, improving the antioxidant defense system, expressing genes related to plant stress acclimation, and protecting pigments (Junior et al., 2021).

Heat Shock Factors and Heat Shock Proteins

Heat stress activates all the plasma membrane sensors and generates signals from different transcriptional regulators of HSR (heat shock response) (RbohD, MBF1c, and HSRs) through different kinases. The chloroplast is projected as a heat sensor as its translation ability of proteins triggers retrograde signals to heat receptive genes, which are HsfA2-dependent themselves (Liu et al., 2015). HSFs (heat shock transcription factors) are activated by calmodulin, Hsp90, and mitogen-activated protein kinases. Signal transduction includes various phases like activation of HSFs and their expression, which leads to the onset of thermo-tolerance (Saidi et al., 2011).

A prominent event is heat-tolerance acquisition, transcription, and translation of heat shock factors (HSFs)

and heat shock proteins (HSPs). Consequently, the constitutive over-expression of these genes and proteins is well-established to enhance heat tolerance (Vierling, 1991). Heat shock factors (HSFs) and dehydration responsive element-binding (DREB) protein families were also identified in *Brassica juncea* (Bhardwaj et al., 2015). The promoter regions of the soybean HSFs contained cis-elements that likely participate in drought, low temperature, and ABA stress responses. GmHsp90A2, GmRAR1, GmSGT1, GmSBH1P, and GmSBH1 are essential chaperones of the protective stress response in soybean (Chen et al., 2019; Huang et al., 2019b), while LusHSF responds in linseed (Saha et al., 2019). These regulators play their role in making the interaction between the MBF1c ethylene activated pathway and HSP allied signaling. This coordination and members of the DREB family facilitate the responses against heat stress. The plant's highly conserved heat stress response has four putative sensors that initiate the heat stress response (Mittler et al., 2012). GmHsp90A2 *was* identified as a positive regulator under heat stress in soybean, which interacted with GmHsp90A1 and exhibited increased tolerance to heat stress through higher chlorophyll and lower malondialdehyde (MDA) contents in plants (Huang et al., 2019b). High temperature can significantly affect gene expression during flowering as thermo-sensitive genic male sterility (TGMS) provides an adequate foundation for male fertility research in *B. napus*. We also found that transcription factor box transcription factor (MADS), Nuclear transcription factor Y (NFY), heat shock transcription factor (HSF), MYB/C, and WRKY might play a crucial role in male fertility under the high-temperature condition (Gao et al., 2021). *BnaMYBs* improve tolerance to cold, heat, drought, and salinity by regulating ROS defense genes (Chen et al., 2016; Hajiebrahimi et al., 2017). Demirel et al. (2014) studied 25 ESTs (express sequence tags), out of which 16 were homologous to known genes. The genes, namely RPS14, CTL2, LSm8, ABCC3, and CIPK, were downregulated, while FPGS, TH1, GhHS128, GhHS126, and IAR3 were upregulated, but expressions of psaB-rps14 and PP2C were not altered, owing to short-term heat stress in cotton. Hence these putative sensors activate heat stress-responsive genes to enhance thermo-tolerance, but the hierarchical order and relation between these pathways remain unclear.

The most recognized putative heat sensors in the plasma membrane are Ca^{2+} channels known as cyclic nucleotide gated calcium channels (CNGCS), a nucleosome containing histone variant (H2A.Z), and unfolded protein sensors; (a) ER-UPR and (b) Cyt-UPR as depicted in **Figure 1**. In a calcium signaling pathway, calcium interacts with the number of signaling molecules inside the cell to trigger the heat stress response. To operate HSPs expressions, Ca^{2+} interacts with HSFs (heat stress transcription factors), *via* CBK (Ca^{2+}/CaM3 binding protein kinases) and CaM3 (calmodulin 3). Ca^{2+} is required for the activity of RbohD against ROS stress (respiratory burst oxidase homolog D) or CDPKs (calcium-dependent protein kinases). It can repair the membrane with synaptotagmin A (SYTA) (Sajid et al., 2018). Calcium-dependent protein kinases (CDPKs), identified only in plants, are a vital regulatory protein decoding calcium signals activated by various environmental stimuli. However, only CDPK 8 from the CDPK family has been reported to have a role in abiotic stress response via scavenging H_2O_2 by catalase-3 (Zou et al., 2015). The expressions of other HSPs and HSFs initiated by Ca^{2+} regulate master HSPs and HSFs and trigger the enzyme activity to prepare the plant for heat tress tolerance, as shown in **Figure 2**. An alternative complementing heat-sensing mechanism proposes that the primary temperature sensor of the cell is located in the plasma membrane and that Ca^{2+} permeable channels act as the earliest temperature-sensing component of the plant heat stress response (Saidi et al., 2009). Heat stress activates cell sensors, and among those, plasma membrane sensors activate calcium channels which causes the inward flux of calcium.

HEAT STRESS AT GERMINATION AND VEGETATIVE STAGE

The germination and emergence ratio of a crop is the critical phenomenon to get the optimum planting density and crop performance in the field. High temperature resulted in poor germination and poor stand establishment in the Indian Brassica germplasm (Azharudheen et al., 2013). Recently, induction of varying degrees of secondary dormancy at sub and supra-optimal temperature regimes were detected among rapeseed cultivars (Gorzin et al., 2020). Heat stress damages plant morphology and is manifested by symptoms on vegetative parts such as leaf sunburn, scorching effects of heat on leaves, twigs, buds, branches, stems, and fruits, reduction in root to shoot ratio, affects plant meristems, and leaf senescence (De la Haba et al., 2014) with subsequent abscission and ultimate reduction in seed yield (Bita and Gerats, 2013). As temperature increases, the plant development builds up to a certain extent and decreases afterward (Wahid et al., 2007). The impaired growth and development symptoms were observed in Brassica (Angadi et al., 2000), soybean (Piramila et al., 2012), and linseed (Gusta et al., 1997) under high-temperature stress. Ahmad et al. (2021a) reported that high temperature (35°C) during anthesis reduces chlorophyll content, photosynthetic rate, and leaf water status in camellia and canola genotypes, leading to reduced plant growth and seed yield. Canola growth was negatively affected above 28°C by reducing plant height, root length, and biomass accumulation due to impaired photosynthetic rate and stomatal conductance (Waraich et al., 2021b). The consequences of heat stress for plant growth and development are presented in **Figure 3**.

HEAT STRESS AT REPRODUCTIVE STAGE

All the plant growth stages could be poorly affected by thermal stress, but the biggest concern of the agricultural world is the reproductive phase. The central part of the world's food supply comes from the flowering plant through sexual reproduction. The first few hours of the reproductive phase are important in fertilization, as a small spell of heat stress occurs, which can be fatal to the whole process (Xi, 1991). Similarly, the reproductive stage is considered the most sensitive stage to be affected by heat stress in Brassica (Young et al., 2004; Ihsan

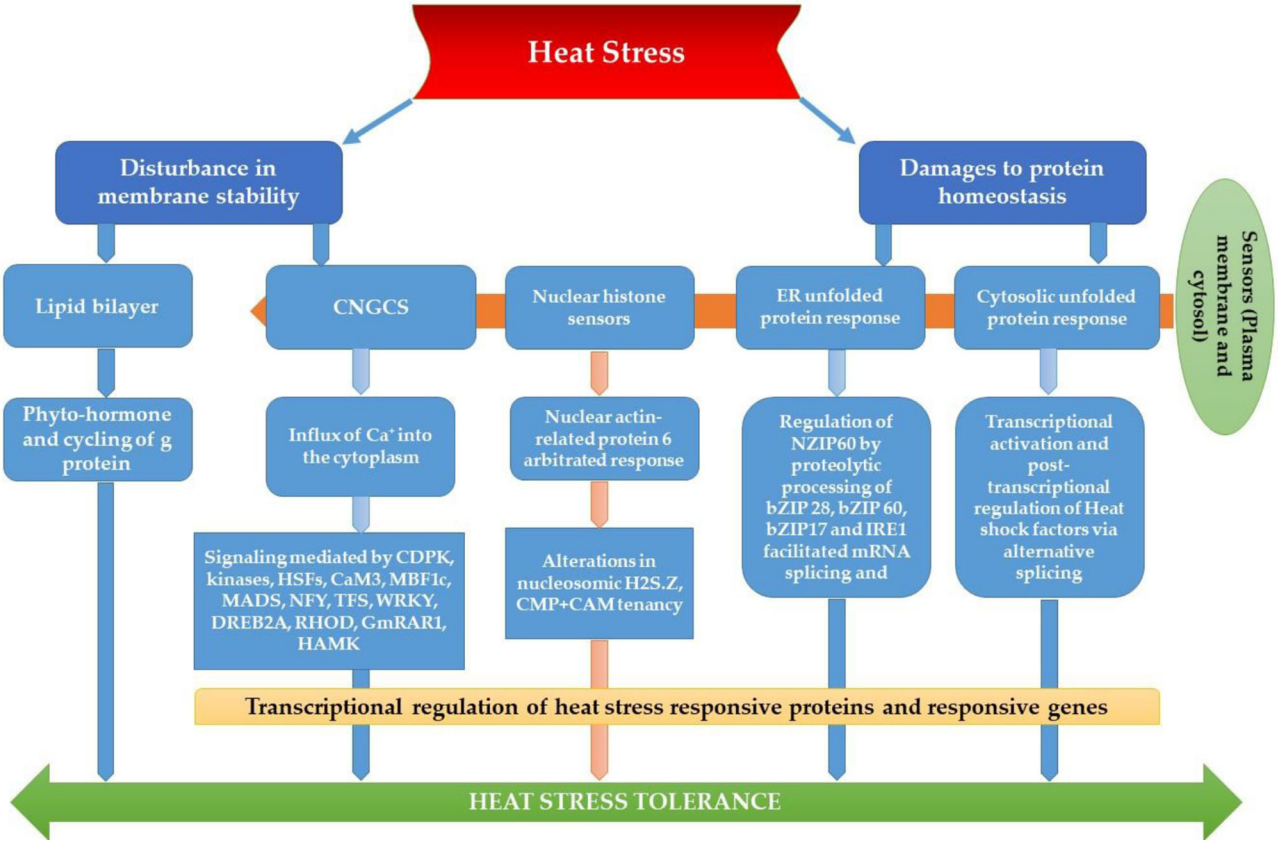

FIGURE 1 | Plant thermo-sensors and main signal transduction pathways implicated in heat stress response and thermo-tolerance (modified from Bokszczanin and Fragkostefanakis, 2013).

et al., 2019). The disruption of the plant's metabolic functions due to high temperature is associated with a consequent reduction in light interception due to a shortening of the growth phases in terms of both size and time. It also has an adverse effect on carbon assimilation, leading to the formation of small and deformed organelles (Maestri et al., 2002). A very fatal heat stress response has been observed in male and female reproductive parts, which impairs pollen viability and germination, inhibits pollen tube growth, impairs receptivity and function of the stigma and ovary, causes fertilization arrest, inhibits embryogenesis, impairs egg viability, and induces ovarian abortion and poor seed set. Brassica plants have shown a poor seed set when exposed to heat stress (Angadi et al., 2000; Morrison et al., 2016). It has been observed that late flowering to early seed setting is the most susceptible growth stage to heat stress in groundnut (*A. hypogaea*) (Prasad et al., 1999). One more example of this occurs in cotton. In this case, the most heat-sensitive stages in cotton are pollen and pollen tube development and fertilization in reproductive growth. High-temperature stress reduces the number of sympodial and monopodial branches, number of bolls, seeds per boll and their weight, and the boll development process (Ekinci et al., 2017; Rahman et al., 2019). The canola grain yield drastically reduced when exposed to high night temperatures during the reproductive stage (Pokharel et al., 2020; Chen et al., 2021b).

OIL QUANTITY AND QUALITY

Among the plant reserves, oils are the most energetic reserves, providing humans with many essential fatty acids and calories that must be part of the daily diet. It is synthesized in plastids, oil bodies, and triacylglycerol (TAG) molecules that accumulate outside the plastids in the endoplasmic reticulum (ER). Exposure to heat episodes has detrimental effects on cell organelles (plastids, ER, and oil bodies), it also induces the denaturation of enzymes, which could lead to the impairment of the mechanism of oil synthesis (Haung et al., 2019). Although under heat stress conditions, the full mechanism of oil accumulation and photosynthesis in *B. napus* remains unclear, it is known that under these conditions, the sugar content increases because seed oil accumulation is reduced, leading to impaired carbohydrate incorporation into TAG (Haung et al., 2019). Heat stress lessens the role of a number of sugar transporter genes, resulting in the imperfect incorporation of carbohydrates into triacylglycerol's units. Taken together, the results confirmed that perturbations in the mechanism of seed photosynthesis, impaired integration of carbohydrates into triacylglycerol, and

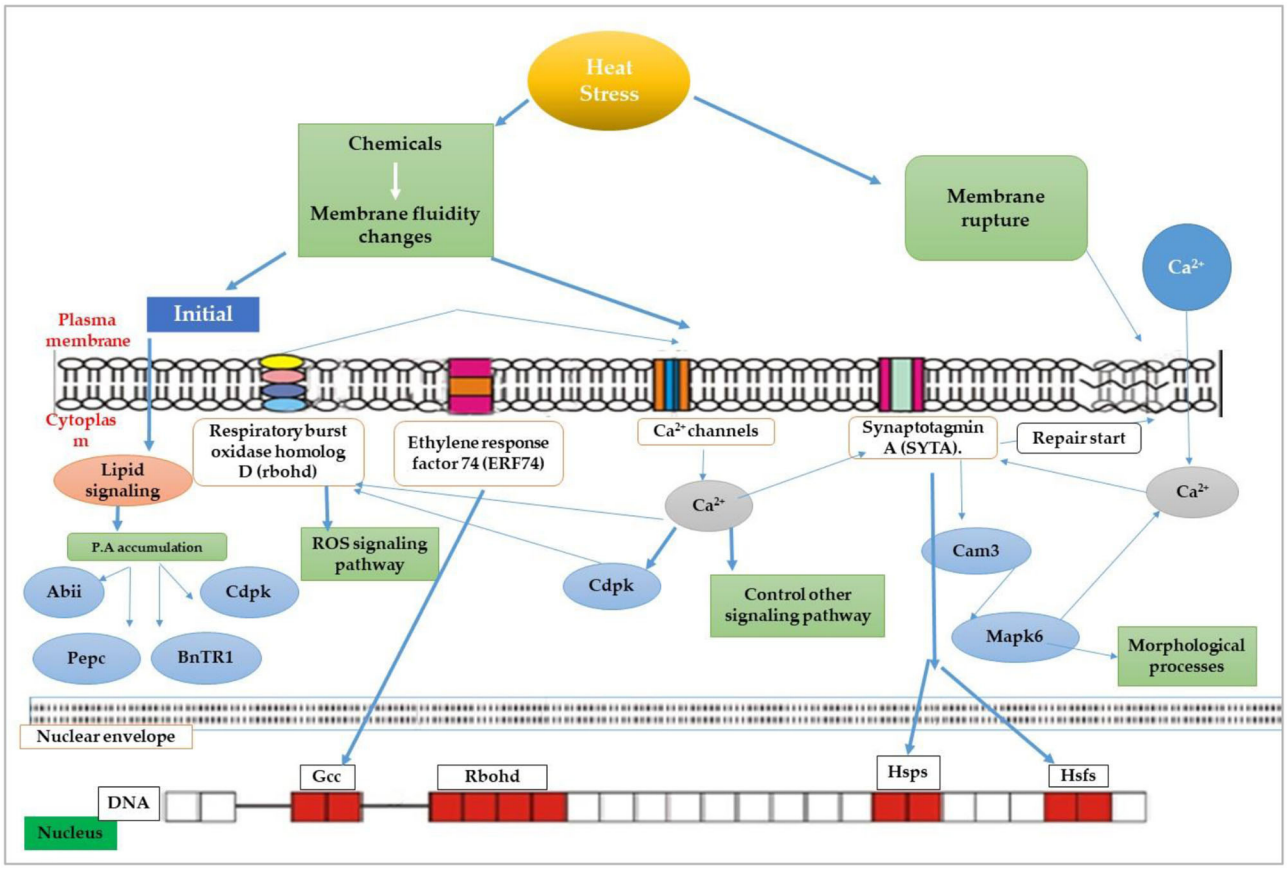

FIGURE 2 | Membrane heat sensors and signal transduction pathways through various receptors across the plasma membrane.

transcriptional deregulation of the BnWRI1 pathway due to heat stress are the main reasons for less oil accumulation (Haung et al., 2019). The relationship of oil concentration with temperature is linear. As temperature increases, the concentration of oleic acid increases linearly, and at the same time, linoleic acid decreases linearly (Thomas et al., 2003; Lanna et al., 2005).

Additionally, linoleic and linolenic acids, isoflavones content, and iodine number also decreased. All these factors added to reducing oil content in soybean seeds (Lanna et al., 2005). The oil yield showed a linear correlation to thousand seed weight, pod length, and seeds per pod of the Brassica species accessions in tropical environments, especially under high-temperature regimes indicating a promising potential as alternative oilseed crops for biodiesel production in tropical conditions (Bassegio and Zanotto, 2020).

PHYSIOLOGICAL AND METABOLIC BASIS FOR REPRODUCTIVE FAILURE UNDER HEAT STRESS

The vulnerability of plants to heat stress varies with the different growth stages. At the same time, the reproductive phase is also susceptible due to its sensitive organelles that surrender to heat changes. Heat stress reduces the plant's photosynthetic capacity, resulting in a lack of resources for the reproduction process in the genotypic and reproductive tissues (Ahmad et al., 2021a). Some causes of reproductive failure and male sterility in plants are related to the genes responsible for the tapetum and pollen functioning, which are altered by heat stress occasioning their degradation. Carbohydrate metabolic enzymes, including sucrose synthase, vacuolar inverses, and sugar transporters, are influenced by heat stress reducing the pollen viability (Zandalinas et al., 2018). The accumulation of soluble carbohydrates in pollen is reduced by low sucrose-starch turnover due to downregulation of the enzymes sucrose synthase and invertase (Hedhly, 2011). Under heat stress, cell proliferation arrest produces changes in chloroplast development, abnormalities in mitochondria, and distended vacuoles (Sakata et al., 2010; Wani and Kumar, 2020). In stigmatic tissues and pollen grains, carbohydrate accumulation is disrupted due to changes in the partitioning of assimilates between the apoplast and symplastic phloem filling, which impairs pollen grain viability. High-temperature stress leads to inhibition of starch production in oilseeds (Thuzar et al., 2010), associated with seed setting and oil accumulation under heat stress. The drastic reduction in grain weight was directly linked with electrolyte leakage and membrane damage resulting in low seed yield under terminal heat stress in *B. juncea* (Kavita and Pandey, 2017).

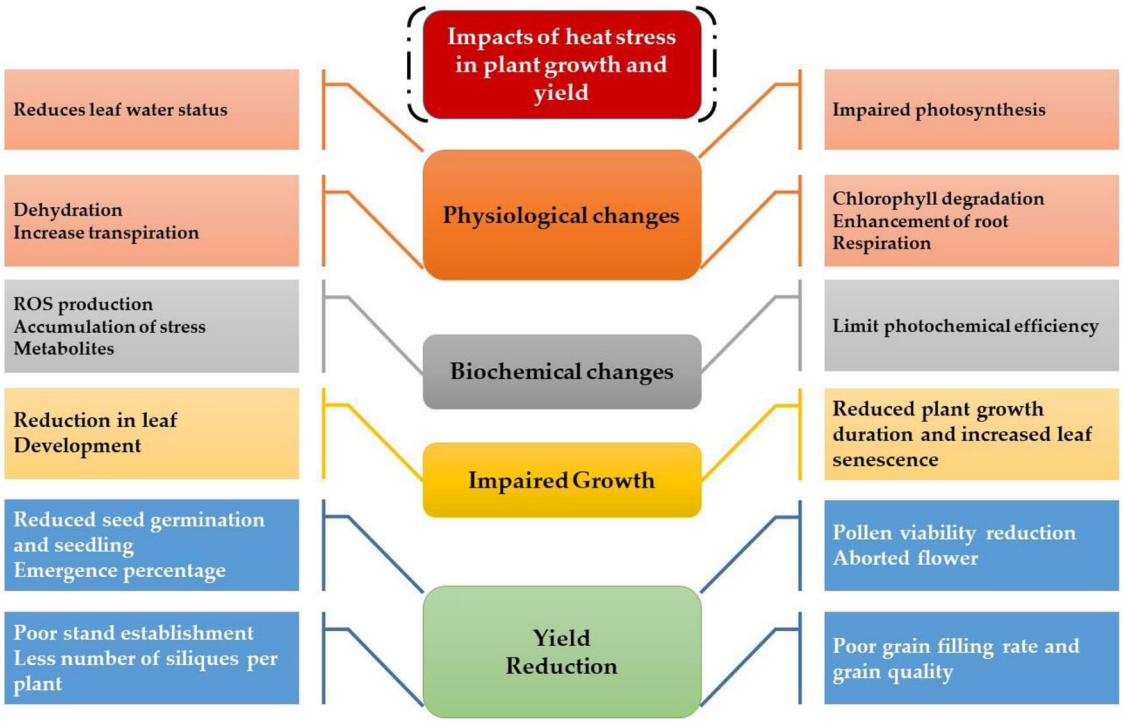

FIGURE 3 | Impact of heat stress on physiological, biochemical, growth, and yield responses in plants.

PHYSIOLOGICAL RESPONSES

Initially, heat stress damages the chloroplast proteins complex and inhibits the enzymatic activity (Ahmad et al., 2010; Hasanuzzaman et al., 2020a). Under high-temperature environments, the chloroplasts are unfolded and vulnerable to rapid degradation of chloroplast proteins (Dutta et al., 2009), particularly one of the significant core subunits in photosystem II (PSII), protein D1, is the most vulnerable to heat stress damage. The impact of heat stress at the cellular level is devastating because it damages membrane stability, inactivates enzymes in chloroplasts and mitochondria, also promotes protein and enzyme degradation, decreases carbohydrate and protein synthesis, reduces carbon metabolism, and alters microtubule organization by expanding and elongating cells, ultimately damaging the cytoskeleton (Bita and Gerats, 2013). Heat stress affects all the physiological processes, but the most sensitive among all is photosynthesis (Crafts-Brandner and Salvucci, 2002; Hasanuzzaman et al., 2020b) as shown in **Figure 4**. The effect of elevated temperatures on photosynthesis can be seen in several instances. Heat disrupts the integrity of the thylakoid membrane and damages photosystems I and II as well as the oxygen complex, affecting phosphorylation (Rexroth et al., 2011). PSII is the most sensitive photosystem (Bibi et al., 2008); thus, severe thermal damage to PSII results in disrupting electron transport and ATP synthesis during the photosynthetic process (Wang et al., 2018a).

ROS production has detrimental effects on photosynthetic machinery and PSII (Bita and Gerats, 2013; Elferjani and Soolanayakanahally, 2018). Leaf chlorophyll contents have a negative relation with heat stress resulting in less photosynthesis at 38/32°C that lowers the chlorophyll content leading to a decrease in the sucrose content. In addition, it suppresses the process of carbon fixation in photosynthesis by reducing chlorophyll content (Liu and Hang, 2000; Ahmad et al., 2021a), reducing the quantum yield of photosystem II (Bibi et al., 2008), reducing e-transport (Wise et al., 2004) due to leakage in the thylakoid membrane, inactivating rubisco activation, and increasing cyclic photophosphorylation. Heat stress affects the plant in many ways. For example, excess water can also be siphoned off due to increased transpiration, leading to reduced plant turgidity and disruption of physiological processes (Tsukaguchi et al., 2003). Heat stress affects water relations in the plant by affecting osmotic adjustment due to the poor photosynthetic capacity of the plant (PSII is the most sensitive part), reducing sugar content, decreasing the osmotic potential of leaves, and increasing transpiration rate (Hemantaranjan et al., 2018).

HEAT STRESS AND PHYTOHORMONES/SIGNALING MOLECULES

The plant faces several external and internal stimuli during its lifespan. Therefore, they need to regulate their growth and development in reaction to these stimuli (Li et al., 2021a). A small group of signaling molecules known as phytohormones

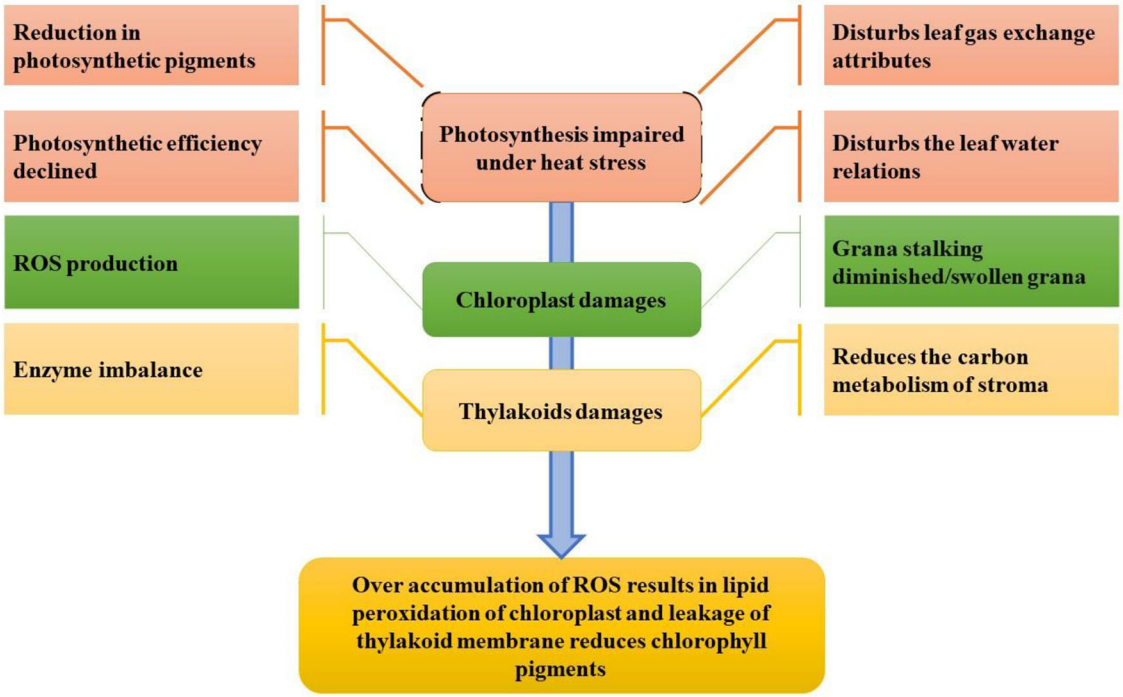

FIGURE 4 | Impact of heat stress on photosynthesis and the photosynthetic system (conceived from Nadeem et al., 2018).

(abscisic acid, brassinosteroids, cytokinin, salicylic acid, jasmonate, and ethylene) present in small quantities in the cell and help to mediate the response to stimuli. Although ABA is the primary regulator of the response to abiotic stress among phytohormones, increasing evidence points to the involvement of other phytohormones. The nature of phytohormone-mediated regulation of heat stress tolerance is a complex phenomenon, as they can act either directly respond or orchestrate the response to high-temperature stress by engaging other phytohormones, including reactive oxygen species, MAP kinases, soluble sugars, and secondary messengers through crosstalk networks (Smékalová et al., 2014; Ljung et al., 2015). The role of phytohormone-induced regulation of stress tolerance has been extensively reviewed (Peleg and Blumwald, 2011; Balfagón et al., 2019). These phytohormones play a crucial role in acclimatizing plants to rapidly changing environmental conditions by regulating transitions between sources and sinks, growth and development, and well-known nutrient distribution (Nazar et al., 2017). Plant hormones mediate soybean plant tolerance to high-temperature stress by enhancing plant growth and development through regulation of the antioxidant defense system, interaction with plant hormones, and reorganization of biochemical metabolism (Imran et al., 2021).

Plants are sessile organisms and their survival in elevated temperature depends upon various factors. One of the most vital substances used in response to external stimuli is endogenously produced phytohormones, which regulate different molecular and physiological reactions (Li et al., 2021b). Phytohormones might act where they were synthesized or can be transported wherever needed (Peleg and Blumwald, 2011). Some of the critical roles phytohormones play to face abiotic stress tolerance include, for example, the increment in the synthesis of cytokinins (CKs) under water stress conditions for better functioning. Melatonin supplementation regulates the plant defense system by improving the activity of antioxidants (superoxide dismutase, ascorbate peroxidase, peroxidase, and catalase) and their genes (GmPOD1, GmSOD, GmAPX, and GmCAT1), biochemicals (phenolic substances, flavonoids, and proline), and polyamines (spermine, spermidine, and putrescine), and also by downregulating stress hormone biosynthesis including abscisic acid content, downregulated gmNCED3 (abscisic acid biosynthesis gene), and upregulated catabolic genes (CYP707A1 and CYP707A2) in soybean. In addition, melatonin induced the expression of heat shock transcription factor (gmHsfA2), heat shock protein 90 (gmHsp90), and indicated detoxification of reactive oxygen species through the H_2O_2-mediated signaling pathway (Imran et al., 2021). Kazan and Manners (2013) delineates the evolutionary role that a second phytohormone auxin (IAA) plays in stress tolerance because of its biosynthesis, signaling, and transportation apparatus present in the cell; some other studies found evidence of the role of gibberellins (GAs) to ameliorate adverse circumstances, for instance Colebrook et al. (2014) found that gibberellins role in abiotic stress tolerance has been increasing with time; another phytohormone that takes part in the response to abiotic stress is abscisic acid (ABA), the level of ABA upsurges in plants under unfavorable conditions modifying gene expression and activating signaling pathways (O'Brien and Benková, 2013) as shown in **Figure 5**. Ethylene (ET) also plays

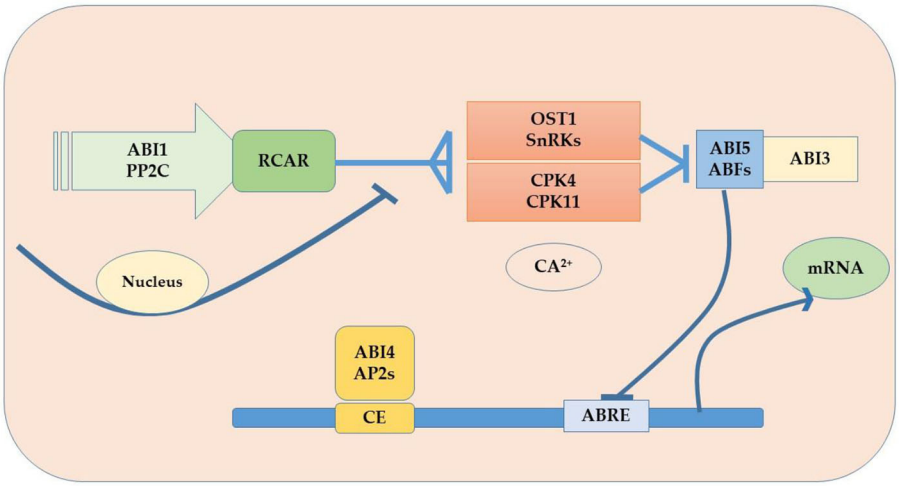

FIGURE 5 | ABA signaling pathway in oilseed crops.

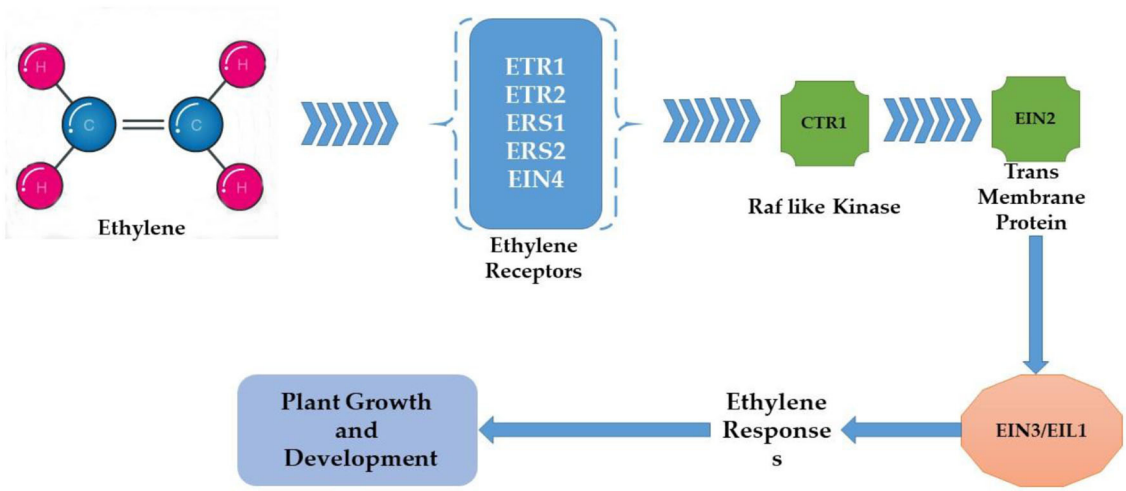

FIGURE 6 | Ethylene signaling pathway under heat stress.

an important role in the acclimatization in adverse conditions (Gamalero and Glick, 2012) and also modulates gene expressions as shown in **Figure 6** (Klay et al., 2014). Jasmonates (JAs), strigolactone (SL), and salicylic acid (SA) govern growth and development and fruit ripening in abiotic stresses (Rivas-San Vicente and Plasencia, 2011); jasmonate (JAs) regulates plant defense to stressful conditions, and brassinosteroids (BRs) are an essential phytohormone that have a role in the heat stress tolerance in plants (Bajguz, 2011; Janeczko et al., 2011). Endogenous abscisic acid concentration was significantly elevated by heat stress (45°C) alone and doubled by heat stress plus brassinosteroids. These results suggested that the well-known enhancement of heat stress tolerance was obtained due to brassinosteroid-induced elevation in endogenous abscisic acid concentration (Kurepin et al., 2008). Jasmonate is required to regulate specific transcriptional responses unique to the heat and high light stress combinations in the chloroplast, especially D1 protein in PSII (Balfagón et al., 2019). All these are key players to provide developmental plasticity in plant growth. The role of phytohormones in oilseed crops also extraordinarily starts at the biosynthesis of oil content using a signal transduction mechanism and has a role in the performance of many growth and developmental processes. It is well-documented that exogenous application of phytohormones mitigates the negative effects of heat stress in canola (Kurepin et al., 2008). Similarly, supplementation with brassinolide at the seedling stage can enhance thermo-tolerance by increasing endogenous ABA levels. However, treatment with 24 epibrassinolides also increases heat stress tolerance (Kagale et al., 2007). qRT-PCR analysis showed that the expression levels of gibberellin biosynthesis

pathway genes (GmGA3ox1, GmGA3ox2, and GmGA3) and auxin biosynthesis pathway genes (GmYUCCA3, GmYUCCA5, and GmYUCCA7) significantly increased upon interaction with high temperature and supplementation of gibberellins and auxin, which improved the performance of soybean plants by improving hypocotyl elongation under high-temperature stress (Bawa et al., 2020).

MECHANISM OF HEAT STRESS TOLERANCE IN PLANTS

The global climate is changing due to various anthropogenic factors that influence temperature regimes (Ahmad et al., 2020). There are several mechanisms, including phenological, physiological, morphological, and biochemical mechanisms, that plants exhibit for their survival under high-temperature conditions (Ghaffar et al., 2020) since plants are sessile in nature, which limits them to a specific range of responses to external stimuli that vary at different stages of growth and have flexible relevance to physiological and cellular mechanisms of protection and acclimatization (Ahmad et al., 2021a). The prime stress indications (e.g., variations in temperature, ionic effects, osmotic effects, membrane uncertainty) would activate the signaling and transcript control that triggers the mechanism of stress responses to restore the homeostasis and repair the plasma membrane. Bohnert et al. (2006) examined cell death due to devastation in the structural and functional proteins and irreversible damage in the homeostasis of the cell because of an insufficient response at different steps of signaling and gene expression processes. Understanding the various mechanisms of the reaction of plants to stress and their importance in the acquisition of thermo-tolerance is of great importance. Under heat stress, plants activate a variety of mechanisms, including accumulation of metabolites (HSPs, osmoprotectants, antioxidants), ion carriers, late embryo abundant proteins, free radical scavengers, transcriptional control, and factors involved in signaling, which are fundamentally very important for stress alleviation (Bokszczanin and Fragkostefanakis, 2013). By observing the heat, signaling, and metabolite production that help the plant survive adverse conditions, a chain of mechanisms and variations began. The impacts of heat stress are evident at different stages and in aspects such as plasma membrane fluidity, biochemical mechanisms in cytoplasmic organelles, and cytosol (Sung et al., 2003). The primary sight of damage resulting from heat stress is plasma-lemma which results in damage to the lipid bilayer plasma membrane. This implies the initiation of cytoskeleton reorganization and Ca2+ influx, leading to the regulation of CDPK and MAPK.

Heat stress results in the production of ROS in various organelles (peroxisomes, chloroplasts, and mitochondria), which are important in the signaling mechanism, activation of antioxidant enzymes, HSPs, and restoring the balance of osmolyte concentration that maintains the water balance of the cell (Bohnert et al., 2006). Plants can adopt several stress mechanisms, with the ability being associated with acquiring thermo-tolerance (Maestri et al., 2002). During conditions of heat stress, HSP chaperones play essential roles in signal transduction and gene expression, as well as in the regulation of cellular redox balance, protection of photosynthesis, protein and membrane repair, osmolyte production (Diamant et al., 2001), and antioxidant production. The response to heat shock can be controlled at the transcriptional and translational levels. The cis-acting DNA sequence, heat shock element (HSE), and LusHSF genes have been found to play an important role in heat-induced transcription (Nover and Baniwal, 2006; Saha et al., 2019). During heat episodes, a protective mechanism is also associated with increased thermo-tolerance of the photosynthetic apparatus (Hemantaranjan et al., 2014). Consequently, the induction of thermo-tolerance for plant protection under such conditions is directly linked to the ability to detoxify and scavenge radical ROS, leading to plant thermostability (Hameed et al., 2012). Although many attempts have been made, Iaccthere is still very little literature on ROS production and scattering. The saturation of membrane lipids under heat stress tolerance increases the content of trans-3-hexadecanoic acid (among phospholipids) and linolenic acid (among galactolipids). However, it is still unclear whether either low membrane lipid saturation or higher membrane lipid saturation is beneficial in mitigating heat stress (Klueva et al., 2001). Total soluble proteins play a vital role in improving heat stress tolerance in oilseed crops, including camellia and oilseed rape by improving plant water relations and gas exchange properties that help improve vegetative and reproductive growth under high heat stress (Ahmad et al., 2021b,c). During heat stress, the photosynthetic electron transport chain is protected by the localization of LMW-HSPs with the chloroplast membrane (Heckathorn et al., 1998). Variations in expressions of genes are a vital part of the heat stress tolerance response. Yang et al. (2006) observed a rapid shift of gene expression under heat stress in inhibiting HSP complement expression. The splicing of many mRNAs could be restrained by heat stress. Unfavorable high-temperature heat stress conditions may also destabilize the non-heat stress-induced proteins encoded by mRNAs. Investigations show that the presence of introns in the HSPs is why the mRNAs were sliced properly compared to proteins with no introns. Accordingly, a number of genes were identified to confer thermo-tolerance in plants, i.e., regulated glutaredoxin, ascorbate peroxidase, heat shock factors, heat shock proteins, and downregulated FAD3-2 and FAD7 to improve resistance in plants against heat shocks induced by high-temperature stress (Murakami et al., 2000; Lwe et al., 2020). However, further studies are needed to elucidate the mechanism of professional transcriptional alteration and transformations of HSP-encoding mRNA under lethal high temperatures. Although, Ca^{2+} acts as a regulator of many physiological and biochemical processes in response to high-temperature stress in plants (Yang et al., 2013), transient elevation of free Ca^{2+} in the cytoplast can be detected in plants in response to various stresses, such as high temperatures. The fact that Ca^{2+} improves plant resistance has been linked to the maintenance of higher photosynthetic rates under stress, histone sensors, and unfolded protein response sensors in the endoplasmic reticulum (ER), RBOHD, plasma membrane channels (which transiently open and induce Ca^{2+} entry flux into the cytosol),

phytochrome B (Mittler et al., 2012; Vu et al., 2019), PSII reaction center stability, ROS detoxification, and high light-induced Ca^{2+} influx into chloroplasts, which regulates antioxidant processes to mitigate high temperature-induced oxidative stress (Yang et al., 2013; Gilroy et al., 2016). For example, the superoxide anion, the initial product of photoreduction of O_2, is dismuted by superoxide dismutase to H_2O_2 and O_2 (Noctor and Foyer, 1998). H_2O_2 is then converted into water by ascorbate peroxidase (APX). Furthermore, exogenous Ca^{2+} could improve the non-photochemical quenching of chlorophyll fluorescence (Ai et al., 2007), protecting the photosynthetic machinery from inactivation and damage caused by excess irradiance (Horton et al., 1996). In addition, a class of HSF family TFs (e.g., HSFA1s) and the Ca^{2+} /CaM signal transduction pathway regulate plant responses to high temperatures (Yang et al., 2013; Cortijo et al., 2017; Ohama et al., 2017). Pretreatment of plants with hydrogen peroxide or phytohormones increases the expression of genes encoding enzymes such as catalase, which scavenges ROS, and redox regulators such as glutaredoxin, which improve plant temperature tolerance (Wang et al., 2014; Devireddy et al., 2021).

Acquired Thermo-Tolerance

In plants, the capability to survive under lethal high-temperature stress following adaptations with sub-lethal high temperature or the capability of a living thing to survive in a severely high temperature is referred to as acquired thermo-tolerance (Jagadish et al., 2021). Like other organisms, plants cope with severe high temperatures by acquiring thermo-tolerance within a few hours; they also have an inherent characteristic to survive in lethal temperatures (Lin et al., 2014). Stress memory is defined as the process of storage and retrieval of information acquired during initial exposure to stress (Crisp et al., 2016; Hilker and Schmülling, 2019). Naturally, plants face different gradual temperature ranges and acclimatize to these otherwise lethal ranges, which is an independent cellular phenomenon of thermo-tolerance acquisition that results from the pretreatment under high temperature for short periods correlated with a higher activity of antioxidant enzymes (Collado-González et al., 2021). This acquisition of thermo-tolerance is used as a yardstick to evaluate the thermo-tolerant and thermo-sensitive genotypes of oilseeds like groundnut and sunflower (Awais et al., 2017b), and elaborate the functions of different stress genes. Thermo-tolerance acquisition is not a single-step phenomenon; rather, it has different phases. Certain factors affect the acquisition of thermo-tolerance like growth stage, acclimation methods, and crosstalk between acquired thermo-tolerance and stress tolerance. These key factors protect the cells from the detritus impacts of heat stress in acquired thermo-tolerance (Jespersen, 2020). A piece of useful information regarding heat stress effects can be revealed by the inspection of all the hostile effects instigated by extreme heat as the responses of thermal stress in the plant are related to other types of stress (Rahaman et al., 2018). The HSRs, referred to as a transitory restructuring of gene expressions, are a preserved biological response of different organisms and cells to eminent temperatures (Schöffl et al., 1999). The upregulated genes under high-temperature stress encoded many heat shock factor (HSF)-like proteins such as HsfB2A (Bra029292) and heat shock proteins (HSPs), including high molecular weight HSPs. Heat stress also upregulated some components of HSR, including ROS-scavenging genes such as protein kinases, glutathione peroxidase 2 (Bra022853, BrGPX2), and phosphatases. At the same time, CYP707A3 (Bra025083, Bra021965) was involved in membrane leakage, but many transcription factor (TF) genes, including DREB2A (Bra005852), were involved in the acquisition of heat stress tolerance in bryophytes (Dong et al., 2015). HSP is a vital apparatus to examine the molecular mechanism of heat stress tolerance and gene-expression regulation in plants. The total temperature needed for the initiation of HSR accords with the optimum temperature of a specific species, which is 5–10°C more than normal thermic conditions. It involves the education of HSPs and, therefore, a higher level of thermo-tolerance acquisition. In the transitory synthesis of HSPs, results showed that the signal that triggers the reaction is either lost, deactivated, or not documented (Burke, 2001; Lwe et al., 2020). The direct role of HSPs in thermo-tolerance is challenging to determine, but its involvement in acquired heat tolerance is a logical model (Burke, 2001). So, the acquired thermo-tolerance in plants obtained through natural phenomenon prompted by a gradual acquaintance to heat periods or biological synthesis of pertinent compounds, although cost-intensive, is a vital and hypothetically a critical strategy. This mechanism is primarily associated with the display of HSR and acquired by the restructuring of gene expression, letting plants survive under the sub-lethal temperature. Edelman et al. (1988) performed studies in soybean seedlings and revealed that as the temperature reached 40°C, the protein pattern shifted from normal proteins to HSPs to acquire thermo-tolerance. Remarkably, a minimal but significant level of acquired thermo-tolerance can be achieved in plants by inducing the expression of a small number of genes regulated by other transcription factors, such as the NAC069 TF (Wang et al., 2016a), MYB-related genes (FAR1, bZIP, and mTERF) (Zhou et al., 2016), MADS-box, MYB41 (Wu et al., 2018), NAC (Saha et al., 2021), and CWM-related genes (Wang et al., 2016b). Kinases including MAPKKK (mitogen-activated protein kinase) (Sun et al., 2014) and superoxide dismutase (SOD)-related genes (BnSOD) (Su et al., 2021), CBL (calcineurin B-like proteins) and CIPK (CBL-interacting protein kinases) (Yuan et al., 2014), and CPK (calcium-dependent protein kinase) and transporters including SUT/SUC and SWEET (Jian et al., 2016) have been identified and found to provide genetic resources for improving high temperature tolerance traits in *Brassica*.

Antioxidant Defense in Response to Heat-Induced Oxidative Stress

Plants can only survive during unfavorable heat conditions if, somehow, they protect themselves from heat-induced oxidative stress. ROS over-accumulation during stress conditions results in the oxidation damage of vital molecules such as DNA, proteins, and lipids. This condition is termed oxidative stress in plants (Mittler et al., 2012). Plants increase the content of antioxidants through different physiological and biochemical mechanisms to overcome oxidative stress caused by heat

stress and scavenge oxygen radicals. Additional enzymes and metabolites participated in the antioxidant defense mechanism. Ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase, peroxiredoxins, and tocopherols are antioxidant enzymes involved in protecting cells from excess ROS (Lin et al., 2010; Tang et al., 2021). Camelina has shown some resilience to high-temperature stress. However, there is no stability in generating and detoxifying the oxygen radicals under heat stress, as heat stress increases the ROS content. Such an inequity may be the increasing amount of H2O2 under heat stress, which creates oxidative damage in the plant (Ahmad et al., 2021c). The activities of antioxidants (catalase, protease, and ascorbate peroxidase), osmolytes (GB and proline), soluble proteins, and sugars increased under heat stress, which subsequently reduced the H2O2 levels in stressed plants (Sarwar et al., 2018; Ahmad et al., 2021c). Further, thiourea improved the defense system of camellia plants by catalase, protease, ascorbate peroxidase, proline, and glycine betaine activities under heat stress in cotton (Majeed et al., 2019, 2021). Application of thiourea regulated the redox state in plant cells, modulated antioxidant activities, and led to the reduction of lipid peroxidation products (Goyal and Asthir, 2016). Improvement in *B. napus* metabolism due to thiourea application was considered critical to mitigate heat stress damage by regulating photosynthetic pigments and photosynthetic efficiency (Waraich et al., 2021b), which has an important role in redox control during phenological and physiological development and oxidative stress homeostasis (Mhamdi and Van Breusegem, 2018). Non-enzymatic antioxidants marked a decrease in ascorbic acid and total soluble sugars in response to heat stress compared to the non-stressed control (Hameed et al., 2013). In addition, the data also revealed a direct relationship between the activities of antioxidant enzymes (superoxide dismutase, peroxidase, glutathione reductase, ascorbate peroxidase, monodehydroascorbate peroxidase) (Wilson et al., 2014) and the relative expression of genes (heat shock proteins, osmotin, dehydrin, leaf embryogenesis protein, aquaporin), under heat stress (Razik et al., 2021). Seedlings exposed to heat stress with the addition of thiourea significantly improved ascorbic acid content compared to seedlings exposed to heat stress without thiourea (Ahmad et al., 2021a). Irenic improvement in catalase, ascorbate peroxidase, ascorbic acid, proline, and glycinebetaine was observed in response to thiourea supplementation compared to the control (without thiourea application) under heat stress (Catiempo et al., 2021). Proline accumulation is one of the early stress-induced plant responses that acts as a selective trait suitable for assessing abiotic stress tolerance. The ascorbate (AsA) and glutathione (GSH) cycles are fundamental for scavenging ROS (**Figure 7**). The activation and functions of antioxidants are sensitive to temperature ranges, but their concentration increases as temperature increases. Chakraborty and Pradhan (2011) assert that the concentration of APX (ascorbate peroxidase), SOD (superoxide dismutase), and CAT (catalase) increased at 50°C. Still, the concentration of GR (glutathione reductase) and POX (peroxidase) changes when the temperature ranges from 20 to 50°C in experiments performed using *Lens culinaris*. Consequently, the activity of the antioxidants depends upon the susceptibility and tolerance of plants, time of the season, and their growth stages (Almeselmani et al., 2006). Rani et al. (2013) exposed tolerant and susceptible genotypes of *B. juncea* to the high temperature of (45.0 ± 0.5°C), also observing the high content and activity of POX, APX, CAT, GR, and SOD in tolerant genotypes and less so in susceptible genotypes. Higher concentrations and activities of enzymatic and non-enzymatic antioxidants could be responsible for quenching the reactive oxygen species that help alleviate the negative impact of heat stress-induced oxidative stress.

CRISPR Technology

Abiotic stresses such as heat, salinity, drought, and waterlogging are critical limiting factors that affect growth, development, seed yield, and quality in oilseed crops (Boem et al., 1996; Purty et al., 2008; Elferjani and Soolanayakanahally, 2018). To date, several mechanisms have been discovered to analyze the mechanism of heat stress tolerance, including overexpression of various miRNAs (Arshad et al., 2017), antioxidant enzymes (Saxena et al., 2020), as well as genes encoding many transcription factors (Hao et al., 2011; Zhu et al., 2018), proteins involved in antioxidant activities (Kim et al., 2019) or osmoprotectants, and proteins facilitating phytohormonal signaling pathways (Sahni et al., 2016) in oilseeds. The success of conventional plant breeding techniques has been extensively studied to regulate heat stress tolerance mechanisms in various crops including oilseeds, but these techniques are very time consuming and cumbersome. As an alternative, genome editing using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) has been raised as an innovative technique for precise and efficient genetic manipulations in plant genomes (Subedi et al., 2020). Although, there is a discrete lack of information regarding negative regulators within the heat stress response, and thus studies involving CRISPR/Cas-mediated enhancement of high-temperature stress tolerance mechanisms remain scarce. The multiplex CRISPR/Cas9 system in the regulation of abiotic stress tolerance has been thoroughly reviewed in oilseed rape (Chikkaputtaiah et al., 2017). In this paper, we provide an overview of CRISPR/Cas GE technology in genome editing in oilseed crops, including primary editing (PE), base editing (BE), tissue-specific editing (CRISPR-TSKO), epigenome editing, and inducible genome editing (CRISPR-IGE), which can help to obtain resistant varieties that can tolerate the deleterious effects of high-temperature stress (Chennakesavulu et al., 2021) and has three dimensions, including adoption, crRNA biogenesis, and interference (Gasiunas et al., 2012; Jinek et al., 2012). Synthetic 20-nucleotide guide crRNA or RNA (gRNA) and Cas proteins are introduced into plants via a plasmid. Then, the crRNA or gRNA guides the effector nuclease Cas to identify and alter target DNA sequences in the plant genome. Subsequently, depending on DNA-RNA recognition and cleavage of the designated DNA sequences, CRISPR/Cas technology can be readily designed to induce double-strand breaks (DSBs) at any target site in the genome.

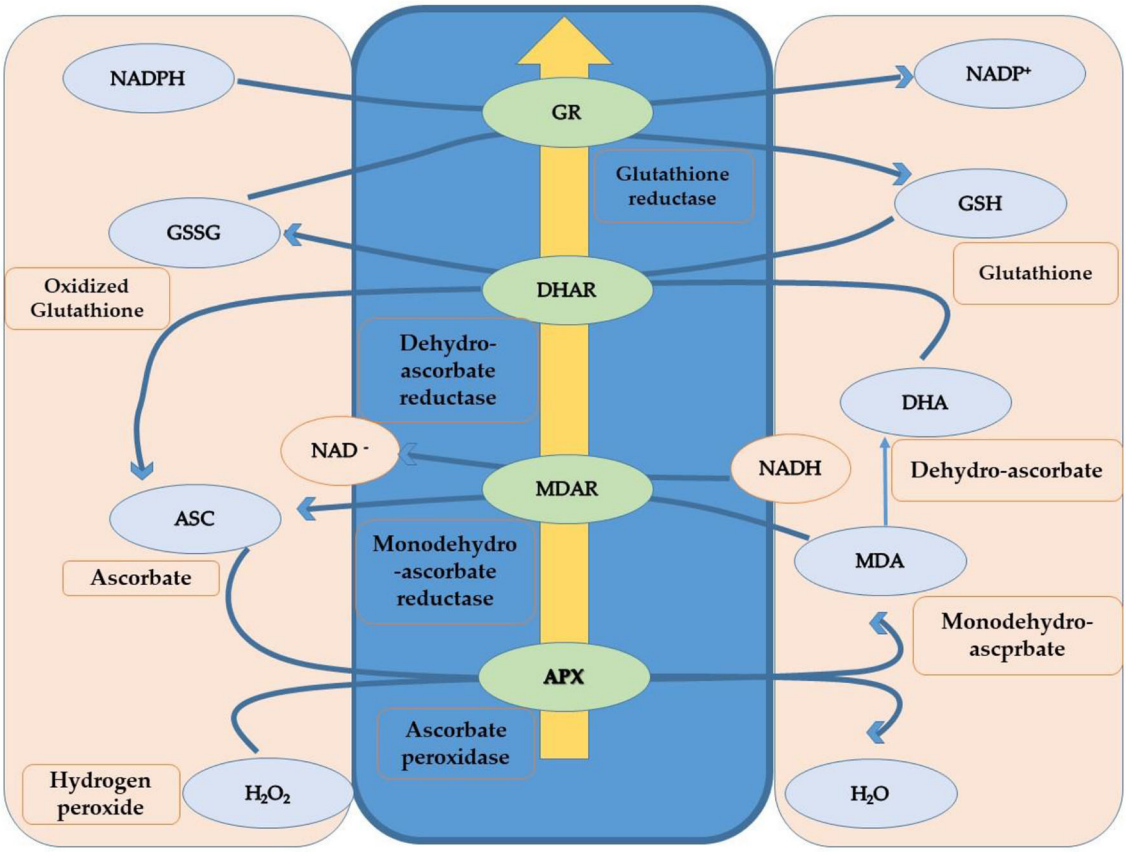

FIGURE 7 | Schematic diagram to show the ASC-GHS cycle to scavenge ROS.

DNA and RNA Base Editing

Genome-wide studies report that agronomically essential plant traits, including tolerance to abiotic stress, are conferred by introducing these beneficial alleles to one or more single nucleotide polymorphisms (SNPs) in plants, which takes breeders several years (Ren et al., 2010; Li et al., 2015; Singh et al., 2015). The error-free homology-driven repair in plants mediated by CRISPR/Cas allows for accuracy in genome editing by introducing these alleles, but with less effort and efficiency in delivering donor repair templates (DRTs). The discovery of CRISPR/Cas9 technology is a simple, easy, and versatile procedure for genome editing in *B. napus* and *B. oleracea* (Song et al., 2016; Li et al., 2021a). Two groups of base editors have been used (Komor et al., 2016), including the cytidine base editor (CBE), which performs C-G to T-A base substitutions, and the adenine base editor (ABE) is designed for A-T to G-C substitutions. The cytidine base editor consists of a cytidine deaminase (rAPOBEC1) fused to a Cas9 nickase (nCas9) carrying a D10A mutation that inactivates the RuvC domain yet is capable of binding with sgRNA (Komor et al., 2016). Adenine base editors contain an adenine deaminase fused to nCas9 to convert A-T bases to G-C via adenine (A) deamination (Gaudelli et al., 2017). Clustered, regularly interspaced short palindromic repeats (CRISPR) are repetitive and short DNA sequences of 29 nucleotides in length are separated by non-repetitive 32-nt spacer sequences integrated in the anterior portions of protospacer adjacent motifs (Song et al., 2016). Genome editing with CRISPR/Cas9 and its advanced versions have been intensively investigated with many applications: activation or repression of gene expression, gene mutation, and epigenome editing. In plants, the application of CRISPR/Cas9 technology is just emerging due to its high efficiency and simplicity (Song et al., 2016).

DNA Prime Editing

CRISPR/Cas9 and CRISPR/Cas12a arbitrated genome editing induces a DSB at the targeted sites (Manghwar et al., 2019), leading to unintentional changes or production of abnormal protein(s) due to random insets or removals in the plants. Though genome editing in base editing technology can be performed without double-strand breaks, base editors cannot perform additions, subtractions, and all types of base conversions (Mishra et al., 2020). To overcome these problems, a new approach to genome editing based on CRISPR, called primary editing (also known as genome editing by search and replace), has been discovered (Anzalone et al., 2019), because this approach

can write new genetic information by allowing all 12 base-to-base conversions, adding and removing desired nucleotides (up to 44 bp, respectively, 80 bp) in the plant genome without the need for double-strand breaks or donor DNA templates.

Epigenome Editing

Epigenetic editing includes DNA methylation and histone modifications and controls a plethora of critical procedures in plants, including stability of the genome, imprinting of genes, and expression of different genes under stressful environments (Zhang et al., 2018). Abiotic stress induces histone modifications and hyper/hypo-methylation of DNA, resulting in pressure inducible genes' activation or repression (Sudan et al., 2018). Song et al. (2012) reported that DNA methylation and histone modification might have a mutual effect on the stress-responsive genes in soybean.

Tissue-Specific Gene Knockout (CRISPR-TSKO)

Essential cellular functions, including growth and development and reproduction, depend upon some highly essential gene families. The removal or absence of these gene families might negatively impact plant performance or even become lethal to the plants (Lloyd et al., 2015). Hence, assessing the role of these genes in plants has rarely been undertaken to date (Lloyd et al., 2015). Researchers discovered a new genome-editing technique known as CRISPR-based tissue-specific knockout (CRISPR-TSKO) (Decaestecker et al., 2019). The Cas9 protein under CRISPR-based tissue-specific KO is expressed in the cell/tissue-specific promoter which leads to the spatial and temporal regulations of gene editing (Decaestecker et al., 2019).

Tissue Culture-Free Genome Editing

The delivery of CRISPR/Cas9 cassettes is often required in plant genome editing of the explant or recipient tissues in a culture which needs to be treated with many exogenous plant hormones to distinguish them in a whole plant that is expensive, time-consuming (Hiei and Komari, 2008), and only suitable for a limited number of species. The complex process of genome editing has been simplified successfully due to the introduction of new tissue culture methods or, in some cases, avoiding the step of tissue culture. In light of this new technique, the gene-edited somatic cells are reprogrammed into the meristematic cells by the co-expression of developmental regulators (DRs) with genome editing machinery (Maher et al., 2020) that helps to make genome editing faster and more straightforward.

Inducible Genome Editing (CRISPR-IGE)

The examination of gene functionality largely depends upon plant phenotypic analysis of the loss of function mutants. The knockout or mutations of several genes can be lethal for the plants during different growth stages throughout the life cycle (Lloyd et al., 2015). Therefore, the development of non-viable phenotypes hinders the comprehensive analysis of such vital genes. Whereas cell or tissue type-specified genome editing methods, like CRISPR-TSKO, exist for plants (Decaestecker et al., 2019), there is no method to eliminate a gene in a specific cell or tissue type in a conditional way. Recently, a new and inducible genome editing (IGE) technique has been discovered by merging CRISPR/Cas9 and a well-known XVE (LexA-VP16-ER) inducible technology (Wang et al., 2020). For instance, a heat shock-inducible CRISPR/Cas9 (HS-CRISPR/Cas9) system has been discovered by researchers to generate genetic mutation. The soybean heat-shock protein (GmHSP17.5E) gene promoter and rice U3 promoter (Czarnecka et al., 1989) were used to express Cas9 and sgRNA, respectively (Nandy et al., 2019). Thus, genome editing in Cas9 can only be performed after inclusion with exogenous heat-shock treatment.

DNA-Free Genome Editing

In CRISPR/Cas technology, target specificity has been provided by the Cas protein and sgRNA. These molecules are usually integrated in the plant genome through the biolistic approach or agrobacterium-mediated transformation technique. The unanticipated changes in the genome created by particular transgenes causes difficulty in upholding a stable phenotype of edited plants (Woo et al., 2015). Furthermore, the presence of CRISPR components in crop plants for a prolonged period may enhance off-target effects; therefore, the transgenes must be eliminated from the plant genome (Woo et al., 2015). Researchers have developed a new approach to solve this problem where rather than recombinant plasmids, preassembled gRNA-Cas9 protein ribonucleoproteins (RNPs) are delivered into the protoplast or *in vitro* zygote (Woo et al., 2015) via a gene gun or transfection. Consequently, the gRNA can direct Cas9 to simplify targeted gene editing without integrating a transgene (Woo et al., 2015), and endogenous proteases degrade Cas9 proteins in plant cells to minimize off-target effects.

The ERA1 (enhanced response To ABA1) and FTA (farnesyl transferase A) genes encode the α and β subunits of farnesyltransferase, which plays a role in ABA signaling, and transformation of these genes leads to hypersensitivity to abscisic acid and reduced stomatal conductance and transpiration rate (Allen et al., 2002; Wang et al., 2009). Stress-induced mutation of both BnERA1 and BnFTA genes in canola has been found to improve the proximity of yield protection under stress conditions (Wang et al., 2005). In addition, in allotetraploid cotton, simultaneous mutation of two paralogous GhARG genes mediated by CRISPR/Cas9-based non-homologous end-joining led to plants with high nitric oxide content and better lateral roots (Wang et al., 2017). For example, the salt overly sensitive (SOS) pathway consists of three major components: the protein kinase SOS2, the calcium-binding protein SOS3, and the plasma membrane Na+/H+ antiporter SOS1 (Zhu, 2002; Guo et al., 2004). Under conditions without a limiting growth environment, gigantea (GI), which is mainly associated with photoperiodic control of flowering and is a major component of stress tolerance (Ke et al., 2017), fixes SOS2 and arrests SOS1 activation. Other potential candidates for NHEJ-based CRISPR/Cas regulation of stress tolerance are specific members of the stress associated protein (SAP) gene family of oilseed species, which have A20/AN1 zinc finger domains and are often differentially defined under stress conditions (Xuan et al., 2011; Dixit et al., 2018). Overexpression of some SAP genes induces widespread

improvement in stress tolerance in many plant species (Dixit et al., 2018; Zhang et al., 2019).

Omics–A Fundamental Approach in Plant Breeding to Improve Abiotic Stress Tolerance

For several decades, scientists have focused on improving the outcome of significant crops under an ever-increasing abiotic stress environment. Even though the demand for oilseed crops has increased at a rapid rate, researchers never gave much attention to oilseed crops that can ensure food security and nutrition. This part of the article is focused on a fundamental breeding approach aiming to improve the performance of oilseed crops under abiotic stress. Therefore, the importance of omics technology in this context is peerless. Presently, almost 80–85% of rapeseed and soybean reference genomes have been sequenced (850 and 950 megabases, respectively) (Gupta et al., 2017). Similar to these efforts, widespread omics datasets have become available from different seed filling stages in other oilseed crops. Transcriptomic and proteomic studies have detected the majority of starch metabolism and glycolysis enzymes as the possible cause of higher oil in *B. napus* compared to other crops (Gupta et al., 2017). However, gaining insights through discrete omics approaches will never be sufficient to address research questions, whereas assimilating these technologies could effectively decode gene function, biological pathways and genome structures, and the metabolic and regulatory networks underlying complex traits. Hence the integration of omics technologies namely genomics, transcriptomics, proteomics, phenomics, ionomics, and phenomics, has a vital role in crop improvement (**Figure 8**).

Conventional Breeding Strategies

The main objective of traditional breeding is to develop high-yielding cultivars under normal environmental conditions. So, the determination of breeders leads the world to produce high-yielding varieties to enhance overall agriculture production (Warren, 1998). High-temperature stress due to climate change may affect the productivity of oilseed crops. Different investigations have been made by breeders and physiologists to develop heat stress tolerance traits in oil crops. They found that these traits are complicated to create because several genes are involved in controlling one specific feature (Blum, 1988). Therefore, stress quantification has a lot of serious issues. In field studies, natural selection is a tough job because of a number of environmental factors that could ruin the accuracy of what is needed for the breeding program. Under field conditions, heat stress is not a consistent phenomenon; it might occur or not which could mislead the breeders to find or develop a resistant trait (Driedonks et al., 2016).

Under a stressed environment scenario, genetic engineering is one of the best economic approaches to develop heat stress tolerance (Blum, 1988). The assessment, identification, characterization, and manipulation through genetic engineering for heat tolerance traits must be evaluated individually for the specific stages through the ontogenesis of crop plants. The heat sensitivity also varies among different species. One example of this is the changes in temperature threshold for groundnut at different growth stages ranging from germination (14–41°C), vegetative development (29–33°C), and reproductive growth (22–28°C) (Prasad et al., 1999, 2000) showing that reproductive growth is more sensitive to heat stress. Plant breeding has advanced to develop tolerant lines for heat stress in many crops, but the range of tolerance and genetic basis still needs to be revealed. The process of the development of new varieties is very costly and time-consuming; therefore, understanding the tolerance mechanisms might help to develop strategies for germplasm screening for the traits which are related to heat tolerance in different oilseeds. Some efforts have been made to build heat tolerance in oilseeds in recent times, for instance, in sunflower (Senthil-Kumar et al., 2003) and cotton (Rodriguez-Garay and Barrow, 1988). Breeders will be encouraged, if the availability of potential donors is good, to deploy such innovative sources in breeding directly but also to exhume the most robust alleles that have the ability to tolerate stress. Consequently, breeding mechanisms for thermo-tolerance are a new approach, and will require a lot of attention in the future. Nonetheless, if the objective is to speed up the progress in the breeding section, most of the emphasis must be placed on (i) the development of a precise/proper procedure for screening; (ii) identifying and characterizing thermo-tolerant genetic resources; (iii) every stage of growth and development of the plant on a genetic basis must be discerned; and (iv) for the transfer of tolerant genes to commercial crops, one must screen and prepare a vast breeding population (Siddique et al., 1999). Progressive molecular biology techniques might enable the development of plants with better thermo-tolerance.

Quantitative Train Locus

The breeders identified several tolerant genes and their inherent patterns through traditional breeding programs (Wahid et al., 2007). Conventional breeding and transgenic approaches helped us to understand the multi-genic trait phenomenon of heat stress tolerance. Multiple genes control different components, which are very important for heat tolerance in some other tissues and growth stages of the plant (Howarth, 2005). Current developments in genotyping assays and marker discovery set the basis toward the accurate chromosomal positioning determination of QTLs accountable for the heat tolerance in plants (Maestri et al., 2002). The discovery of QTL-linked markers empowers the breeding of stress tolerance pyramiding/uniting of QTL-associated tolerance to different stresses. Numerous major or minor QTLs and associated markers for thermo-tolerance have been recognized in oilseed crops such as groundnut (Selvaraj et al., 2009), sesame (Wang et al., 2016b), and soybean (Guo et al., 2010). Jha et al. (2014) prepared and summarized a list of QTLs linked with heat resistance of many crops with details of the total number of QTLs discovered, mapping of used population, PVE, positions of chromosomes, and associated markers. There are a number of proteins identified which are influenced by heat stress and cause floral abortion, including the cytochrome P450 family, associated with a reduction in the number of silique and abortion of pollens. Adenosine kinase-2 (Radchuk et al., 2006),

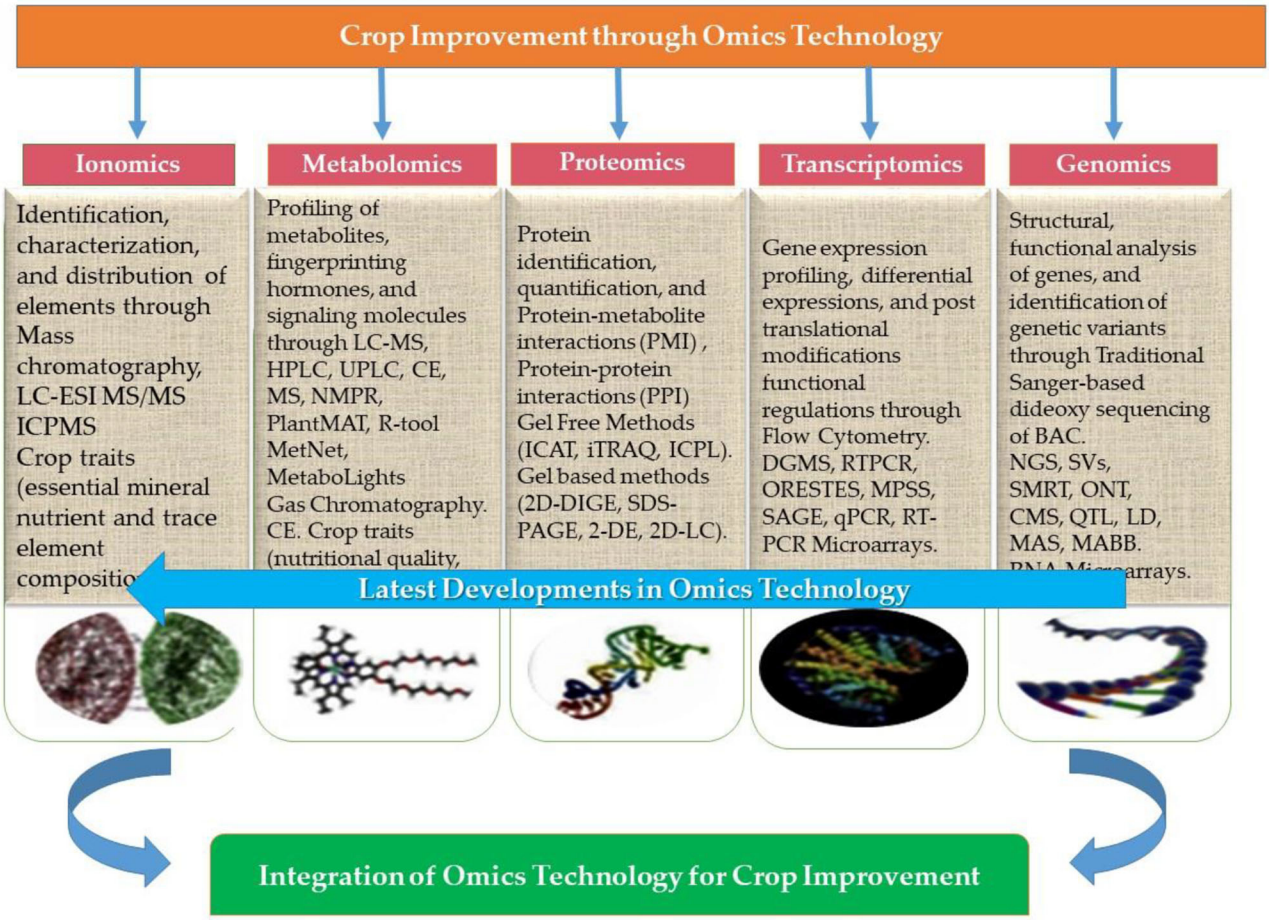

FIGURE 8 | Integration of omics approaches (genomics, transcriptomics, proteomics, metabolomics, and ionomics) for crop improvement (modified form of Zargar et al., 2016).

a protein of pentatricopeptide repeat-containing family (PPR) is linked to the obstruction of flower and pod growth via embryonic abortion, and proteins of the MATE efflux family are associated with embryo development (Zhao et al., 2015). Embryonic and seed abortion-related proteins were also found like pyruvate kinase family protein (Radchuk et al., 2006), phosphatidic acid phosphohydrolase 2, lysine methyltransferase family protein, RGA-like protein 3 (Fischinger and Schulze, 2010), and phosphoenolpyruvate carboxylase 3. There are a few candidate genes that have been identified which were associated with QTLs under artificial heat stress conditions for different oilseed traits.

QTL Mapping/Linkage Mapping and Linkage Disequilibrium (LD)/Genome-Wide Association Mapping

For QTL mapping, one must have the genomic resources in the shape of genome maps and molecular markers and genetic resources in the condition of the bi-parental mapping population. For the major oilseed crops, some genome maps and molecular markers have been identified (Sun et al., 2007; Xia et al., 2007; Chen et al., 2014; Wang et al., 2016b; Talukder et al., 2019). QTL mapping has been adopted for the genes of complex traits in a number of oilseed crops, for seed and oil yield (Shi et al., 2009) and abiotic stress tolerance (Kiani et al., 2007). An alternate approach of QTL mapping which is now being used in crop science known as LD-based association mapping (AM) was utilized in genetic studies of humans in the early days of its inception. The basis of AM in the germplasm collection is the correlation between phenotype and genotype. The use of AM in QTL detection has many advantages on bi-parental linkage mapping, such as (1) manipulation in all events of recombination that happened in the crops' evolutionary history that resulted in the much-advanced resolution of mapping; (2) in this case, there is a need to prepare a particular population that shortens the time required in QTL mapping, and (3) as linkage mapping is suitable for the study of only two alleles, AM can detect/study a higher population of alleles (Neale and Savolainen, 2004). However, AM has its drawbacks, including a specious/false-positive linkage between a trait and a marker. Many statistical tools have been developed to address the problem

of hidden population structures (Falush et al., 2003). The leading causes for these problems are mating systems, genetic drift, and incorrect selection (Flint-Garcia et al., 2003). The genome-wide association mapping system under high-temperature stress is being used in several oilseed crops like soybean (Li et al., 2014), rapeseed (Cai et al., 2014; Zhu et al., 2017), cotton (Sun et al., 2019), sunflower (Dowell et al., 2019), groundnut (Jiang et al., 2014), and sesame (Wei et al., 2013).

Transcriptomics: A Key to Understanding Abiotic Stress Responses in Plants

The study of the transcriptome from a specific tissue, a particular organ, or organism under specified circumstances is known as transcriptomics. Transcriptomics characterizes the transcriptome as a whole where all the expressed genes have been studied at one term, underneath a particular physiological condition or developmental stage. It paved the way to understand how the plant responds to abiotic stresses. The transcriptomic approach is much more complicated than the genome that encodes it as a number of the same types of mRNA can be produced by one gene that encodes various proteins through alternate splicing. In *B. napus*, the levels of DNA methylation increased more in a heat-sensitive than a heat-tolerant genotype under heat stress (Gao et al., 2014). Transcriptomic analysis by next-generation sequencing (NGS) and RNA–seq for sRNAs has primarily improved genomic resources since it was used in genomics research (Ulfat et al., 2020). In contrast to the past, sequencing-based and hybridization-based approaches can help understanding of the gene expression of multiple genes at a time at the whole-genome level. Microarray technology is a leading technology in hybridization-based methods that was used in oilseed crops for large-scale gene expression, for example, *B. napus* (Raman et al., 2012), sunflower (Fernandez et al., 2012), soybean (Ding et al., 2021), and groundnut (Guo et al., 2011; Xiao-Ping et al., 2011). We have better alternatives of gene expressions through sequence-based approaches like serial analysis of gene expression (SAGE), expressed sequence tags (ESTs), open reading frame EST (ORESTES), and digital expression analysis (RNA-seq) by utilizing generation sequencers and massively parallel signature sequencing (MPSS) (Marioni et al., 2008; Campobenedetto et al., 2020). Whole-genome RNA-seq became more convenient, and the gene expression at the whole genome level was rapid due to the emergence of NGS. This is helpful in organisms with a limited genome and some non-model lacking reference genes (Strickler et al., 2012). RNA-seq has been used in many oilseeds like in canola (Jiang et al., 2013), soybean (Kim et al., 2011; Ding et al., 2020), oil palm (Shearman et al., 2013), sunflower (Fass et al., 2020), and groundnut (Chen et al., 2013). Phylogenetic, collinearity, and multi-plesynteny analyses exhibited dispersed, segmental, proximal, and tandem gene duplication events in the HSF gene family. Duplication of gene events suggests that the HSF gene family of cotton evolution was under strong purifying selection. Expression analysis revealed that GhiHSF14 is upregulated in heat stress in cotton (Rehman et al., 2021). The microarray data characterize numerous tissues, developing stages, and ecological situations as shown in **Table 2**.

Proteomics Approach

An emerging technology that can provide a precise and tremendous amount of information regarding various metabolites and proteins generated due to abiotic stresses is proteomics (Rodziewicz et al., 2014). The role of proteomics is to decipher the importance of redox homeostasis, chaperons or heat shock proteins, proteins essential in signal transduction, and metabolic pathways during heat stress. It endorses the amount of protein present and sends direct information, giving more precise knowledge and a level of understanding compared to genomics. The sustainability and crop improvement in oilseeds can be achieved by integrating proteomics and genetic data of root systems under high temperatures in oilseeds (Valdés-López et al., 2016). The primary problem of proteomics is the presence of multiple genes at one time that have gone through PTMs.

Nevertheless, this technology is emerging fast with a principal focus on protein interactions, protein quantity, and post-translational modifications (Champagne and Boutry, 2013). Generally, proteomics can be used for proportional expression analysis of two or more protein samples, for understanding post-translational variations, for proteome profiling to recognize how proteins perform biological progressions, for learning of protein-protein relations, and for ascertaining novel biomarkers to sense and screen exact stress expressions (Chandramouli and Qian, 2009). Several significant experiments have been conducted using the proteomics approach in oilseed crops (**Table 2**). In oilseed crops, the best way to improve stress tolerance is to associate different candid proteins that are physiologically significant. In oilseed crops, proteomic identification has been made by using both the gel-free and gel-based proteomics approaches (Chandramouli and Qian, 2009), and separation is done by using the most frequently used gel-based strategies, including one-dimensional gel electrophoresis (SDS-PAGE) (Han et al., 2013; Messaitfa et al., 2014; Li et al., 2020) and 2D-polyacrylamide gel electrophoresis (2-DGE) (Ghaffar et al., 2020). MS techniques like ESI and MALDI TOF proteomic-based experiments are very accurate and more precise due to the accessibility of many genomic sequences of many organisms and EST information. Though, it has some technical issues like restricted dynamic resolutions when a substantial number of proteins are drawn to analyze the identification and separation of hydroponic proteins and obtainability of the pure proteome. These challenges can be overcome by using fluorescent dyes, application of affinity chromatography, reverse phase HPLC, and explicit fractionation techniques. Besides, we can characterize the complete proteome through high-throughput techniques (i.e., robotics, spectrometers, and multi-dimensional chromatography). These valuable tools have already been tested. For instance, the functions of different proteins have been evaluated using proteomics in other plants like soybean (Mooney et al., 2004; Natarajan et al., 2006). In addition to all that, new gel-free, highly efficient methods have been developed for proteomic analysis. This discovery opens the possibility of identifying many genes and replacing the

TABLE 2 | Omics studies on heat stress tolerance in different oilseed crops.

Crop	Temperature (°C)	Omics techniques	Plant part	Method	Trait/treatment stage	No. of proteins/genes differentially identified	Location	References
Soybean	42	Genomics	Seeds	RT-PCR, qRT-PCR analyses	HSF family genes	38	China	Li et al., 2014
Canola	40	Genomics	Seeds	RNA-seq and qRT-PCR analysis	HSF gene family	64	China	Zhu et al., 2017
Soybean	35	Transcriptomic	Seeds	RNA-Seq analysis	Biostimulant mechanism	879	Italy	Campobenedetto et al., 2020
	38/32	Transcriptomic	Male organ	Real-time PCR (qRT-PCR)	Cytoplasmic male sterility (CMS)-based hybrid (F1)	8,784	China	Ding et al., 2020
	38/32	Transcriptomic	Male organ	qRT-PCR	Cytoplasmic male sterility (CMS)-based hybrid (F1)	1,145	China	Ding et al., 2021
Sunflower	45	Transcriptomic	Seedlings	qRT-PCR	Phenological traits	97	Argentina	Giacomelli et al., 2012
Cotton		Transcriptomic	Seeds	Multiple sequence alignments (MSA), NA-seq expression	Cis-regulatory elements	79	China	Rehman et al., 2021
Canola	40/30	Proteomics	Leaf	RPLC, LC-MS/MS	Carbohydrate metabolism, HSPs, and chaperones	1,022	China	Yuan et al., 2019
Soybean	37	Proteomics	Anther	SDS-PAGE	Reproductive organs	371, 479, and 417	China	Li et al., 2020
	40	Proteomics	Roots	LC-MS/MS	Root hairs and stripped roots	1,849 and 3,091	USA	Valdés-López et al., 2016
Sunflower	33/29	Proteomics	Leaf	HPLC	Reproductive stage	2,343	Spain	De La Haba et al., 2020
Soybean	42/26	Metabolomics	Seed	UPLC/MS/MS2, UP LC/MS/MS2, GC/MS	Oil	275	USA	Chebrolu et al., 2016
	43/35	Metabolomics	Leaf	UPLC/MS, GC/MS				Das et al., 2017
Canola	31/14	Metabolomics	Floral buds	Gas chromatography–mass spectrometry GC–MS	Heat	25	Canada	Koscielny et al., 2018
Soybean	45/28	Phenomics	Leaf	OJIP protocol of a Fluorpen Z995-PAR	Vegetative (4th leaf stage)		USA	Herritt and Fritschi, 2020
	42/28	Phenomics	Leaf	PAM fluorometer, SPAD	Germination		India	Jumrani et al., 2017
Brassica	35/25	Phenomics	Leaf	Scanalyzer, LC, PRI, Qy	Reproductive stage		Australia	Chen et al., 2019
Cotton	38	Phenomics	Leaf	IRGA, Spectro-photometer	CMT, CSI		USA	Singh et al., 2013

low-throughput techniques. The gel-free techniques currently used for oilseed crops include ICAT (isotope-coded affinity tagging) (Oh et al., 2014), MudPit (multidimensional protein identification technology) (Agrawal and Rakwal, 2008), iTRAQ (isobaric tagging for relative and absolute quantitation) (Li et al., 2020), and SILAC (stable isotope labeling by amino acids in cell culture) (Zargar et al., 2013). The novel techniques of the long-column method, 2D-LC, and iTRAQ OFFGEL fractionation, have been developed to identify low abundance proteins. Progressive automatic peptide purification systems with great accuracy and more reproducibility are a crucial task in plant proteomics (Zargar et al., 2013). Additionally, iTRAQ is more precise and consistent for protein quantitation than traditional 2-DE analysis (Qin et al., 2013).

Metabolomics Approach

The genes and proteins that play a crucial role in plant stress responses are identified using genomics, transcriptomics, and proteomics. The boundary of metabolic pathways and regularity networks responding to a specific stressor or a number of simultaneous stresses is needed for the proper understanding of stress response in plants. A new zenith has been provided by metabolomics for stress-related studies in crop plants and has become a crucial tool to understand the molecular mechanisms underlying stress responses (Weckwerth and Kahl, 2013). Targeted organism's metabolomics is a non-biased, comprehensive, and high-throughput analysis of the complex metabolite mixture. This is an important technique that, in collaboration with genomics, transcriptomics, and proteomics, can provide a missing link in functional genomics, offer new insights into the study of systems biology, and can more accurately elucidate biological mechanisms (Saha et al., 2019). With further advances in proteomics, metabolomics is a dynamic technique to functional genomics that allows us to recognize and quantify metabolomes within a single cell, organ, or organism (Chebrolu et al., 2016). Plant metabolism undergoes specific configurational changes to achieve metabolic homeostasis. It synthesizes different compounds to mitigate the adverse effects of any stress it may experience in its life cycle. There have been significant advances in metabolomics that could provide greater insight into the various mechanisms of thermo-stress tolerance at the metabolic level (Bokszczanin and Fragkostefanakis, 2013). For example, in studies on soybean, multiple antioxidants have been found to play a role in improving thermo-tolerance through metabolite studies (Chebrolu et al., 2016). Metabolomics techniques include some separations approaches like HPLC, capillary electrophoresis (CE), gas chromatography (GC), mass spectroscopy (MS), ultra-performance liquid chromatography (UPLC) along with detection techniques like nuclear magnetic resonance (NMR) (Das et al., 2017). In metabolite studies, one has to be focused on all the metabolites at one time because metabolism in plants is very dynamic and every single aspect might be linked with some other metabolites with different expressions; moreover, it might produce multiple metabolites at certain times (Fischbach and Clardy, 2007). Several key proteins involved in seed storage proteins, fatty acid metabolism, allergens, and toxins connected with the development of castor oil seeds were recognized by engaging an isobaric tag for relative and absolute quantification (iTRAQ) and isotope-coded protein labels (ICPLs) and technologies. Understanding the major metabolites in soybean plants in response to high-temperature conditions can help in the development of heat-resistant varieties. The concentrations of flavonoids, ascorbate (AsA) precursors, and tocopherols were higher in heat-tolerant genotypes than in heat-sensitive ones (Feng et al., 2020), and these metabolites can alleviate the adverse effects caused by damage from heat-induced reactive oxygen species (ROS) during seed maturation under high temperatures (Das et al., 2017). Many studies have shown that ROS-scavenging mechanisms play an important role in protecting plants from heat stress (Xu et al., 2016).

Ionomic Approach

This refers to an omics study that deals with all the quantitative documentation of the whole set of ions in an organism under various external stimuli and then quantifies the changes in ion production. The production of these ions can be elucidated by different biochemical pathways that play a vital role in mineral transport—enzyme catalysis is a cofactor in some important regularity pathways—and maintaining the integrity of the cell. Therefore, any variation or change in the process of ion production results in serious changes in metabolic processes. The plant ionome must be studied to understand the key role of ions to carry life processes. Therefore, a good understanding of gene regulation can be achieved by ionomic-based studies. These ionomic studies are known to differentiate natural alleles and different mutants (that might have variation in one or several elements) (Chen et al., 2009). Ionomics is very important to understand the elemental composition profile and their role in the nutritional requirement and physiological and biochemical functionality. It was observed that these two genes alter the ionome and elements that present in the leaf. So, these variations caused the shift in gene expression and change the multi-elemental profile due to variation in water and ion transport (Ziegler et al., 2013). Additionally, multi-elemental profiling helps to detect mutants with various numbers of ions in soybean seeds (Ziegler et al., 2013). The plant elemental profile is controlled by a number of factors like availability of elements, their uptake, their transport, and external conditions that carry out evapotranspiration. These factors have made the ionome plan very specific and very sensitive so that the elemental composition shows several different states. Silicon is found to have a good role in the abiotic stress tolerance in plants (Liang et al., 2007) but soybean cannot accumulate silicon due to some genetic differences, except recently a silicon transporter gene was introduced in soybean by using ionomic technologies (Deshmukh et al., 2013).

Phenomics Approach

In recent years, great progress was made in the field of genomics with the objective of understanding and unraveling crop genomes. It incorporates the development of various kinds of genotyping platforms: molecular markers and the study of marker-trait associations lead to the innovation of genes/QTLs,

and genetic mapping procedures, which make main crop genome sequences accessible and garners improvements in sequencing technologies, lead to a decline in the costs of sequencing. The sequencing methods have certain improvements that have made the crop genomes and plant sequencing genomes monotonous (Jackson et al., 2011). Additionally, it is possible to identify allelic variation through crop genome sequencing (Furbank and Tester, 2011). Some additional costs of genotyping of the plant genome could be reduced with the advancement in high-throughput markers and genotypic platforms (Jumrani et al., 2017).

Furthermore, for the more accurate results from the other omics techniques like proteomics, transcriptomics, genomics, etc., one must link their information with proper phenotyping. The bottlenecks in phenotyping are (i) phenotyping in replicated trials in numerous situations over years; (ii) sluggish and expensive phenotyping; (iii) for QTL/gene discovery, the phenotyping of large mapping populations, followed by the cloning of significant QTLs; (iv) less accurate approximations of phenotypic data for testing allelic disparities of a candidate gene in a germplasm set; and (v) destructive tools used in phenotyping at static times/growth stages (Furbank and Tester, 2011). All of these are responsible for the gap created between genotypes and phenotypes referred as the GP gap. For the resolution of this problematical bottleneck, the phenomics revolution is the need of the hour. Efforts are made worldwide to overcome this problem by evolving plant phenomics amenities that can scan and measure data for hundreds of thousands of plants in a day in a cultured way (http://www.plantphenomics.org.au/). These efficient phenomics amenities make use of good non-invasive imaging, image analysis, spectroscopy, high-performance computing facilities, and robotics hence saving labor, cost, and time. Consequently, combined with all other omics approaches, phenomics has a considerable future in plant breeding and genetics (**Figure 9**).

AGRONOMIC APPROACHES

Nutrient Applications

High-temperature stress that could lead to nutritional deprivation is a significant factor contributing to impaired plant growth and development. At the same time, exogenous application of nutrients may alleviate the negative impacts of heat stress coupled with fulfilling the nutritional requirement. Studies have revealed the ameliorating effects of nutrient applications. Nevertheless, foliar or extracellular application of nitrogen (N) and potassium (K) may improve the ability of the plant to tolerate high-temperature stress (Hammac et al., 2017; Muhammad et al., 2019). The application of micronutrients, such as Se (selenium), B (boron), Mn (manganese), and macronutrients, such as nitrogen (N), potassium (K), sulfur (S), and calcium (Ca^{2+}) can modulate leaf water status, stomatal regulation, and upregulation of physiological and metabolic processes that increase heat stress tolerance (Waraich et al., 2012). Seed priming with potassium nitrate played an important role in mitigating heat stress by increasing the concentration of nitrate reductase, catalase, peroxidase, proline enzymes, and chlorophyll content, which helped sesame to maintain its performance under stress conditions (Kumar et al., 2014, 2016). High-temperature stress reduces net carbon gain and dry matter production in soybean under both P application and P deficiency conditions and reduces net carbon gain (Singh et al., 2018). Increased S supply has been shown to lead to higher levels of total glucosinolates in *Brassica rapa* (Li et al., 2007) and individual glucosinolates such as glucoraphanin and glucoraphasatin (Krumbein et al., 2001), sinigrin, glucobrassicanapin, gluconapin, and progoitrin in *Brassica juncea* (Kaur et al., 1990), which helped induce heat tolerance at elevated temperature. In addition, the application of sulfur at high temperatures increased the activity of various enzymes, including nitrate reductase, glutamine synthase, and glutathione dehydrogenase, which are essential in nitrogen metabolism in sunflower (Ahmad et al., 2020). Foliar-applied sulfur alleviated the deleterious impact of high-temperature stress in canola by increasing plant gas exchange attributes including photosynthesis and stomatal conductance which depends upon the water status in the plant cells and regulates the gaseous exchange to improve yield and yield components in camelina (Waraich et al., 2021b). In oilseed crops, the role of sulfur is undeniable as it helps to improve the seed quality parameters. In contrast, in the absence of sulfur, the seed oil content decreased in oilseed crops under average or heat stress conditions (Brunel-Muguet et al., 2015). However, sulfur with expected thermo-sensitization effects may also have the impact a few nutritional (fatty acids, seed storage protein concentration) and physiological (IAA, SA, ABA: GA3 ratio) quality criteria, as well as the antioxidant capacity in *B. napus* (de Almeida et al., 2021).

Plant Growth Regulation

With a naturally induced defense system, many chemical compounds regulate the plant activity under heat stress at physicochemical levels (Ahmad et al., 2021a). To improve the growth and productivity of oilseed crops under environmental stress, the supplementation of plant growth regulators (PGRs) either through seed or on foliage holds a superior position. The plant growth regulators are chemicals that may regulate the growth, physicochemical attributes, and stress tolerance mechanisms under stressful environments (Shah et al., 2021). Thiourea, as a plant growth controller, may mediate plant growth under high-temperature stress. Thiourea (TU) is a growth promoter under stress conditions due to its redox regulatory property imparted by the –SH group and regulates the cell homeostasis to induce stress tolerance (Sahu, 2017; Wahid et al., 2017). Waraich et al. (2021a) revealed the role of thiourea to upregulate the gas exchange and water relations in camelina genotypes grown under high-temperature stress. The results of the current study showed that application of thiourea (applied either at the vegetative or reproductive stage) improved the growth and yield under heat stress by maintaining the gas exchange traits, antioxidant enzyme activities, and osmoprotection in sunflower (Akladious, 2014), canola (Ahmad et al., 2021a,c), and camelina (Ahmad et al., 2021b) as shown in **Figure 10**. Exogenously applied abscisic acid enhances plant defenses by regulating the accumulation of soluble sugars that improve the lipid profile in castor bean (Chandrasekaran et al.,

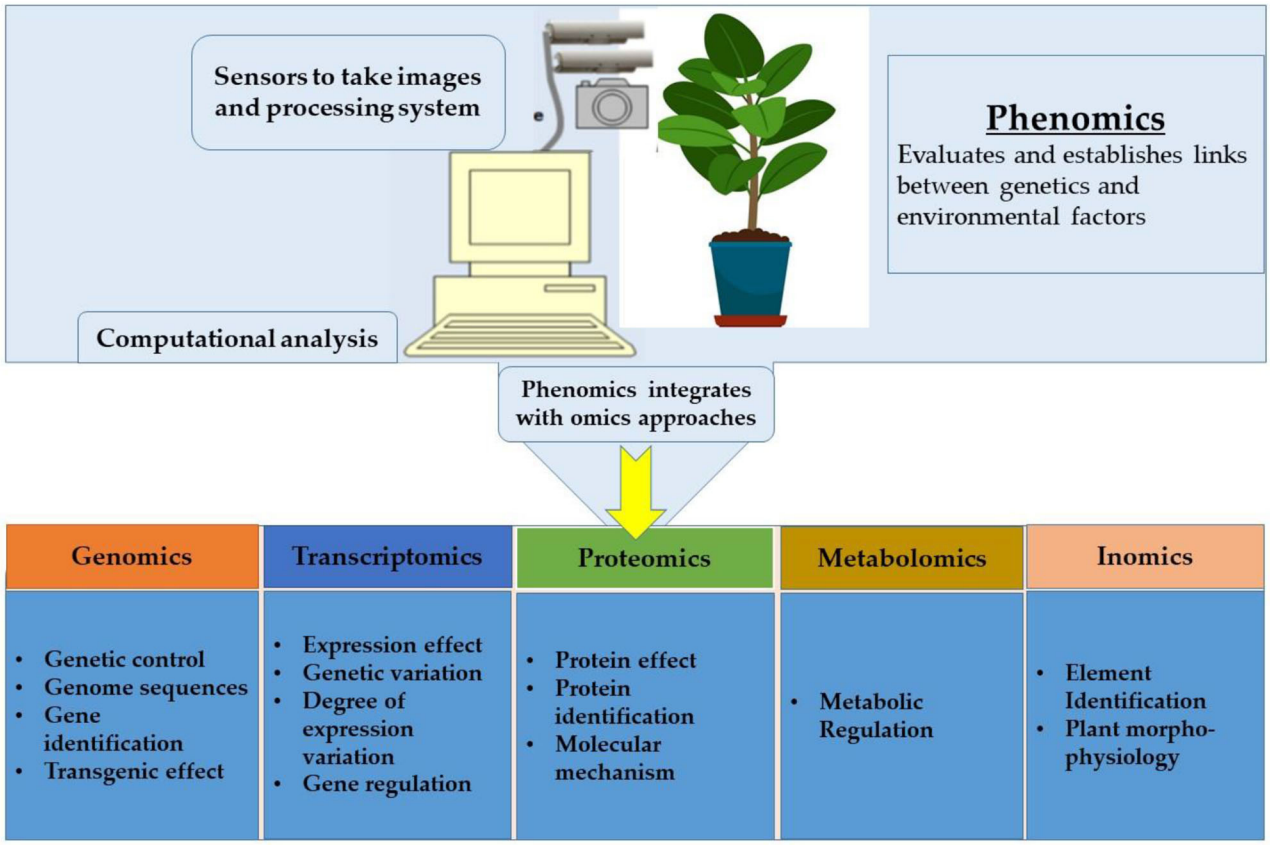

FIGURE 9 | Phenomics and its integration with other omics approaches (adopted from Deshmukh et al., 2014).

2014), while in *Brassica napus* it increases the accumulation of a synthetic brassinosteroid (24-epi-BL) that induces heat tolerance (Kurepin et al., 2008). γ-aminobutyric acid (GABA) significantly improved the accumulation of osmolytes including proline, soluble proteins, and sugars, activities of antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, glutathione reductase, peroxidase, monodehydroascorbate peroxidase), and relative gene expression (dehydrin, heat shock proteins, osmotin, leaf embryogenesis protein, aquaporin) (Razik et al., 2021), which helped to reduce hydrogen peroxide and malondialdehyde content in GABA-treated plants compared to untreated plants under heat stress with an increase in the levels of gene transcripts encoding antioxidant enzymes, suggesting that GABA regulated antioxidant defenses and could be partly responsible for the improved heat tolerance in sunflower (Razik et al., 2021). Heat stress is one of the causes of gamma-aminobutyric acid (GABA) accumulation in sesame (*Sesamum indicum* L) plants (Bor et al., 2009). Similarly, Bor et al. (2009) reported that a short heat shock interval also increased the endogenous GABA content in pea and sesamum indicum plants. Pre-treatment of soybean seeds with 1 mM of putrescine (Put), spermidine (Spd), and spermine (Spm) alleviated heat stress-induced damage by improving growth parameters and antioxidant defense compared to water-sprayed control (Amooaghaie and Moghym, 2011). Brassinosteroids positively affect plant responses to abiotic stresses by maintaining Na+ homeostasis, metal sequestration, increasing heat shock protein synthesis, enhancing GRX (glutaredoxin) and NPR1 (non-expressor of pathogenesis-related genes 1) for redox signaling, and increasing the activities of enzymes involved in the ascorbate-glutathione cycle (Ahammed et al., 2020).

Microbial Inoculation

A number of microorganism-based stress mitigation mechanisms have been proposed for plant species. Microbes belonging to different genera of *Achromobacter, Variovorax, Azospirillum, Bacillus, Azotobacter, Enterobacter, Klebsiella, Aeromonas*, and *Pseudomonas* have demonstrated the ability to enhance plant growth even under adverse environmental conditions (Arkhipova et al., 2007) including high-temperature stress (de Zelicourt et al., 2013; El-Daim et al., 2014). Microbial inoculation enhances the regulation of the plant defense system by improving the production of enzymatic and non-enzymatic antioxidants along with the production of osmolytes under high-temperature stress. Endophytic fungus has been found to stimulate vegetative growth and biomass production due to its role in mediating the photosynthetic system including chlorophyll content compared to non-inoculated plants (Ismail

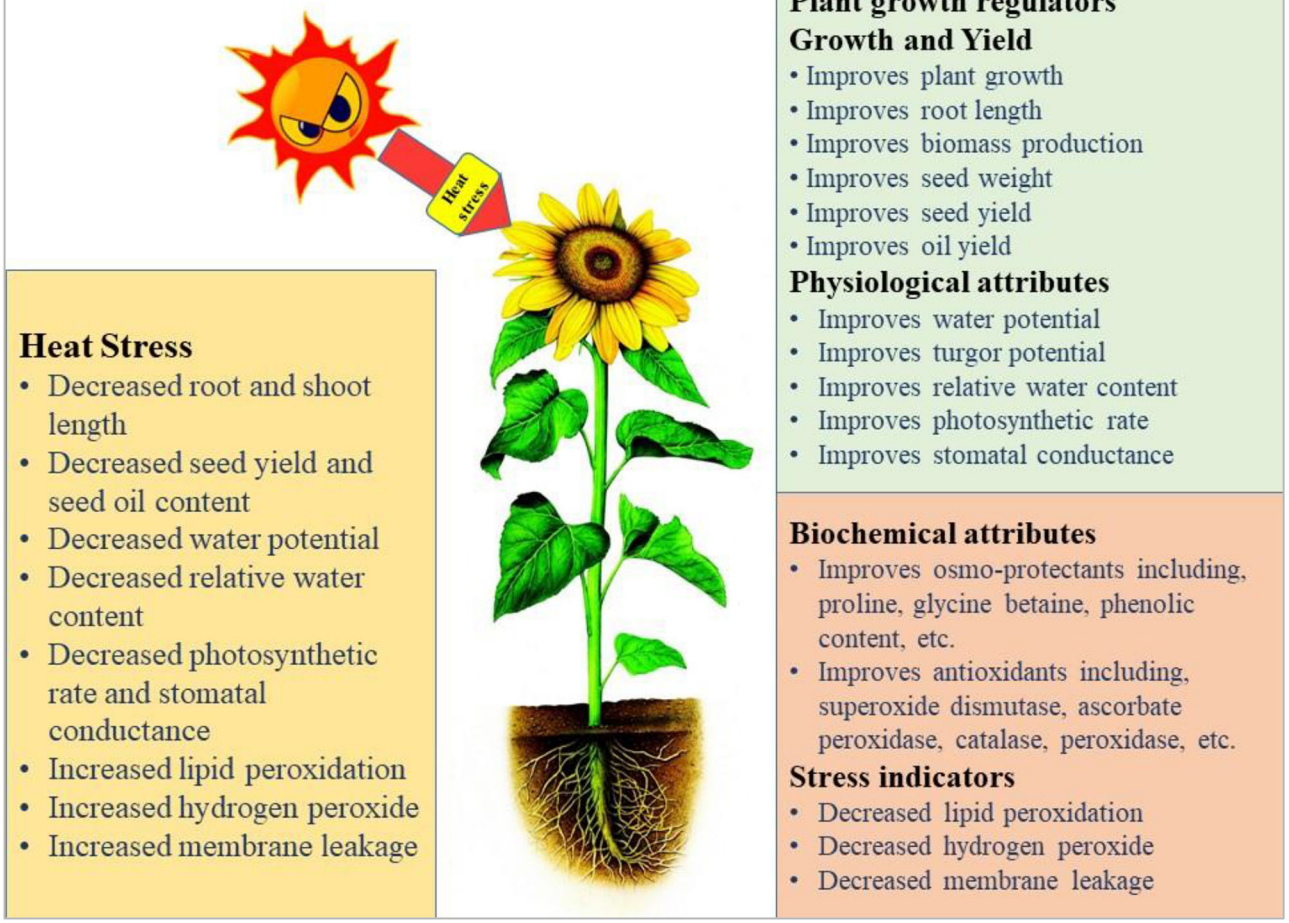

FIGURE 10 | Effect of heat stress and application of plant growth regulators on phenological, physiological, and biochemical properties of oilseed plants.

et al., 2020). Several microbes have been found to play an ameliorative role at elevated temperature by improving the antioxidant content of plants, viz., *Bacillus tequilensis* (SSB07) was very promising for mitigating the negative effects of climate change on crop production as it improved root/shoot length, biomass, leaf development, the content of photosynthetic pigments, endogenous jasmonic acid, and salicylic acid in the phyllosphere, and significantly reduced stress-responsive ABA overproduction (Kang et al., 2019). The improvement in plant growth was reflected by greater plant height, leaf area, biomass, and photosynthetic pigment production under heat stress and expected conditions in the inoculated plants. Under stress conditions, *Glomus intraradices* and *G. mosseae* were found to improve seed oil content in *B. napus* (Keshavarz, 2020). Application of *Bacillus cereus* SA1 under high-temperature stress enhanced the defense system of soybean plants by increasing superoxide dismutase activity, ascorbic acid peroxidase and glutathione content, and expression of heat shock proteins (GmLAX3 and GmAKT2), which have been linked to reduced detoxification of reactive oxygen species, increased potassium gradients, and altered auxin and ABA stimuli, and which are critical for plants under heat stress (Khan et al., 2020). *Bacillus tequilensis* (i.e., SSB07) improved the growth of Chinese cabbage seedlings and produced the gibberellins GA_1, GA_3, GA_5, GA_8, GA_{19}, GA_{24}, and GA_{53}, as well as indole-3-acetic acid and abscisic acid. The application of *B. tequilensis* SSB07 was also found to increase the shoot length and biomass, leaf development, and photosynthetic pigment contents of soybean plants. Under heat stress, SSB07 injection significantly increased the endogenous jasmonic acid and salicylic acid contents of the phyllosphere and significantly downregulated the production of stress-responsive ABA. Thus, *B tequilensis* SSB07 shows promise for countering the harmful effects of climate change on crop growth and development (Kang et al., 2019).

Smart Agricultural Technology

The latest technologies applied to crops can reveal plant responses to various biotic and abiotic stresses. However, the practical application of these technologies is not widespread

among stakeholders due to their high cost. Among irrigation techniques, sprinklers, gravity irrigation, subsurface drip irrigation, and center pivot irrigation can cool plants at elevated temperatures, which would be the preferred method for commercial growers to manage crop losses due to heat stress to reduce water losses from traditional irrigation methods. Soil moisture is critical during heat stress. Shade covers, made of lightweight materials or those commercially available in various materials, are an inexpensive strategy applied on a large scale to manage heat stress. In addition, remote sensing technologies that use thermal imaging, satellite imaging, thermal imaging, and hyperspectral sensing are being used to detect heat stress before symptoms are detectable, thus preventing agricultural losses (Hernández-Clemente et al., 2019). An airborne infrared/visible imaging spectrometer was used to quantify heat stress tolerance based on changes in soil surface temperatures (Shivers et al., 2019). The photochemical reflectance index (PRI) measured by aerial hyperspectral scanners reveals the moisture status of crop plants concerning heat stress to allow gradual feeding without adverse effects on proper growth and development (Suárez et al., 2008). New discoveries in remote sensing and plants genomics enable climate-smart agriculture by developing climate-resilient crops (Jumrani et al., 2017; Hossain et al., 2021). Remote sensing techniques may help obtain accurate calibrated measurements of environmental factors that affect the performance of oil crops over a range of spatial and temporal resolutions and thus help sustain agricultural productivity under heat stress. Chlorophyll fluorescence measured using a spectro-radiometer and chlorophyll fluorometer under high-temperature stress may help induce a heat tolerance mechanism in plants in cotton (Van der Westhuizen et al., 2020). Therefore, chlorophyll fluorescence techniques may help in non-invasive eco-physiological studies to access responses of plants against high-temperature stress (Jumrani et al., 2017). However, the fluorometer in the leaf chamber is a pulse amplitude modulation (PAM) fluorometer to measure leaf fluorescence in light and dark-adapted leaves, which can be used to recognize the basis of photosynthesis and plant responses to environmental changes (Khan et al., 2020). Along with physiological observations, plant morphological attributes, such as leaf curling early in the morning at low air temperature, which indicate the onset of high-temperature stress, can be used to identify the negative effects of heat stress in oilseed plants. Curled leaves impair the transpiration rate by reducing leaf surface area, which reduces light interception, affecting the water and nutrition uptake. Cell sap observations in the early morning can also be used to identify the impact of high-temperature stress. However, the crop stage at the onset of heat stress is imperative for determining the type of treatment to alleviate the impact of high-temperature stress. Under the shadow of smart technologies, genome editing (GE) is one of the most powerful techniques to improve heat stress tolerance by manipulating the genome sequence in plants. Genome editing may help improve crop performance under high temperature, and has shown a remarkable potential to tackle the insecurities of the food industry and develop a climate-smart agriculture system globally (Liu et al., 2013). On the other hand, plant nutrition also has an important role in heat stress in oilseeds because foliar spraying of Zn regulated the physiological properties of plants which helped to increase the number of siliques per plant, number of seeds per silique, thousand seed weight, seed yield, seed oil content, and linoleic acid content. In contrast, erucic acid, stearic acid, and glucosinolate were decreased (Rad et al., 2021). However, the improvement of Zn supplementation may increase seed oil content due to the production of auxin biosynthesis, chlorophyll content, nitrogen uptake, phosphorus uptake, and a reduction in sodium concentration in the plant tissues. Another important consideration for the development of climate-smart oilseed cultivars is that the vast majority of studies in which abiotic stress tolerance has been assessed thus far have been based upon the effect of a single form of stress. While prolonged or acute exposure to any single abiotic stress can be enough to devastate oilseed crop yields in the field, several stresses often co-occur in various combinations and at varying levels, which can compound the resulting negative effects (Elferjani and Soolanayakanahally, 2018). The precise molecular effects of these interactions are not well-understood. Therefore, a better understanding of the mechanisms of response to abiotic stresses under complex growing conditions will be fundamental to maximizing our ability to ensure future oilseed improvement using any breeding platform.

CONCLUSION AND FUTURE PERSPECTIVES

Oilseeds are an important source of food for human consumption, and are used as fuel for biodiesel and as various industrial products. Under a climate change scenario, there is a high probability that the temperature will exceed the threshold for oilseeds. Plant responses to heat stress vary from symptomatic to quantitative. Although, the reproductive stage, the outcome of which symbolizes the economic value of oilseeds, is specifically more susceptible to high heat stress, which directly affects the male and female reproductive parts. Lipid peroxidation leading to excessive ROS production, changes in antioxidants, and reconfiguration of metabolite synthesis also plays a significant role. In response to high-temperature stress, a few adaptive mechanisms are manifested in plants, including a wide range of morphological, physiological, and molecular mechanisms that enable plant survival. Physiological and molecular mechanisms are essential to help breeders develop better genotypes that can perform better under heat stress. At present, the physiological mechanisms of heat stress are reasonably well-understood, but more profound knowledge is needed in several areas, particularly to better understand the physiological basis of the source-to-sink partitioning of assimilates. The introduction of signaling cascades leads to profound changes in uncharacterized gene expression that are central to adaptation to heat stress. Although several signaling molecules are activated/expressed at high temperatures, Ca2+ regulation remains critical. Expression of HSPs, HSFs, and other stress-related chaperones that serve to fold and unfold basic proteins under stress confirm the three-dimensional assembly of membrane proteins for sustained cellular function and persistence under high-temperature stress.

The potential applicability and popularity of genome editing enables sustainable development of plant resistance to abiotic stress. Although the use and development of CRISPR/Cas-based technologies in oilseed crops is still in its infancy, it is clear that these high-precision molecular breeding tools have the potential to provide unprecedented levels of productivity improvement in agronomically valuable oilseed crops and could thus contribute significantly to our ability to sustainably meet future demand for oilseed-derived products. Omics has gained momentum in the last few decades and has become a tool for crop rescue in the context of climate change. The combination of multi-omics approaches will play a major role in identifying stress-responsive genes and identifying the role of different genes in metabolic pathways and the use of this information in the rapid development of climate-resilient oilseeds. Thus, the application of genomics, transcriptomics, proteomics, phenomics, and ionomics approaches seems to be more appropriate to better understand the molecular basis of oilseed response to heat stress in addition to plant tolerance to heat stress. Study evolution is expanding the gene pool by using advanced biotechnological tools using omics, which is the best way to increase productivity. The CRISPR/Cas9 genome editing system and omics technologies promise a future for agricultural biotechnology in sustainable improvement of qualitative and quantitative agronomic traits of significant crops to sustain crop productivity in a rapidly changing global climate. Agronomic strategies including nutrient management, microbial inoculation, plant growth regulation, and innovative agricultural technologies play an essential role in mitigating the detrimental effects of heat stress. However, future studies are urgently needed to understand the mechanisms behind heat stress reduction through microbial treatments. All these efforts will undoubtedly help to mitigate the negative effects of heat stress and contribute to improved plant productivity and food security under current scenarios of climate change and global warming.

AUTHOR CONTRIBUTIONS

MA generated the idea and made the 1st draft of the manuscript. All the other authors listed have made a substantial, direct, and intellectual contribution to improve the work and approved it for publication.

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A Ratiometric Calcium Reporter CGf Reveals Calcium Dynamics Both in the Single Cell and Whole Plant Levels Under Heat Stress

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Land plants evolved to quickly sense and adapt to temperature changes, such as hot days and cold nights. Given that calcium (Ca^{2+}) signaling networks are implicated in most abiotic stress responses, heat-triggered changes in cytosolic Ca^{2+} were investigated in *Arabidopsis* leaves and pollen. Plants were engineered with a reporter called CGf, a ratiometric, genetically encoded Ca^{2+} reporter with an m**C**herry reference domain fused to an intensiometric Ca^{2+} reporter **G**CaMP6**f**. Relative changes in $[Ca^{2+}]_{cyt}$ were estimated based on CGf's apparent K_D around 220 nM. The ratiometric output provided an opportunity to compare Ca^{2+} dynamics between different tissues, cell types, or subcellular locations. In leaves, CGf detected heat-triggered cytosolic Ca^{2+} signals, comprised of three different signatures showing similarly rapid rates of Ca^{2+} influx followed by differing rates of efflux (50% durations ranging from 5 to 19 min). These heat-triggered Ca^{2+} signals were approximately 1.5-fold greater in magnitude than blue light-triggered signals in the same leaves. In contrast, growing pollen tubes showed two different heat-triggered responses. Exposure to heat caused tip-focused steady growth $[Ca^{2+}]_{cyt}$ oscillations to shift to a pattern characteristic of a growth arrest (22%), or an almost undetectable $[Ca^{2+}]_{cyt}$ (78%). Together, these contrasting examples of heat-triggered Ca^{2+} responses in leaves and pollen highlight the diversity of Ca^{2+} signals in plants, inviting speculations about their differing kinetic features and biological functions.

Keywords: calcium, mCherry fused GCaMP6f, whole rosette imaging, pollen tube imaging, single cell imaging, ratiometric calcium reporter CGf, heat stress

ONE-SENTENCE SUMMARY

This paper shows that heat stress can trigger cytosolic Ca^{2+} signals in seedling leaves and suppress the growth associated patterns of Ca^{2+} oscillations in pollen tubes.

INTRODUCTION

An important adaptive trait for many land plants is an amazing ability to sense and adapt to changing temperatures (Lamers et al., 2020; Hayes et al., 2021; Mareri et al., 2021;

Nishad and Nandi, 2021). Nevertheless, global climate change is predicted to make periods of heat stress increasingly detrimental to plant growth and reproduction (Challinor et al., 2014; Zhao et al., 2017; Cohen et al., 2021; Zandalinas et al., 2021). The threshold at which different plants succumb to heat stress varies and can be influenced by combinatorial stresses, such as drought, light intensity, and nutrition (Lamers et al., 2020; Hayes et al., 2021; Zandalinas et al., 2021). In *Arabidopsis,* optimal growth occurs around 16–25°C (Calhoun et al., 2021). As temperatures rise to 30–37°C, *Arabidopsis* plants activate heat stress response pathways (Hayes et al., 2021). While long term exposure to temperatures around 40°C will ultimately cause cell death, *Arabidopsis* can still complete its life cycle with a diurnal stress regime that includes a 1-h mid-day 40°C heat stress, albeit with a major reduction in seed set (Tunc-Ozdemir et al., 2013).

As plants are exposed to heat stress, multiple cellular processes are disrupted, including protein folding, cytoskeletal organization, membrane stability, regulation of reactive oxygen species (ROS), and ion homeostasis (Lenzoni and Knight, 2019; Lamers et al., 2020; Zandalinas et al., 2020; Hayes et al., 2021). Multiple mechanisms for heat sensing have been proposed, including direct changes to membrane fluidity, photosensors, and transcription factors (Lamers et al., 2020; Zandalinas et al., 2020; Hayes et al., 2021). Temperature sensing likely occurs independently in different organelles, for example, in the chloroplast and ER (Lenzoni and Knight, 2019; Malini et al., 2020; Li and Howell, 2021; Singh et al., 2021). In theory, most macromolecules in a cell can be structurally or kinetically altered by heat, which invites consideration that heat sensing thresholds might occur as meta-phenomena that evolved without dedicated sensors.

Ca^{2+} signaling networks are implicated in most abiotic stress responses in plants (Atif et al., 2019; Tang et al., 2020; Alves et al., 2021; Ma et al., 2021; Noman et al., 2021). However, there is mixed evidence to directly support a role for Ca^{2+} signals as an initial heat sensing response (Gao et al., 2012; Tunc-Ozdemir et al., 2013; Finka and Goloubinoff, 2014; Lenzoni and Knight, 2019). For example, a recent study failed to detect a heat-triggered Ca^{2+} signal in the cytosol of cotyledon staged seedlings, but did observe a strong signal in the chloroplast (Lenzoni and Knight, 2019). Some of the mixed results might be explained by a reliance on the Ca^{2+} reporter aequorin. Aequorin has relatively weak affinity for Ca^{2+} ($K_D \sim 7$–13 μM) (Costa et al., 2018), which makes it suboptimal for detecting Ca^{2+} signals in the low to mid nM range (i.e., near resting $[Ca^{2+}]_{cyt}$ around 50–100 nM).

Here, we used a new design for a genetically encoded ratiometric Ca^{2+} reporter to investigate heat-triggered $[Ca^{2+}]_{cyt}$ changes in leaves and pollen tubes from *Arabidopsis thaliana.* This reporter, called CGf, was engineered with an m**C**herry fused to the N-terminal end of an intensiometric Ca^{2+} reporter **G**CaMP6**f**, and incorporates a ratio design feature similar to other ratiometric reporters comprised of two tandem fluorescent proteins (Cho et al., 2017; Waadt et al., 2017; Luo et al., 2019). The Ca^{2+} sensor domain is derived from GCaMP6f, which is a well-established Ca^{2+} reporter with a K_D = 220–375 nM (Chen et al., 2013; Badura et al., 2014; Helassa et al., 2016; Costa et al., 2018).

While GCaMP Ca^{2+} reporters are useful for detecting qualitative changes in $[Ca^{2+}]_{cyt}$, without an internal reference for normalization, it is sometimes difficult to know whether intensity differences might be caused by varying levels of reporter expression or localization rather than changes in Ca^{2+} dynamics. Using mCherry as an internal reference, CGf's ratiometric feature provides an opportunity to compare Ca^{2+} signaling between different tissues, cell types, or subcellular locations. CGf was used here to reveal three different heat-triggered cytosolic Ca^{2+} signals in leaves, as well as a very different heat-induced suppression of tip-focused Ca^{2+} oscillations in growing pollen tubes. The observation that pollen failed to show the same heat stress signals as seen in leaves highlights the need to consider how different plant cells sense and respond to heat.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Hygromycin-resistant *Ubiquitin10promoter:mCherry-GCaMP6f* (plasmid stock 2935) was transformed into *Arabidopsis thaliana* COL-0 using *Agrobacterium tumefaciens* (GV3101 strain) floral dip method (Clough and Bent, 1998). Sterilized seeds were sown on square Petri dishes containing 0.5x Murashige and Skoog medium (Phytotechnology Laboratories, pH 5.7), 0.05% (w/v) MES, 25 mg/L hygromycin B (Gold Biotechnology), and 1% (w/v) agar. After 48–72 h of stratification in the dark at 4°C, seeds were transferred to room temperature conditions with constant light for 10 days. Thereafter, seedlings were transplanted into soil prepared according to manufacturer guidelines (Sunshine SMB-238 SunGro Horticulture, Marathon pesticide, Cleary Turf and Ornamental Systemic Fungicide). Plants were grown to maturity in growth chambers at 22°C with 70% humidity and 16 h light (∼125 μmol m^{-2} s^{-1}) followed by 8 h dark. For whole-plant heat stress imaging, seedlings grown in the above conditions for 10 days were transplanted to hydrated 36 mm diameter Jiffy-7 Peat pellets (Jiffy group, Manitoba, Canada) and placed in Turface MVP (Profile Products LLC, Buffalo Grove, IL) in Magenta vessel GA-7-3 (MilliporeSigma, Burlington, MA). Plants grew additional 2 weeks (or until ready for imaging) under long day light cycles (16 h-light/8 h-dark) at 22°C.

Plasmid Construction

For plant expression, *Ubiquitin10promoter:mCherry-GCaMP6f* (plasmid stock 2935) was constructed through standard molecular techniques using a pGreenII vector system (Hellens et al., 2000) with a hygromycin resistant (HygR) selection marker for plants and a kanamycin resistance (KanR) selection marker for *E. coli*. For *in vitro* analyses, coding sequence for *mCherry-GCaMP6f* (plasmid stock 3007) and *GCaMP6f* (plasmid stock 3221) were cloned separately into a KanR pET28-Novagen vector (Sigma-Aldrich). Promoters used included a Ubiquitin 10 promoter (UBQ10; Norris et al., 1993) for pGreenII vector and a

T7 lac promoter (Studier and Moffatt, 1986) for pET28 vectors. The DNA sequence is provided in **Supplementary Figure 5** for *mCherry-GCaMP6f* (plasmid stock 2935).

Genetics and Seed Set Analyses

Transgene transmission was measured by scoring hygromycin resistance of F1 progeny from reciprocal outcrosses. Seeds were processed as described in section "Plant materials and Growth Conditions." Statistical significance was determined using Pearson's chi-squared test (X^2) unless stated otherwise. For seed set analyses, mature siliques were cleared by incubation in 70% ethanol at room temperature over 24 h.

Imaging Equipment

Whole-plant images were collected using an AxioZoom V16 fluorescent microscope with a PlanNeoFluar Z 1.0x/0.25x objective (Carl Zeiss, Inc., Thornwood, NY, United States) and ORCA-Flash4.0 V2 Plus sCMOS digital camera (Hamamatsu Photonics Inc., San Jose, CA). For ratio imaging, separate signals from GCaMP6f and mCherry domains were detected with filter set 38 eGFP shift free (Ex 470/40 nm, dichroic mirror 495 nm; Em 525/50 nm) and filter set 63 HE mRFP shift free (Ex 572/25 nm, dichroic mirror 590 nm; Em 629/62 nm).

The same imaging equipment was used for pollen heat stress assays except with a PlanNeoFluar Z 2.3x/0.57x objective and a W-view Gemini Image Splitting Optics for simultaneous two fluorescence imaging (Hamamatsu Photonics Inc., San Jose, CA) equipped with emission filter sets (Chroma ET510/20 nm 25 mm diameter for GFP signal and Chroma ET632/60 nm 25 mm diameter for mCherry signal) and a 25.5 × 36 × 2 mm (W x L x H) T560lpxr-UF2 dichroic mirror (Chroma, Bellows Falls, VT).

For high resolution imaging, pollen time lapse images were captured using a Leica DMi8 inverted microscope fitted with a Yokogawa CSU-W1 spinning disk confocal scanner module and a CCD camera. Images were captured with 63X/1.4 NA objective with filter switching (GFP laser Ex 488 nm Em 525 nm/50 m OD8; RFP laser Ex 561 nm Em 610 nm/75 m OD8).

Rosette Imaging and Analysis of Blue Light- and Heat-Triggered Ca^{2+}_{cyt} Increases

Blue light- and heat-triggered $[Ca^{2+}]_{cyt}$ changes were monitored using wild type COL-0 plants stably expressing CGf. Analyses shown were conducted using ∼3-week-old rosettes when plants showed a minimum of 8–9 true leaves. Plants were allowed to adapt to dim light for at least one hour prior to the start of a blue light exposure at time –120 min (Ex470/40nm with 24 μmol m-2s-1 intensity). Images corresponding to GCaMP6f and mCherry were obtained every 5 sec for 5 h.

Heat stress exposures were achieved by placing ∼3 week-old, soil-grown plants in a custom-made heat chamber with temperatures increasing to ∼40°C in ∼6 min after turning the heat pad on. The custom-made heat chamber was built by installing an 8 inch diameter digital heat pad at the bottom of a 4-inch high domed container constructed with a viewing port covered with thin plastic wrap (**Supplementary Figure 4**).

Fluorescent intensities of GCaMP6f and mCherry were acquired from individual GFP and RFP channel images at each time point using ImageJ software (Abràmoff et al., 2004). Intensity changes for GCaMP6f and mCherry were individually calculated using single wavelength quantification equations. For mCherry, the calculation was F_t/F_{basal}, where 'F_t' is fluorescence measured at a given time point within the time course. For GCaMP6f, the calculation was $\Delta F/F_{basal}$, where ΔF is $F_t - F_{basal}$, and F_{basal} is mean value of pre-heat period between –10 to 0 min. A CGf ratio was calculated as GCaMP6f fluorescent intensity ($greenF_t$) divided by mCherry fluorescent intensity ($redF_t$). Percentile (%) CGf max in leaves was calculated using a CGf max ratio divided by the CGf max ratio 2.15 (**Supplementary Figure 3** and **Supplementary Movie 2**).

In vitro Pollen Germination

Arabidopsis pollen grains from 1 to 2 day old flowers were germinated on an agar surface containing 1.5% low melting agarose, 10% (w/v) sucrose, 2 mM calcium chloride ($CaCl_2$), 0.004% (w/v) boric acid (H_3BO_3), 2 mM potassium chloride (KCl), 0.4 mM magnesium sulfate ($MgSO_4$), and pH 7.5 using potassium hydroxide (KOH). Pollen grains germinated in a humidity chamber in the dark at 22°C for 1–3 h. Glass coverslips were placed over pollen tubes on the agar surface prior to imaging. Only pollen tubes between 100 and 400 μm in length were used for analysis.

Pollen Heat Stress Assay and Time Series Analysis

Pollen heat stress experiments were imaged using PlanNeoFluar Z described above in section "Imaging Equipment" Both red and green fluorescence signals were simultaneously imaged using an optical splitter at 500 ms $frame^{-1}$ for 4 min. Control (no heat treatment) pollen tubes were imaged at room temperature (22°C). Time course for heat treatment was applied as follows: initial 22°C for 1 min, temperature ramping from 22 to 32°C in 1 min, and 32°C for remaining 2 min. Heat treatment was applied using a COSORI digitally controlled cup warmer (Catalog#CO194-CW).

Pollen time lapse images were adjusted using ImageJ plugins Rolling Ball Background Subtraction and 3D Drift Correction (Parslow et al., 2014). After image adjustment, Multiple Kymograph plugin was used to generate kymographs (average pixel neighborhood = 5) for individual fluorescent channels. Kymograph text files were analyzed using CHUKNORRIS web interface[1] for single channel kymographs (Damineli et al., 2017). A raw ratio was calculated using CHUKNORRIS-derived time series (ROI.ts) data by dividing GCaMP6f fluorescent intensities ($greenF_t$) by mCherry fluorescence ($redF_t$). Raw ratios were converted to a % maximum of the CGf reporter using the maximum raw ratio observed during pollen tube bursting events.

High resolution pollen imaging was performed using a spinning disk confocal described above in section "Imaging Equipment" using 1 s intervals. Time series analysis was

[1]https://feijolab.shinyapps.io/CHUK/

performed as described above. The region of interest for tip-focused imaging was 10 μm (pollen apex), whereas flicker ratios were determined from a 50 μm region behind the pollen apex.

E. coli Protein Expression, Purification, and Characterization

CGf and single fluorescent GCaMP6f proteins were cloned into pET expression vector and transformed into T7 expression cells (NEB cat# C2566). Mid-log phase liquid cultures in 2xYT media were induced with 0.5 mM IPTG and grown at 30°C for 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM MOPS pH 7.2, 500 mM NaCl, 10% glycerol (w/v), 10 mM Imidazole, 1.5 mg/mL Lysozyme, 1 mM PMSF) and frozen at –20°C. Upon thawing, cells were lysed by the addition of 0.4% Triton x100 and sonication. Lysates were cleared by centrifugation at 10,000 rpm for 30 min at 4°C. Cleared cell lysate was applied to Ni-NTA beads (Qiagen, 700 μl packed bead volume) and were washed by 10 column volumes of each of the following buffers: 6xHis MOPS Wash Buffer (20 mM MOPS pH 7.2, 100 mM NaCl, 10 mM Imidazole), 6xHis MOPS Wash Buffer with 1 mM EGTA, and 6xHis MOPS Wash Buffer with 0 mM EGTA. Proteins were eluted with 200 mM Imidazole and concentrated using Pierce Concentrator 10K MWCO filters (Thermo Scientific). Eluted proteins were stored in 60% glycerol (w/v) and stored at –20°C.

Protein concentrations were measured using Bradford reagent (BioRad) and Pre-Diluted BSA Protein assay standards (Thermo Fisher Scientific) in 96 well plates analyzed by Spectromax M5 plate reader at 595 nm absorbance. Protein purity was determined using SDS-PAGE (AnyKD Bio-Rad) and total protein stain (Gel Code Blue, Thermo Fisher Scientific).

To measure *in vitro* Ca^{2+} binding affinities, fluorescence intensities were measured at various free [Ca^{2+}] concentrations using a Calcium Calibration Buffer Kit (Invitrogen) with 30 mM MOPS pH 7.2, 100 mM KCL, and varying additions of 10 mM EGTA or 10 mM CaEGTA. Dilution series were performed as directed by the manufacturer's protocol with absorbance measured using Shimatzu RF-6000 Spectrofluorometer. Fluorescence intensities were measured for GFP at Ex 488 nm/Em 512 nm.

In planta Protein Stability Analysis

Leaf tissue from 10-day-old seedlings, grown as described in section "Plant Materials and Growth Conditions" was collected and frozen in liquid nitrogen. Frozen tissue was ground into a frozen powder using prechilled mortar and pestle, adding homogenization buffer (HB) as needed (i.e., 100 μL HB per 100 mg tissue). Homogenization buffer consists of 100 mM Tris pH 7.5, 150 mM NaCl, 290 mM sucrose, 10 mM imidazole, 10% glycerol, 0.1% Tween-20, EDTA-free protease inhibitor cocktail V (cat# P50900-1, Research Products International), and 1 mM PMSF (phenylmethylsulfonyl fluoride). Plant extracts were filtered through cheesecloth prewet with HB. Filtered extract was spun in glass Corex tubes at 6,000 g for 15 min at 4°C to remove cellular debris. Crude plant extracts were analyzed using SDS-PAGE precast gels (AnyKD, Bio-Rad), followed by Western blot analysis of reporters using primary RFP monoclonal antibody (Thermo Fisher cat # 200-301-379S) and secondary F(ab')2-Goat anti-Mouse IgG (H + L) antibody (HOURP, Thermo Fisher Cat# A24512).

RESULTS

A Fusion of mCherry to GCaMP6f Generates a Robust Ratiometric Ca^{2+} Reporter

A ratiometric Ca^{2+} reporter was generated with an N-terminal m**C**herry followed by a linker and an intensiometric Ca^{2+} reporter **G**CaMP6**f** (which has a calmodulin binding sequence "M13" followed by a circularly permutated eGFP and a terminal calmodulin domain (Chen et al., 2013; **Figure 1A**). When Ca^{2+} binds to the calmodulin domain, the protein undergoes a conformational change that promotes intra-molecular binding to the M13 peptide sequence (**Figure 1B**). This conformational change results in an increase in GFP fluorescence proportional to [Ca^{2+}] (**Figure 2**).

To determine whether the Ca^{2+} affinity of GCaMP6f was altered by a fusion with mCherry, both CGf and an unfused GCaMP6f reporter were separately expressed in *E. coli* and purified. Spectral analyses indicated that both CGf and GCaMP6f showed very similar Ca^{2+} titration curves (**Figures 2A,B**). CGf bound Ca^{2+} with an apparent K_D in the range of 203–223 nM (**Figure 2C**), which is similar to other measurements in the literature for GCaMP6f that ranged from 220 to 375 nM (Chen et al., 2013; Badura et al., 2014; Helassa et al., 2016; Costa et al., 2018). An approximate K_D of 220 nM is used here as a point of overlap between our measured K_D and the lower end of the range reported in the literature. The Hill coefficients for both CGf and GCaMP6f were in the range of 1.8–2.1 (**Figure 2C**), which indicates that the designed fusion did not significantly alter the expected cooperative binding of Ca^{2+} to the calmodulin domain. These Ca^{2+} binding analyses indicate that the addition of an mCherry to the N-terminal end of GCaMP6f did not dramatically alter basic kinetic features previously established for GCaMP6f.

To address the potential concern that the mCherry domain might dampen GCaMP fluorescence because of Förster resonance energy transfer (FRET), the spectra for the Ca^{2+} titration curves was evaluated over an expanded emission range to include potential fluorescence from mCherry (emission 600–650 nm) (**Figures 2A,B**). An alternative fusion design was previously reported to show energy transfer to mCherry and up to a 50% quenching of the GCaMP6 signal (Cho et al., 2017; Luo et al., 2019). In contrast, the CGf design here showed relatively little evidence of quenching, with an estimated 1.3% of the total spectral fluorescence (at the K_D [Ca^{2+}] of 220 nM) resulting from a potential Ca^{2+} dependent energy transfer from GCaMP6f to mCherry (**Figure 2B**, emissions between 600 and 650 nm). Thus, in the context of using the mCherry fluorescence as a

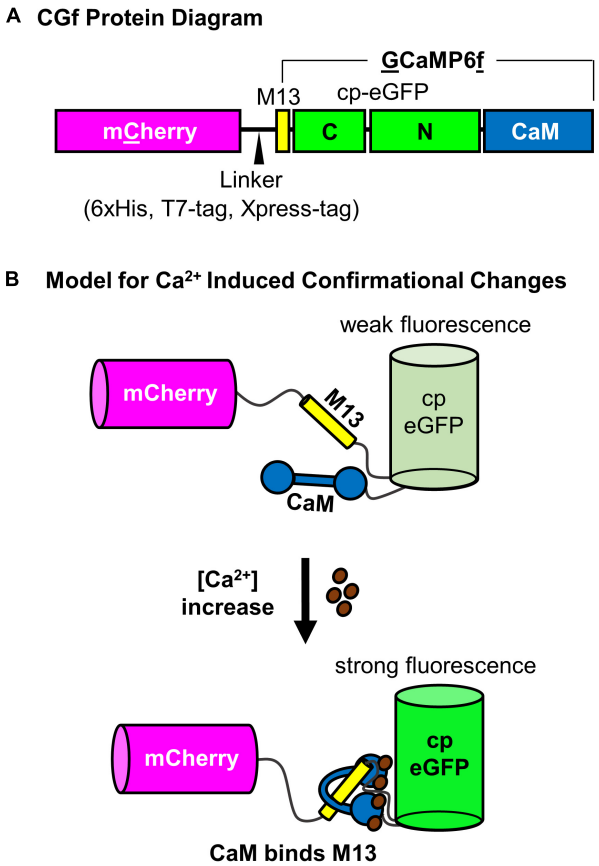


FIGURE 1 | Schematic diagram of CGf, a ratiometric GCaMP6-based Ca^{2+} reporter. **(A)** CGf protein diagram of m**C**herry fusion to N-terminal end of **G**CaMP6**f**, which is comprised of an M13 binding site for calmodulin, a cp-eGFP, and calmodulin (CaM). The linker between mCherry and GCaMP6f includes a 6xHis, T7-Tag, and Xpress Tag (MedChemExpress, LLC). **(B)** Model for Ca^{2+} induced conformational changes. (Top) Without Ca^{2+}, the calmodulin domain is dissociated from the M13 peptide, which results in a conformation that allows solvent access to protonate the chromophore and quench eGFP fluorescence (weak fluorescence) (Ding et al., 2014). As [Ca^{2+}] increases, Ca^{2+} binding to CaM promotes CaM-M13 binding interactions, which blocks solvent access, promotes deprotonation of the chromophore, and increases eGFP fluorescence (strong fluorescence). mCherry fluorescence is not dependent on Ca^{2+} binding, serving as an internal reference for comparison to the Ca^{2+} dependent changes in eGFP fluorescence.

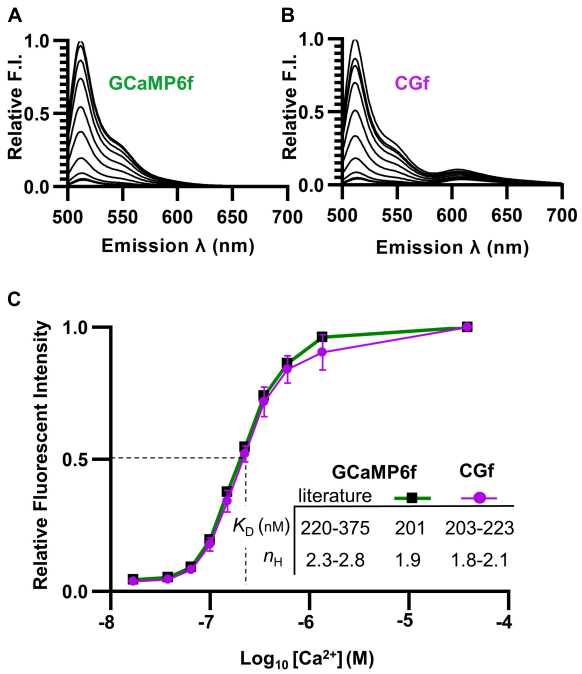


FIGURE 2 | Spectral comparisons showing that the fusion of mCherry to GCaMP6f did not alter Ca^{2+} binding. **(A,B)** Emission spectra comparing relative brightness of GCaMP6f **(A)** to CGf **(B)** across varying Ca^{2+} concentrations from 0.017 to 39 μM. **(C)** Ca^{2+} titration curves comparing GCaMP6f to CGf. These results were used to calculate Ca^{2+} binding affinities (apparent K_D) and Hill equation fits (n_H). Comparisons are provided to previously reported ranges for GCaMP6f (Chen et al., 2013; Badura et al., 2014; Helassa et al., 2016).

normalization baseline, this small amount of FRET (1.3%) did not appear to represent a major concern.

To evaluate whether the CGf fusion was proteolytically stable when expressed in plants, stable transgenic plants were generated that expressed CGf under the control of a UBQ10 promoter. Plants were chosen for analysis using fluorescence microscopy to confirm strong expression throughout the plant, including leaves and pollen. Protein extracts from leaves were subjected to SDS-PAGE. A Western blot analysis was conducted using a primary antibody recognizing mCherry. A single band was detected at a size expected for an intact mCherry-GCaMP6f fusion (**Supplementary Figure 1**). This provided corroboration that the fusion was proteolytically stable, which was considered important because a confident interpretation of the ratiometric output for CGf relies on the maintenance of a 1:1 stoichiometry between the reference mCherry and CGaMP6f.

CGf Can Be Expressed Without Disrupting Plant Development

A general concern for constitutive expression of any bioreporter is whether the reporter itself might significantly impact an organism's development or responses to environmental stimuli. This concern was especially relevant here because previous reports of potential phenotypic impacts were reported from the over-expression of calmodulin (Yang et al., 2018), as well as several Ca^{2+} reporters that harbor a calmodulin domain (Costa et al., 2018). However, for the CGf construct used here, the frequency of plants with obvious phenotypes appeared to be less than 1 in 10.

For the selection of plant lines for Ca^{2+} imaging, additional characterizations were done to confirm the absence of serious phenotypic problems (**Figure 3**). With the same transgenic lines used for imaging rosettes, plants were grown side by side in the same pots with wild type controls (**Figure 3A**). In these paired growth comparisons, we failed to observe any statistical differences in rosette sizes, root growth on agar plates, average seed numbers per silique, or total seed yield per plant (**Figure 3**

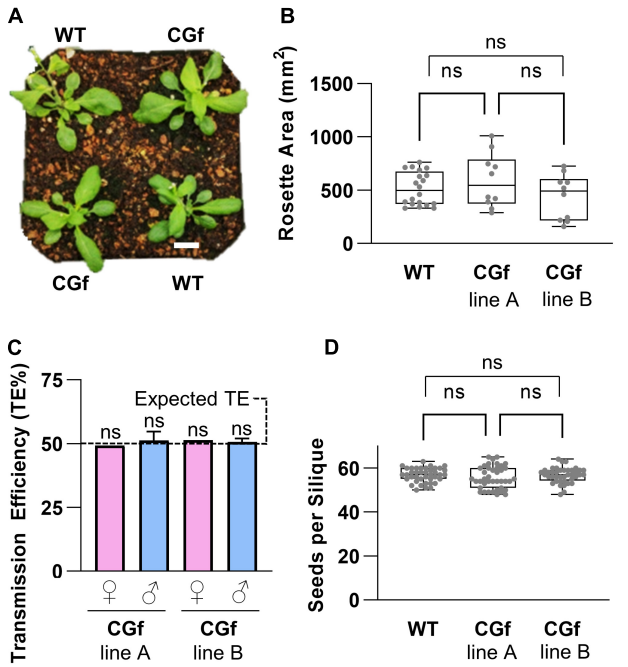


FIGURE 3 | CGf expression did not alter plant development and reproduction. **(A)** Visual representation of 5-week-old wild type (WT) and CGf plants at the time of bolting. Scale bar = 5 mm. **(B)** Total rosette area of 5-week-old plants. WT n = 20; Two independent CGf lines **(A,B)** n = 10 each; from 3 experimental replicates. **(C)** CGf genetics. Reciprocal outcrosses were performed on plants harboring a heterozygous hygromycin-resistant transgene. Progeny were scored for hygromycin resistance to determine transmission efficiency (TE) of the transgene. TE = # resistant seedlings/total # seedlings. Female (♀) or male (♂) refers to outcross transmission of gamete harboring CGf transgene. WT pollen were outcrossed to CGf ovules. CGf pollen were outcrossed to *male sterility 1* (*ms1-1*) plants. TE% was not significantly (ns) different than expected 50% TE, as determined by the Pearson's Chi-square test (χ^2) with a minimum threshold set at $p \leq 0.05$. ♀ $n > 50$; ♂ $n > 300$ for each independent line (seed stocks 2540 and 2640). **(D)** Seed set analyses showing no statistical significance between WT and CGf. n = 40 siliques for each group; 3 replicates. NS determined using One-way ANOVA multiple comparisons with the Turkey post-hock test. Error bars show min to max values of representing data sets (gray dots). Unless stated otherwise, CGf line A is seed stock (ss) 2543. CGf line B is ss2544.

and **Supplementary Figure 2**). Similarly, for transgenic lines used to image pollen, pollen outcrosses from heterozygous plants failed to reveal any non-Mendelian distortion in pollen transmission efficiencies (**Figure 3C**).

As commonly observed for many transgenic plants, some of the plant lines maintained over multiple generations appeared to segregate rare examples of gene silencing, as detected by screening seedlings for consistently high levels of mCherry fluorescence. As a best practice, good expressor lines were maintained by germinating seeds on plates with a hygromycin selection for the transgene, and by screening seedlings for strong mCherry fluorescence signals in roots and leaves. Thus, while caution is always needed to avoid potential reporter expression artifacts and gene silencing, healthy plant lines were readily identified and maintained for multiple generations, unlike concerns reported for several other Ca^{2+} reporters with alternative designs (Ast et al., 2017; Waadt et al., 2017).

Calibrating CGf Ratios Based on a Maximum Calcium Saturated Signal

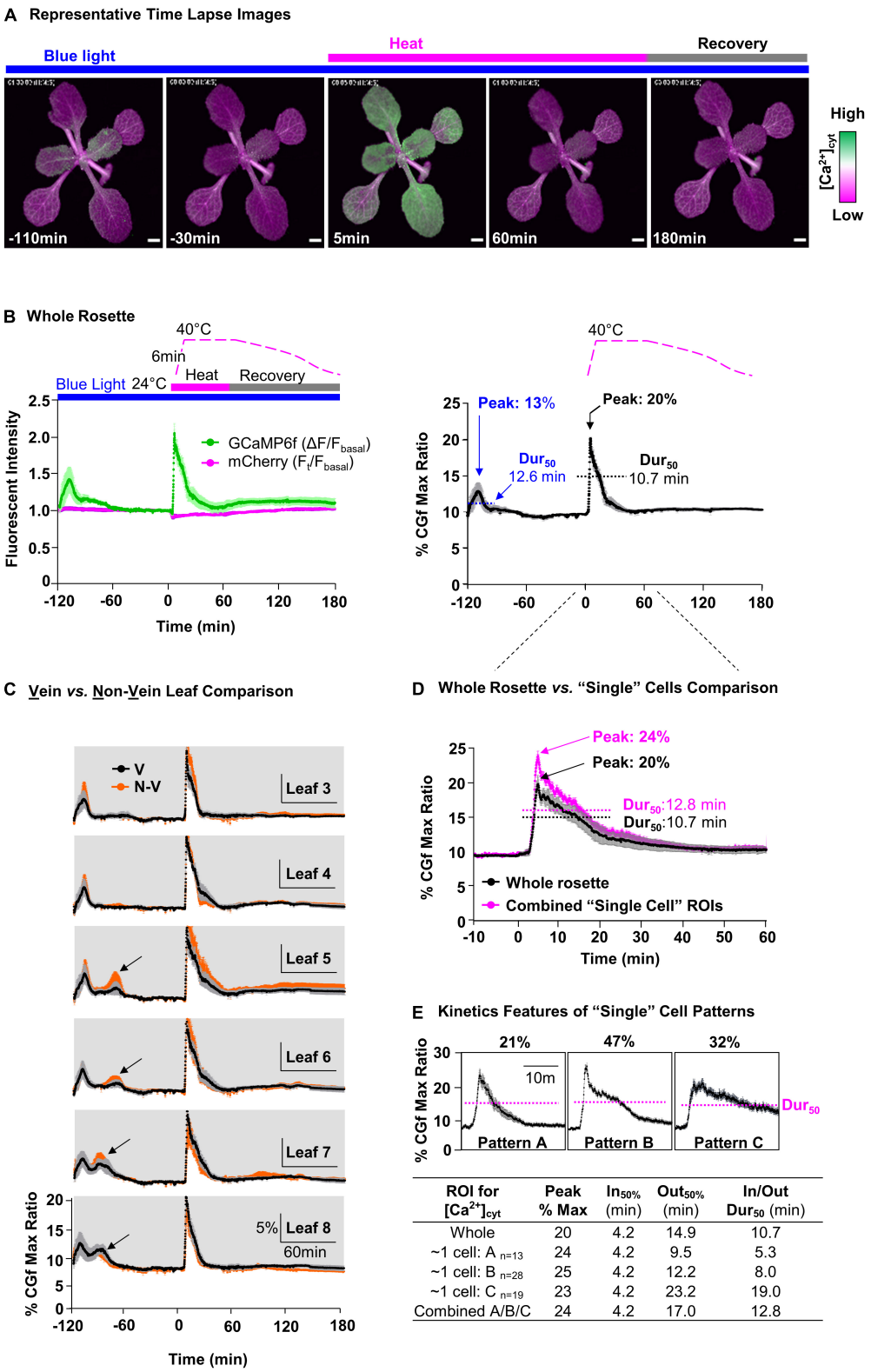
The ability to monitor the ratio between GFP fluorescence and a baseline mCherry signal creates an opportunity to more reliably compare relative magnitudes of Ca^{2+} signals between different tissues, cell types, and within different subcellular locations. Without an internal baseline reference as provided here by the mCherry domain, it is not possible to know if different intensities from the GFP fluorescence are due to different amounts of reporter, or differences in $[Ca^{2+}]_{cyt}$. However, a caveat to using a ratiometric strategy is that the individual fluorescence intensities for both mCherry and GCaMP6f domains can vary under different imaging acquisition parameters, as occurs with adjustments of exposure times for each fluorophore, differences in excitation or emission wavelengths, or different background corrections appropriate for different tissues or conditions. Thus, it is important to first optimize imaging parameters and then apply those parameters across all experiments to reliably compare Ca^{2+} signals.

Another method to consider is normalizing ratio signals to a maximum ratio observed in a given tissue or cell type. For CGf expression in leaves, a 100% max signal was estimated by creating a wound site that disrupted the integrity of cellular Ca^{2+} stores and created a long-lived $[Ca^{2+}]_{cyt}$ increase that drove the ratiometric output to a maximum (**Supplementary Figure 3**). Using this approach, the heat-triggered Ca^{2+} signals described below were estimated to be approximately 20% of the maximum saturated potential for the CGf reporter (**Figure 4**). However, caution is required to not over-interpret the precision of estimating true *in vivo* concentrations, as unknown differences between *in vivo* and *in vitro* conditions can dramatically alter calibrations. Regardless, a peak signal of 20% of CGf's maximum still represents more than a 2-fold relative increase in $[Ca^{2+}]_{cyt}$ over unstimulated resting levels.

Heat-Triggered Cytosolic Calcium Signals Throughout the Rosette

To visualize heat-triggered Ca^{2+} signals in rosette leaves, seedlings were first adapted to dim-light to ensure a reproducible starting point for imaging. The imaging time course was then initiated by the exposure of plants to blue light (Ex470/40 nm with 24 $\mu mol\ m^{-2}s^{-1}$ intensity) to excite the GFP domain of CGf (**Figures 4A,B**, and left). This initial blue light stimulation reliably triggered Ca^{2+} signals that reached an average magnitude around 13% of CGf's maximum ratio, with 50% durations measured around 12.6 min (**Figure 4B**, right, and **Supplementary Movie 1**). A 50% signal duration was calculated starting at the time Ca^{2+} influx reached 50% of its maximum peak.

The heat stress was initiated 2-h after the start of imaging to ensure that plants had recovered from the initial blue light exposure and other unintended stimuli. The heat stress was introduced using a heating pad inside an enclosure (**Supplementary Figure 4**). The temperatures ramped from ~ 24



ROI for $[Ca^{2+}]_{cyt}$	Peak % Max	$In_{50\%}$ (min)	$Out_{50\%}$ (min)	In/Out Dur_{50} (min)
Whole	20	4.2	14.9	10.7
~1 cell: A $_{n=13}$	24	4.2	9.5	5.3
~1 cell: B $_{n=28}$	25	4.2	12.2	8.0
~1 cell: C $_{n=19}$	23	4.2	23.2	19.0
Combined A/B/C	24	4.2	17.0	12.8

FIGURE 4 | Comparative measurement of blue light- and heat-triggered Ca^{2+} changes in whole rosettes and "single cell"-sized areas of leaves. **(A)** Representative time lapse images of blue light and heat-triggered Ca^{2+} responses in plants expressing CGf. Magenta bar = heat stress. Gray bar = post-heat stress recovery. Blue bar indicates a continuous blue-light exposure (ex440/40 nm with 24 μmol $m^{-2}s^{-1}$ intensity). Scale bar = 1 mm. **(B)** Time series analysis of blue light and heat

(Continued)

Figure 4 | stress $[Ca^{2+}]_{cyt}$ responses in the rosette. 'B, Left' shows GCaMP6f (green) and mCherry (magenta) signals. Time course for heat treatment: 2 h pre-heat acclimation (–120 to 0 min), 1 h heat stress (0–60 min), and 2 h post-heat stress recovery periods (60–180 min). "B, Right" shows % of CGf max ratio. 50% durations (Dur_{50}) were calculated as the time from the $T_{1/2}$ of influx to the same concentration during efflux. The blue light peak is marked at –110 min and heat stress peak just after 0 min. **(C)** Time series analysis for leaves with a comparison of Ca^{2+} responses between vein (V, black) and non-vein (N-V, orange) tissues. Black arrows mark additional blue light-specific Ca^{2+}peaks that are not present in the oldest leaves. Leaf numbers indicate order of development, from the oldest (Leaf 3) to the youngest (newest) detectable (Leaf 8). **(D)** Comparative analysis of heat-triggered Ca^{2+}changes between whole rosette (black) and combined average of "single cell"-sized ROIs (magenta). Error bars are SEM of n = 5 independent plants (whole rosette) or n = 60 ROIs from the same set of 5 plants. **(E)** Characterization of three distinct heat-triggered Ca^{2+} patterns (A,B,C) in "single cell"-size ROIs with respective n, influx rate ($In_{50\%}$), and efflux rate ($Out_{50\%}$), and Dur_{50}.

to 40°C over a short 6 min period. However, even before reaching 40°C, the changing temperatures triggered a rapid and steady rise in $[Ca^{2+}]_{cyt}$ with a peak corresponding to 20% of CGfs maximum ratio, which was approximately 1.5-fold higher than the blue light triggered signals at the start of the imaging experiments. While the 40°C temperature was maintained for a full hour, the peak $[Ca^{2+}]_{cyt}$ immediately began a relatively slow decline to produce a transient with a 50% duration of 10.7 min, which was slightly faster than the 12.6 min for blue light-triggered signal (**Figure 4B**, right).

Heat Stress $[Ca^{2+}]_{cyt}$ Signals Occur With Similar Kinetics in All Rosette Leaves

To evaluate whether there were differences between the heat stress responses in different leaves, whole plant responses were reanalyzed at the level of individual leaves. The signal traces were grouped according to leaf age from the oldest (Leaf 3, first detectable true leaf) to the newest (e.g., Leaf 8) (**Figure 4C**). This analysis indicated that all leaves in a rosette showed a high degree of similarity for their heat sensing threshold, as well as their peak magnitudes and 50% signal durations. In addition, very similar kinetic profiles were observed for signals corresponding to veins and non-vein regions of the leaves. However, a leaf-age dependent variation was observed for the initial blue light signals, with the youngest (newest) leaves showing an additional second peak (see newer leaves L5, L6, L7, L8, in **Figure 4C**).

Heat Stress $[Ca^{2+}]_{cyt}$ Signals in Leaves Occurred With Three Different Kinetic Patterns

To evaluate whether there were signaling differences at the "single cell" level, images were also analyzed with smaller regions of interest (ROI)s in non-vein tissues (**Figure 4C**). While the ROIs were approximately the surface area of a single pavement cell, the signals collected from these ROIs actually represented several cells because the fluorescence contributed from the underlying cell layers. For the "single cell" ROIs selected, the summation of their combined signal traces was very similar to the average for the whole rosette (**Figure 4D**). However, in contrast to whole rosette or individual leaf analyses (**Figures 4B,C**), signals from these "single cell" ROIs showed variations that could be clustered into three patterns with distinct kinetic features (**Figure 4E**). A key kinetic difference was in the 50% durations, which were 5.3, 8.0, and 19 min, respectively (**Figure 4E**).

CGf Detected Two Types of Ca^{2+} Oscillations in Pollen Tubes Under Normal Conditions

In evaluating CGf's ability to detect subcellular Ca^{2+} signals in growing pollen tubes, two patterns of tip-focused $[Ca^{2+}]_{cyt}$ oscillations were observed (**Figure 5** and **Supplementary Movies 3, 4**), similar to those previously reported using YC3.6 (K_D 250 nM) (Damineli et al., 2017) and GCaMP5 (K_D 450 nM) (Akerboom et al., 2012). These two patterns are referred to here as steady growth Ca^{2+} (SGC) oscillations and arrested growth Ca^{2+} (AGC) oscillations. Time lapse images and a representative kymograph show a transition from SGC to AGC oscillations within the same pollen tube over a 18 min imaging window at room temperature (**Figures 5A,B** and **Supplementary Movie 3**). SGC oscillations were rapid, shallow oscillations with a high baseline average $[Ca^{2+}]_{cyt}$, while AGC oscillations showed peaks with higher magnitudes and clear intervening periods of very low resting baseline $[Ca^{2+}]_{cyt}$ (**Figure 5C**).

It appeared that tip-focused Ca^{2+} oscillations often displayed peak signal intensities that ranged from 70 to 100% of the Ca^{2+} saturated maximum. While these high signal ratios were outside the linear calibration range for CGf, they suggest that many signals reached magnitudes equal or greater than 960 nM $[Ca^{2+}]_{cyt}$, which corresponds to CGf's estimated Ca^{2+} saturated maximum.

In addition to confirming two common patterns of tip-focused Ca^{2+} oscillations, rapid $[Ca^{2+}]_{cyt}$ transients of equal magnitude were also observed at dispersed locations in the pollen tube shank, as shown in time lapse images (**Figure 5A**) and time series analysis (**Figure 5D**). Described here as flickers, these signals often showed 50% durations as rapid as 0.5 s, suggesting these signals could easily be missed if intervals between image captures are longer than 2 s. Flickers did not appear to be synchronized with tip oscillations, nor necessarily show repeating oscillations at the same subcellular locations (**Supplementary Movie 4**). While a survey of the literature indicates that flickers are often observed in time lapse images (Keinath et al., 2015; Diao et al., 2018), there has been little discussion about their kinetic features or speculation about their biological meaning.

Arabidopsis Pollen Responds to Temperature Increases by Dampening Tip-Focused Ca^{2+} Dynamics

To investigate the effects of heat stress on calcium dynamics in pollen tubes, we imaged and analyzed pollen tubes showing

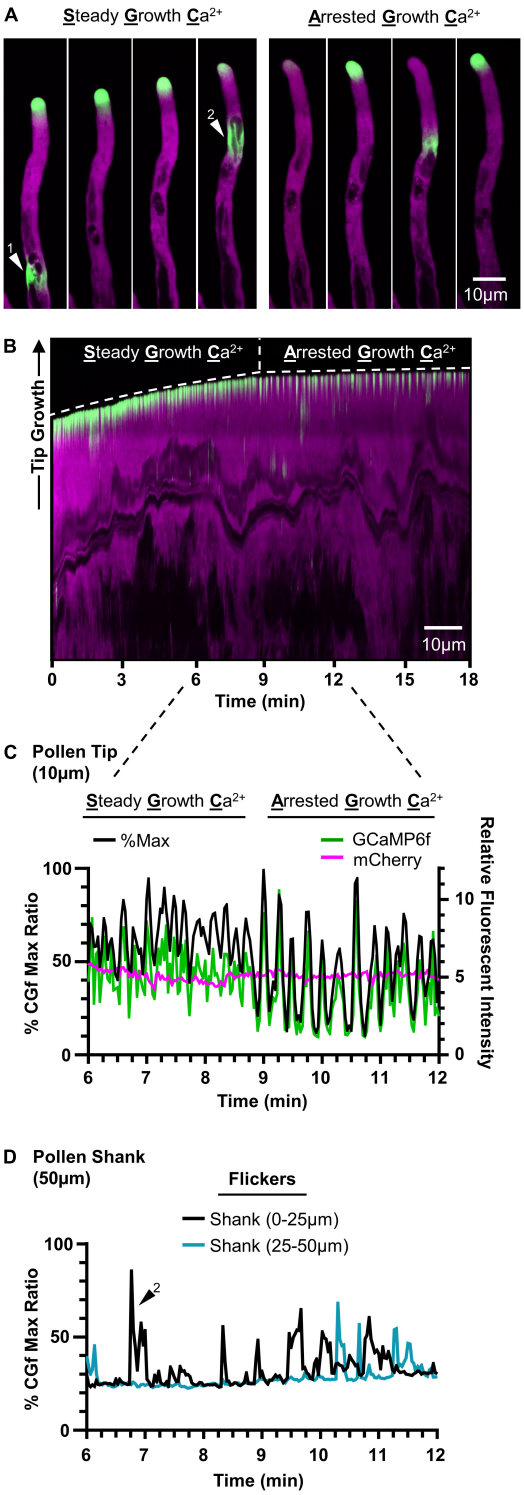


FIGURE 5 | CGf detects Ca^{2+} signals associated with steady growth, growth arrest, and flickers in *Arabidopsis* pollen tubes. Representative time lapse images **(A)** and kymograph **(B)** showing steady growth tip-focused Ca^{2+} (SGC) oscillations, arrested growth Ca^{2+} (AGC) oscillations, and Ca^{2+} flickers in the pollen shank within the same pollen tube over 18 min. Example flickers are marked by white triangles 1 and 2. **(C)** Comparison of pollen tip oscillatory

(Continued)

FIGURE 5 | patterns and magnitudes between SGC and AGC oscillations using CHUKNORRIS (Damineli et al., 2017). Relative fluorescent intensities (right) of GCaMP6f (green) and mCherry (magenta) with corresponding % of CGf max (black, left). Tip = first 10 µm. **(D)** Ca^{2+} flickers display similar magnitudes to tip-focused ratios, as shown by representative flicker 2, and are observed as localized changes scattered throughout the pollen shank. Shank = 50 µm following the 10 µm tip region.

steady growth tip-focused Ca^{2+} (SGC) oscillations prior to the heat exposure. Considering that a 35°C heat stress increased bursting frequency 100-fold, a milder heat stress exposure was applied by increasing the temperature from 22 to 32°C (1°C per 6 s for 1 min) (**Figure 6A**), a stress temperature also used in other studies (Muhlemann et al., 2018; Luria et al., 2019). In this study, a 32°C heat stress increased the frequency of a Ca^{2+}-pattern shift by eightfold (**Figure 6B**).

Imaging with CGf revealed that SGC oscillations shifted to one of two patterns under heat stress, an **a**rrested **g**rowth **C**a^{2+} (AGC) or a **d**ampened **C**a^{2+} (DC) oscillation, as shown in representative kymographs (**Figure 6C**). Time series analysis revealed heat-triggered AGC oscillations (**Figure 6D** and **Supplementary Movie 5**) appeared to have similar kinetic features to those that were occasionally observed under non-stress conditions (**Figure 5C**), as characterized by high magnitudes and clear intervening periods of very low resting baseline $[Ca^{2+}]_{cyt}$. DC oscillations were characterized by very shallow oscillations on top of an almost undetectable baseline $[Ca^{2+}]_{cyt}$ (**Figure 6E** and **Supplementary Movie 6**). Additionally, DC oscillations were observed 3.5-fold more often than AGC oscillations (**Figure 6F**). The average $[Ca^{2+}]_{cyt}$ associated with DC oscillations was reduced by at least 50% compared to pre-stressed SGC oscillations, as confirmed in all three independent transgenic lines evaluated (**Figure 6G**). To ensure that heat-triggered dampening of tip-focused $[Ca^{2+}]_{cyt}$ was not an artifact of cell death, heat-stressed pollen tubes were allowed to recover and imaged thereafter. In all cases pollen tubes remained viable, as indicated by growth or the appearance of $[Ca^{2+}]_{cyt}$ oscillations. For a subset of tubes, further imaging was conducted after a 1 h recovery period. For the 6 tubes that showed a DC oscillation pattern during heat stress, after a 1 h recovery period they all showed a restoration of $[Ca^{2+}]_{cyt}$ oscillations (e.g., **Supplementary Movie 7**) and growth rates between 0.25 and 2.6 µm/min, which overlaps with growth rates for unstressed-pollen tubes grown in parallel (**Supplementary Figure 6**).

In contrast to heat-triggered signals observed in leaves (**Figure 4**), we failed to see any evidence for a sustained heat-triggered increase in $[Ca^{2+}]_{cyt}$ in the pollen tube shank (**Figure 6C**).

DISCUSSION

The CGf Ca^{2+} reporter developed here was used to conduct ratiometric imaging of heat stress triggered $[Ca^{2+}]_{cyt}$ dynamics in whole seedlings, leaves, "single cell"-sized regions of interest, and single cell pollen with subcellular resolution (**Figures 4**, **6**).

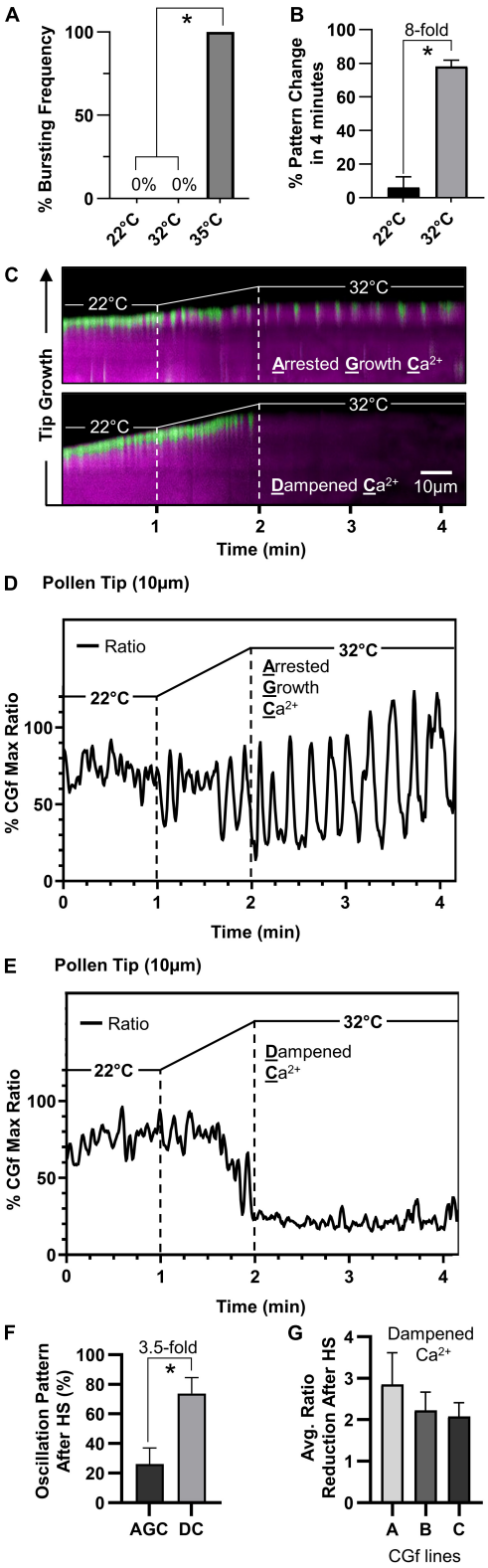


FIGURE 6 | *Arabidopsis* pollen respond to temperature increases by attenuating tip-localized Ca^{2+} dynamics. **(A)** Pollen tubes did not rupture at 32°C compared to 100% bursting at 35°C ($n = 76$, $p < 0.001$). **(B)** 32°C heat stress results in significantly more shifts in tip-focused oscillation patterns compared to 22°C controls ($n = 51$, $p < 0.001$). **(C)** Kymograph representation of steady growth tip-focused Ca^{2+} (SGC) oscillations shifting to one of two patterns under heat stress, an arrested growth Ca^{2+} (AGC) oscillation or a dampened Ca^{2+} (DC) oscillation. Time course: 22°C for 1 min, 22–32°C in 1 min, followed by 2 min at 32°C. **(D,E)** CHUKNORRIS time series analysis of tip-focused $[Ca^{2+}]_{cyt}$ shifting from SGC oscillations to **(D)** heat-triggered AGC oscillations or **(E)** heat-triggered DC oscillations. CGf Ratios are shown as a% of Max. **(F)** At 32°C, DC oscillations are observed 3.5 times more often than AGC oscillations ($n = 30$, $p < 0.01$). **(G)** The average tip-focused $[Ca^{2+}]_{cyt}$ during SGC oscillations is decreased by more than twofold in all three reporter lines analyzed. Error bars are SEM of $n = 3$ pollen tubes for each independent line (CGf line A ss2540, line B ss2640, and line C ss2641). * = statistical significance for each respective panel.

The reporter's design provides a stable mCherry-red fluorescence as a reference for normalizing dynamic changes in green fluorescence caused by Ca^{2+} interactions with the GCaMP6f domain (**Figure 1**). Without a normalization control for GCaMP6f, it is difficult to distinguish between real Ca^{2+} dependent changes and artifacts of reporter abundance in a particular leaf, cell type, or subcellular region. Here we used the ratiometric features of CGf to identify five different patterns of heat-triggered modifications to Ca^{2+} dynamics in *Arabidopsis*.

The Expanding Palate of Ca^{2+} Reporters

CGf represents a new addition to a growing diversity of Ca^{2+} reporters, such as the YCnano65 (Horikawa et al., 2010), Matrosky (Ast et al., 2017), and R-GECO1-mTurquoise (Waadt et al., 2017). Each of these reporters has advantages for different imaging equipment and specific biological applications. A reporter that most closely resembles CGf was similarly designed as a fusion of GCaMP6f with an mCherry, but differs in that its mCherry domain was fused to the C-terminal instead of N-terminal end of GCaMP6f (Waadt et al., 2017). A concern raised about this previous design was its potential to cause growth deficiencies. While stable overexpression of any reporter has potential for negative impacts, the frequency of independent transgenic lines showing an obvious phenotypes for CGf was estimated here at less than 1 in 10, with normal growth and reproduction confirmed by quantification of rosette sizes, root growth rates, seed numbers per silique, total seed set, and reciprocal crosses to test for normal Mendelian segregation of the transgene (**Figure 5** and **Supplementary Figure 2**).

The fusion of an mCherry to GCaMP6f did not appear to significantly alter GCaMP6f's Ca^{2+} affinity, as indicated by a side-by-side Ca^{2+} titration comparison with a single fluorescent reporter (**Figure 2**). An apparent K_D of 220 nM was estimated for CGf, which represents an overlap between the ranges measured here for CGf and GCaMP6f (201–223 nM, **Figure 2**) and the low end of the 220–375 nM range reported in the literature for GCaMP6f (Helassa et al., 2016; Chen et al., 2013).

CGf's ratiometric design represents an important feature that permits different Ca^{2+} signals to be compared for relative differences in peak magnitudes and signal durations. The various signal intensities observed here were calculated as a percentage of CGf's Ca^{2+} saturated maximum based on imaging conditions

used in this study. The heat stress signals in leaves were estimated at around 20–25% of the CGf maximum (**Figure 4**), whereas signals observed in pollen often reached magnitudes close to 100% (**Figure 5**). With the caveat that *in vitro* calibration curves cannot precisely predict an *in vivo* $[Ca^{2+}]_{cyt}$, a 50% maximum signal *in planta* will likely be close to the reporters K_D concentration (**Figure 2**). Thus, the heat stress signals observed in leaves showed magnitudes that appeared to be less than CGfs K_D around 220 nM, whereas signals in pollen showed magnitudes that likely rose to near or above the estimated 960 nM needed to reach CGf's maximum.

Heat Stress Triggers Multiple Ca^{2+} Signals in Leaves

CGf-based analyses in leaves provide strong evidence that heat stress can induce cytosolic Ca^{2+} signals in plants (**Figure 4**). Prior studies with aequorin provided mixed results (Gao et al., 2012; Finka and Goloubinoff, 2014; Lenzoni and Knight, 2019). In a recent study using aequorin, a heat-triggered Ca^{2+} signal was identified inside chloroplasts, but not in the cytoplasm, using young cotyledon staged seedlings (Lenzoni and Knight, 2019). Another aequorin study with *Arabidopsis* seedlings reported a gradual heat-dependent increase in $[Ca^{2+}]_{cyt}$, but this analysis was not extended for a sufficient period of time to confirm that $[Ca^{2+}]_{cyt}$ returned to a baseline resting level (Gao et al., 2012), as expected for a stereotypical Ca^{2+} signal. In *Physcomitrella*, heat stress also failed to induce a typical Ca^{2+} transient in wild type cells, although an increase in $[Ca^{2+}]_{cyt}$ was observed for a heat-sensitive mutant with a deletion of a cyclic nucleotide gated channel (Finka and Goloubinoff, 2014). These mixed results might be explained by aequorin's relatively weak affinity for Ca^{2+} ($K_D \sim 7$–13 μM) (Costa et al., 2018), which makes it a suboptimal reporter for detecting the types of low nM $[Ca^{2+}]$ signals reported here using a CGf ($K_D \sim 220$ nM). In addition, the aequorin reporter requires the addition of a substrate. Therefore, mixed results may be due to different efficiencies of substrate loading or other constraints on substrate/aequorin interactions.

The CGf reporter also showed that heat-triggered signals were approximately 1.5-fold greater in magnitude than the blue light signals in the same leaves (**Figure 4**). Blue light signals were previously documented using aequorin (Harada et al., 2003), YCnano65, and a GCaMP6 (Ishka et al., 2021). Evidence indicates that blue light activates phototropin receptors that trigger a Ca^{2+} induced Ca^{2+} release from internal Ca^{2+} stores (Harada and Shimazaki, 2007). Interestingly, there is also evidence that phototropins contribute to temperature perception (Hayes et al., 2021). However, they are reported to have increased activity at lower temperatures, which argues that they are unlikely candidates for mediating a heat-induced Ca^{2+} influx. In addition, the kinetic differences seen here between blue light and heat-triggered signals suggests that the cellular machinery involved in generating each of these signals is either different or subject to different regulatory controls. While several candidate channels for heat-triggered Ca^{2+} entry have been proposed, such as cyclic nucleotide gated channels (Tunc-Ozdemir et al., 2013; Wang et al., 2021), it is not yet clear which channels might actually contribute to the heat stress signatures identified here in leaves.

Heat Stress Suppresses Normal Growth Associated Ca^{2+} Signals in Pollen

The CGf analyses with pollen provided an example of a heat stress response that appears very different than leaves. Unlike leaves, pollen tubes failed to show a heat-triggered increase in $[Ca^{2+}]_{cyt}$, either at the tip or elsewhere in the tube shank (**Figure 6**). Instead, the normal steady growth Ca^{2+} oscillations at the pollen tube tip shifted to a new oscillation pattern typical of a growth arrest (22% of cases) or a severely dampened oscillation with a nearly undetectable baseline $[Ca^{2+}]_{cyt}$ (78% of cases).

While pollen fertility is considered highly vulnerable to heat stress, the underlying causes remain speculative and are likely different during various phases of pollen development and fertilization (Johnson et al., 2019). It is noteworthy that experiments here revealed that a relatively small increase in the maximum heat from 32 to 35°C was accompanied by a more than 100-fold increase in the frequency of pollen tube tip ruptures (**Figure 6A**). This suggests that rapid tip growth processes represent a point of thermo-vulnerability, possibly because heat stress disrupts the precise coordination required to stabilize newly delivered membranes and cell wall structures at the growing tip. Regardless, the observed heat stress suppression of tip-focused $[Ca^{2+}]_{cyt}$ oscillations occurred within a minute, correlating with a rapid and potentially thermo-protective switch to a pause in tip growth.

Using Ratiometric Reporters to Catalog the Diversity of Stimulus-Specific Ca^{2+} Signals in Plants

CGf and other ratiometric reporters are often brighter and provide stronger signals than FRET-based sensors such as YCnano65. Thus, CGf-like reporters represent an important experimental opportunity to compare and classify different stimulus-specific Ca^{2+} signals. While it is not yet clear how many functionally different Ca^{2+} signals are generated in plants, pollen cells alone express at least 36 potential Ca^{2+}-permeable ion channels, along with multiple kinetic-modifying Ca^{2+} pumps and exchangers located in various compartments including the vacuole, ER, plasma membrane and other membrane organelles (Johnson et al., 2019). This complexity of cellular machinery creates an expectation for a large diversity of Ca^{2+} signals throughout the plant.

While three kinetically distinct heat-triggered $[Ca^{2+}]_{cyt}$ signatures were identified in leaves (**Figure 4**), it is likely that additional signals will be uncovered as more individual cell types are examined and different heat stress scenarios are considered. Importantly, heat stress in the real world is often accompanied by additional combinatorial stress factors, such as drought, high light, and nutritional limitations (Zandalinas et al., 2021), all of which are expected to uniquely impact the transcription and regulation of the machinery coding and decoding Ca^{2+} signals in different cells.

The current study suggests that both heat stress and blue light signals in vegetative cells occur through a rapid $Ca^{2+}{}_{cyt}$ influx followed by a relatively slow efflux, with 50% durations ranging from 5 to 19 min (**Figure 4**). In contrast, $[Ca^{2+}]_{cyt}$ signals in pollen were as much as 2,000-times faster, with 50% durations ranging from 0.5 to 5 s (**Figure 5**). These kinetic differences likely have profound consequences in the context of downstream signaling events. For example, a 5–19 min continuous elevation in $[Ca^{2+}]_{cyt}$ provides ample time for Ca^{2+} to diffuse throughout the cell and create long-lasting physiological changes, such as sustained activation of Ca^{2+}-dependent phosphoregulatory networks and transcriptional changes leading to a long-term acclimation response (Liu et al., 2018; Alves et al., 2021; Damaris and Yang, 2021; Noman et al., 2021). In contrast, the rapid and highly localized $[Ca^{2+}]_{cyt}$ signals at the pollen tube tip are likely to have more restricted tip-focused functions related to rapid growth, such as regulating dynamics of secretion, actin filaments, and other components playing key roles in the tip-focused growth machinery (Qian and Xiang, 2019). The observation that heat stress suppresses these growth-associated $[Ca^{2+}]_{cyt}$ signals (**Figure 6**) supports a model in which the most urgent need for heat stress signaling in pollen tubes is to shift $[Ca^{2+}]_{cyt}$ oscillations at the growing tip into a growth arrest mode because a failure to do so could lead to an asynchronization of the growth machinery and an increased frequency of pollen tube ruptures.

These contrasting examples of heat-triggered $[Ca^{2+}]_{cyt}$ responses in leaves and pollen highlight the diversity of Ca^{2+} signals generated in plants, and more so, a need to better understand the underlying channels, transport systems, and signal transduction networks responsible for creating and decoding Ca^{2+} signals in plants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CW led the experiments on pollen imaging and helped design all experiments. S-HK conducted whole-plant imaging in response to heat stress and data analyses. EB conducted the *in vitro* spectrofluorometer studies. EM conducted effects of CGf expression to root, rosette, and seed sets development. MM conducted Western Blot analyses. GM and JH initiated the CGf reporter design for pollen. JH led the development of transgenic lines, reporter constructs, and genetic analyses. W-GC led the experiments on seedling imaging. W-GC and JH designed the experiments and supervised the overall project. All authors contributed constructive comments on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.777975/full#supplementary-material

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