HORMONAL CROSSTALK ON THE REGULATION OF STRESS RESPONSES

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HORMONAL CROSSTALK ON THE REGULATION OF STRESS RESPONSES

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Editorial: Hormonal crosstalk on the regulation of stress responses

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Editorial on the Research Topic
Hormonal crosstalk on the regulation of stress responses

Plant biotic and abiotic stress poses significant challenges to the agroecosystem in the age of rapid global climate change, thus affecting crop growth and posing a serious risk to agricultural productivity and food security worldwide. Several studies have demonstrated that plant stresses trigger the production of phytohormones leading to an alteration in their balance, which contributes to plant adaption to numerous external stimuli. Although significant progress has been made in the characterization of stress-triggered changes in hormone metabolism in plants, many unknowns remain. This Research Topic reflects the latest updates on hormonal regulation of the complex signaling networks and metabolic pathways and provides future perspectives on the hormonal crosstalk in regulating stress responses and tolerance mechanisms.

Exogenous hormone applications may improve abiotic and biotic stress tolerance by regulating reactive oxygen species (ROS), antioxidant enzyme activity, photosynthesis, soluble sugars, and nitrogen metabolism. Nagar et al. examined the role of a plant hormone gibberellic acid (GA) in terminal heat stress tolerance of wheat genotypes upon spraying GA_3 or its biosynthesis inhibitor paclobutrazol (PBZ). The authors found that exogenous GA_3 did not increase thermotolerance, whereas PBZ's application enhanced thermotolerance by increasing antioxidant enzyme activity and photosynthesis, and decreasing lipid peroxidation and ion leakage under heat stress These results suggest that the thermotolerance is closely associated with alternative mechanisms caused by PBZ application but not inhibition of GA biosynthesis. Within the same species, Kaya et al. explored the effects of the combined application of another plant hormone, methyl jasmonate (Me-JA) and nitric oxide (NO)–donor sodium nitroprusside (SNP) in cadmium (Cd) stress mitigation. The results revealed that exogenously applied Me-JA and SNP, either alone or combined, improved nitrogen metabolism in plants grown under Cd stress. Compared to individual applications of Me-JA or SNP, the combined application resulted in more prominent plant growth-promoting effects and Cd level reduction in plant

tissues, implying synergistic effects of the two compounds in alleviating Cd toxicity. Additionally, Zhang, Chen, et al. investigated exogenous brassinolide (BR)-induced calcium nitrate stress tolerance in tomato through proteomics analysis, uncovering improved photosynthesis and antioxidant metabolism, and reduced ROS and lipid peroxidation. A study by He et al. implicated the small auxin up-regulated RNA (SAUR) family member *AtSAUR32* in abscisic acid (ABA) signaling under drought stress. *AtSAUR32* participated in drought stress tolerance by regulating transcription factors (DREB, WRKY, and NAC) in ABA-independent signaling pathway and by interacting clade-A PP2Cs proteins in ABA-dependent signaling pathway.

Other research articles in this collection evaluated the impact of exogenous compounds like melatonin, cytokinin 6-benzyladenine (6-BA), and diethyl aminoethyl hexanoate (DA-6) in regulating plant responses to environmental signals. The work by Zhang, Fan, et al. expands our knowledge about the molecular mechanism of melatonin in regulating the cotton response to salt stress using RNA-seq analysis. Exogenous melatonin activated antioxidant enzyme activity and Ca^{2+} signal transduction, which in turn reduced excess production of ROS. In addition, expression of transcription factors (AP2/ERF-ERF, WRKY, NAC, C2H2, etc.) and redox-related genes was upregulated by melatonin under salt stress. The obtained data suggest that melatonin plays a crucial role in regulating the complex network for the salt stress tolerance. In another work published in this research collection, Wang et al. showed the effects of the exogenous application of synthetic 6-BA on plant growth under waterlogging stress in waxy corn. Exogenous 6-BA alleviated the increase of chlorosis, necrosis, ROS and membrane lipid peroxidation by activating antioxidant enzyme activities (ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase) and the ascorbate-glutathione cycle system in waterlogging-stressed waxy corn, consequently improving waterlogging stress tolerance. Similarly, Hassan et al. showed the pretreatment effect of DA-6 on seed germination of white clover under drought stress. DA-6 pretreatment enhanced indole-3-acetic acid, cytokinin and GA content, but suppressed ABA content, leading to an increase in the ROS scavenging system, osmotic adjustment, dehydrin protein and transcript accumulation.

Furthermore, Aslam et al. reviewed the roles of jasmonate (JA), Ca^{2+} and glutathione (GSH) in abiotic stress tolerance, and their interconnected signaling pathways. Ca^{2+} signaling leads to JA biosynthesis and GSH accumulation, thereby maintaining cellular homeostasis and consequently improving abiotic stress tolerance. This interaction between the JA, Ca^{2+}, and GSH represents a novel abiotic stress tolerance mechanism in plants. In the other review article in this collection, Singhal et al. discussed the crosstalk of important signaling compounds (NO, hydrogen sulfide, hydrogen peroxide, Ca^{2+}, and ROS) and phytohormones, and their role in salinity stress tolerance. Further, the recent advances are described in integrative multi-omics approaches, which are crucial to leverage to better understand the molecular basis of salinity tolerance and develop new promising salt-tolerant varieties.

And the final article in this collection sheds fresh light on ferroptosis, i.e., iron-dependent-cell death in plants (Dixon et al., 2012). Ferroptosis is mostly regulated by ROS, GSH and Fe^{2+} levels under abiotic and biotic stress (Dixon and Stockwell, 2019). The study by Riyazuddin and Gupta suggests a potential association between gaseous plant hormone ethylene and ferroptosis.

Overall, these articles provide illustrative examples of recent advances in the research area of hormone crosstalk and highlight the complexity of connections between key signaling and metabolic pathways in plants. We hope that these compiled articles will give a new insight into this topic and useful information to expand the research area in the future.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Arabidopsis SMALL AUXIN UP RNA32 Protein Regulates ABA-Mediated Responses to Drought Stress

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SMALL AUXIN UP-REGULATED RNAs (SAURs) are recognized as auxin-responsive genes involved in the regulation of abiotic stress adaptive growth. Among the growth-limiting factors, water-deficit condition significantly affects plant growth and development. The putative function of SAUR family member *AtSAUR32* has the potential to diminish the negative impact of drought stress, but the exact function and mode of action remain unclear in Arabidopsis. In the current study, *AtSAUR32* gene was cloned and functionally analyzed. *AtSAUR32* localized to the plasma membrane and nucleus was dominantly expressed in roots and highly induced by abscisic acid and drought treatment at certain time points. The stomatal closure and seed germination of *saur32* were less sensitive to ABA relative to *AtSAUR32*-overexpressed line (OE32-5) and wild type (WT). Moreover, the *saur32* mutant under drought stress showed increased ion leakage while quantum yield of photosystem II (ΦPSII) and endogenous ABA accumulation were reduced, along with the expression pattern of ABA/stress-responsive genes compared with WT and the OE32-5 transgenic line. Additionally, yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays showed that AtSAUR32 interacted with clade-A PP2C proteins (AtHAI1 and AtAIP1) to regulate ABA sensitivity in Arabidopsis. Taken together, these results indicate that *AtSAUR32* plays an important role in drought stress adaptation via mediating ABA signal transduction.

Keywords: ABA, Arabidopsis, *AtSAUR32*, drought, PP2C

INTRODUCTION

Being sessile in nature, plants adopt sophisticated mechanism to overcome unavoidable harsh environmental challenges such as extreme temperature and drought, which adversely affect plant growth, development, and productivity. Among these mechanisms are regulation of various metabolic pathways, cellular processes, and activation of stress-resistant genes. Such genes that participate in stress responses can be used to boost crop stress tolerance (Liu et al., 2020). During the period of adaptation, several plant hormones play pivotal functions in stimulating or inhibiting plant development, growth, and stress responses. Among all phytohormones, abscisic acid (ABA)

is well known as a stress plant hormone that regulates various molecular and cellular processes including stoma aperture (to control transpiration rate), and transcript levels of stress-responsive genes throughout development (Kuromori et al., 2018; Yoshida and Fernie, 2018) in response to osmotic and drought stress conditions. As an endogenous signal for growth, ABA plays a vital function in the germination of seeds, seedling growth, and development under normal conditions (Humplík et al., 2017). Additionally, when plants experience water deficit, ABA biosynthesis occurs rapidly, moves from the roots to shoots, regulates leaf growth, and induces stomatal closure to counter the stress (Guo et al., 2020).

Furthermore, ABA-dependent and -independent pathways play significant roles in drought stress responses (Shinozaki and Yamaguchi-Shinozaki, 2007). In the ABA-dependent pathway, ABA accumulates during stresses and pyrabactin resistance/pyrabactin resistance-like/regulatory components of ABA receptors (PYR/PYL/RCAR) bind to ABA and clade-A phosphatase type 2C (PP2C.A) to form a PP2C-ABA-PYL ternary complex (Cutler et al., 2010). Binding of ABA-bound PYR/PYL/RCAR to PP2C inhibits the phosphatase activity of PP2C thereby activating SUCROSE NONFERMENTING-1-related protein subfamily 2 kinases (SnRK2s) to phosphorylate ABF/AREB/ABI5 family transcription factors and activates ABA-induced gene expression (Fujita et al., 2009), and ultimately stomatal closure is promoted to overcome the stress (Cutler et al., 2010). Members of clade-A PP2C include *ABI1* (ABA INSENSITIVE 1), *ABI2*, *HAB1* (HYPERSENSITIVE to ABA1), *HAB2*, *AHG1* (ABA HYPERSENSITIVE GERMINATION1), *HAI1* (HIGHLY ABA-INDUCED 1), *HAI2*, and *AIP1* (AKT1-Interacting Phosphatase1) (Hirayama and Shinozaki, 2007). It has been proved that these PP2C genes are generally negative regulators of ABA signaling pathways, which are involved in many ABA-regulated responses, such as rhizogenesis, seed germination, stomatal closure to reduce transpiration rate, inhibition of vegetative growth, and drought-induced resistance (Kim et al., 2013). ABI1 and ABI2 have been verified as negative regulators of ABA signaling and drought stress response. The null mutants of PP2C.A and HAB1 increases ABA response and drought tolerance (Lim et al., 2014, 2015). *hai1* mutant exhibits increased plant sensitivity to ABA signaling, lowers water loss, and results in increased drought tolerance compared to WT (Zhang et al., 2013). Members of the PP2C.A subfamily in rice have also been proposed to mediate drought resistance (Singh et al., 2010). Therefore, PP2C.A members are widely believed to serve very important roles in drought stress responses.

Auxin is vital for regulating plant growth, development, and adaptation to fluctuating environment. However, SMALL AUXIN UP RNAs (SAURs) are primary auxin-responsive genes that are critically involved in auxin signaling pathway and are quickly induced by auxin treatment. Previous studies revealed that SAURs regulate plant developmental and physiological processes (Wu et al., 2012). In Arabidopsis, overexpression of SAUR41 and stabilized fusion proteins of SAUR19 and SAUR63 promote hypocotyl elongation and leaf growth (Chae et al., 2012; Spartz et al., 2012; Kong et al., 2013). Studies have proved that SAUR19 in Arabidopsis can inhibit PP2C.D phosphatases to regulate plasma membrane H^+-ATPases to promote cell expansion (Spartz et al., 2014). *AtSAUR36* was reported to negatively regulate cell expansion and inhibit leaf growth (Hou et al., 2013). *AtSAUR76* confers reduction in leaf size, but may positively regulate root growth (Stamm and Kumar, 2013). Overexpression of *AtSAUR36* promotes leaf senescence (Hou et al., 2013). Meanwhile, some SAUR genes were proved to participate in tropic growth, apical hook development, seed germination, shade avoidance responses, calcium signaling, and so on (Ren and Gray, 2015). However, functional studies of SAURs were limited in some subgroups and there are few reports on the roles of SAUR in stress responses. Under high temperature, *SAUR19* gene functions downstream of PIF4 to regulate hypocotyl growth (Franklin et al., 2011) and *Pro35S*:GFP-*AtSAUR19* expression confers drought hypersensitivity in tomato (Spartz et al., 2017). The expression levels of many SAUR genes were reduced in response to stress-related hormones: jasmonate and ABA, as well as drought and osmotic stresses (Kodaira et al., 2011). These results indicate that SAUR genes play multiple functions in plant development and defense responses.

Therefore, we conducted this research to functionally characterize a novel gene (*AtSAUR32*) in Arabidopsis plant. We investigated the role of *AtSAUR32* in ABA signaling and drought tolerance through generating *saur32* mutants and overexpressed lines via transgenic approach. Further, AtSAUR32 was verified to interact with HAI1 and AIP1 proteins since their expression levels were increased in *saur32* mutants, while yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays were used for further confirmation. Besides, we investigated the physiological responses and changes triggered by drought stress in SOD, proline, H_2O_2 accumulation, and ion leakage. Together, these findings provide a solid background to understand the vital role of *AtSAUR32* gene in drought stress tolerance.

MATERIALS AND METHODS

Web-Based Analysis of AtSAUR32

For the identification of *cis*-regulatory elements, 1.5 k bp upstream from the start codon (ATG) of the Small auxin up-regulated RNA (SAUR) gene, *AtSAUR32*, was obtained from the Arabidopsis information resource (TAIR)[1] and queried against the PlantCARE[2] (Lescot, 2002) (**Supplementary Table 1**).

saur32 Mutants and Transgene Constructs

Arabidopsis thaliana plants used in this study were of the Columbia-0 ecotype (Col-0) background. One T-DNA insertion mutant *saur32* (*SALK_033535*) was obtained from the Arabidopsis Biological Resource Center, which has been previously reported to lack *AtSAUR32* expression (Park et al., 2007). In order to identify the homozygous T-DNA insertion,

[1]https://www.arabidopsis.org/index.jsp

[2]http://bioinformatics.psb.ugent.be/webtools/plantcare/html/

the genomic DNA of mutant seedlings were submitted to PCR genotyping using the primer list in **Supplementary Table 2**. The seeds were surface-sterilized, stratified at 4°C for 3 days, and germinated on 1/2 MS medium for 10 days. Seedlings were then transferred to a growth chamber at 22/18°C cycle with a photoperiod of 12/12 h (light/dark) under 60% relative humidity for further growth.

To construct vectors for *AtSAUR32* overexpression, the coding region was cloned into the PB7YWG2.0 Gateway vector (Invitrogen) fused with an enhanced yellow fluorescent protein (YFP) tag and driven by the constitutive 35S promoter (primer pairs listed in **Supplementary Table 3**). Then, the recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium*-mediated transformation was performed using wild-type (WT) Arabidopsis by floral dip method (Zhang et al., 2006; Ali et al., 2020b). Transgenic plants were selected and then screened with 0.1% Basta for their ability to overexpress *AtSAUR32*. The T_4 homozygous transgenic plants were used for all experiments.

Y2H and BiFC Assay

The full-length coding sequence of *AtSAUR32* was amplified by PCR for Y2H assay, using the primer pairs listed in **Supplementary Table 4**. *AtSAUR32* sequence was ligated into the pGBKT7 vector by restriction site *EcoR* I and *Sma* I, while *HAI1* and *AIP1* were cloned into pGADT7 with the same enzyme sites to form pGADT7-*HAI1*/*AIP1* vectors as prey vector. Two pairs of vectors (pGBKT7-*AtSAUR32*+pGADT7-*HAI1*, pGBKT7-*AtSAUR32*+pGADT7-*AIP1*) and control vectors (pGBKT7-53+pGADT7-T1, pGBKT7-Lam+pGADT7-T1) were transformed into yeast strain AH109 according to the manufacturer's protocol (Clontech, Beijing, China). The transformed yeast was selected on SD/-Leu-Trp-His-Ade dropout medium. The colonies that contained two interacting proteins grew up after incubation at 30°C for 3 days.

For the BiFC assay, the coding sequence of *AtSAUR32* was fused with N-YFP to generate N-terminal in-frame fusions with N-YFP, and CDS of *HAI1* and *AIP1* were fused with C-YFP to generate C-terminal in-frame fusions with C-YFP as described earlier (Cui et al., 2007). The plasmids were introduced into *A. tumefaciens* (C58C1) as verified through sequencing, and infiltrated into *Nicotiana benthamiana*. After 48 h of co-infiltration, the tissues were analyzed. The fluorescence signal was visualized using a Zeiss LSM710 confocal microscope, and images were superimposed using ZEISS LSM710 software.

Protein Localization Assay

The coding sequence of AtSAUR32 was amplified and cloned into pFGC-eGFP vector by using the standard molecular techniques (primers, **Supplementary Table 5**) (Ouyang et al., 2016). The transient expression of AtSAUR32 tagged with GFP was achieved in tobacco epidermal cells. In detail, the construct was transferred into GV3101-competent cells followed by harvesting cells and then added 200 μM acetosyringone, 10 mM MES (pH 5.5), and 10 mM $MgCl_2$ and subsequently injected into 4-week-old tobacco seedlings through the leaves using needless syringe. The tobacco plants were kept in the dark for 48 h and then shifted to growth chamber for further 2–3 days. The tobacco leaf cells were visualized using a fluorescent confocal microscope (OLYMPUS BX63, Tokyo, Japan) with an emission of 509 nm and a 488-nm excitation wavelength.

Measurement of Ion Leakage and Water Loss

The ion leakage rate was measured as described by Liu et al. (2017) with a little modification (Liu et al., 2017). Leaf samples were submerged in 10 ml of distilled water followed by incubation in boiling water (100°C) for 20 min; the conductivities C_1 (initial before heating) and C_2 (after cooling) were measured using a conductivity meter (Model DDS-11A, Shanghai Leici Instrument Inc., Shanghai, China). The ion leakage was calculated as $(C_1/C_2) \times 100$.

The water loss assay was assessed in detached leaves of 4-week-old *AtSAUR32*-overexpressed, *saur32*, and WT plants placed in a growth chamber with 40% relative humidity. The fresh weight (FW) was recorded immediately after the designated interval of time [0, 0.5, 1, 2, 4, 8, and 12 h post-treatment (hpt)]. The proportion of water lost was calculated as described by Yoo et al. (2010), and the experiment comprised three replicates (Yoo et al., 2010).

$$\text{Water loss rate } \frac{\text{Initial FW} - \text{Final FW}}{\text{Initial FW}} \times 100$$

ROS Detection by Histochemical Staining

The accumulation of hydrogen peroxide (H_2O_2) was performed as previously described (Ali et al., 2020a). The fully expanded leaves from the top of control and drought-stressed WT, *ATSAUR32*-overexpressed, and *saur32* mutant plants were detached and accumulation of H_2O_2 in leaves was detected by staining with diaminobenzidine (DAB) in 50 mM *Tris*-acetate (pH 3.8) after incubation at 25°C in the dark for 8 h. Chlorophyll was removed by immersing in 80% ethanol, at 70°C for 10 min and stained images were photographed.

Seed Germination Experiment

The seeds of WT and *saur32* were surface-sterilized, stratified at 4°C for 3 days, and then grown on 1/2 MS medium (0 and 1 μM ABA); seed germination rates were measured at 12-h intervals (0, 12, 24, 36, and 48 h), and seed germination percentage was calculated using the formula below. Further, to observe seedlings in response to ABA, the WT and *saur32* seeds were germinated on 1/2 MS medium supplemented with 0, 0.2, 0.4, and 0.8 μM ABA. After 10 days, seedlings with expanded cotyledon were counted to calculate the green cotyledons. Each test was performed in more than three biological repeats.

$$\text{Germination percentage } \frac{\text{Number of germinated seeds}}{\text{Total number of platted seeds}} \times 100$$

Stomatal Aperture Conductance

Stomatal aperture of epidermal peels was assessed according to Zhang et al. (2012) with little modifications. Leaves from 4-week-old plants were removed and epidermal peels were incubated

in buffer containing a solution of 50 mM KCl, 0.2 mM $CaCl_2$, and 10 mM 2-(N-morpholino)-ethane-sulfonic acid (MES)-KOH (pH 6.15) at 22°C under white light (150 pmol m^{-2} s^{-1}) for 2 h to open the stomata. The leaves were then transferred to MES-KCl buffer containing 10 μM ABA for 1 and 3 h. During analysis, light was reduced so that it does not affect the stomata size. The length and width of stomatal pore were measured using ImageJ tool (Schneider et al., 2012) and used to calculate the stomatal aperture. The experiment was conducted with three biological replicates.

Phytohormone Extraction and Quantification

The extraction of the endogenous hormones: abscisic acid (ABA), jasmonic acid (JA), and Auxin (IAA) from *saur32* and WT plants were done according to the described methods with little modifications (Fu et al., 2012; Pan et al., 2019). Plant samples (0.1 g) were ground in liquid nitrogen and homogenized in 1 ml ethyl-acetate having 25 μl solution of standard d6–ABA (OlchemIm Ltd., Czechoslovakia), d5-JA (QCC), and d2-IAA (Sigma-Aldrich). The samples were vortexed, agitated at 140 rpm at 4°C for 12 h, and centrifuged at 12,000 rpm for 10 min, and extraction was repeated with agitation for 1 h. The supernatants were collected and evaporated to dryness using nitrogen gas. 0.5 ml of 70% methanol was added to the dried samples, vortexed, and centrifuged at 12,000 rpm for 10 min at 4°C. 0.2 ml of sample supernatants was placed in snap-cap vials and analyzed using Agilent 1290 infinity HPLC system coupled with Agilent 6460 Triple Quad liquid chromatography-mass spectrometry device (Agilent Technologies, Germany). The Agilent Zorbax XDB C 18 column (150 × 2.1 mm, 3.5 μm) was used for HPLC analysis as described in the previous study (Chen et al., 2018).

RNA Isolation and Library Preparation

Total RNA was isolated from *saur32* and WT plants (4 weeks old) using TRIzol reagent (Invitrogen, Germany) according to the manufacturer's instructions. RNA quality was checked by 1% agarose gel electrophoresis, and the concentration was determined by Nanodrop 1000 spectrophotometer (Thermo Scientific Inc.). The RNA samples (two replicates) were sent to Biomarker Company (Beijing, China) for reverse transcription to cDNA and then sequenced on an Illumina HiSeq^{TM} 2000 platform (Jiang et al., 2011). Analysis and identification of differentially expressed genes (DEGs) ($\log_2|$ Fold Change$| \geq 1$ and FDR < 0.01) and Gene Ontology (GO) functional and pathway enrichment analyses of DEGs were also carried out by the same company. Raw sequence reads were uploaded into the NCBI Sequence Read Archive with accession number PRJNA688697.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from all the samples using TRIzol reagent (Invitrogen, Germany), according to the manufacturer's instructions. The RNA extraction procedure and cDNA synthesis followed the same procedures as detailed in our recently published papers (Ali et al., 2018, 2020a). For qRT-PCR, specific primer sets were designed and checked for primer specificity in Arabidopsis genome (**Supplementary Table 6**). qRT-PCR was performed in CFX96 Real-Time System machine (Bio-RAD, Hercules, CA, United States) using SYBR Premix Ex Taq^{TM} II (TaKaRa). Relative expression levels were normalized by Arabidopsis *ACTIN7* gene and calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Data were presented as means and +SD of three replications obtained from three independent biological experiments.

Drought Stress and ABA Treatment

For drought treatment, 4-week-old well-watered Arabidopsis *saur32* mutant, *AtSAUR32*-overexpressed, and WT plants were used. The plants were deprived of water for 15 days in the greenhouse. Three biological replicate experiments were conducted for each line. For ABA treatment, 4-week-old Arabidopsis plants were sprayed with 10 μM ABA and control plants were separately sprayed with mock solution and maintained under the same growth environment.

Determinations of Proline and SOD Content

Free proline content was quantified following the procedure described by Bates et al. (1973). Leaf sample (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid using mortar and pestle, and the homogenate was filtered through Whatman filter paper no. 2. Ninhydrin solution (2.5%) was prepared in glacial acetic acid with further addition of 6 M phosphoric acid. The reaction mixture containing the plant extract (2 ml), glacial acetic acid (2 ml), and ninhydrin fresh solution (2 ml) was mixed and boiled at 100°C for 1 h in a water bath. Toluene (4 ml) was added in cooled reaction mixture tubes and thoroughly shaken. The optical density (OD) values were measured at 520 nm and the proline content (μmol g^{-1} FW) was calculated from standard curve.

Superoxide dismutase (SOD) activity was measured by photoreduction of NBT (Nitro-Blue Tetrazolium). 0.5 g of fresh leaf was ground in mortar with 5 ml of PBS (50 mM PBS, 25 mM NBT, 0.003 mM Riboflavin, and 0.1 mM EDTA, pH 7.8). The extract was centrifuged for 15 min under 4°C at 12,000 rpm. The supernatant was illuminated at 4000 lux for 20 min. SOD activity was spectrophotometrically quantified at 560 nm. Control was measured in the dark according to the abovementioned procedure while the SOD activity was calculated by the method of Dionisio-Sese and Tobita (Stewart and Bewley, 1980).

Statistical Analysis and Data Plotting

Each experiment was conducted in replicates for authenticity, and data were analyzed by IBM SPSS Statistics 25 while Duncan Multiple Range (DMR) test was applied. The plotted data are the means of three independent experiments and the standard deviation (±SD). Asterisk symbols ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) indicate the significant difference in comparison to WT. Graphs were drawn by GraphPad Prism 8.0 (GraphPad

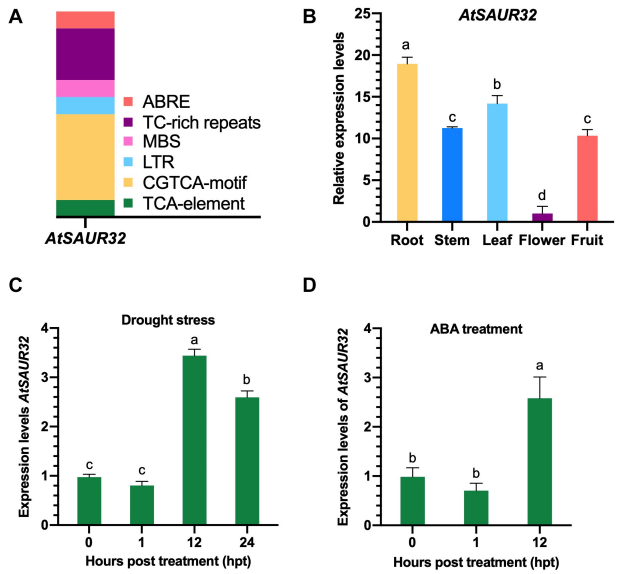

FIGURE 1 | Transcriptomic and qRT-PCR analysis of *AtSAUR32* gene. **(A)** *Cis*-regulatory elements involved in the stimulation of *AtSAUR32* used 1.5 k upstream region and analyzed by the online tool PlantCARE. **(B)** The tissue-specific expression levels of *AtSAUR32* (Arabidopsis plants were grown under normal condition) were examined using qRT-PCR. **(C)** The expression pattern of *AtSAUR32* under drought treatment performed by qRT-PCR analysis. **(D)** The expression pattern of *AtSAUR32* under ABA treatment, examined by qRT-PCR. Mean values and standard deviation (±SD) are plotted as analyzed by Duncan's multiple range (DMR) test ($P < 0.05$); lowercase letters (a–d) indicate significant difference.

Software, Inc., La Jolla, CA, United States), and PhotoScape X (Pro version 4.0) was used for designing.

RESULTS

In silico and Expression Analysis of *AtSAUR32*

In silico analysis of *AtSAUR32* indicated that *cis*-elements conferring response to plant hormones and abiotic stresses were found in the promoter region. Noticeably, the *AtSAUR32* promoter region carried ABA responsiveness elements (ABRE), MeJA-responsiveness elements (CGTCA-motif), and salicylic acid responsiveness elements (TCA-element) shown in **Figure 1A** and **Supplementary Table 7**. Besides phytohormone responsive element, some abiotic stress responsive regulatory elements were also detected, such as MYB binding site involved in drought-inducibility (MBS), LTR (low-temperature responsiveness), and TC-rich repeats (defense and stress responsiveness). All of the anticipated *cis*-elements were involved in responses to signaling molecules and stresses.

The qRT-PCR analysis revealed that *AtSAUR32* transcript abundance was evaluated in vegetative (root, stem, and leaf) and reproductive (flower and fruit) tissues in WT Arabidopsis plants. The results showed dominant expression in root (19-fold) followed by leaf (14-fold), while the lowest expression, 1-fold, was recorded in the flower (**Figure 1B**), implying significant contribution of *AtSAUR32* during the seedling stage of Arabidopsis. For further insight into the transcriptomic features of *AtSAUR32* in Arabidopsis leaves and roots under drought conditions, we performed *in silico* analysis from publicly accessible transcriptomic database of Arabidopsis (Berardini et al., 2015). The diagrams showed the index of expression level ranging from yellow (0) to red (570) (**Supplementary Figure 1**). The qRT-PCR results indicated that the expression level of *AtSAUR32* in Arabidopsis leaves exhibited higher expression levels at certain time points under drought stress conditions. As demonstrated in **Figure 1C**, *AtSAUR32* expression was initially at a basal and non-significant level in the first 1 h of drought stress and abruptly increased at 12 hpt to a higher peak (>3.6-fold) and then slightly reduced after 24 h. Overall, *in silico* and qRT-PCR analyses revealed that *AtSAUR32* showed remarkable response to drought at the transcriptional level.

In order to examine whether ABA regulates *SAUR32*, we retrieved publicly available data from Arabidopsis database (Berardini et al., 2015) and confirmed through qRT-PCR analysis. The results revealed high response of *AtSAUR32* to ABA treatment at 3 hpt. The diagrams display the index of expression levels ranging from yellow (0) to red (332) (**Supplementary Figure 2**). Furthermore, qRT-PCR analysis exhibited that after 12 h, the expression level of *AtSAUR32* was significantly higher compared with 0 and 1 hpt (**Figure 1D**).

AtSAUR32 Protein Localized in Cell Membrane and Nucleus

The pFGC-eGFP expression vector containing 35S promoter and green fluorescence protein (GFP) reporter gene was recombined with the ORF portion of AtSAUR32. The *N. benthamiana* plants that expressed as red nuclear marker RFP-H2B protein were infiltrated with pFGC-eGFP and pFGC-eGFP-AtSAUR32 fused plasmids for AtSAUR32 expression in ephemeral tissue. The confocal laser micrographs showed that 35S::AtSAUR32::GFP fused protein was mostly localized in the cell membrane and nucleus of the cell (**Supplementary Figure 3**).

Effect of Drought on *AtSAUR32* Knockout Arabidopsis Plants

To explore the protective role of *AtSAUR32* in Arabidopsis against water-deficit conditions, we examined the effects of *AtSAUR32* gene loss of function in knockout mutant. The T-DNA insertion mutant of *AtSAUR32* was determined in SALK collection (Columbia background), corresponding to the donor stock accession SALK_033535, which was named *saur32*. The sequencing of the T-DNA flanking region in *saur32* showed that the insertion was localized on the 5′ UTR region (**Figure 2A**). Furthermore, we confirmed the *saur32* mutant through qRT-PCR exhibited lack of *AtSAUR32* expression in *saur32* (**Figure 2B**) in line with Park et al. (2007). Hence, the *saur32* mutant and WT Arabidopsis plants were used for further study. The phenotypes shown in **Figure 2C** revealed that after rewatering, more WT than *saur32* plants recovered from the drought stress with survival percentage of 39 and 21, respectively (**Figure 2D**), indicating

that *AtSAUR32* loss of function significantly increases the susceptibility of Arabidopsis plants to drought stress. Moreover, the quantum yield of photosystem II electron transport (ΦPSII) and ion leakage were examined in untreated healthy and drought-treated plants. Under drought conditions, the value of ΦPSII was significantly decreased (0.28) in *saur32* at 7 days post-treatment (dpt) as compared with WT (0.39), while it was further decreased with increased time interval (15 dpt) in *saur32* and WT plants (**Figure 2E**). On the other hand, ion leakage in mutant (*saur32*) plants increased after drought stress, and a highly significant leakage was observed at 7 and 15 dpi in *saur32* plants, which were > 10 and 26% higher than in WT plants, respectively (**Figure 2F**). However, excessive ion leakage > 72% was recorded at 25 dpt. The determination of ion leakage was an indirect assessment of plasma membrane injury caused by drought stress. These results suggest the prominent role of *AtSAUR32* against drought as the *saur32* knockout mutant lost the ability to survive longer and less defensive in nature against abiotic stresses.

AtSAUR32-Overexpressed Arabidopsis Plants Exhibited Enhanced Drought Tolerance

To confirm the positive role of the drought stress-responsive *AtSAUR32* gene, it was overexpressed in Arabidopsis under the control of cauliflower mosaic virus 35S promoter. The 35S:*AtSAUR32* overexpressed lines were confirmed by PCR. To choose the best drought-resistant homozygous T_4 lines for further investigation, we detected the relative expression level of *AtSAUR32* in WT and three selected *AtSAUR32*-overexpressed (*AtSAUR32*-OE) Arabidopsis lines (OE32-5, OE32-6, and OE32-10) under normal growing conditions. As presented in **Figure 3A**, the transcript level of *AtSAUR32* in OE32-5 and OE32-6 lines was substantially higher compared with WT and OE32-10. Thus, the OE32-5 and OE32-6 were selected for further investigation.

To determine drought tolerance, *AtSAUR32*-overexpressed (OE32-5 and OE32-6) and WT plants were grown for 4 weeks under normal conditions, then subjected to drought stress for 15 days, and rewatered, and survival percentage was estimated 7 days after rewatering. Results revealed that *AtSAUR32* overexpressed lines (OE32-5 and OE32-6) had significant higher survival rates than the WT plants (**Figure 3B**). Seven days after rewatering, the survival percentage in the OE32-5 line was > 60%, while the OE32-6 line had 50% and were screened out for further study. The transpiration rate was measured in detached rosette leaves to determine the degree of water retention following drought stress and found that the weight loss in the OE32-5 was consistently (2–5 h) significantly lower than in the leaves of *saur32* mutant and WT plants (**Figure 3C**), indicating that the increased drought resistance of OE32-5 plants might be due to reduced rates of leaf transpiration. Moreover, ABA signaling regulates drought response by regulating stomatal aperture. The stomatal aperture of the *saur32*, OE32-5, and WT plants leaves was measured after ABA treatment (10 μM). At 1 h ABA treatment, the stomatal aperture of *saur32* leaves was significantly enhanced (0.39) relative to WT (0.29), while OE32-5 leaves showed a slight reduction (0.26) (**Figure 3D**). However, with increasing treatment period, the stomatal aperture was significantly increased in *saur32* as compared with WT and OE32-5, which were 0.41, 0.15, and 0.18, respectively. These results are consistent with water loss assay (**Figure 3C**).

To evaluate physiological changes, the contents of free proline and SOD were measured following drought stress. In the *AtSAUR32* transgenic line OE32-5, free proline concentrations were approximately one half lower than those in the *saur32* mutants (**Figure 3E**), while in WT plants, the accumulation was 32% lower. Comparison of SOD contents in *AtSAUR32* transgenic and *saur32* mutant plants showed remarkably lower (38.9%) levels in OE32-5 transgenic plants relative to *saur32* mutant, while WT is 17.2% lower (**Figure 3F**). The higher accumulation of proline and SOD in the *saur32* mutants revealed that the effect of drought stress on *saur32* mutants was significant as compared with the WT and OE32-5 line, which retained their ability to curb the drought stress. Moreover, as an important indicator of hydrogen peroxide (H_2O_2) production/damage, DAB staining was carried out. Clearly, more intense DAB staining (brownish color) was observed in *saur32* mutant plant after 10 days of drought stress, which indicated H_2O_2 accumulation (**Figure 3G**). On the contrary, OE32-5 and WT plants showed lower accumulation of H_2O_2, which displayed minimum damage under stress conditions. Surprisingly *AtSAUR32* plays a negative role in the development of plant considering that the size, number, and fresh weight of leaves were significantly higher in the *saur32* mutant plants than those in the WT plants throughout the period of investigation, although comparable values were observed for fresh leaf weight at 30 days after germination (**Supplementary Figure 4**).

AtSAUR32 Increases Sensitivity to ABA Responses

The phytohormone ABA plays a prominent role in the development of plant and generally regulates drought response (Zhu et al., 2020). We detected the seed germination rate of *saur32* mutant and WT in the presence of ABA (1 μM) and mock media (0 μM ABA). The germination rate of *saur32* was substantially higher than WT on the medium containing ABA at 12 and 24 hpt, which indicated that *saur32* plants are insensitive to exogenous ABA application (**Figure 4A**). However, in later hours (36 and 48 h), the effect was decreased and no obvious differences were noticed in treated and untreated seeds. Further, different concentrations of ABA (0, 0.2, 0.4, and 0.8 μM) were used to assess the green cotyledon of *saur32* and WT plants. After 10 days of treatment, the WT seedlings showed a dramatic decrease in green cotyledon percentage in media supplemented with 0.4 and 0.8 μM ABA as compared with *saur32* seedlings (**Figures 4B,C**). However, PAM chlorophyll fluorometer results displayed a remarkable phenotypic difference between WT and *saur32* seedlings grown on medium with 0.8 μM ABA while those on 0 and 0.2 μM had no significant difference phenotypically (**Figure 4C**). These results indicated that *AtSAUR32* had a negative function in green cotyledon and seedling growth under ABA treatment. Together, these results implied that *AtSAUR32* significantly alters some physiological

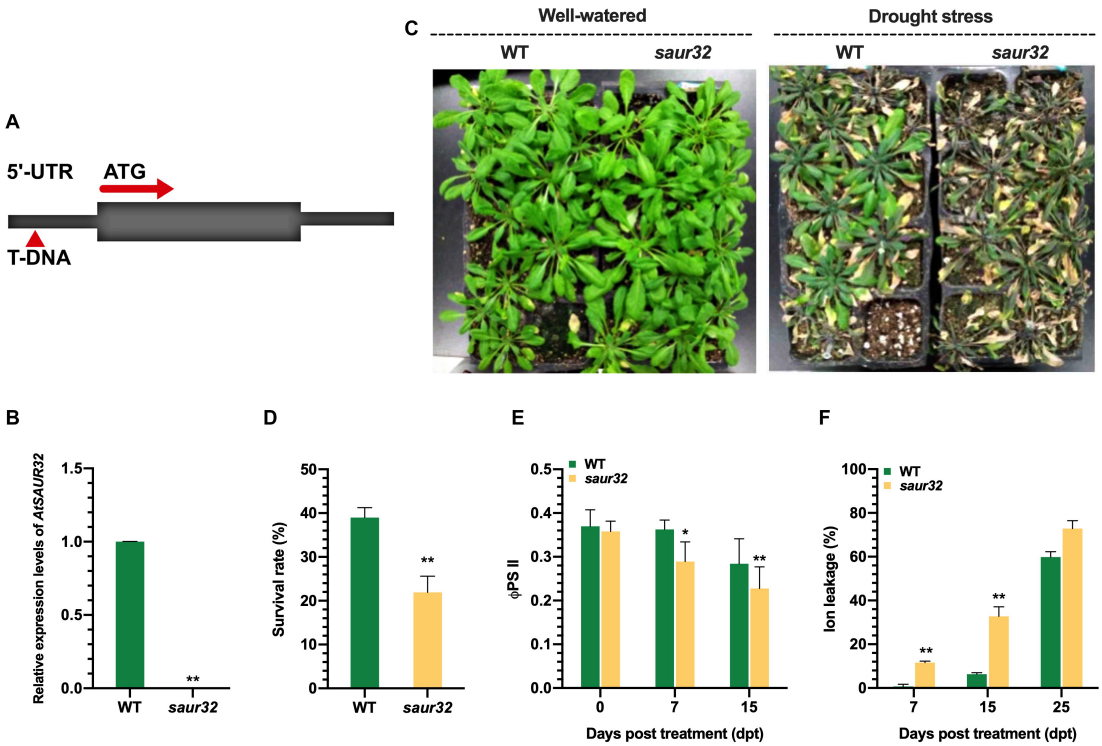

FIGURE 2 | *AtSAUR32* knockout mutant increased sensitivity to drought stress. **(A)** Schematic diagram of T-DNA insertion in *AtSAUR32*. **(B)** Transcript level of *AtSAUR32* in *saur32* mutant and WT plants. **(C)** Phenotypes, well-watered and drought stress recovery assay of *saur32* mutant and WT plants 7 days after rewatering. **(D)** Survival percentage of WT and *saur32* mutant plants 7 days after rewatering. **(E,F)** Effect of drought stress on quantum yield of photosystem II electron transport (ΦPSII) and ion leakage of *saur32* mutant and WT plants, respectively. Mean values and standard deviation (±SD) were plotted as analyzed by Duncan's multiple range (DMR) test; asterisk (*$p < 0.05$; **$p < 0.01$) indicates significant difference.

processes, confirming its active roles in Arabidopsis growth and development. Additionally, investigation of the physiological basis revealed that accumulation of ABA was significantly lower (fourfold) in *saur32* mutants compared with WT while the indole acetic acid (IAA) level was barely changed in both lines (**Figure 4D**). Although there were reductions in JA and JA isoleucine (JA-Ile) contents in saur32, no substantial differences were observed.

RNA-Seq and Expression Analysis of Stress-Related Genes

In order to investigate defense-responsive genes from the transcriptomes of *saur32* mutant and WT plants, total RNAs were isolated and sequenced using an RNA-seq approach. A total of 124 DEGs were identified in which 102 were up-regulated while 22 were down-regulated (**Supplementary Table 8**). These genes were subjected to KEGG pathway analysis and the mainly enriched pathways associated with these DEGs included "plant hormone signal transduction" (ko04075), "nitrogen metabolism" (ko00910), "alanine, aspartate and glutamate metabolism" (ko00250), and so on. Besides, the transcription of some stress-responsive and phytohormone-related genes including *NAC29*, *bHLH100*, *WRKY40*, *SAG13*, *HSP70*, *HSP20*, *HSP20*, *JAZ23*, *ARR6*, *ARR15*, *HAI1*, and *AIP1* was alerted. We confirmed the expression of these DEGs through qRT-PCR analysis and similar patterns of expression were observed as exhibited in RNA-seq (**Supplementary Figure 5**). Furthermore, the functions of the DEGs were classified according to GO classifications. In GO annotations, most of them were enriched in cell, cell parts, binding, catabolic activity, cellular process, and single-organism process (**Supplementary Figure 6**).

Further, *saur32* and WT plants were treated with ABA to examine the transcript levels of defense and ABA-responsive genes through qRT-PCR analysis. The analysis demonstrated that distinctive responses were observed in different genes (**Figure 5A**). Among all 16 genes, 9 were up-regulated in most of the tested time points in *saur32* relative to WT plants. Additionally, *DREB1* exhibited a remarkable increase in transcript level in WT (333) at 4 hpt than *saur32* (272). The *HAI1* and *AIP1*, which are clade-A PP2C genes and actively participate in ABA signal transduction, were significantly expressed in *saur32* plants as compared with WT during ABA treatment. So, the PP2Cs were found to be greatly induced in *saur32* relative to WT. Moreover, four genes were selected on the basis of their strong response to ABA and subjected to drought stress to quantify their transcripts by qRT-PCR. The analysis displayed that the expression of *HAI1*, *AIP1,* and *AREB1* was substantially induced by drought treatment in all lines (WT, *saur32*, and OE32-5), while in *saur32* mutants, the expression was high at several

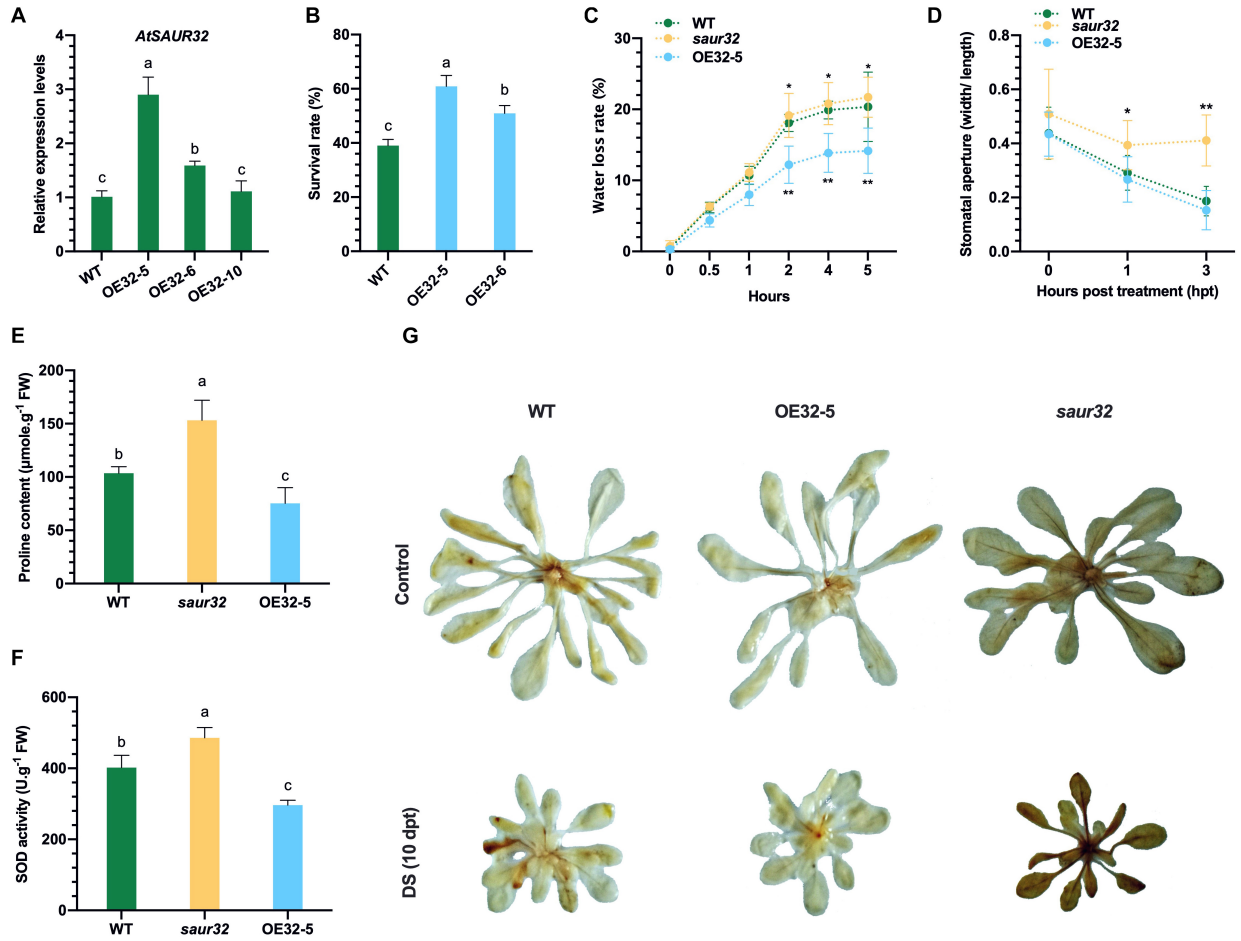

FIGURE 3 | *AtSAUR32* loss-of-function and gain-of-function effect on Arabidopsis plant under drought stress conditions. **(A)** Transcript levels of *AtSAUR32* in wild type (WT) and *AtSAUR32*-overexpressed lines (OE32-5, OE32-6, and OE32-10). **(B)** Survival rates of WT and *AtSAUR32*-overexpressed lines (OE32-5 and OE32-6), 1 week after rewatering. **(C)** Transpiration water loss from WT, *saur32*, and OE32-5 Arabidopsis plant leaves at various time points after detachment. Leaves of similar developmental stage were detached and weighted at the mentioned time; leaves' initial weight was used as a fresh weight to calculate water loss. **(D)** Stoma aperture of leaves of WT, *saur32*, and OE32-5 Arabidopsis plants under 10 μM ABA treatment. **(E)** Proline accumulation. **(F)** SOD content in WT, *saur32*, and OE32-5 plants. **(G)** Phenotypes showed DAB (3,3-diaminobenzidine) staining, indicating H_2O_2 accumulation after drought treatment. Mean values and standard deviation (±SD) were plotted as analyzed by Duncan's multiple range (DMR) test ($P < 0.05$); lowercase letters (a–d) and asterisk (*) indicate significant difference.

time points (**Figure 5B**). As a drought marker transcription factor, *DREB1* was greatly induced by drought stress in OE32-5 compared with WT and *saur32* at 12 hpt. Thus, the variation in expression levels of key genes was closely linked with drought tolerance of the *saur32* mutant.

AtSAUR32 Protein Interacts With PP2C.A

RNA-seq and expression data demonstrated that the transcript of ABA-responsive genes *HAI1* and *AIP1*, members of PP2C.A family, was significantly induced in the *saur32* mutant. So, we carried out Y2H assay to verify the potential AtSAUR32-interacting partners. The results showed that the yeast strain containing AtSAUR32-HAI1 and AtSAUR32-AIP1 proteins grew well in the selection media SD/-Leu-Trp-His-Ade, indicating strong protein–protein interaction (**Figure 6A**). To further confirm whether AtSAUR32 and PP2C.A genes (*HAI1* and *AIP1*) physically interact, we performed BiFC assay. AtSAUR32 infused with the N-terminal fragment of yellow fluorescence protein (YFP) could be co-expressed with AIP1 and HAI1, which were infused with the C-terminal, to produce the AtSAUR32-2YN+HAI1-2YC and AtSAUR32-2YN+AIP1-2YC vector. These vectors were cotransformed into tobacco cells, resulting in strong fluorescence signals. Confocal laser scanning microscopy displayed fluorescence at the nucleus, indicating localization to the nucleus of the cell (**Figure 6B**) and confirming that the AtSAUR32-HAI1 and AIP1 interaction occurred *in vivo*. Both Y2H and BiFC results indicated that AtSAUR32 actually could interact with AIP1 and HAI1 proteins.

DISCUSSION

Plants recruit various protective strategies to adjust themselves to unfavorable environmental conditions to complete the life

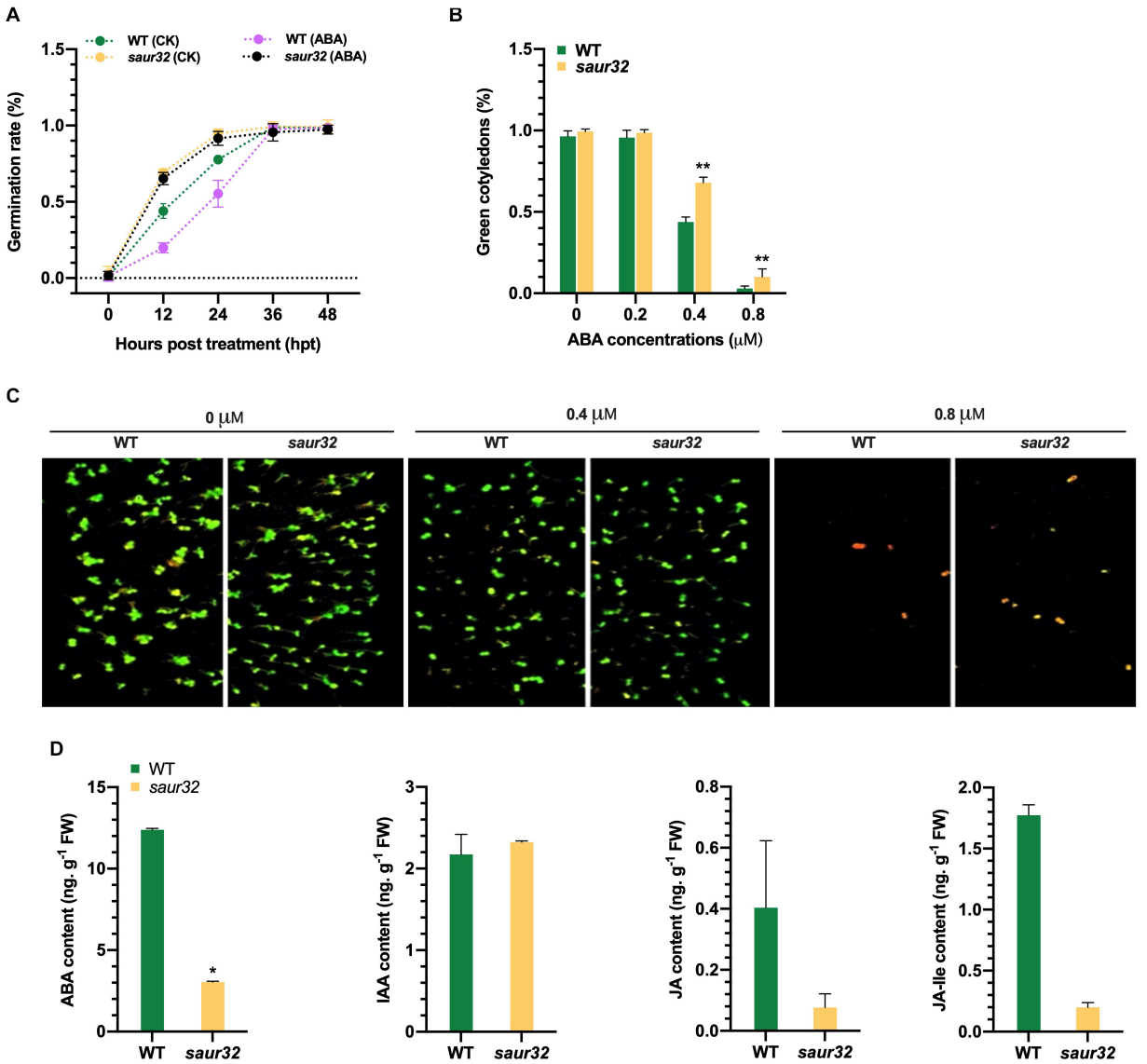

FIGURE 4 | Effect of exogenous ABA application on WT, *saur32,* and OE32-5 plants. **(A)** Effect of ABA treatment (1 μM) on the germination rate of *saur32* mutant and WT; 0 μM ABA was used as control. **(B)** Effect of ABA concentrations (0, 0.2, 0.4, and 0.8 μM) on green cotyledons of WT and *saur32* at 5 days post-germination. **(C)** The cotyledons' growth was observed in the 1/2 MS medium with 0, 0.4, and 0.8 μM ABA using Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer (Note: 0.2 μM ABA phenotypes are not added because phenotypically they are the same as 0 μM and have no significant difference). **(D)** Accumulation of endogenous hormones in *saur32* and WT plants. Mean values and standard deviation (±SD) are plotted as analyzed by Duncan's multiple range (DMR) test ($P < 0.05$); asterisk (*) indicates significant difference.

cycle. Additionally, plants sense internal and external stress signals and induce a defensive system to sustain growth and adaptation. Among the different proteins involved in the defense system, SAUR proteins greatly share a common function in cell elongation, induce plant growth by regulating cell wall acidification, and regulate growth dynamically in response to environmental cues (Stortenbeker and Bemer, 2019). For example, Arabidopsis transcriptional profiles showed that the SAUR genes are strikingly induced by the growth-inhibiting hormone ABA (Ren and Gray, 2015; Qiu et al., 2020). However, the present study revealed that the SAUR family member *AtSAUR32* plays an important role in drought tolerance via ABA signal transduction, and it seems promising to identify a new player in ABA pathways.

The protein sequence of SAUR32 is highly conserved within the A1 subgroup of the SAUR family due to the presence of auxin_inducible domain and sequence similarities (Wu et al., 2012). As shown in **Figure 1A**, the phytohormones including ABA responsiveness elements (ABRE) were determined in the promoter of *AtSAUR32*, and several abiotic stressor responsive elements were also found such as MYB binding site (MBS), which is drought-inducible, LTR (low-temperature responsiveness),

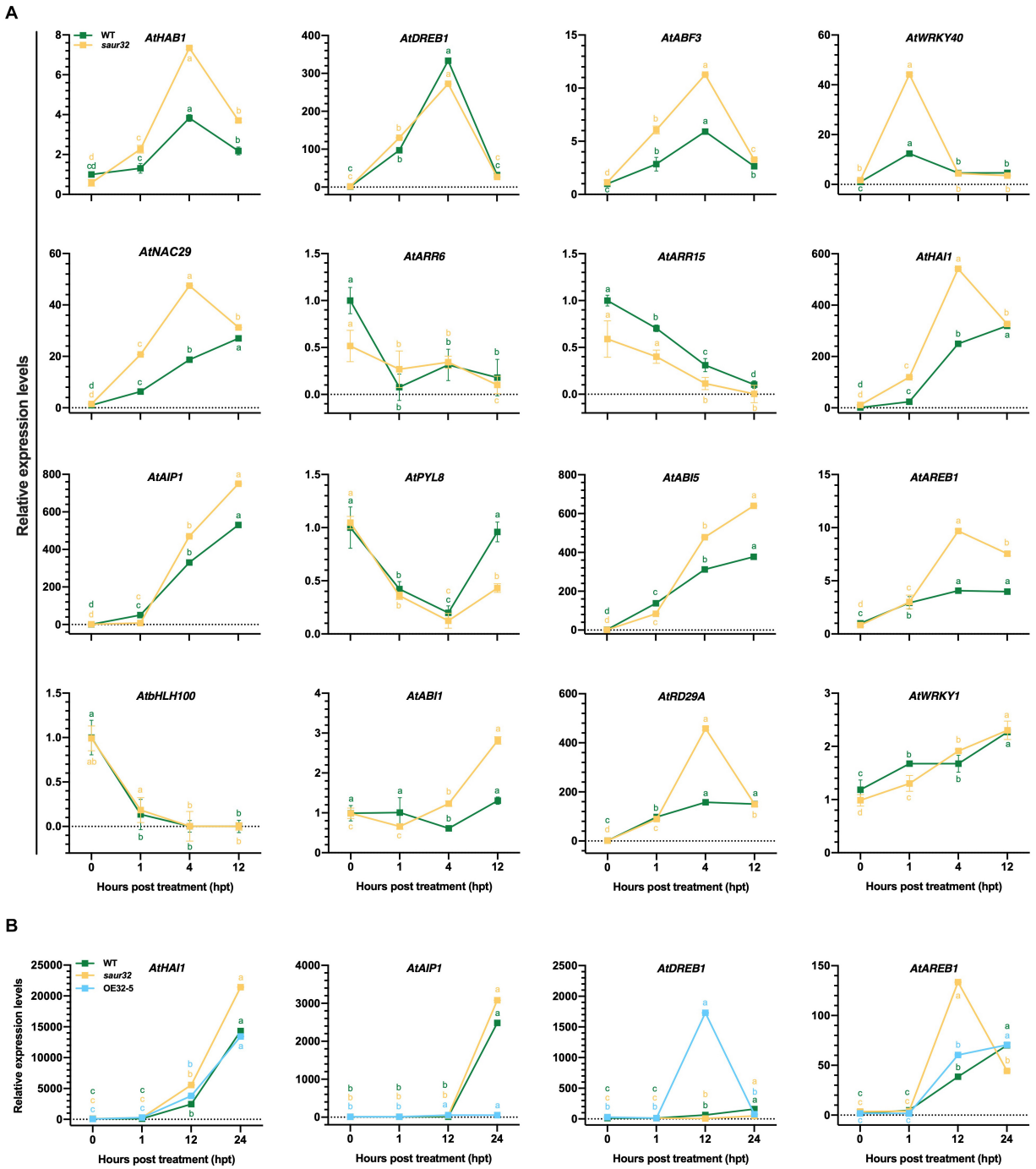

FIGURE 5 | The expression levels of different genes in response to ABA and drought stress treatment. **(A)** Transcript levels of different genes during ABA treatment in WT, *saur32* plants. **(B)** Expression of genes under drought stress in WT, *saur32,* and OE32-5 plants. Mean values and standard deviation (±SD) plotted as analyzed by Duncan's multiple range (DMR) test ($P < 0.05$); lowercase letters (a–d) indicate significant difference.

and TC-rich repeats (defense and stress responsiveness). The transcriptomic analysis displayed a significant increase in the transcript of *AtSAUR32* to drought and ABA, which were further confirmed by qRT-PCR analysis (**Figure 1**), while in contrast, some other SAUR genes were repressed by the application of ABA (Kodaira et al., 2011; Ren and Gray, 2015). Altogether, these outcomes demonstrate that our target gene has the potential to participate in different mechanisms in plants.

Combining studies of genome, proteome, and metabolome carried out over the last decade have revealed that SAURs regulate

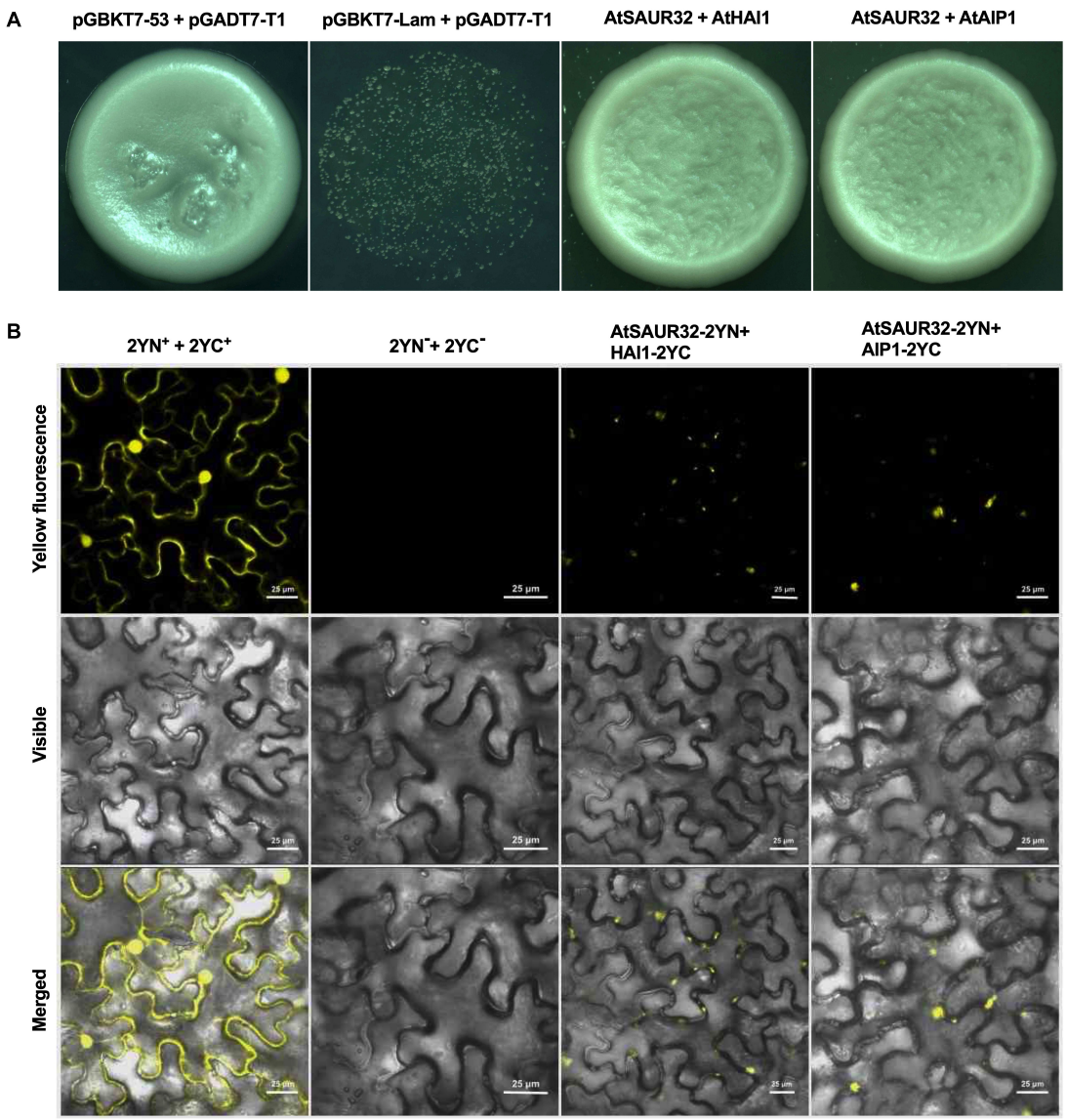

FIGURE 6 | Identification of AtSAUR32 interacting proteins in Arabidopsis and their colocalization analysis. **(A)** Interactions of AtSAUR32 proteins with two PP2C.A (HAI1 and AIP1) in yeast two-hybrid assay. Growth of transformants on SD/-Leu-Trp-His-Ade indicates interaction. **(B)** For bimolecular fluorescence complementation (BiFC) analysis, plasmids were co-infiltrated into *Nicotiana benthamiana* and were observed after 48 h of agroinfiltration under a confocal microscope.

plant developmental and physiological processes, including leaf growth and senescence, hypocotyl growth, floral organ expansion, root growth, and development (Chae et al., 2012; Li et al., 2015; Ren and Gray, 2015). The tissue-specific expression results showed that *AtSAUR32* was highly expressed in different organs of the plant under normal growing conditions, suggesting that it plays a vital role in plant development (**Figure 1B**). Another study discovered that *SAUR36* is regulated by both auxins and gibberellins while its overexpression increased hypocotyl growth in light-growth conditions (Stamm and Kumar, 2013). These findings suggest that Arabidopsis SAUR proteins in different subfamilies may function in a similar manner and influence plant growth in various aspects.

The plant hormone ABA has dual functions as a growth inhibitor or stress indicator and is considered as a major signaling molecule in abiotic stress response (Gomez-Cadenas et al., 2015; Wang et al., 2018). ABA simultaneously controls stomatal closure and lateral bud dormancy, thus confirming the sensitivity to ABA of these traits (Shimizu-Sato and Mori, 2001; Arend et al., 2009). In the current study, loss of function of *AtSAUR32* weakened ABA sensitivity by increasing stomatal aperture and green cotyledon percentage as compared with WT and the *AtSAUR32* transgenic line (**Figures 3D**, **4B**). Additionally, the seed germination of the *saur32* mutant was accelerated compared to that of control, though the seeds of *saur32* were insensitive to ABA during germination (**Figure 4A**). These findings implied

that *AtSAUR32* probably participates in seed germination and stomatal movement by interaction with ABA signal transduction.

AtSAUR32 overexpression enhanced drought tolerance in Arabidopsis while *AtSAUR32* knockout plants displayed contrasting results. Under drought conditions, OE32-5 had a higher *AtSAUR32* expression level, survival rate, and lower water loss rate, compared with WT and the *saur32* mutant (**Figures 3A–C**). During water-deficit conditions, cell membrane injury occurred and subsequently membrane lipid peroxidation was remarkably enhanced (Shi et al., 2014). We observed that the degree of cell membrane damage for *AtSAUR32* knockout plants was higher and their photosynthetic efficiency rate was on decline with increase in time (**Figures 2E,F**). Drought stress can also trigger the accumulation of reactive oxygen species (ROS), and SOD is the first defense line of action of the plant ROS defense (Huang et al., 2016). *AtSAUR32* knockout (*saur32* mutant) exhibited a higher level of osmotic regulatory substance (proline) content and SOD activity to quickly overcome stress and increase cell membrane stability (**Figures 3E,F**), thereby proving defensive reaction under stress (Ighodaro and Akinloye, 2018; Mohseni et al., 2018). As demonstrated in our study, loss of function of *AtSAUR32* decreased defense response to drought stress, which may be associated with higher SOD activity in the initial stage of ROS scavenging; similar findings were also noticed by Wang et al. (2016). Later, the over-accumulation of H_2O_2 occurred in the *saur32* mutant relative to WT and OE32-5 plants under drought conditions (**Figure 3G**), which exhibited that the *saur32* mutant suffered more seriously under drought while *AtSAUR32* (OE32-5) plays a positive role in stress. Therefore, the content of H_2O_2 is often used as an indicator of the level of damage to plant cells (Li et al., 2018). Proline is one of the most essential compatible osmolytes for cellular osmotic adjustment in many plant species during drought stress, high salinity, and other environmental stressors (Szabados and Savouré, 2010; Kumar et al., 2017). Additionally, the accumulation of proline eliminates the damage caused by ROS (Schöffl et al., 1999). So, the overall results showed that *AtSAUR32* could enhance drought tolerance to a significant level.

AtSAUR32 probably regulates drought tolerance mediated by ABA-independent pathways and other drought-responsive factors. RNA-seq and qRT-PCR analysis demonstrated that the expression of some stress-responsive genes such as *AtNAC29*, *AtWRKY40*, *AtbHLH100*, *AtARR15*, *AtSAG13*, *AtJAZ23*, *AtOSM34*, *AtHSP20*, and *AtHSP70* showed obvious changes in the *saur32* mutant (**Figure 5A** and **Supplementary Figure 5**). *AtNAC29* and *AtWRKY40* were notably induced by ABA, while *AtbHLH100*, *AtARR6*, and *AtARR15* were severely repressed by ABA in *saur32* compare to WT. These genes probably participated in ABA and drought stress responses. Arabidopsis *AtWRKY40* was proved to be a negative regulator of ABA responses and bound to the promoters of multiple stress-inducible transcription factor genes and repressed their expression at low ABA concentrations (Xu et al., 2006). NAC transcription factors play an important role in stress response via both ABA-independent and ABA-dependent pathways. Thus, *NAC016*-, *NAC019*-, *NAC055*-, and *NAC072*-overexpressed plants exhibit strong drought tolerance, indicating that NAC TFs positively regulate drought stress-responsive signals (Fujita et al., 2004; Tran et al., 2004). The *ANAC072/RD26* overexpression trigger ABA sensitivity, though transgenic plants in which its activity was repressed were insensitive (Nakashima et al., 2012). The transcript levels of *bHLH122* were strongly induced by drought, and up-regulation of *bHLH122* substantially increased cellular ABA levels, which demonstrated that *bHLH122* functions as a positive regulator of water-deficit conditions (Liu et al., 2014). Heat shock proteins (HSPs) are induced by drought and correspond to enhanced tolerance (Aneja et al., 2015). Previous studies found that overexpression of *HSP17.6A* in Arabidopsis resulted in gain of tolerance in these plants during water-deficit conditions, possibly through protein stabilization (Sun et al., 2001). Hence, the above results suggest that *AtSAUR32* positively participates in drought response via ABA-independent and ABA-dependent pathways, while these findings will help to understand the complex auxin and ABA signaling interaction network.

Plant reduces water loss by ABA accumulation, which triggers ABA-dependent signaling that causes the activity of clade-A PP2Cs such as HAI1 and AIP1, to be inhibited through their interaction with ABA stimulated PYL. This, in turn, allows SnRK2 kinase to regain activity and phosphorylates its targets such as ABF transcription factors that control ABA-dependent gene expression and influence stomatal aperture. As clade-A PP2Cs, the HAI1 gene has a more prominent role in drought response compared to AIP1; however, HAI mutants enhanced proline and osmoregulatory solute accumulation at low water potential (Bhaskara et al., 2012). The interactive behavior of HAI1 with PYL5 and PYL7–10 of the PYL/RCAR ABA receptor family participates in feedback regulation of ABA signaling, which is quite possible due to dephosphorylation of SnRK2s (Fujita et al., 2009; Antoni et al., 2012). In our study, the PP2C.A subfamily genes including *HAI1* and *AIP1* were identified as DEGs using RNA-seq analysis and qRT-PCR analysis. Furthermore, the expression of *HAI1* and *AIP1* was up-regulated and was notably induced by ABA and drought stress in the *saur32* mutant (**Figures 5A,B**). The expression pattern of

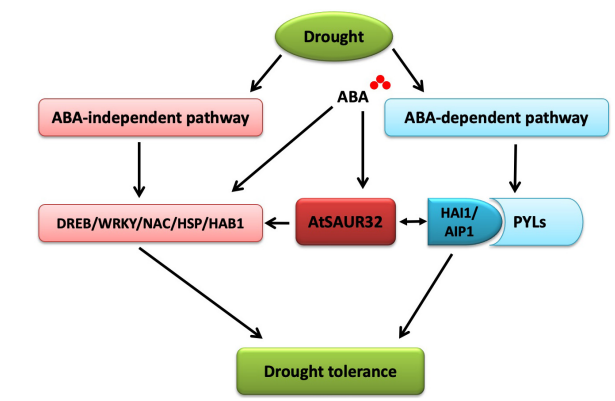

FIGURE 7 | Proposed model for the roles of *AtSAUR32* in drought stress resistance in Arabidopsis.

some ABA and drought marker genes such as *AREB1*, *ABF3*, and *DREB1* was altered under ABA and drought treatment. Meanwhile, Y2H and BiFC analyses indicated that HAI1 and AIP1 proteins highly interacted with AtSAUR32 (**Figures 6A,B**); however, further work may help to support the potential *in vivo* interaction of SAUR32 with these phosphatases. A previous study showed that AtSAUR19 subfamily proteins were upstream regulators and interacted to repress the expression of clade-D PP2Cs. Here, AtSAUR32 is speculated to interact with clade-A PP2Cs participating in the model of ABA-dependent PYL-PP2C-SnRK2s interaction in ABA signaling (**Figure 7**).

CONCLUSION

In conclusion, *AtSAUR32* transcription is sensitive to exogenous ABA application and enhances drought resistance via ABA signal transduction. The physiological and morphological attributes implied that *AtSAUR32* is more sensitive to ABA; however, under drought stress conditions, the *saur32* mutant suffered more seriously as compared with WT and OE32-5 plants. So, *AtSAUR32* might be positively involved in response to drought conditions; however, further genetic analyses would help to confirm these results. Moreover, RNA-seq and qRT-PCR analysis identified the co-regulatory defensive genes, PP2C.A subfamily members that were remarkably induced by ABA and drought in *saur32* relative to WT. Y2H and BiFC analysis revealed that some PP2C.A protein AHI1 and AIP1 interacted with AtSAUR32. Considering that, PP2C.A genes in Arabidopsis might be regarded as key regulators of ABA signal transduction, while in drought response, AtSAUR32 was found to mediate ABA-dependent signal transduction through AHI1 and AIP1. Besides, the transcripts of stress-responsive genes (*WRKY40*, *NAC29*, *bHLH100*, and *HSPs*) were altered, and their response patterns to ABA were changed with loss of function of *AtSAUR32*. Put together, these results suggest that *AtSAUR32* probably regulates drought tolerance through ABA-dependent and -independent pathways.

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

GL and YH conceived the study. YH, YL, and ML generated transgenic lines and performed phenotypical observation. YH, DY, and AL-S carried out the cytological analysis. MA, YH, MI, IJ, and XY performed the data analysis. MA and GL wrote and revised the manuscript. All authors discussed and commented on 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.2021.625493/full#supplementary-material

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OPINION
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Plausible Involvement of Ethylene in Plant Ferroptosis: Prospects and Leads

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INTRODUCTION

Plants are continuously exposed to various biotic and abiotic stressors that limit their productivity. Any kind of stressor leads to the generation of Reactive Oxygen Species (ROS), majorly, through the activity of plasma membrane-localized respiratory burst oxidase homolog (RBOH) proteins that generate $O_2^{\bullet-}$ in the apoplast (Mittler, 2017). Owing to its reducing properties, $O_2^{\bullet-}$ is highly toxic and is involved in the peroxidation of membrane lipids and the conversion of Fe^{3+} to ferrous ions (Fe^{2+}). In addition, $O_2^{\bullet-}$ can also be converted into H_2O_2 by the activity of superoxide dismutase (SOD), which may enter the cytosol through aquaporins to trigger signaling cascades (Rodrigues et al., 2017). Further, Fe^{2+} can directly interact with H_2O_2 through Fenton reaction and accelerates the production of hydroxyl radical (OH•), which is another lethal ROS (Mittler, 2017). Since ions of Fe play a major role in ROS production, plants keep a check on the concentration of these ions to maintain ROS homeostasis. In plants, two major groups of proteins participate in iron (Fe^{2+}/Fe^{3+}) sequestration including ferritin and plant defensin proteins. The latter group of proteins also participate in the plant defense and inhibit the growth of fungal pathogens. Recently, a pivotal role of iron in the hypersensitive response induced cell death, during plant defense signaling, was reported in rice upon infection with the fungal pathogen, *Magnaporthe oryzae* (Dangol et al., 2019). This type of iron-dependent cell death was first reported in mammalian tumor cells and was termed "ferroptosis" by Dixon and co-workers in 2012 (Dixon et al., 2012). Ferroptosis is, genetically, morphologically, and biochemically, distinct from other types of known cell death including apoptosis, necrosis, and autophagy (Dixon et al., 2012). The onset of ferroptosis requires high levels of ROS and Fe^{2+} and depletion of reduced glutathione (GSH) (Dixon and Stockwell, 2019). Subsequently, a GSH-dependent glutathione peroxidase-4 (GPX-4) was identified as a key player of mammalian ferroptosis (Angeli et al., 2014; Yang et al., 2014). GPX enzymes catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced GSH or thioredoxin (TRX) (Yang et al., 2016).

In plants, ferroptosis was first reported by Distéfano and co-workers in 2017 in *Arabidopsis thaliana* under heat stress conditions, and, similar to the animals, the plant ferroptosis was also found to be dependent on the GSH depletion, lipid peroxidation, and accumulation of ROS and ions of Fe (Distéfano et al., 2017). Although the literature evidence on plant ferroptosis is limited, it has been observed that it can be triggered by both abiotic (heat stress) and biotic stressors (*M. oryzae)*, and both plant and animal ferroptosis exhibit similar morphological characteristics, such as cytoplasmic retraction, normal nuclei, and the formation of small lytic vacuoles (Distéfano et al., 2021). In addition, a noticeable shrinkage of mitochondria along with increased mitochondrial membrane density and decreased mitochondrial cristae has been observed in animal ferroptosis

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(Dixon et al., 2012); however, no such observation has been reported in the case of plants. By utilizing several agonists (**Supplementary Table 1**), Dangol et al. characterized the detailed process of plant ferroptosis, and their results collectively suggested the role of plasma membrane-localized NADPH-oxidases (RBOHs), NADP-malic enzyme, polymerization of actin microfilaments along with the depletion of GSH, peroxidation of membrane lipids, and accumulation of ions of Fe and ROS (Dangol et al., 2019). Similar to the animals, the plant ferroptosis also seems to be dependent on the activity of GPX-4 as treatment for erastin, an inducer of ferroptosis and inhibitor of GPX-4, resulted in the depletion of reduced and total GSH and accumulation of ROS and Fe^{3+} to result in ferroptosis in rice sheath cells (Dangol et al., 2019). Correspondingly, treatment of Arabidopsis cells with another GPX-4 inhibitor RSL3 led to cell death, which was prevented by the treatment of ferroptosis inhibitors including liproxstatin-1 and ferrostatin-1 (Hajdinák et al., 2019). A recent study demonstrated that silencing of *GPX4* in *Nicotiana benthamiana* resulted in enhanced ferroptosis in response to *Tobacco mosaic virus* 24A+UPD infection (Macharia et al., 2020). However, some of the recent evidence suggests the possible involvement of others GPXs, such as membrane-localized GPXL5, in the plant ferroptosis (Meyer et al., 2020). Overall, these results suggest that ferroptosis is a highly regulated cell death process that is induced by both biotic and abiotic stressors. Since the analysis of ferroptosis during incompatible rice–*M. oryzae* interactions suggested a positive role of ferroptosis in preventing the infection by avirulent strains of the fungus, the process of ferroptosis can be manipulated in the future to develop the biotic and abiotic resilient crops (Dangol et al., 2019; Kazan and Kalaipandian, 2019).

Evidence in Support of Ethylene Mediated Regulation of Ferroptosis

Although the direct involvement of any phytohormones in the plant ferroptosis has not been reported yet, a growing body of evidence suggests the possible involvement of ethylene in the plant ferroptosis. It has been observed that all the components of ferroptosis, including GSH, ions of Fe, and ROS, are linked to the gaseous plant hormone ethylene, and thus, we hypothesize the involvement of ethylene in the regulation of plant ferroptosis based on the following literature evidence.

1. There is ample evidence that suggests ethylene induces the biosynthesis of GSH. For instance, exogenous treatment of ethylene has been shown to significantly elevate the levels of GSH and ascorbate (AsA) in *Zea mays* seedlings under cadmium (Cd) toxicity (Liu et al., 2019). In addition, the exogenous application of ethephon, an ethylene precursor, increased the amount of GSH in Cd-treated *Brassica juncea* (Khan et al., 2016), while the application of ethylene inhibitor Aminoethoxyvinylglycine (AVG) resulted in decreased GSH levels (Masood et al., 2012). Similarly, ethephon treated *Glycine max* plants showed an accumulation of GSH together with the increased activity of glutathione reductase (GR) under waterlogging conditions (Kim et al., 2018). In contrast, treatment using ethylene biosynthesis inhibitor resulted in reduced levels of GSH in Cd-exposed *Lycium chinense* plants compared with untreated plants, further confirming a direct role of ethylene in the regulation of cellular GSH levels (Guan et al., 2015).
2. The connection between Fe^{2+} and ethylene is well-established, and it has been known for years that excess ions of Fe trigger ethylene biosynthesis in plants (Peng and Yamauchi, 1993; Becker and Asch, 2005; Majerus et al., 2007). The ethylene biosynthetic pathway is relatively simple, taking place *via* only two committed enzymatic reactions catalyzed by 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) (Riyazuddin et al., 2020). Both ACS and ACO are controlled at the transcriptional and post-translational levels, which enables a tailored regulation of ethylene production in plants (Pattyn et al., 2021). The final regulatory step of the ethylene biosynthetic pathway is the conversion of ACC to ethylene and is catalyzed by ACO, which requires Fe^{2+} as the active-site cofactor. Therefore, Fe^{2+} plays a critical role in ethylene biosynthesis by regulating the activity of ACO (Houben and Van de Poel, 2019). Subsequently, the deficiency or accumulation of Fe^{2+} may directly affect cellular ethylene production. In other words, Fe^{2+} may be the limiting factor in ethylene production, and the excess could result in higher production of ethylene (Peng and Yamauchi, 1993; Li et al., 2015). For instance, excess Fe^{2+} resulted in the upregulation of the transcript level of ethylene biosynthesis genes, such as *AtACS2*, *AtACS7*, *AtACS8*, *AtACS11,* and *AtACO1* and *AtACO2*, and contributed to higher ethylene production in Arabidopsis (Li et al., 2015). In turn, ethylene, thus, produced triggered the expression of genes encoding Fe-sequestering ferritins, such as *FER1*, *FER2*, *FER3*, and *FER4,* and minimized Stelar and xylem Fe^{2+} concentrations to limit Fe accumulation and toxicity both in shoots and roots (Li et al., 2015). Further, *FER1* and *FER3* were found to be significantly elevated in the roots of Arabidopsis ethylene-overproduction mutant (*eto1-1*) as compared with wild type (WT) during Fe^{2+} toxicity (Li et al., 2015).
3. The interconnection between ethylene and ROS homeostasis is quite evident, and it is well-known that ethylene maintains ROS homeostasis by activating the enzymatic and non-enzymatic antioxidant defense to limit the accumulation of ROS and subsequent peroxidation of membrane lipids that result in ferroptosis (Riyazuddin et al., 2020). At first, it was shown that the exogenous application of ethylene precursor ACC resulted in the enhanced activities of ascorbate peroxidase (APX), catalase (CAT), SOD, and peroxidase (POX), and reduced the lipid peroxidation in creeping bentgrass (Larkindale and Huang, 2004). Similarly, exogenous application of ACC has been shown to improve the heat stress tolerance in rice seedlings by reducing lipid peroxidation and relative electrolyte leakage during heat stress (Wu and Yang, 2019). Correspondingly, the exogenous application of ethephon, another ethylene precursor, to Ni-treated *Brassica juncea* plants resulted in significantly increased activity of SOD, APX, GR, GPX, and the accumulation of proline (Khan et al., 2020). Similarly, exogenous application of ethephon

substantially induced the activity of enzymatic and non-enzymatic antioxidants, such as SOD, APX, GR, GSH -S-transferase (GST), GPX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), AsA, and GSH, under Zinc toxicity in *B. juncea* (Khan et al., 2019). In *Lactuca sativa*, the application of exogenous ethylene increased the activity of antioxidant enzymes, such as SOD, CAT, and APX, and reduced the H_2O_2 content (Ma et al., 2013), while in *Nelumbo* sp., ethylene treatment maintained endogenous ROS levels by regulating the AsA–GSH antioxidant system under Cd stress (Yuan et al., 2018). In *Dendrobium nobile*, ethephon treatment led to an increase in the contents of AsA and GSH, while exogenous treatment of ethylene inhibitor resulted in decreased contents of both

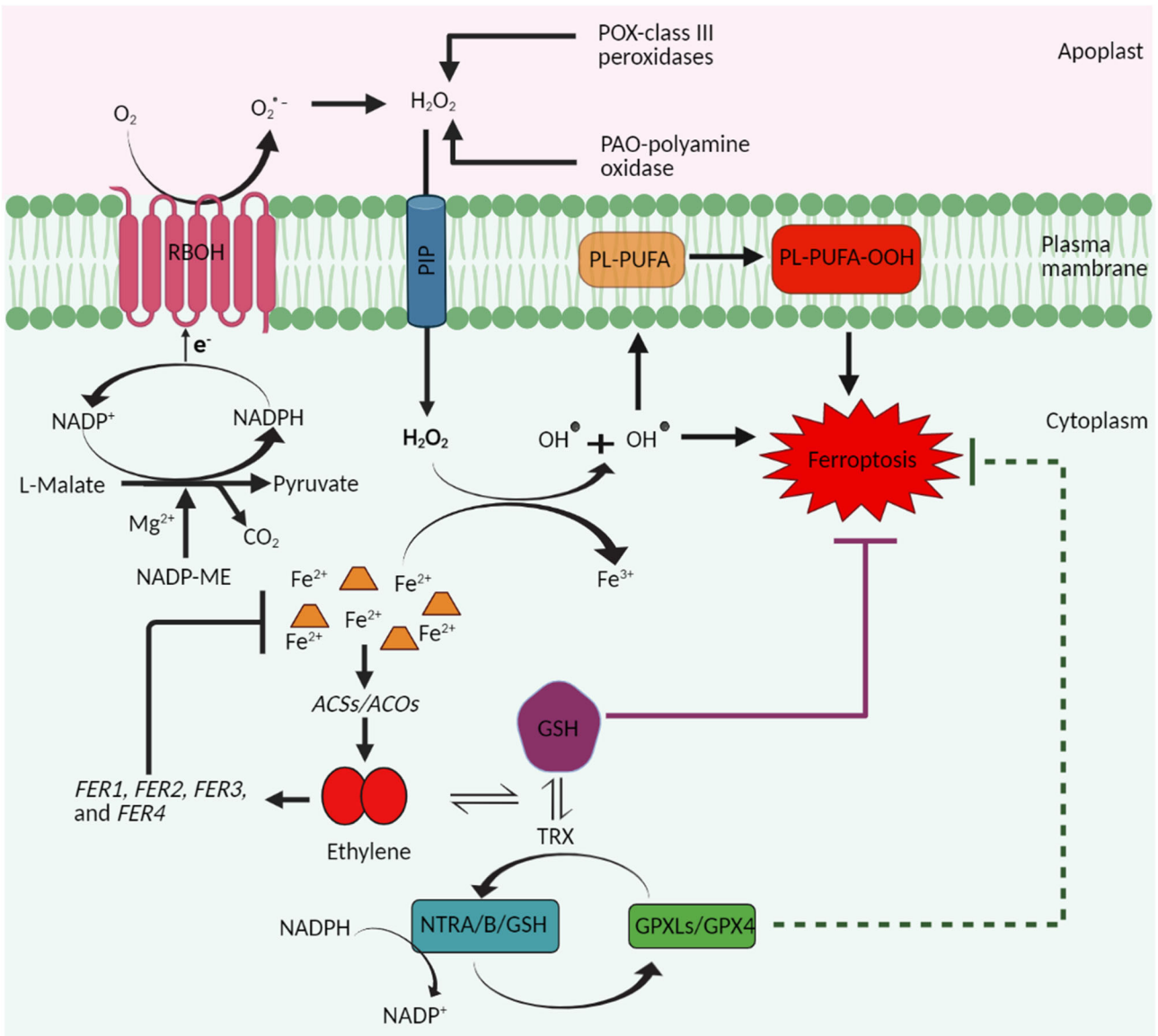

FIGURE 1 | A schematic representation highlighting the proposed role of ethylene in ferroptosis cell death in plants. Plant ferroptosis is characterized by the increase in ROS and subsequent peroxidation of membrane lipids, accumulation of iron, and depletion of reduced glutathione (GSH). Stress-induced accumulation of ROS is mainly dependent on the activity of plasma membrane-localized NADPH-oxidase (respiratory burst oxidase homolog, RBOH) proteins. NADP-malic enzyme supplies electrons to the RBOH proteins and thus contributes indirectly to ROS production and thus to plant ferroptosis. H_2O_2 produced in the apoplast, because of the conversion of superoxide radicals ($O_2^{\bullet-}$) and/or the activities of the class III peroxidases (POX) and polyamine oxidases (PAO), enters the cytosol through plasma membrane intrinsic proteins (PIP; aquaporins) and mediates the conversion of Fe^{2+} to Fe^{3+} through the Fenton reaction. Fe^{2+} are otherwise involved in the biosynthesis of ethylene by functioning as a cofactor of enzyme ACC oxidase (ACO). Ethylene so produced triggers the synthesis of ferritin proteins that participate in the sequestration of ions of iron and thus prevent their accumulation, which is a prerequisite for ferroptosis cell death. In addition, ethylene also induces the synthesis of GSH and thus helps to maintain the GSH levels to prevent ferroptosis cell death.

of these antioxidants. In addition, APX and GR also showed a significant change under the ethylene regulator treatments (Zhang et al., 2021). Further, a growing body of evidence suggests that Ethylene response factors (ERFs) play a role in linking redox and hormonal regulation in plant responses to abiotic stresses. The overexpression of *ERF96* gene enhanced selenium tolerance in Arabidopsis *via* elevation of CAT, GPX activities, and GSH content to cope with H_2O_2 compared with those in WT (Jiang et al., 2020). Similarly, overexpression of *ERF38* resulted in reduced contents of MDA and H_2O_2 in the transgenic poplars as compared with WT by elevating the expression of POD and SOD and accumulation of proline and soluble proteins (Cheng et al., 2019). Furthermore, overexpression of *ERF1* in tomatoes resulted in higher proline accumulation and lower lipid peroxidation as well as increased the activity of antioxidant enzymes (POD and SOD) under salt stress (Hu et al., 2014). ERF3 also regulated ROS metabolism in tobacco resulting in lower accumulation of ROS (Wu et al., 2008). Overall, all of these results confirm a tight correlation between ethylene levels and ROS homeostasis in plants.

4. In addition to regulating the enzymatic and non-enzymatic antioxidants, ethylene has also been shown to regulate the activities of NADPH-oxidases (RBOHs) to limit ROS production. At first, ethylene was shown to be involved in the regulation of H_2O_2 signaling by controlling the expression of *Rboh* genes under hypoxia stress in Arabidopsis (Yang and Hong, 2015). In addition, ACS1 mediated early ethylene production has been shown to temporarily inhibit the expression of NADPH-oxidase (RBOH-D and RBOH-F) genes to prevent the ROS burst in *Brassica oleracea* (Jakubowicz et al., 2010). On the contrary, reduced expressions of ethylene biosynthesis genes including *ACS7* and *ACS8* and ethylene signaling genes including *ERF73* were observed in the *rbohd*-knockout mutants, further indicating an intricate relationship between NADPH-oxidase, ROS production, and ethylene (Yang and Hong, 2015).

CONCLUSION

Based on the presented literature evidence, it can be speculated that ethylene controls and inhibits the ferroptosis process in plants by at least three ways: (1) by limiting the excess accumulation of Fe^{2+} in the cells via increasing the iron sequestrating ferritin proteins and thus inhibiting the generation of excess ROS based on Fe^{2+} accumulation, (2) by activating the antioxidant defense mechanism to limit the excess ROS accumulation in the cells, and (3) by facilitating the synthesis of GSH (**Figure 1**). However, further experimentations are required to investigate the ethylene biosynthesis and/or signaling during iron- and ROS-dependent ferroptosis in plants.

AUTHOR CONTRIBUTIONS

RR and RG conceived the idea. Both the authors were involved in the writing of the manuscript, contributed equally to the drafts, and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

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Melatonin Improves Cotton Salt Tolerance by Regulating ROS Scavenging System and Ca^{2+} Signal Transduction

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As one of the cash crops, cotton is facing the threat of abiotic stress during its growth and development. It has been reported that melatonin is involved in plant defense against salt stress, but whether melatonin can improve cotton salt tolerance and its molecular mechanism remain unclear. We investigated the role of melatonin in cotton salt tolerance by silencing melatonin synthesis gene and exogenous melatonin application in upland cotton. In this study, applicating of melatonin can improve salt tolerance of cotton seedlings. The content of endogenous melatonin was different in cotton varieties with different salt tolerance. The inhibition of melatonin biosynthesis related genes and endogenous melatonin content in cotton resulted in the decrease of antioxidant enzyme activity, Ca^{2+} content and salt tolerance of cotton. To explore the protective mechanism of exogenous melatonin against salt stress by RNA-seq analysis. Melatonin played an important role in the resistance of cotton to salt stress, improved the salt tolerance of cotton by regulating antioxidant enzymes, transcription factors, plant hormones, signal molecules and Ca^{2+} signal transduction. This study proposed a regulatory network for melatonin to regulate cotton's response to salt stress, which provided a theoretical basis for improving cotton's salt tolerance.

Keywords: cotton, melatonin, salt stress, Ca^{2+}, ROS

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INTRODUCTION

Cotton is an industrial and cash crop with important cash value (Sunilkumar et al., 2006). During the growth and development of cotton, it is often subjected to some abiotic stresses, such as drought, salinity, high temperature, and low temperature. Soil salinization is one of the biggest challenges facing world agriculture (Zhu, 2001), because salinization hinders the growth and development of plants and reduces crop yields (Shahzad et al., 2021). The saline soil contains excessive neutral salt, mainly NaCl and Na_2SO_4, which cause salt stress. The harmful effects of NaCl on plants are due to the accumulation of sodium in the soil, which reduces water availability and the toxic effects of sodium and chloride ions on plants (Van-Zelm et al., 2020). Plants use a variety of biochemical and

molecular responses to cope with stress, including selective formation or elimination of salt ions, control of root absorption of ions and transport to leaves, synthesis of compatible osmotic agents, stimulation of hormones, regulation of gene expression, etc. (Parida and Das, 2005). More than 800 million hectares of land throughout the world are salt affected. This amount accounts for more than 6% of the world's total land area (Munns and Tester, 2008). Therefore, the yield of salinized farmland is increased by improving the salt-tolerance of cotton, studying the salt tolerance of cotton has become more and more important.

Many plant growth regulators have been used to improve the salt tolerance of crops to achieve agricultural sustainability, such as SA (salicylic acid) (Bastam et al., 2013). Melatonin is a growth regulator and stress regulator, which can improve plant performance and yield (Arnao and Hernández-Ruiz, 2014). Both exogenous application of melatonin and genetic transformation of melatonin synthesis genes to regulate endogenous melatonin concentration can alleviate the effects of biotic and abiotic stress. Melatonin has been reported to improve plant tolerance to salt stress. Exogenous melatonin alleviates reactive oxygen species (ROS) accumulation and protects photosynthetic activity in Maize Seedlings under salt stress by activating antioxidant enzymes (Chen et al., 2018). Melatonin improved tolerance to salt stress by promoting growth, root yield and sugar content, chlorophyll synthesis, photosynthetic system II (PS II) activity, and changed in gas exchange parameters in sugar beet seedlings (Zhang et al., 2021). Melatonin enhances Phaseolus vulgaris salt tolerance by enhancing ROS metabolism, expression of antioxidant defense related genes, and photosynthetic capacity (ElSayed et al., 2021). Melatonin affects rice photosynthesis under salt stress by increasing total antioxidant capacity, promoting the xanthophyll cycle, increasing xanthophyll pool size to dissipate excess light energy, increasing key photosynthetic enzyme activities, and maintaining a low ROS state (Yan et al., 2021). Interaction of Ca/CaM and melatonin is involved in overcoming salt-induced ionic, osmotic, and oxidative damages and Ca and melatonin may act as long-distance signals for inducing systemic salt tolerance in *Dracocephalum kotschyi* (Vafadar et al., 2020). The increase in melatonin caused by the high expression of *MzASMT9* leads to *Arabidopsis* strains with higher salt tolerance than wild-type plants, which can be proved by reducing ROS, reducing lipid peroxidation and enhancing photosynthesis (Zheng et al., 2017).

Melatonin (N-acetyl-5-methoxytryptamine) is a metabolite derived from tryptophan, and it is widely distributed in primitive photosynthetic bacteria and higher plants, including algae and fungi (Kanwar et al., 2018). In 1995, melatonin was first discovered in plants (Dubbels et al., 1995; Hattori et al., 1995), since then, the research on plant "melatonin" has started. Melatonin is related to many physiological functions of plants, including seed germination, growth, rooting, photosynthesis and stress resistance. It is considered to be a multi-regulatory molecule and may play a role in plant master regulation (Arnao and Hernandez-Ruiz, 2019). In plants, melatonin is synthesized from tryptophan through four successive enzyme reactions (Kang et al., 2011), including tryptophan decarboxylase (TDC) (Luca and Brisson, 1989), tryptamine 5-hydroxylase (T5H) (Fujiwara et al., 2010), serotonin N-acetyltransferase (SNAT) (Lee et al., 2014), N-acetylserotonin methyltransferase (ASMT) (Kang et al., 2011). It is reported that caffeic acid O-methyltransferase (COMT) also has ASMT enzyme activity in Arabidopsis (Byeon et al., 2014). Although there has been a lot of research on melatonin in abiotic stress, the molecular mechanism of melatonin in regulating cotton response to NaCl stress is still unclear.

In this study, 20 μM melatonin was used to pre-treat cotton to explore the molecular mechanism of melatonin improving salt tolerance of cotton. We analyzed the differentially expressed genes (DEGs) induced by melatonin in cotton involved in salt stress by RNA-seq technology, and found that DEGs are involved in phosphoinositide metabolism and signal transduction, hormone synthesis and signal transduction, and redox reactions, some transcription factors were only induced by melatonin. Inhibition of the expression of the melatonin synthesis gene *GhCOMT* by virus induced gene silencing technology (VIGS) suppressed the endogenous melatonin content in cotton, resulting in the silenced plants being more sensitive to salt stress, and exogenous supplementation of melatonin to the silenced plants alleviated the salt sensitivity of the silenced plants. Meanwhile, there were differences in melatonin content between two cotton materials with different salt tolerance (Zhong9807 and GK50), and Zhong9807 melatonin contents were higher in cotton materials with high salt tolerance than in GK50. This study aims to use melatonin to improve the salt tolerance of cotton, to explore the mechanism of melatonin in regulating cotton's response to salt stress, to discover salt tolerance genes regulated by melatonin, and to provide a new basis for improving the salt tolerance of cotton.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Taking upland cotton cultivar Zhong9807 and GK50 as experimental materials, Zhong9807 has higher salt tolerance than GK50 (Wang et al., 2016). The cotton seeds were sown on a 1:1.5 medium substrate of sand and vermiculite, and grown in an indoor incubator at 25°C for 16 h during the day and 8 h at night. In the treatment of cotton seedlings, the washed cotton seedlings were placed in 300 ml volumetric Erlenmeyer flasks with 10 seedlings in each Erlenmeyer, and the Erlenmeyer flasks were placed in a room incubator at 25°C for 16 h during the day and 8 h during the night.

Pre-treatment of Cotton With Exogenous Melatonin

To explore the suitable concentration of melatonin for improving salt tolerance in cotton, we washed three leaf stage cotton seedlings, moved to Erlenmeyer flasks containing 300 ml capacity of distilled water, and 10 seedlings in each Erlenmeyer flask. Cotton seedlings were treated with different concentrations of melatonin (0, 20, 50, 100, and 200 μM) and sprayed once a day for three consecutive days in a constant temperature incubator

at 25°C for 16 and 8 h during the day/night. The melatonin pre-treated cotton seedlings were retransplanted to 100 mM/L NaCl solution Erlenmeyer flasks, 0 μM concentration of melatonin treated cotton seedlings as the blank control (CK), water-treated wild-type seedlings were used as reference for normal growth (CK0) and phenotypic changes were observed.

cDNA Library Preparation and Transcriptome Sequencing

The true leaf samples of cotton seedlings pre-treated with 0 μM or 20 μM melatonin were collected for transcriptomic sequencing 12 h after salt treatment. Cotton seedlings pre-treated with 0 μM melatonin were used as controls and three biological replicates were set. Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer®spectrophotometer (IMPLEN, Westlake Village, CA, United States). RNA concentration was measured using Qubit®RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, Carlsbad, CA, United States). RNA integrity was assessed by using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext®Ultra™ RNA Library Prep Kit for Illumina®(NEB, United States) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and paired-end reads were generated.

RNA-Seq Data Analysis

Raw data (raw reads) of FASTQ format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, Q30, GC content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

The clean reads were mapped to the reference genome sequence, and reads that were perfectly matched or contained one mismatch were further analyzed and annotated on the basis of the reference genome. We used HISAT2 tools to map the reads to the reference genome. Gene expression levels were estimated as fragments per kilobase of transcript per million fragments mapped (FPKM).

Differential expression analysis of two conditions/groups was performed using the DESeq R package (1.10.1). DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed. Consequently, DEGs were obtained of three biological conditions. Fold Change ≥ 2 and FDR < 0.01 were taken as the thresholds for determining whether a gene had differential expression.

Gene Ontology (GO) enrichment analysis of the DEGs was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. KEGG (Kanehisa et al., 2007) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies[1]. We used KOBAS (Mao et al., 2005) software to test the statistical enrichment of differential expression genes in KEGG pathways.

Real-Time PCR

Randomly selected 15 different genes and used the same sample to perform qRT-PCR to verify the RNA-seq data. The total RNA was extracted with EASYspin Plus plant RNA rapid isolation kit (Aidlab Co., Ltd., Beijing, China). The pure RNA was reverse-transcribed using Transcript United States II one-step gDNA removal and cDNA synthesis supermix (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Primer Premier 5 software was used to design gene-specific primers, details of primers are shown in **Supplementary Table 2**. qRT-PCR assays were performed on the Bio-Rad 7500 fast fluorescence quantitative PCR platform with TransStart®top green qPCR supermix (TransGen Biotech Co., Ltd., Beijing, China) in accordance with the manufacturer's protocol, three biological replicates, the $2^{-\Delta\ \Delta\ Ct}$ method is used to measure the relative expression level of genes (Livak and Schmittgen, 2002). Internal control is *GhUBQ7* (Tan et al., 2013), it is stably expressed in cotton plants and is not affected by treatment and genotype. Perform correlation analysis between qRT-PCR and RNA-seq.

Effect of Salt Stress on Melatonin in Cotton

To explore the change of melatonin content in cotton under salt stress, we washed Zhong9807 cotton seedlings of three leaf stage size, transplanted them into a 300 ml volumetric Erlenmeyer flask containing 100 mM/L NaCl solution, and at 0 and 12 h of salt treatment, the true leaves were taken as samples for melatonin determination in three biological replicates.

Determination of Melatonin Content in Different Cotton Materials

In order to explore whether the endogenous melatonin level will affect the salt tolerance of cotton varieties, we selected medium Zhong9087 and GK50 cotton seedlings of the three-leaf stage size,

[1]http://www.genome.jp/kegg/

and took true leaves as samples for determining the melatonin content, three biological replicates for each sample.

Endogenous Melatonin Content Detection

Samples needed to measure endogenous melatonin levels were taken, and used the Plant Melatonin (MT) ELISA Kit (Ziker, ZK-P7490, Shenzhen, China) to measure the endogenous melatonin content. The kit uses double-antibody one-step sandwich enzyme-linked immunosorbent assay (ELISA). Add 0.1 g of the sample to an appropriate amount of physiological saline, mash it, centrifuge at 3000 rpm for 10 min, and take the supernatant. The assay was performed according to the instructions of the Plant Melatonin (MT) ELISA Kit, with three biological replicates for each sample.

Detecting of Antioxidant Enzyme Activity

Samples needed to determine the peroxidase (POD) activity and superoxide dismutase (SOD) activity were taken, and used the POD activity detection kit (SinobestBio, YX-W-A502, Shanghai, China) and the SOD activity detection kit (SinobestBio, YX-W-A500-WST-8, Shanghai, China) to determine the antioxidant enzyme activity, respectively. Weighed about 0.1 g of tissue and added 1 mL of extract for ice bath homogenization; centrifuged at 8000 $\times$ *g* at 4°C for 10 min, supernatant was placed on ice and measured according to the instructions. Three biological replicates were performed for each sample.

Detecting of Ca^{2+} Content

The determination of Ca^{2+} content refers to EDTA titration method in GB 5009.92–2016 "Determination of Calcium in Food of National Standard for Food Safety." Samples that needed to be assayed for Ca^{2+} content were cleaned by ddH_2O, placed in an oven, oven dried at 110°C for 10 min, 80°C until constant weight, accurately weigh 0.2 g of sample into a graduated digestion tube, add 10 ml 10% nitric acid, and digest in an adjustable electric furnace (reference conditions: 120°C/0.5 h to 120°C/1 h, increase to 180°C/2 h to 180°C/4 h, and increase to 200–220°C). The digestion solution appeared colorless and transparent or slightly yellow. Constant volume to 25 ml with water after cooling, then dilute as needed for the actual assay, and add a volume of lanthanum solution (20 μg/L) to the dilution to a final concentration of 1 μg/L and mix for further use, this being the sample to be tested. Pipette 1 ml of the sample to be tested and a blank into a test tube and add one drop of sodium sulfide solution (10 g/L), 0.1 ml of sodium citrate solution (0.05 mol/L), 1.5 ml of potassium hydroxide solution (1.25 mol/L), and three drops of calcium red indicator. Titrate immediately in a 10 fold dilution of EDTA solution until the indicator changes blue from purple red and record the volume of EDTA solution consumed with a 10 fold dilution.

VIGS Technique Silenced the Melatonin Synthesis Gene

In order to investigate whether the content of endogenous melatonin in cotton has an effect on the salt tolerance of cotton, the melatonin synthesis gene was silenced by VIGS. We used *Arabidopsis AtCOMT* (AT5G54160) (Byeon et al., 2014) as the query sequence and BLAST to the cotton genome to obtain the putative *GhCOMT* (Gh_D12G2680) in cotton. Vector was a pYL156 vector maintained in our laboratory, and BamHI and SacI restriction enzymes were selected for double digestion, Use the online tool SGN–VIGS[2] design length in 300 bp silence about the size of the fragment. The in-Fusion primers for *GhCOMT* were designed manually, and the primers for the *GhCOMT* silencing fragment were as follows: forward primer, 5′-AGAAGGCCTCCATGGGGATCC ATGGGTTCAACCGGTGAAACCCAAAT-3′; reverse primer, 5′-TGCCCGGGCCTCGAGACGCGTGAGCTCGCCATCAGGA AGAGTGCGCA-3′. Using cotton leaf cDNA as template, the silenced fragment was amplified, and the VIGS expression vector *pYL156:GhCOMT* was constructed by in-Fusion ligation technique. The constructed expression vectors were transformed into *Escherichia coli*, and after correct sequencing, they were transformed into Agrobacterium using the freeze-thaw method. The virus mediated gene silencing (VIGS) system consisted of a recombinant vector, the negative control pYL156, the positive control *pYL156:PDS*, and the helper vector pYL192. Silencing of the phytoene dehydrogenase gene (*PDS*) resulted in white leaves, so the whole system was judged to be correct by observing whether the VIGS plants with *pYL156:PDS* had true leaves turned white. After the cotyledon of cotton seedling was flattened, it was prepared for infection. The night before infection, sufficient water was poured, and the Agrobacterium solution was handled well. After the injection was completed, cotton seedlings were incubated normally after they were protected from light for 24 h. Cotton was treated with 100 mm NaCl when grown to the three leaf stage.

Statistical Analysis

The GraphPad Prism 8.0 software was employed to analysis (ANOVA) the results. Duncan's Multiple Range Test was used to compare the least significant difference of means ($P < 0.05$).

RESULTS

Exogenous Application of Melatonin Improves Cotton Salt Tolerance

Cotton seedlings at three leaf stages were pre-treated with different concentrations of melatonin (0, 20, 50, 100, and 200 μM), and then treated with 100 mM NaCl solution, 0 μM concentration of melatonin treated cotton seedlings as the blank control (CK), water-treated wild-type seedlings were used as reference for normal growth. Different responses to salt stress were observed in melatonin-pre-treated and control seedlings (**Figure 1a**). At 12 h salt treatment, the cotyledons of the control seedlings lost luster and wilting more severely than the pre-treated seedlings. With the increase of salt stress time, the true leaves of control seedlings wilted at first, and the wilting degree was more serious than that of pre-treated seedlings. At 4 days

[2]https://vigs.solgenomics.net/

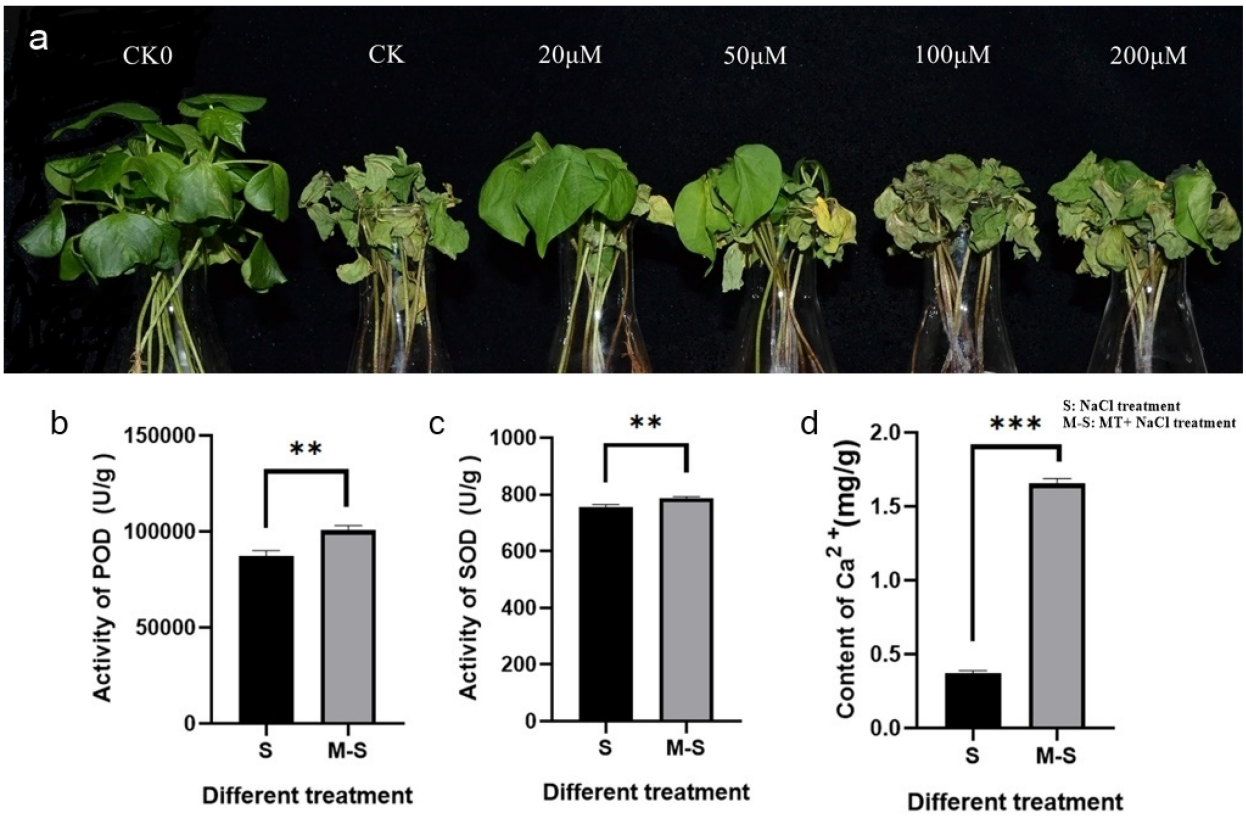

FIGURE 1 | Melatonin enhances salt tolerance in cotton. **(a)** Wilting symptoms 4 days after cotton salt pre-treatment with different concentrations of melatonin (0, 20, 50, 100, and 200 μ M); **(b)** Melatonin enhances the activity of POD; **(c)** Melatonin enhances the activity of SOD; **(d)** Melatonin enhances the content of Ca^{2+}. $^{**}p < 0.01$ and $^{***}p < 0.001$.

of salt stress treatment, almost all the control seedlings died, but the seedlings treated with 20 or 50 μM melatonin did not die completely. Although all the cotyledons of the seedlings treated with 20 μM melatonin wilted, the true leaves only partially wilted and did not die. Therefore, Therefore, 20 μM melatonin was preliminarily selected as the treatment concentration based on phenotypic changes.

In order to test whether 20 μM melatonin could improve salt tolerance of cotton, antioxidant enzyme activity and Ca^{2+} content were measured. The antioxidant enzyme activity and Ca^{2+} content of seedlings treated with 0, 20 μM melatonin were determined after salt stress. The POD activity (**Figure 1b**), SOD activity (**Figure 1c**), and Ca^{2+} content (**Figure 1d**) were significantly different among different treatments. Under salt stress, the POD activity, SOD activity, and Ca^{2+} content of plants treated with exogenous melatonin increased significantly.

Analysis of the Response of DEGs to Salt Stress Under Melatonin Pre-treatment

In order to explore how exogenous melatonin regulates cotton's response to salt stress and thus improves salt tolerance, we compared the RNA-seq data of CK VS S and S VS M-S. We used the salt stress treatment for 12 h as the sampling time. The samples included the control group (CK) and the treatment group (S, M-S), and three biological replicates. Transcriptome analysis of nine samples, a total of 58.98 Gb Clean Data was obtained, and the Clean Data of each sample reached 5.83 Gb, and the Q30 base percentage was 93.03% and above (**Supplementary Table 1**). Clean Reads of each sample were sequentially aligned with the specified reference genome, the comparison efficiency varies from 96.17 to 97.26%. Based on the results of the comparison, we performed alternative splicing prediction analysis, gene structure optimization analysis, and discovery of new genes. 7608 new genes were discovered, of which 6588 were functionally annotated.

In the process of DEGs detection, Fold Change ≥ 2 and FDR < 0.01 are used as the screening criteria to obtain the differential gene expression (DEGs) between the two treatments (CK VS S and S VS M-S) (**Figure 2A**). In the CK VS S library, we detected 9199 DEGs that responded to salt stress (compared to CK, 3837 genes were up-regulated and 5362 genes were down-regulated). In the SV S M-S library, a total of 786 DEGs were detected (relative to S, 356 genes were up-regulated and 430 genes were down-regulated). **Figure 2B** showed the differences in DEGs among the three treatments. We found that 305 genes were differentially expressed jointly between S and M-S, 8894 genes were specifically expressed in the treatment group S, and 481 genes were specifically expressed in the treatment group M-S. These DEGs are related to the relief of salt

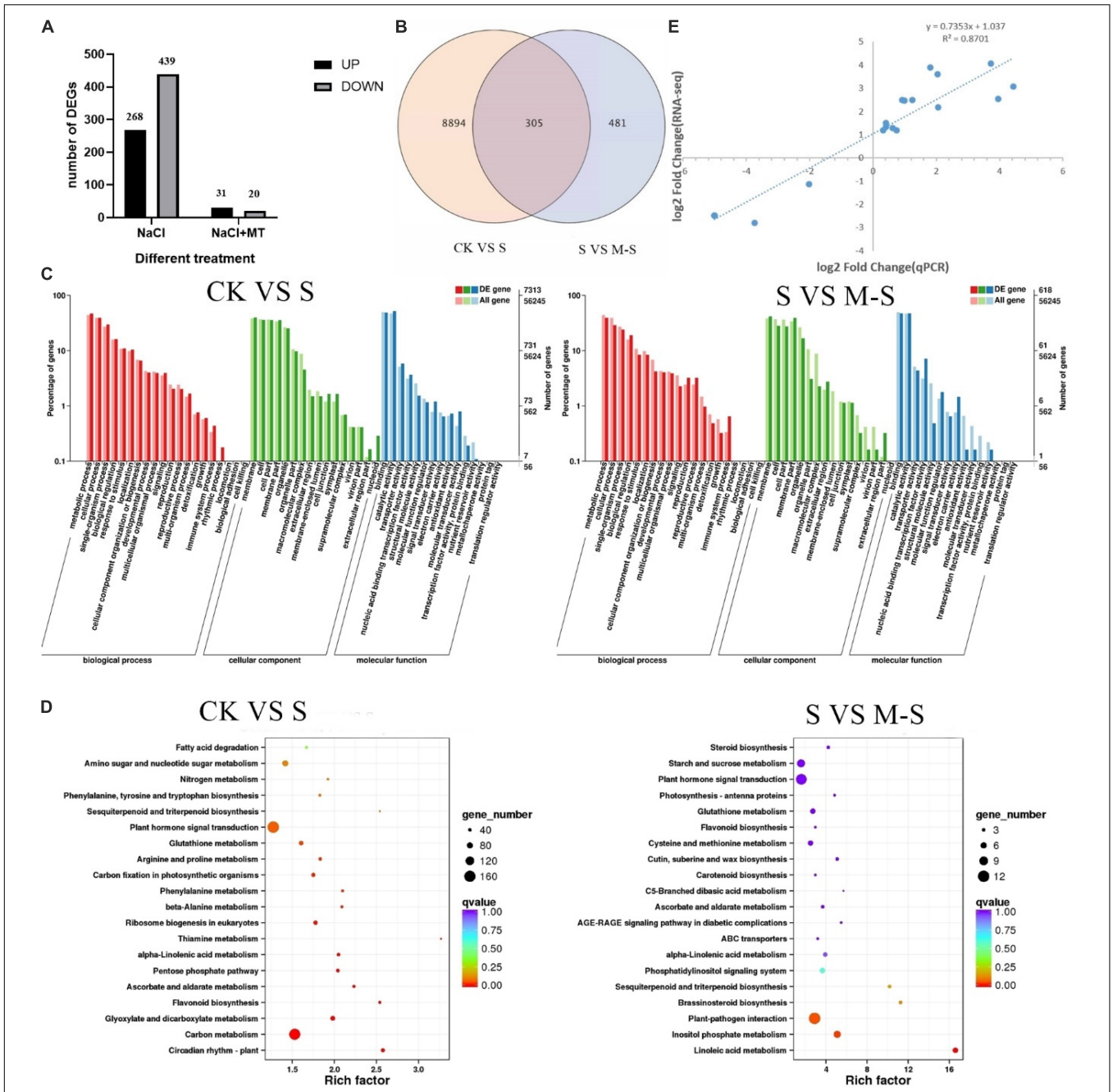

FIGURE 2 | Results of RNA-seq data analysis. **(A)** Number of different gene expression (DEGs) in CK VS S and S VS M-S. **(B)** Venn diagram of DEGs. **(C)** Summary of Gene Ontology (GO) categories of the DEGs. **(D)** Summary of KEGG categories of the DEGs. **(E)** Relationship between RNA-seq and quantitative real-time PCR (Q-PCR) expression data ($\log_2$ fold change) (R^2 = 0.8701) Note: CK: Blank Control; S: Salt stress treatment; M-S: Melatonin and salt stress treatment.

stress by melatonin. These genes that respond to exogenous melatonin were considered to be important candidate genes for further research.

According to the classification method of GO, the functions of each classification system are classified (Young et al., 2010). A total of 7,313 DEGs between CK and S were annotated by GO, most of which were rich in functional categories such as metabolic process, cell process, membrane component, binding and catalytic activity. Between S and M-S, 618 DEGs were co-enriched by GO annotation (**Figure 2C**), which were mainly divided into metabolic process, cell process, membrane part, cell part, binding activity and catalytic activity, etc.

Differentially expressed genes were performed KEGG enrichment analysis to determine the main pathways for the enrichment of melatonin-induced cotton leaves under salt stress (**Figure 2D**). Between CK and S, 1872 DEGs enriched

to 121 KEGG pathways, mainly enriched in the following pathways: there were 163 genes in plant hormone signal transduction (ko04075) (38 genes were up-regulated, 125 genes were down-regulated); 156 genes were enriched in carbon metabolism (ko01200), and there were 120 genes in starch and sucrose metabolism (ko00500). Between S and M-S, there were 12 genes (11 genes down-regulated, 1 gene up-regulated) in plant-pathogen interaction (ko04626), and 11 genes (8 genes up-regulated, 3 genes down-regulated) were enriched to plant hormone signal transduction (ko04075), there were seven genes (six up-regulated and one down-regulated) in inositol phosphate metabolism (Ko00562). A total of five genes (three up-regulated and one down-regulated) were enriched to phosphatidylinositol signaling system (KO04070) and five genes up-regulated in Glutathione metabolism (Ko00480). These pathways were main enrichment pathways.

To verify the transcriptome data, we used qRT-PCR to explore the expression of 15 different genes randomly selected. Perform correlation analysis on the two sets of data, qRT-PCR and RNA-seq were used to compare the gene folding changes (FC) between the two treatment groups. As shown in the **Figure 2E**, the qRT-PCR data were consistent with the RNA-seq data, and the significant positive correlation (R^2 = 0.8701) supports the reliability of the RNA-seq data.

Melatonin Regulates the Expression of Redox-Related Genes to Relieve Salt Stress

In order to explore the oxidoreductase related genes involved in salt stress regulation by melatonin, we found that 707 genes were enriched in oxidoreductase activity (GO:0016491), 439 genes were down-regulated, and 268 genes were up-regulated. Between S and M-S, 51 genes were found to have the molecular function of oxidoreductase activity (GO:0016491), of which 20 genes were down-regulated and 31 genes were up-regulated. In total, 50 of these genes were assigned to the oxidation-reduction process (GO:0055114), and they were differentially expressed in each treatment. Among them, 18 genes were down-regulated under salt stress, but were up-regulated by melatonin under salt stress; six genes were up-regulated under salt stress, but were induced down-regulated by melatonin under salt stress (**Supplementary Table 3**). The specific expression of seven genes was induced by melatonin under salt stress. We found that 20 redox-related genes were not expressed under salt treatment alone, but were regulated by melatonin under salt stress and their expression is up-regulated. These results indicated that melatonin can improve cotton salt tolerance by regulating some redox-related genes.

Melatonin Regulates Transcription Factors in Response to Salt Stress

Transcription factors play a vital role in regulating plant stress tolerance (Gruber et al., 2009). In order to discover the transcription factors induced by melatonin under salt stress, a total of 98 transcription factors were differentially expressed between S and M-S, including AP2/ERF-ERF, WRKY, NAC, and C2H2 and other transcription factor family members (**Figure 3**).

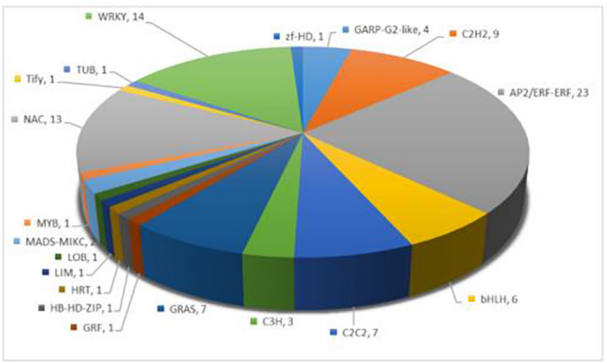

FIGURE 3 | Annotation of specific expression of transcription factors induced by melatonin under salt stress.

Interestingly, 60 transcription factors were not differentially expressed between S and CK, but were differentially expressed between S and MS. There were 23 transcription factors that were up-regulated by melatonin under salt stress, such as AP2/ERF-ERF, C2H2, bHLH, and WRKY. These transcription factors were not induced by salt stress, but were regulated by melatonin under salt stress, and participate in cotton's resistance to salt stress.

Melatonin Co-ordinates Other Plant Hormones in Cotton's Resistance to Salt Stress

As we all know, plant hormones play a very important role in plant growth and stress response. The idea of melatonin as a plant hormone is gradually accepted by everyone (Arnao and Hernandez-Ruiz, 2019). In order to explore whether melatonin is involved in regulating other plant hormones in the process of improving cotton tolerance to salt stress, we analyzed the expression of genes related to the hormone pathway. We have observed that 11 hormone-related genes are differentially expressed by melatonin under salt stress (**Figure 4**), and five genes have decreased expression under salt stress, but are induced by melatonin to undergo differential changes under salt stress. Four genes (GH_A03G2091.gene, GH_A07G0419.gene, GH_D08G2786. gene, and GH_d01G1143. gene) were induced by melatonin to up-regulate expression, and 1 gene (GH_A08G2800.gene) was down-regulated and increased by melatonin. Six genes were not differentially expressed under salt stress, but they were induced by melatonin under salt stress. Four genes (GH_A13G0422.gene, GH_D04G1779.gene, GH_D05G2097.gene, and GH_D13G0417.gene) were up-regulated, and two genes (GH_A09G2113.gene and GH_A09G1643.gene) down-regulate the expression. These genes were involved in the signal transduction process of auxin, ABA, ethylene, brassinosteroid, and jasmonic acid.

We also found that in the brassinolide biosynthetic pathway, three genes were induced by melatonin, one gene (GH_A10G2603.gene) was down-regulated, and two genes (GH_A05G1364.gene and GH_D05G1368.gene) were up-regulated. At the same time, there were

four genes (GH_A13G0422.gene, GH_D04G1779.gene, GH_D05G2097.gene, and GH_D13G0417.gene) in the signal transduction pathway of brassinolide under salt stress induced by melatonin to up-regulate their expression, but they were not induced by salt stress. Based on these results, we speculated that melatonin may act synergistically with brassinosteroid to improve cotton's tolerance to salt stress.

Melatonin-Regulated Genes Involved in Phosphoinositide Metabolism and Its Signaling System

In order to explore which molecular mechanism of melatonin regulating to improve the salt tolerance of cotton, we enriched the differential genes between S and M-S into KEGG pathways. We found that seven genes (six genes up-regulated and one gene down-regulated) were enriched in Inositol phosphate metabolism (ko00562), and five genes (three genes up-regulated, two genes down-regulated) were enriched in phosphatidylinositol signaling system (ko04070). There were three genes that are commonly enriched in these two pathways (GH_A05G0889.gene, GH_A06G1884.gene, and GH_D05G1952.gene), all of which were up-regulated by melatonin under salt stress (**Figure 5**). **Figure 5** showed the expression pattern of nine genes induced by melatonin in the process of phosphatidylinositol signaling system in cotton resisting salt stress. These genes were specifically expressed by melatonin under salt stress, indicating that melatonin participates in the resistance of cotton to salt stress by inducing phosphoinositide metabolism and the expression of genes related to signaling system.

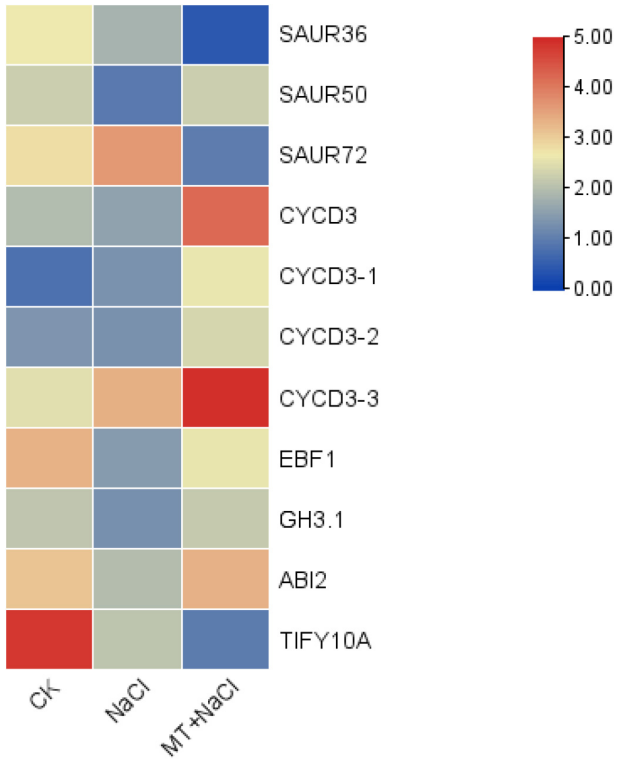

FIGURE 4 | Effect of melatonin on gene expression related to cotton hormone signal transduction.

Detection of Endogenous Melatonin Content in Upland Cotton

In order to explore whether the endogenous melatonin content is related to the salt tolerance of cotton, the endogenous melatonin levels of two cotton variety (Zhong9807, GK50) at the three-leaf stage seedlings were measured (**Figure 6A**). By comparing two cotton varieties with different salt tolerance, the melatonin content in Zhong9807 roots and leaves of the better salt tolerance is higher than GK50. We speculated that the salt tolerance of cotton may be related to the endogenous melatonin content. At the same time, in order to explore whether the endogenous melatonin content of cotton is involved in the resistance of salt stress, we treated the cotton seedlings at the three-leaf stage with salt, and measured the endogenous melatonin content after 12 h, and treated the water as a control. The results showed that salt treatment induced an increase in endogenous melatonin content (**Figure 6**). In short, cotton endogenous melatonin participates in cotton's resistance to salt stress and has a certain relationship with cotton's salt tolerance.

Inhibition of Melatonin Made Cotton More Sensitive to Salt Stress

In order to investigate whether the change of endogenous melatonin content would affect the salt tolerance of cotton, VIGS technique was used to silence the melatonin synthesis gene *GhCOMT:pYL156:PDS* causes chlorosis and blanching of leaves, and the success of the experiment could be judged by observing whether the leaves of *pYL156:PDS* transformed plants turned white, with pYL156 empty injected plants serving as a blank control. About 2 weeks after VIGS infection, the leaves of *PYL156:PDS* plants became albino, indicating that our silencing system was stable. When the cotton seedlings grew to the three-leaf stage, the expression of *GhCOMT* was detected, and PYL156 plants were taken as the control, and it was found that the expression of *GhCOMT* was significantly decreased (**Figure 7a**). Meanwhile, the content of endogenous melatonin was significantly decreased in the silenced plants (**Figure 7b**), indicating that gene silencing was successful. Subsequently, the silencing plants were selected and subjected to salt stress, and the pYL156 and *pYL156:GhCOMT* plants were washed and transplanted into 300 mL conical flask containing 100 mM/L NaCl solution. At the same time, some of the *pYL156:GhCOMT* plants were exogenous with 20 μM melatonin, and the phenotype was obvious after 3 days of salt treatment (**Figure 7c**). The stress of gene silencing *pYL156:GhCOMT* plants was more serious than that of control pYL156 plants, and the degree of true leaf wilting was significantly higher than that of control plants. Meanwhile, the stress symptoms of *pYL156:GhCOMT* plants were alleviated after exogenous melatonin was applied.

Based on our transcriptome data, we found that melatonin enhances cotton salt tolerance mainly by affecting reactive oxygen scavenging system and Ca^{2+} signal transduction pathway.

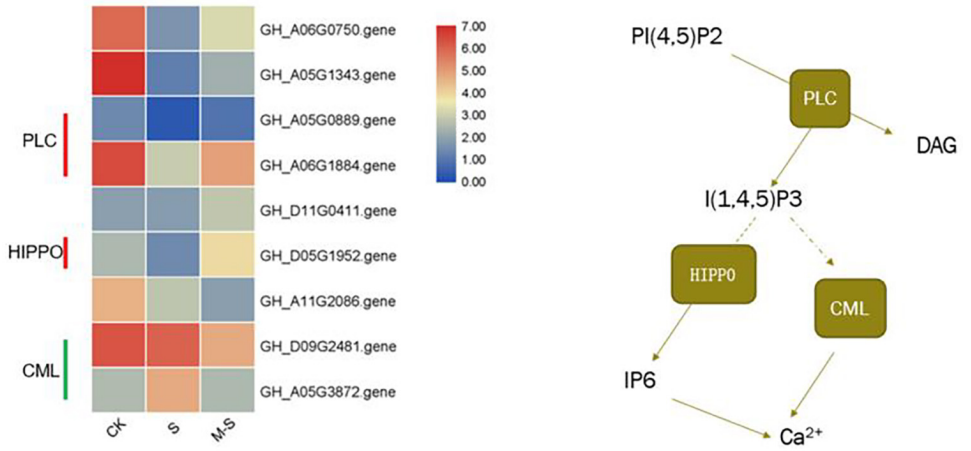

FIGURE 5 | Effect of melatonin on the expression of phosphatidylinositol Signaling system related genes.

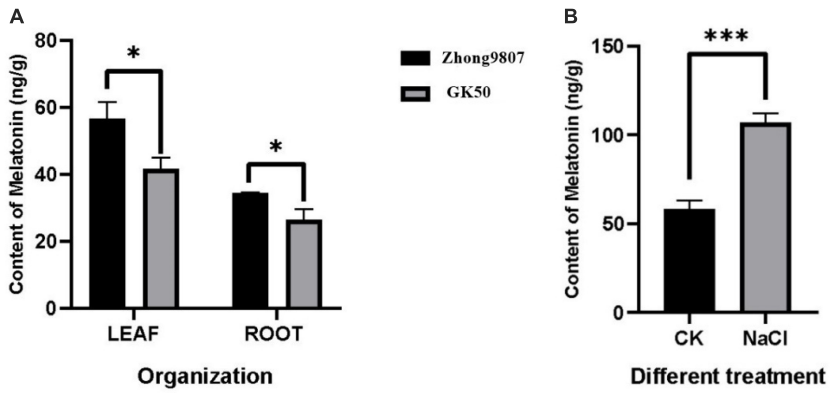

FIGURE 6 | Determination of endogenous melatonin content in cotton. **(A)** Comparison of endogenous melatonin levels between two cotton species (Zhong9807 and GK50). **(B)** Effects of salt stress on endogenous melatonin levels in cotton seedlings (Zhong9807). $^{*}p < 0.05$ and $^{***}p < 0.001$.

We measured biochemical markers POD activity (**Figure 7d**), SOD activity (**Figure 7e**), and Ca^{2+} content (**Figure 7f**) by sampling plants treated with pYL156, *pYL156:GhCOMT*, and *pYL156:GhCOMT*+20 μM melatonin, respectively. The POD activity, SOD activity and Ca^{2+} content of *pYL156:GhCOMT* plants were significantly down-regulated, indicating that the function of reactive oxygen scavenging system was weakened, Ca^{2+} signal translocation was weakened, and the salt tolerance of cotton was decreased. Meanwhile, exogenous melatonin supplementation could alleviate the effect of gene silencing on salt tolerance of cotton.

DISCUSSION

Melatonin Plays an Important Role in Salt Tolerance of Cotton

Since melatonin is involved in many plant development processes and stress responses, melatonin levels in plants will change under different environments, especially under stress. It has been reported that sodium chloride, hydrogen peroxide, drought, pH, cold stress and ultraviolet radiation can all induce the increase of melatonin level in plants (Arnao and Hernández-Ruiz, 2006; Arnao and Hernández-Ruiz, 2013; Riga et al., 2014). Under various stress conditions, the level of melatonin in plants increases significantly (Zhang et al., 2015), which is conducive to anti-stress ability. This is consistent with our results, we found that salt stress can induce the increase of endogenous melatonin levels in cotton. At the same time, the content of endogenous melatonin in Zhong9807 with higher salt tolerance was higher than that in GK50, which also indicated that the content of melatonin in cotton with higher salt tolerance was higher, which was consistent with the conclusion that melatonin levels in *Verticillium dahliae* resistant varieties were higher than those in susceptible varieties (Li et al., 2019). Overexpression of melatonin synthesizing gene Caffeinate O-methyltransferase 1 (*COMT1*) significantly enhanced the ability of tomato to reduce the toxicity and residue of MBC (Yan et al., 2019). After drought treatment, the Pro content of transgenic *TACOMT* was higher than that of WT, while the MDA content was lower than that of

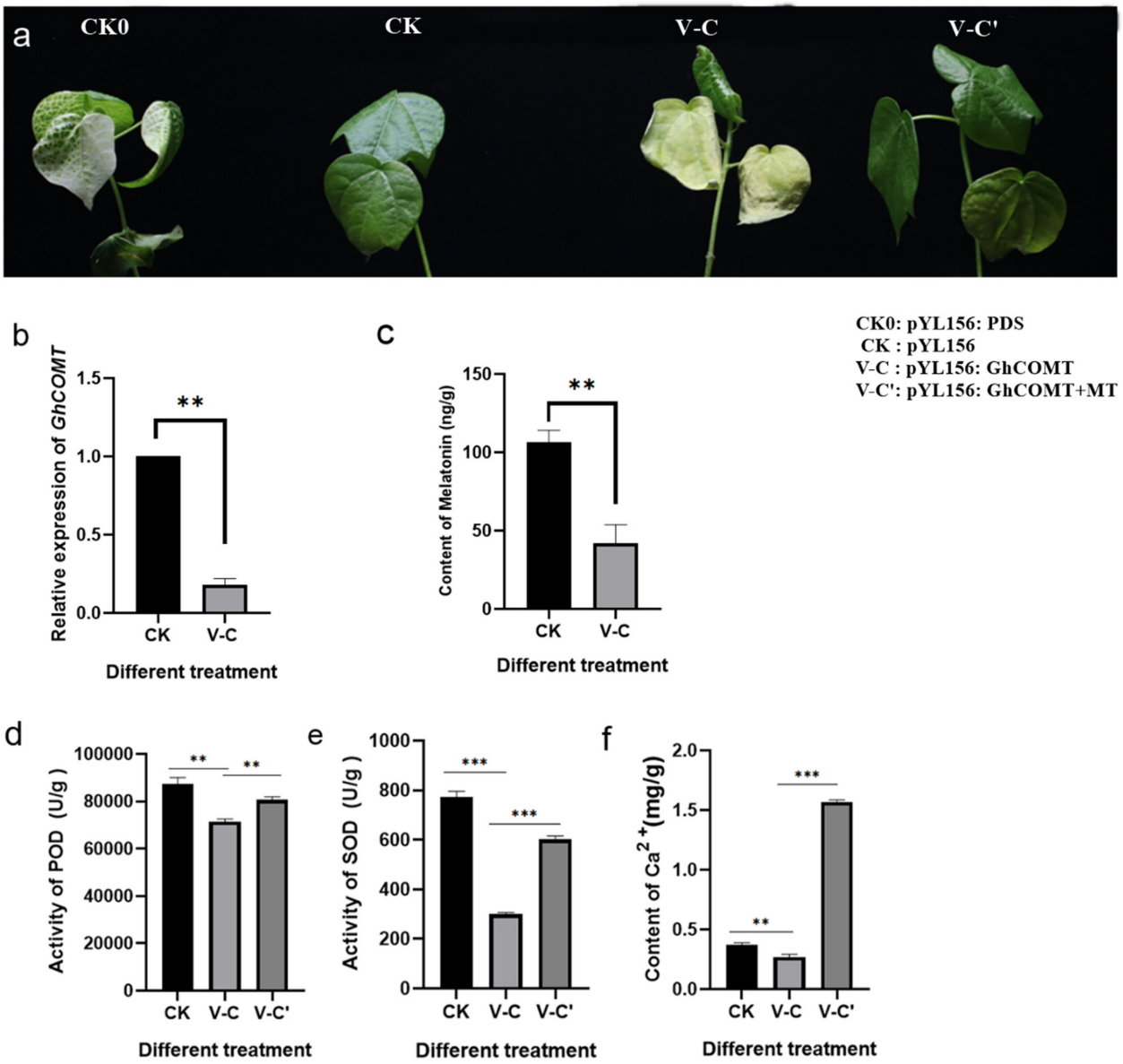

FIGURE 7 | Inhibition of endogenous melatonin reduced the salt tolerance of cotton. **(a)** Salt tolerance of *GhCOMT* silenced plants decreased; **(b)** Detection of *GhCOMT* silencing efficiency; **(c)** Detection of melatonin content in *GhCOMT* silenced plants; **(d)** Detection of POD activity in *GhCOMT* silenced plants; **(e)** Detection of SOD activity in *GhCOMT* silenced plants; **(f)** Detection of melatonin content in *GhCOMT* silenced plants. $^{**}p < 0.01$ and $^{***}p < 0.001$.

WT (Yang et al., 2019). By silencing *GhCOMT*, we reduced the melatonin content of cotton, thus making silenced plants more sensitive to salt stress. These results fully show that melatonin is closely related to salt tolerance of cotton.

Melatonin Acts as an Antioxidant Defense System in Cotton Salt Stress

Plant melatonin is considered to be an antioxidant defense system, which plays an important role in controlling ROS and reactive nitrogen species (RNS) in plant cells, as well as other free radicals and harmful oxidative molecules (Reiter et al., 2014). Studies have found that melatonin may reduce the oxidative damage caused by salt stress by directly enhancing the activity of antioxidant enzymes or removing H_2O_2 (Li et al., 2012). Exogenously apply of melatonin, regulates SOD, CAT, GR, APX, and ASC-GSH pathways, participates in enhancing the detoxification effect of ROS, reducing cell damage and cell death (Siddiqui et al., 2019). Melatonin pre-treatment of apple seedlings under salt stress are in high branch height, leaf number, chlorophyll content and electrolyte permeability affected by salt stress is less than the untreated plant, hydrogen peroxide level by half, to induce ROS metabolism enzyme (ascorbic acid peroxidase, catalase and peroxidase activity), Na^+ and K^+

transporter (NHX1 and AKT1) increases, it all helps to alleviate salinity induced inhibition (Li et al., 2012). The overexpression of *MzASMT9* led to the increase of melatonin, as a result, *Arabidopsis thaliana* overexpressed plant showed higher salt tolerance, lower ROS, lower lipid peroxidation and enhanced photosynthesis than wild-type plants (Zheng et al., 2017).

In this study, melatonin regulated the expression of redox-related genes and up-regulates genes related to the glutathione metabolism (ko00480) pathway to help cotton alleviate salt stress. Based on RNA-seq data, exogenous application of melatonin can change the expression of some genes related to redox and Glutathione metabolism, and even induce the expression of some genes that were not induced by salt stress, and participate in redox reactions. When plants are exposed to abiotic or biotic stress or sense melatonin, melatonin can regulate and rapidly up-regulate the activities of different antioxidant enzymes and stress tolerance related genes, and activate downstream signal transduction pathways (Arnao and Hernández-Ruiz, 2015). Due to its unique redox and nucleophilic properties, glutathione plays an important defensive role against ROS, foreign organisms and heavy metals in biological reduction reactions (Gao et al., 2020). In short, as an antioxidant defense system, melatonin improved the salt tolerance of cotton by scavenging active oxygen.

Melatonin Interacts With Plant Hormones

With the melatonin receptor CAND2/PMTR1 discovered in *Arabidopsis* (Wei et al., 2018), Melatonin began to be considered a plant hormone. The structural similarity between melatonin and indole-3-acetic acid (IAA) (auxin) has prompted botanists to further study its possible role as a regulator of normal plant growth and development (Sun et al., 2020). Some studies have shown that plant melatonin works by regulating various elements related to the redox network or interfering with the activity of other plant hormones (Tan and Reiter, 2020). Melatonin maintains abscisic acid homeostasis by positively regulating its biosynthetic genes and negatively regulating catabolism genes. It effectively down-regulates the abscisic acid synthesis gene MdNCED3 and up-regulates its catabolism genes MdCYP707A2 and MdCYP707A1, resulting in a decrease in abscisic acid (Li et al., 2015). Drought stress up-regulates ABA, BRs, and JA, and down-regulates CKs and GAs, while melatonin increases BRs, GAs, JA, and CKs levels and reduces ABA levels (Moustafa-Farag et al., 2020). The melatonin-jasmonic acid interaction is expressed by regulating molecular transcripts such as JAZs in the jasmonic acid signal (Shi et al., 2015). Drought stress inhibits the biosynthesis of gibberellin, and after melatonin treatment, the biosynthesis of gibberellin is greatly enhanced, thereby improving drought tolerance (Sharma et al., 2020). Application of melatonin significantly increased the zeatin + zeatin riboside (Z + ZR), IAA, gibberellic acid (GA) contents (Ahmad et al., 2020). Melatonin is a positive regulator of dark growth or shade outgrowth by regulating BR biosynthesis in plants (Hwang and Back, 2018). The effect of melatonin on ethylene biosynthesis, ethylene perception and ethylene signal may help tomato fruit ripening and quality improvement (Liu et al., 2019).

Based on transcriptome data analysis, under salt stress, melatonin can induce the synthesis of some hormones (Auxin, ABA, Ethylene, Brassinosteroid, Jasmonic acid) and the expression of signal transduction-related genes. Some genes were not induced by salt stress, but their expression was induced by melatonin under salt stress. We speculated that melatonin may interact with other hormones by regulating the expression of these genes. Exogenous application of melatonin up-regulated the expression of cotton brassinolide synthesis-related genes and their signal transduction-related genes. In our study, two genes in the brassinolide synthesis pathway were up-regulated and expressed by melatonin, and one gene was down-regulated. At the same time, four genes in the brassinolide signal transduction pathway were up-regulated. Exogenous melatonin treatment induces multiple BRs biosynthetic genes, including DWARF4, D11, and RAVL1 (Hwang and Back, 2018). Melatonin regulated brassinolide synthesis, controls stomatal movement, improves cell membrane stability and water absorption, and reduces ion leakage caused by cell membrane damage under water-limited conditions (Moustafa-Farag et al., 2020). BRs can modify enzymatic and non-enzymatic antioxidant systems, which are involved in maintaining ROS homeostasis and protecting cells from ROS-induced damage (Núñez et al., 2003). As a hormone, melatonin is a regulator of many plant hormones (Arnao and Hernández-Ruiz, 2020), co-ordinating other plant hormones in response to salt stress in regulating cotton defense network.

Melatonin as a Regulator Regulates Transcription Factors Involved in Salt Stress

Melatonin appears to be more than just a classic plant hormone, because its role is diverse, its potential to alter gene expression is significant, acting as a biological stimulus in non-biological stress situations, regulating key gene expression against stressors (Arnao and Hernandez-Ruiz, 2019). Transcriptome profile through RNA-sequence analysis identified 1228, 1120, and 1537 DEGs in control plant (Ctr) vs. simulated acid rain stressed plant (P25) comparison, control plant vs. melatonin treatment in simulated acid rain stressed plant (P25M) comparison and P25 vs. P25M comparison, respectively (Debnath et al., 2020). Transcription factors play an important role in stress tolerance. The reported transcriptome analysis confirmed that more than 30 TF family genes are involved in abiotic stress responses, including MYB, WRKY, ERF, bZIP, etc. (Peng et al., 2014). Many transcription factors have been confirmed to be up-regulated in melatonin therapy, most of which are stress-related transcription factors. Exogenous melatonin can regulate the expression of TFs such as bZIP, MYB, WRKY, ERF, promote the expression of genes encoding ROS scavenging enzymes, and improve tolerance to abiotic stress (Liang et al., 2015).

In this study, we found that 21 transcription factor families between S and M-S, with a total of 98 genes differentially expressed, of which 60 transcription factors were not induced by salt stress, but were specifically induced by melatonin under salt stress expression, such as AP2/ERF-ERF, C2H2, bHLH, and WRKY and other transcription factors. It is known that AP2/ERF family transcription factors regulate various environmental stress response processes of higher plants, such as abiotic stress

(cold, heat, drought, salt, and osmotic stress) and biotic stress (herbivorous insects and microbial pathogens) (Feng et al., 2020). Studies have shown that C2H2 plays an important role in the defense and adaptation responses of plants to various environmental stress conditions (Wang et al., 2019). WRKY transcription factors have multiple functions in regulating stress response, leaf senescence, plant growth and development, etc. (Gu et al., 2018). Some bHLH TFs are also believed to be able to cope with a variety of abiotic stresses and improve the drought resistance, salt tolerance and cold tolerance of plants (Sun et al., 2015). These transcription factors induced by melatonin may play an important role in the resistance of cotton to salt stress.

Melatonin as a Signaling Molecule Induces a Second Messenger in Response to Salt Stress

Many lipids and lipid-related molecules are thought to play a role in plant defense signals (Shah, 2005). Membrane-associated phospholipids and soluble inositol phosphates (collectively referred to as phosphoribosyls) are present in all eukaryotic cells and are involved in the response of plants to many environmental stimuli. When plants are under abiotic stress, membrane receptors will be stimulated, and membrane-related inositol phospholipids will transmit cellular information by producing second messengers, lipid-binding DAG and soluble IP3 (Jia et al., 2019). Phospholipase C (PLC) is activated by external stimuli to cause hydrolysis of PtdInsP2, generating soluble second messengers inositol 1,4,5-triphosphate (InsP3) and diacylglycerol (DAG) (Hung et al., 2014). In plants, DAG is converted to phosphatidic acid (PA), which is an important signal molecule in plant abiotic and biotic stress (Testerink and Munnik, 2011), InsP3 may be further phosphorylated to form inositol hexaphosphate (InsP6), both InsP3 and InsP6 are thought to release Ca^{2+} from intracellular storage (Valentová and Martinec, 2007; Yang et al., 2010). When plants are exposed to external stimuli, they increase the intracellular Ca^{2+} concentration, which in turn activates a series of calcium-binding proteins, including Ca^{2+} sensors/decoders, protein kinases and transcription factors (Srivastava et al., 2013). Melatonin can

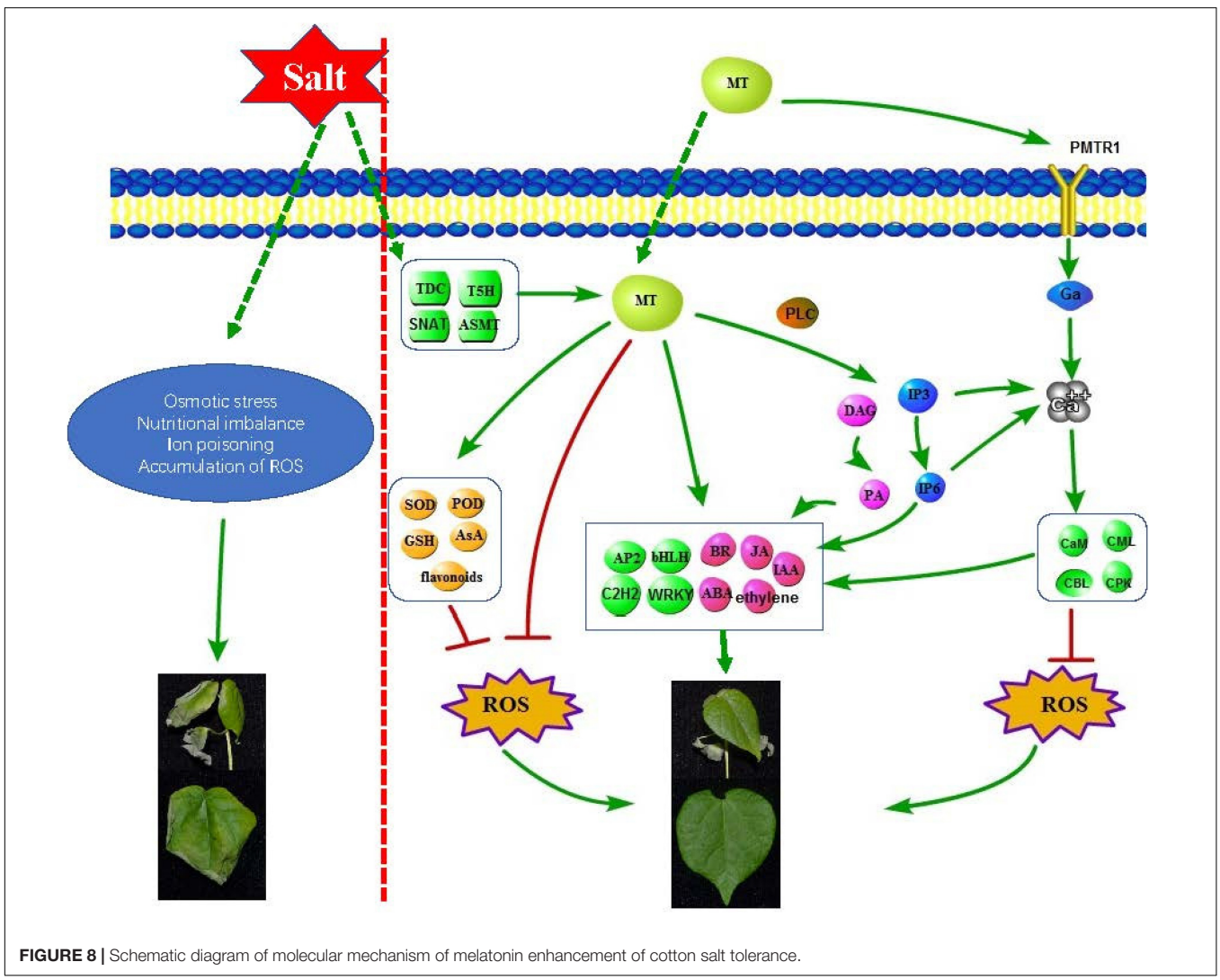

FIGURE 8 | Schematic diagram of molecular mechanism of melatonin enhancement of cotton salt tolerance.

change the expression of genes involved in signal transduction. Six stress receptors and 14 genes involved in calcium-dependent signaling are up-regulated by melatonin (Weeda et al., 2014). The interaction between melatonin and Ca^{2+} calmodulin has been shown to regulate many calcium-dependent cellular functions in animal cells (Krystyna et al., 2009).

In this study, transcriptome data analysis found that *GhPLC2* (GH_A05G0889.gene and GH_A06G1884.gene) and *GhHIPP02* (GH_D05G1952.gene) were down-regulated under salt treatment, but were up-regulated by melatonin under salt treatment. Phospholipase C (PLC) can hydrolyze phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] into two second messengers, inositol-1,4,5-triphosphate (InsP3) and Diacylglycerol (DAG) (Munnik and Vermeer, 2010). *GhHIPP02* can catalyze the production of Inositol hexaphosphate (IP6), which can be used as a second messenger that affects the release of Ca^{2+} in cells, and is also the main storage form of phosphate in plant cells. At the same time, the unique role of InsP6 in plants is related to the potential of regulating plant hormone receptors. It can bind to F-box protein, participate in the reception of auxin, and play a role in auxin signal transduction (Tan et al., 2007). Both *GhCML10* (GH_D09G2481.gene) and *GhCML45* (GH_A05G3872.gene) were down-regulated by melatonin under salt stress. Studies have shown that transgenic lines overexpressing *CML10* show late flowering and reduce seed yield. Compared with WT, the ability to resist osmotic stress is reduced, indicating that *CML10* has a negative effect on the growth and stress response of *Arabidopsis* (Divi et al., 2010). Cotton pre-treated with melatonin can accelerate the synthesis of InsP3 and InsP6 and promote the release of Ca^{2+} by up-regulating InsP3 and InsP6 synthesis-related genes under salt stress. At the same time, melatonin induced the down-regulation of *GhCML* gene under salt stress, leading to the release of Ca^{2+}. As a signal molecule, melatonin promoted the release of Ca^{2+} by regulating the phosphatidylinositol signal system and improves the salt tolerance of cotton.

Melatonin Improves Cotton Salt Tolerance Through a Complex Network

Soil salinization is an increasingly serious global problem, because salinization hinders plant growth and development and reduces crop yields. Soil salt produces osmotic stress and toxic stress to plants, leading to plant growth inhibition, developmental changes, metabolic adaptation, and ion isolation or rejection (Munns and Tester, 2008). The effects of salt stress on plants mainly include osmotic stress, specific ion toxicity, nutritional imbalance and ROS (Abbasi et al., 2016). Exposure of plants to salt stress can cause excessive production of ROS, resulting in damage to plant cell membranes (Mittova et al., 2009). A large number of studies have revealed the important role melatonin plays in improving the salt tolerance of plants.

In this study, combining our results, a complex regulatory network was used to describe the molecular role of melatonin in improving the salt tolerance of cotton (**Figure 8**). We found that salt treatment can induce an increase in the level of endogenous melatonin in cotton seedlings; exogenous application of melatonin can regulate antioxidant enzymes, redox-related genes, glutathione, etc. In order to remove ROS, melatonin can also induce the accumulation of some representative non-enzymatic antioxidants, such as AsA, phenolic compounds, flavonoids and carotenoids (Pardo-Hernández et al., 2020); as a broad-spectrum antioxidant, interacts with ROS and directly eliminates it (Lins et al., 2012); melatonin induced the expression of some hormones (Auxin, ABA, Ethylene, Brassinosteroid, Jasmonic acid) synthesis and signal transduction related genes, and coordinates other hormones in the cotton defense network; melatonin induced the expression of some transcription factors (AP2/ERF-ERF, C2H2, bHLH, WRKY, etc.) to resist salt stress; melatonin induced the expression of genes related to the phosphatidylinositol signaling system and activates some signaling molecules (IP3, DAG, IP6, Ca^{2+}), Phospholipase C (PLC) hydrolyses PtdInsP2 to generate InsP3 and DAG, which can be converted into PA as a signal molecule and PA as a membrane localization signal. Many PA target proteins (AtPDK1, ABI1, CTR1, etc.) involved in environmental stress response have been identified (Hou et al., 2016), and InsP3 generates InsP6 through a series of enzymatic reactions. InsP6 is the main storage form of phosphate in plants and the second messenger of plants. It can regulate Ca^{2+} release, hormone receptor TIR1, gene expression, etc. (Munnik and Vermeer, 2010). When melatonin is sensed by receptor CAND2/PMTR1, it triggers the dissociation of Gα form Gγβ, which activates the downstream H_2O_2 and Ca^{2+} signaling transduction cascade, leading to the phenotype of stomatal closure (Wei et al., 2018). Intracellular Ca^{2+} increases, thereby initiating stimulation-specific downstream signal transduction. This is dependent upon the array of calcium sensors. The calcium sensors are categorized into CaMs (calmodulins), CMLs (CaM-like proteins), CDPKs (Ca^{2+} dependent protein kinases), and CBLs (calcineurin B-like proteins) (Srivastava et al., 2013). These sensor proteins can directly perform their functions or interact with corresponding decoding elements. The application of melatonin and Ca^{2+} inhibits ROS generating enzymes, including NADPH oxidase in the plasma membrane and GOX in peroxisomes, respectively (Siddiqui et al., 2020). In this study, we found that melatonin melanin plays an important role in improving the salt tolerance of cotton, laying a certain theoretical foundation for improving the salt tolerance of cotton.

CONCLUSION

As an antioxidant defense system and signaling molecule, melatonin plays an indispensable role in the response of plants to abiotic stress, and has great potential in improving crop response to abiotic stress. The content of endogenous melatonin in cotton may affect its salt tolerance. In this study, we used exogenous application of melatonin to increase the salt tolerance of cotton, and inhibiting the content of endogenous melatonin decreased the cotton salt tolerance, indicating that melatonin and cotton salt tolerance are closely related. RNA-seq technology was used to explore the mechanism of melatonin regulating the salt stress of cotton at the molecular level. This study found that

melatonin improved cotton's tolerance to salt stress by regulating antioxidant systems, plant hormones, transcription factors, lipid metabolism, signal molecules and other mechanisms. This study is helpful to establish an effective pathway to improve cotton salt-tolerance through melatonin mediation.

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 SRA BioProject, accession no: PRJNA722118.

AUTHOR CONTRIBUTIONS

WY and YZ: conceived and designed the experiments. YZ, XL, XC, and JW: methodology. YZ, YF, CR, HZ, and NX: experiments. MD, DW, JW: analysis of data. YZ: writing—original draft preparation. YZ, QW, SW, CC, LG, and LZ: writing—review and editing. WY: supervision. 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.2021.693690/full#supplementary-material

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REVIEW
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Role of Jasmonates, Calcium, and Glutathione in Plants to Combat Abiotic Stresses Through Precise Signaling Cascade

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Plant growth regulators have an important role in various developmental processes during the life cycle of plants. They are involved in abiotic stress responses and tolerance. They have very well-developed capabilities to sense the changes in their external milieu and initiate an appropriate signaling cascade that leads to the activation of plant defense mechanisms. The plant defense system activation causes build-up of plant defense hormones like jasmonic acid (JA) and antioxidant systems like glutathione (GSH). Moreover, calcium (Ca^{2+}) transients are also seen during abiotic stress conditions depicting the role of Ca^{2+} in alleviating abiotic stress as well. Therefore, these growth regulators tend to control plant growth under varying abiotic stresses by regulating its oxidative defense and detoxification system. This review highlights the role of Jasmonates, Calcium, and glutathione in abiotic stress tolerance and activation of possible novel interlinked signaling cascade between them. Further, phyto-hormone crosstalk with jasmonates, calcium and glutathione under abiotic stress conditions followed by brief insights on omics approaches is also elucidated.

Keywords: abiotic stress, cell signaling, jasmonic acid, glutathione, calcium

INTRODUCTION

Different environmental conditions turn out to be the cause of stress in plants that tend to affect their growth, development, metabolism, and even cause death (Boguszewska and Zagdańska, 2012). These abiotic stresses such as salinity, heavy metals, temperature, drought, etc. are serious threats that affect crop productivity (Asgher et al., 2015; Raza et al., 2020). Plants have mechanisms due

Abbreviations: JA, jasmonic acid; Ca^{2+}, calcium; GSH, glutathione; MeJA, methyl jasmonate; JAZ, jasmonate zip domain; Ile, isoleucine; ROS, reactive oxygen species; Na^{+}, sodium; Pb, lead; CAT, catalase; APX, ascorbate peroxidase; LOX3, lipoxygenase3; AsA, ascorbic acid; Cd, cadmium; HM, heavy metal; Cu, copper; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; SOD, superoxide dismutase; POD, peroxidase; H_2O_2, hydrogen peroxide; jar1, methyl jasmonate resistant1; coi1, coronatine insensitive1; oji1, ozone-sensitive and jasmonate insensitive; OPR3, 12-oxophytodienoate reductase 3; GST, glutathione *s*-transferase; CAM, calmodulins; GR, glutathione reductase.

to which they adapt themselves to different climatic conditions by modulating their growth and physiology. Phytohormones are associated with various physiological and metabolic processes in plants (Kumar et al., 2014; Asgher et al., 2018; Geetika et al., 2020). The phytohormones play notable roles in inducing the numerous complex processes of growth, development, and response to stress by retaliating the signaling cascades in plants. Moreover, it has been suggested that these phytohormones have potential to minimize the ill effects of abiotic stress (Thao et al., 2015; Asgher et al., 2018; Zaid and Mohammad, 2018). Plant growth hormones such as auxin, gibberellin, cytokinin, abscisic acid, salicylic acid (SA), ethylene, JA, and recently studied brassinosteroid, act as components of abiotic-stress signaling (Fahad et al., 2015; Sharma and Laxmi, 2016; Wani et al., 2016). Among these phytohormones JA has gained much importance during the recent years.

JA has a ubiquitous expression in the plant systems. JA and its derivatives do have remarkable roles as plant growth and stress regulators, involved in diverse plant developmental processes such as callus growth, seed germination flowering, primary root growth, and senescence (Fahad et al., 2015). It acts as an important signaling molecule either in biotic or abiotic stress response (Wasternack, 2015; Per et al., 2018; Ali and Baek, 2020; Jang et al., 2020). Some jasmonates are derived from fungus while its methyl ester form, i.e., methyl jasmonate (MeJA) is extracted from petals of jasmine (*Jasminum grandiflorum*) (Avanci et al., 2010). It is usually present in flowers and reproductive tissues, while sparsely present in minute levels in root and mature leaves. JA have the capability to enhance or suppress the plant response (Agrawal et al., 2003; Fahad et al., 2015). JA tends to boost the antioxidant machinery of the plants (Bali et al., 2020).

Ca^{2+} acts as one of the important secondary messengers in all life forms involving many cell signaling cascades (Berridge et al., 2000; Stael et al., 2012). Among the most important nutrient elements, Ca^{2+} has a role under optimal and stressful conditions in plants (White and Broadley, 2003). Different kinds of stimulus are perceived by Ca^{2+} for downstream cellular retaliations via activation of Ca^{2+} channels followed by an increase in Ca^{2+} concentration due to influx of Ca^{2+} thereby inducing Ca^{2+} signaling (Evans et al., 2001; Chinnusamy et al., 2004). During abiotic stresses, Ca^{2+} signaling plays an important role by stimulation of Ca^{2+} channels and causes an increase in cytoplasmic Ca^{2+} levels for further downstream retaliations (McAinsh and Pittman, 2009; Dodd et al., 2010; Sarwat et al., 2013; Liu et al., 2018).

Glutathione one of the non-protein tripeptide thiol compounds, known as "master antioxidant" or "super defender," is ubiquitous in nature and present in all plant cells at relatively high concentrations (Dixon et al., 1998). GSH is known to play a pivotal role in root development, plant disease resistance, protection against chilling damage, cell proliferation, and salt tolerance (Mittova et al., 2003; Gómez et al., 2004; Vivancos et al., 2010). GSH holds a very important position in stress responses by determining the cell redox state of the cell (Noctor et al., 2012).

Recent findings suggest that JA has a prime role at the physiological and biochemical levels that is associated with the plant defense against abiotic stress. However, JA cannot work alone to alleviate abiotic stress but works in concord manner via various signaling cascades. Vast literature is available on role of JA and its crosstalk with other phytohormones under abiotic stress, but there is no literature that documents the role of Jasmonate, Calcium, and Glutathione in plants to combat Abiotic stresses through precise signaling cascade. So, this review exemplifies the role of Jasmonates, Calcium, and glutathione in abiotic stress tolerance and activation of possible novel interlinked signaling cascade between them. Further, phytohormone crosstalk with jasmonates, Calcium, and Glutathione under abiotic stress conditions following with brief insights on omics approaches is also discussed.

BIOSYNTHESIS OF JASMONIC ACID

Jasmonic acid (JA) belongs to the family oxylipins, produced from polyunsaturated fatty acid (PUFA) through its oxidative metabolism (Wasternack and Hause, 2002; Ahmad et al., 2016). Its synthesis occurs via octadecanoic acid pathway involving esterification of α-linolenic acid (C18) in galactolipid membranes of chloroplast (Wasternack and Strnad, 2018; Wang et al., 2020). Phospholipase A causes the release of α- linolenic acid followed by oxygenation by 13-lipoxygenase (13-LOX) to a 13-hydroperoxyoctadecatrienoic acid, which is then converted by a 13-allene oxide synthase (13-AOS) to a highly unstable epoxide. Cyclization of this epoxide to *cis*-(+)-12-oxo-phytodienoic acid (OPDA) by the action of an allene oxide cyclase (AOC). The next half of JA biosynthesis takes place in peroxisomes. Followed by subsequent reduction and three steps of β-oxidation after which shortening of the carboxylic acid side chain to (+)-7-iso-JA, which is released into the cytosol and epimerizes to (−)JA. Then Conjugation with amino acids, such as isoleucine, is catalyzed by jasmonoyl–isoleucine (JA-ile) conjugate synthase (JAR1) which is the most active JA bio-compound (Wasternack and Hause, 2013; Ruan et al., 2019). JA and its metabolites, collectively called jasmonates, have an important role in mediating plant signaling in response to abiotic stress. JA signaling based gene expression is negatively regulated by jasmonate zip domain proteins (JAZ proteins). However, JA preferentially conjugates to isoleucine (Ile) to form Ile-JA or gets converted into MeJA (Wasternack, 2007; Wasternack and Hause, 2013). Ile-JA is in turn perceived Skip-Cullin–F-box complex (SCF^{COI1}) which mediates degradation of the JAZ repressor via 26S proteasome degradation, thereby relieving the repression by JAZ, transcriptional regulator (**Figure 1**) (Melotto et al., 2008; Gfeller et al., 2010; Zhou and Memelink, 2016). The JAZ repressors recruit the protein topless (TPL) and the novel interactor of JAZ (NINJA) together to form transcriptional repression complex that inhibits the expression of jasmonate-responsive genes through formation closed to open complex, thereby favoring further attachment of histone deacetylase 6 (HDA6) and HDA19 (Causier et al., 2012; Chini et al., 2016).

BIOSYNTHESIS OF GLUTATHIONE

Various kinds of stresses tend to fluctuate the status of glutathione (Gómez et al., 2004). The biosynthesis of this non-protein thiol

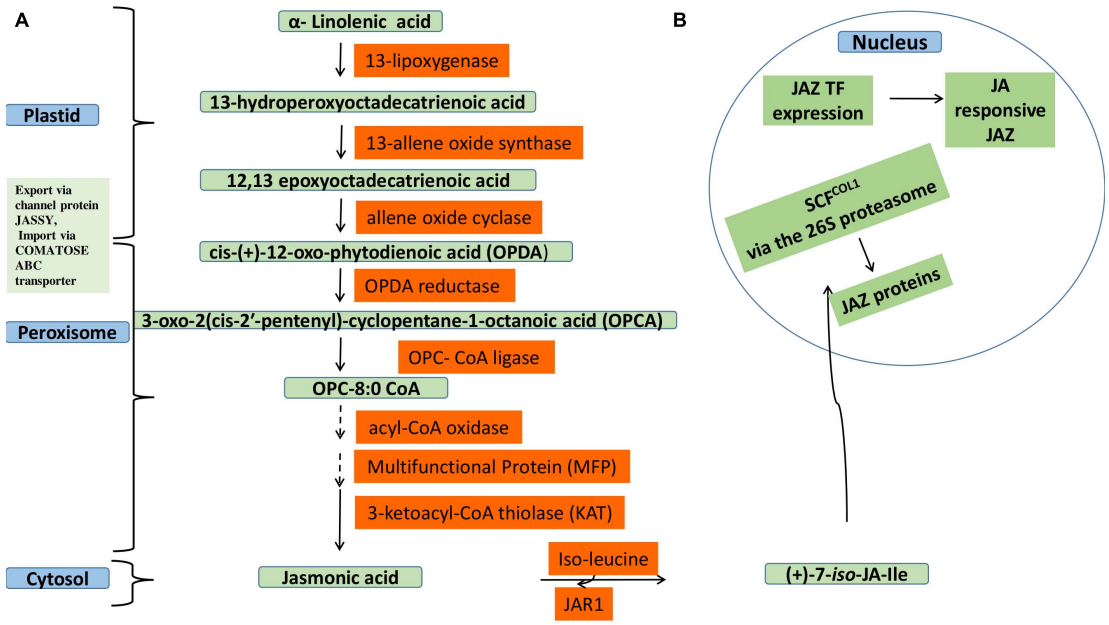

FIGURE 1 | Jasmonic acid biosynthesis and its gene regulation. **(A)** Jasmonic acid biosynthesis in various cellular compartments involving plastid, peroxisome and cytosol. In plastid α-linolenic acid is converted into 12-oxo-phytodienoic acid that is exported via JASSY channel protein and imported via COMATOSE ABC transporter to peroxisome. Subsequent reduction, β-oxidation, and epimerization reactions lead to JA formation and is released into cytosol. Then JA Conjugates with isoleucine in cytosol to form jasmonoyl–isoleucine (Ile-JA). **(B)** Degradation of JAZ repressor proteins via SCF^{COI1} regulating JA gene expression at transcription level Ile-JA is in turn perceived Skip-Cullin–F-box complex (SCF^{COI1}) which mediates degradation of the JAZ repressor via 26S proteasome degradation, thereby relieving the repression by JAZ, transcriptional regulator.

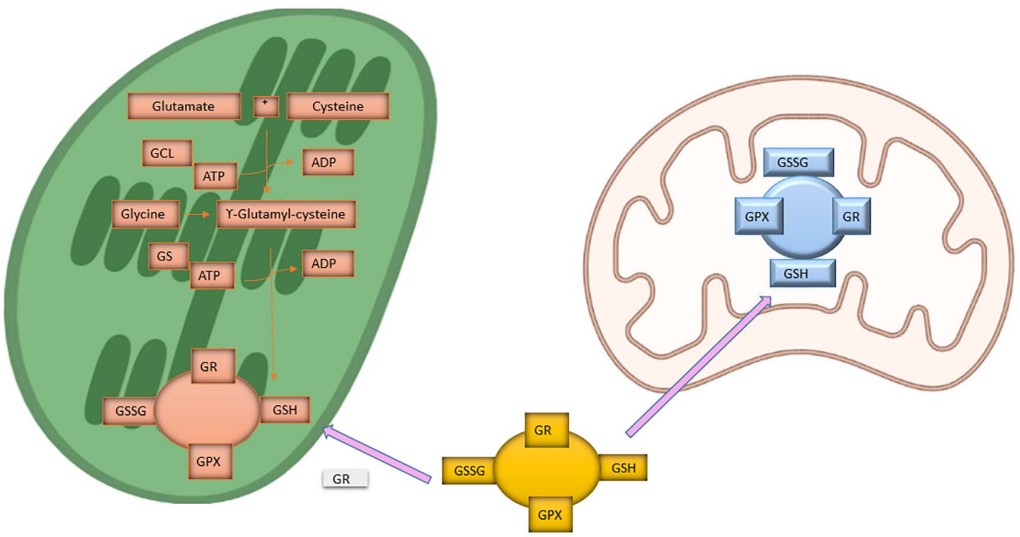

FIGURE 2 | Biosynthesis of Glutathione. The two ATP dependent steps involved in biosynthesis of Glutathione. Glutamate and cysteine leads to the formation of γ-glutamylcysteine and in the next step glutathione formation occurs by addition of glycine to γ-glutamylcysteine. This reduced glutathione (GSH) acts as substrate for numerous cellular reactions in cytosol and mitochondria to yield oxidized glutathione (GSSG).

compound involves two ATP dependent steps as shown in **Figure 2**. First, the reaction is catalyzed by glutamate-cysteine ligase (GCL) involving glutamate and cysteine leading to the formation of γ-glutamylcysteine, and in the next step glutathione formation occurs by addition of glycine to γ-glutamylcysteine in the presence of glutathione synthetase (GS). This reduced glutathione (GSH) acts as substrate for numerous cellular reactions to yield oxidized glutathione (GSSG). The balance between GSH and GSSG acts as an important role in maintaining homeostasis of the cell (Meister, 1995; Noctor et al., 2012).

Availability or localization of GCL and GS plays an important role in glutathione biosynthesis. Early reports suggest that in plant cells, GCL is localized in chloroplast and GS in cytosol as well as in chloroplast cells (Hell and Bergmann, 1988, 1990). Work on Arabidopsis suggest that GCL and GS are encoded by a single gene with alternate start sites leading either cytosolic or plastid targeted protein (Wachter et al., 2005). Glutathione biosynthesis compartmentalization is unique to plant systems (Galant et al., 2011; Noctor et al., 2012). Generally, over-expression of GCL, not GS, in plants raises glutathione content by increasing flux through the pathway. Increase in GCL activity that is from GSH to GSSG form takes place in response to 5 mM H_2O_2 treatment to Arabidopsis seedlings as depicted by immunoblot and activity assays (Hicks et al., 2007). Moreover, addition of cysteine, glutamate, or glycine does not enhance glutathione synthesis suggesting the role of GCL as a metabolic control point in the pathway (Meyer and Fricker, 2002). Metabolic studies suggest that feedback inhibition may not be a major control regulatory feature of glutathione (Meyer et al., 2001). Even though glutathione inhibits both GCL and GS (Jez et al., 2004). According to Previous reports glutathione biosynthesis pathway is controlled in a tightly regulated manner and hence supports the increased expression level of GCL and GS genes under various stress conditions (Xiang and Oliver, 1998; Lu, 2013).

ROLE OF JASMONATES, CALCIUM, AND GLUTATHIONE IN ABIOTIC STRESS TOLERANCE

Abiotic stresses including heavy metals, salinity, temperature, drought, etc. represent a significant threat to plants by causing cellular damage and inhibiting normal physiological activities in plants thereby limiting productivity (Fujita et al., 2006; Asgher et al., 2015; Hasanuzzaman et al., 2017a) the detailed roles of JA, Ca^{2+}, and GSH under different abiotic stress conditions are discussed below.

Salt Stress

Alkaline stress is one of the most important stresses, especially in arid and semi -arid environments, that affects crop productivity at a global level (Parvin et al., 2019). Alkaline salt contamination to agricultural soil has been predicted in the past few decades mostly in Asian countries (Paz et al., 2012). Extreme alkaline stress can promote negative effects on plants at cellular level high sodium (Na^+) concentration, enhancing ionic stress (Chen et al., 2012). About 10% of the world's cultivable land productivity is affected by salt and alkaline stress (Tanji, 2006). Salinity disturbs the allocation of minerals and membrane permeability. It decreases chlorophyll biosynthesis, metabolism of nitrogen, and carbon dioxide (Gupta et al., 2002; Kim et al., 2004). High salinity also causes the production of reactive oxygen species (ROS), hence leading to oxidative stress (Smirnoff, 1993). Application of plant growth regulators including phytohormones help to counter the different environmental stresses in plants. Earlier reports have shown that JA activates expression of α- linolenic acid metabolism genes which is a branch of JA biosynthesis (Wasternack, 2007). Moreover, it has been seen that JA accumulation occurs under salt stress in plant species like *Solanum lycopersicum* (Pedranzani et al., 2003). The exogenous application of JA alleviated salt-induced injury in other variety of plants like barley (Walia et al., 2007), rice (Kang et al., 2005), and wheat (Qiu et al., 2014). Exogenous supplementation of (60 and 120 mM) MeJA increased growth and physiological attributes of *Anchusa italica* (Taheri et al., 2020). It has been recently reported that 45–60 μM MeJA significantly improved SOD, GPX, APX while 15–30 μM increased AsA, CAT, GSH activities in *Glycyrrhiza uralensis* seedlings under salt-stressed conditions, respectively (Lang et al., 2020). JA treatment led to up-regulation of the osmolyte synthesis, antioxidant system, and metabolite accumulation in tomato (Ahmad et al., 2018). Under increasing salt stress conditions abscisic acid (ABA) accumulated in tolerant varieties of rice while decreased in salt intolerant ones. Exogenous application of JA, however, led to an increase in ABA, especially in salt intolerant cultivars of rice, thereby ameliorating the salt stress (Kang et al., 2005). Exogenous application of MeJA effectively safeguards salinity stress symptoms in soybean seedlings by increasing the levels of ABA and relieving the repression of GA biosynthesis (Yoon et al., 2009). The lipoxygenase 3 (LOX 3) enzyme involved in JA biosynthesis of Arabidopsis is induced under salt treatment. However, LOX3 mutant seem to be hypersensitive toward salt treatment and could be complemented by exogenous JA treatment (Ding H. et al., 2016). Moreover, JA increased GSH related gene expression in plants in response to salt stress which in turn increases the antioxidant ability thereby protecting against oxidative stress caused by salt stress as seen in wheat seedlings (Qiu et al., 2014; Mir et al., 2018a). Ion homeostasis seem to be an important factor for plants under salt stress (Hasegawa, 2013). Plants have mechanisms by which they are able to sense salt stress via ionic and osmotic signals (Zhu, 2003). The Salt Overly Sensitive (SOS) pathway is a central mechanism in plant salt tolerance, which includes two calcium sensor proteins, SOS3 and SCaBP8; the protein kinase SOS2; and the Na^+/H^+ anti-porter SOS1. AtANNEXINs are a family of calcium-dependent membrane-binding proteins in plants. AtANNEXIN4 (AtANN4) plays a critical role in generating the calcium signal under salt stress that activates the SOS pathway in Arabidopsis. The SOS pathway suppresses AtANN4-mediated calcium transients through an interaction between SCaBP8 and AtANN4. AtANN4 likely functions during a very early stage of the plant salt stress response by generating a calcium signal. Afterward, an initial calcium signal is created in cells by AtANN4, SCaBP8, and SOS2 under salt stimulus. Phosphorylation of AtANN4 by SOS2 reduces the calcium-binding capability of AtANN4, which might alter calcium perception. The collaboration with SCaBP8 and phosphorylation by SOS2 represses the AtANN4-mediated calcium changes, there by provides a negative regulation loop. The negative feedback regulatory loop involving the SOS pathway decreases cytoplasmic sodium levels and elicits specific, long lasting salt stress reactions in plants (Ma L. et al., 2019). Phosphatases like calcineurin (protein phosphatase B) are important Ca^{2+} sensors and result in a decrease in K^+ current and stomatal closure in plants. The specific to salt stress (SOS3) gene of

Arabidopsis was comprehended to encode calcineurin B homolog of yeast. However, SOS3 mutant acts as a Ca^{2+} sensor to elicit downstream signaling under salt stress conditions which are hypersensitive to Na^{+} and are partially suppressed by increasing the concentration of Ca^{2+}. Therefore, SOS3 mutant helps to unravel the basic role of Ca^{2+} in regulating potassium nutrition and salt stress in plants (Sanders et al., 1999).

Metal(loid) Stress

Metal contamination of agricultural systems has become a worldwide concern due to industrialization and anthropogenic activities like mining (Chary et al., 2008; Noriega et al., 2012). Essential micronutrients like Zn and Cu are required by plants to carry out activities of different metal dependent proteins and enzymes. There accumulation above certain threshold value results in oxidative stress and nutrition stress. It results in alteration in carbohydrate metabolism, inhibition of photosynthesis and transpiration. Changes in plant morphology and physiology are also evident under heavy metal (HM) stress (Qureshi et al., 2016). JA production in response to metal or metalloid has been reported in *Wolffia arrhiza* (Piotrowska et al., 2009), *Arabidopsis thaliana* (Maksymiec et al., 2005), and *Cajanus cajan* (Poonam et al., 2013). Under biotic stress, JA levels increase within seconds to minutes (Chung et al., 2008). The endogenous JA levels also increase after abiotic stress exposure (Wang et al., 2020). However, response of exogenous application of JAs depends on the type of plant species tested or its concentration (Ahmad et al., 2016). Similarly, exogenous application of JA helps in alleviating the toxic effect of HM, depending on the species and the concentration of JA used. JA tends to modulate the HM accumulation by preventing its biosorption, plant growth restoration, and primary metabolite formation (Piotrowska et al., 2009; Farooq et al., 2016). GSH is also reported to have an important role in ROS detoxification and HM chelation. GSH has a central role in both antioxidant defense system and glyoxalase system and therefore provides protection from oxidative damage induced by HM. Moreover, GSH and its metabolizing enzymes such as glutathione-*s*-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR), etc. provides protection against ROS by HM uptake, chelation, and detoxification. GSH also acts as cofactor in ROS scavenger reactions like in the glyoxalase pathway. Proline levels also increase in HM tolerance by maintaining the stringent redox environment of the cell by retaining a higher GSH pool (Hossain et al., 2012). Moreover, exogenous Ca^{2+} treatment increased the tolerance of the plant to HM by inhibiting ion uptake, increasing membrane stabilization, proline, and soluble sugar content (Shen et al., 1998; Kanu et al., 2019; Mukta et al., 2019).

Lead Stress

Lead (Pb) a non-essential element toxic to plants is absorbed by plants via roots, shoots, or foliage. Its entry into the plant cell causes inhibition of enzymatic activities, alteration of mineral nutrition, hormonal status, membrane structure, etc., leading to decreased growth and chlorosis (Seregin and Ivanov, 2001; Küpper, 2017). Change in enzyme activities causes inhibition of seed germination as reported in *Sporobolus alterniflorus* (Mrozek and Funicelli, 1982) and *Eichhornia crassipes* (Malar et al., 2016). Moreover, it is also responsible for inhibition of growth in plants like privet (Zhou et al., 2018). High Pb content leads to generation of ROS that directly or indirectly induces oxidative stress (Verma and Dubey, 2003; Reddy et al., 2005). JA elicits lead detoxification in tomato through production of secondary metabolites and gene expression and by decreasing the expression of the RBO and P-type ATPase transporter genes (Bali et al., 2019a,b). It also leads to the induction of lipid peroxidation, perturbing the level of saturated to unsaturated fatty acids in plant (Verma and Dubey, 2003; Bidar et al., 2008). Pb toxicity in *W. arrhiza* increased when supplemented with 100 μM of JA by the formation of lipid peroxides which resulted in decreased fresh weight, chlorophyll a, carotenoid soluble protein content, and monosaccharide while 0.1 μM of JA protected *W. arrhiza* fern against Pb stress by preventing Pb accumulation, restoring plant growth, and primary metabolite level by promoting the activities enzymatic antioxidants and non-enzymatic antioxidants, such as the content of AsA and GSH (Piotrowska et al., 2009). JA also causes changes in ascorbate glutathione pathway in plants like *Lycopersicum esculentum* under lead stress at various growth stages (Bali et al., 2018). GSH has chelating properties for heavy metals and therefore helps in ROS detoxification (Hossain et al., 2012). GSH also exhibits diverse functional roles in alleviating Pb induced toxicity by increasing activity of antioxidant enzymes like activating GPX and GR that act as ROS scavenger and regeneration of the GSH/GSSG pool of the cell, respectively (Hasanuzzaman et al., 2018). Exogenous application of GSH improved tolerance in *Iris lacteal* var. *chinensis* by mediating Pb accumulation and transport (Yuan et al., 2015).

Cadmium Stress

Cadmium (Cd), a non-redox heavy metal with long biological perseverance is highly toxic to plants (Asgher et al., 2015). It interferes with normal functioning of plants like photosynthesis, mineral, and water uptake (Baryla et al., 2001; Khan N.A. et al., 2016). Its toxicity in plants causes chlorosis, leaf rolling, and reduced growth of stem and root (Smeets et al., 2005; Mishra et al., 2006). It induces oxidative stress via generation of ROS causing serious damage to plants (Gallego et al., 2012). JA positively regulates plants in response to Cd stress (Zhao et al., 2016). Varying concentrations of JA and MeJA tend to alleviate the stress caused by Cd in soybean and *Oryza sativa*. 5 μM of MeJA improved antioxidant response and accumulation of antioxidants under Cd stress in *O. sativa* while 20 μM of JA reduced the damage caused by Pb stress in soybean (Noriega et al., 2012; Singh and Shah, 2014). These phytohormones cause increase in growth and photosynthesis besides changing the activity of different antioxidants and increasing GSH pools (Yan et al., 2013; Singh and Shah, 2014). Tomato seedlings susceptible to Cd show enhanced JA deficiency, this suggests that JA positively regulates the tomato plant to Cd stress (Zhao et al., 2016). Furthermore, JA acts as a signaling molecule for combating Cd stress and is also associated with expression of genes related to GSH biosynthesis (Maksymiec et al., 2007). Heavy metals compete with Ca^{2+} on the plasma membrane by substituting Cd, thereby altering the plant metabolism (Mansour, 2004).

However, exogenous application of Ca^{2+} results in improving biochemical and physiological processes, besides enhancing activity of antioxidant enzymes, which provides tolerance against Cd stress as shown in faba bean (Siddiqui et al., 2012). Up regulation of antioxidant enzymes has been observed upon Ca^{2+} treatment to the *Sesamum indicum* under Cd stress (Abd-Allah et al., 2017). The same positive correlation was observed by exogenously applying GSH in combating Cd induced stress in *O. sativa*. It was reported that the difference in tolerance capability of sensitive and insensitive cultivars of *O. sativa* is associated with the tendency of the plant to elevate its GSH levels. Higher GSH levels halt the translocation of Cd and decreases its lethal effect. GSH treatment also enhances the chlorophyll level, photosynthetic performance and antioxidant capability of plants (Cai et al., 2011a; Fang et al., 2020).

Copper Stress

Copper (Cu^{2+}) is a micronutrient that plays an important role in energy generation by means of ATP synthesis and carbon dioxide assimilation. It also alters ultra-structure and pigment composition of chloroplast. Therefore, being responsible for decline in the rate of photosynthesis via decrease in Ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO) inhibition of the electron transport chain and Photosystem II activities (Rakwal et al., 1996; Gang et al., 2013). High levels of Cu^{2+} exposure to *Theobroma cacao* seedlings caused damaging effects such as absence of starch grains and swelling of chloroplast double membrane (Souza et al., 2014). It has been reported that JA (1 μM, 1 nM, and 1 pM) enhances photosynthetic pigment accumulation and production of hydrogen peroxide (H_2O_2) mitigating enzymes, i.e., superoxide dismutase (SOD) and peroxidase (POD), suggesting that seed priming with JA can decrease the toxic effect of Cu^{2+} (Poonam et al., 2013). Moreover, addition of Ca^{2+} into nutrient solution improved the growth of Cu-treated seedling, by lowering the concentration of polyamines putrescine and increasing the levels of spermine and spermidine in the epicotyl of plants (Shen et al., 1998). Supplementation of Ca^{2+} to pea plants increases the Cu metal bio- absorption and maintains the homeostatic environment of the cell (Ben Massoud et al., 2019). A similar effect has been observed for GSH that helps in alleviating the effect of copper in rice seedlings by reducing copper uptake (Mostofa et al., 2015). Pretreatment with GSH caused the activation of oxidative stress scavenging mechanisms of plant thereby decreasing the level of ROS and Malondialdehyde concentration (Tahjib-Ul-Arif et al., 2020).

Arsenic Stress

Arsenic toxicity poses a serious health threat to all living organisms across the globe associated with anthropogenic activities like mining and smelting operations (Kumar et al., 2015; Singh et al., 2015). The As contamination in groundwater is a worldwide problem. It badly affects crop productivity and accumulates in different plant tissues, including grains, and affects the food chain (Verma S. et al., 2016; Ghosh et al., 2019). Naturally As exists in Inorganic arsenate As (V) and arsenite As (III) forms. Both forms are toxic but As (III) is more toxic than As (V) to plants as it has a tendency to bind proteins with sulfhydryl groups and hinder with their functions (Verma S. et al., 2016). It also leads to ROS generation and inhibition of respiration by binding to vicinal thiols in pyruvate dehydrogenase and 2-oxo-glutarate dehydrogenase (Helleday et al., 2000; Verma S. et al., 2016). Thus, arsenic-induced ROS production causes impairment of normal cellular function and plant metabolism by widespread damage to DNA, lipids, and proteins (de Campos et al., 2019). In order to recognize the possible mechanism involved in mitigating As toxicity in plants, the expression analysis of various genes involved in AsIII translocation and sequestration have been analyzed. MeJA has been reported to alleviate AsIII toxicity in rice through modulating As uptake, translocation, and JA signaling (Verma et al., 2020). JA also regulates the pigment balance, ROS homeostasis, and improvement of the antioxidant enzymatic system, thereby increasing accumulation of As without showing major damage (Coelho et al., 2020). It has also been found that MeJA has improved the growth and yield characteristics of rice varieties under As toxicity by alleviating oxidative stress through increasing the activity of antioxidant enzymes along with ASA–GSH cycle and reducing As accumulation by controlling As transporters (Mousavi et al., 2020). As induces the generation of ROS leading to oxidative stress and lipid peroxidation in plants (Shukla et al., 2018). Arsenic toxicity causes activation of phytochelatins (PCs) produced from GSH. PCs sequester As into the vacuoles make complexes with As, which gets sequestered into the vacuoles through ABCC1/ABCC2 transporters (Schmöger et al., 2000; Dhankher, 2005). Furthermore, Lambda class of GST (GSTLs) has been seen to bind tightly to the flavonols and their derivatives (Chronopoulou et al., 2017). It has been suggested that GSTLs can recycle GSH adducts of oxidized flavonols back to the parent flavonols, maintaining the antioxidant pools (Hernández et al., 2009). Genome wide expression analyses have shown differential expression of OsGSTLs at various stages of plant development as well as under stress conditions (Kumar et al., 2013). However, the exogenous use of GSH in As-treated seedlings decreased As-induced oxidative stress, increased the AsA and GSH contents, and mediated As translocation from the roots to the shoots. Therefore, the results suggest that exogenous GSH application could be a favorable approach to enhance As stress resistance in rice (Farooq et al., 2018; Jung et al., 2019). The identification of calcium-dependent protein kinase CPK31 is a major component controlling As(III) tolerance in Arabidopsis. Genetic and biochemical studies show that CPK31 fulfils this function by interaction with NIP1;1, providing a novel role of CPK31 in controlling As(III) toxicity in plants via Ca^{2+} signaling (Mousavi et al., 2020).

Nickel Stress

Nickel (Ni) is among the common heavy metals that cause serious health complications even in trace quantity (Masindi and Muedi, 2018). Nickel induces the deficiency of Zn and Fe. It also hinders the uptake of other heavy metals such as Cd, Pb, Co, and Cr (Myśliwa-Kurdziel et al., 2004). Nickel toxicity disrupts the important macro and micronutrients uptake by hindering the translocation of these nutrients through root to aerial (Pandey and Sharma, 2002; Chen et al., 2009; Ameen et al., 2019). The

treatment of NiSO4.7H2O has led to decrease in chloroplast size and number. It was also seem to be responsible for the disorganization of ultrastructure of chloroplast like numbers of grana decreased, thylakoids deformation, the development of plasto globuli, and the membrane lipid composition alterations were stated in *Brassica oleracea* plants. These changes were due to the Ni induced drop in cell moisture content or subsequent peroxidation of membrane lipids due to oxidative stress (Ameen et al., 2019). The toxicity of Ni has been associated with oxidative stress in plants (Rao and Sresty, 2000; Boominathan and Doran, 2002). Nickel toxicity like other abiotic stresses led to production of ROS (Gill and Tuteja, 2010). Excessive Ni increases the concentration of superoxide anions, hydroxyl radicals, nitric oxide, and hydrogen peroxide (Stohs et al., 2000). The toxicity of heavy metals is directly linked with overall crop yield. The increasing concentration of Ni has deleterious effects on plants that finally triggered reduction in crop yield (Balaguer et al., 1993). Ni was also reported to be associated with inhibition of germination and production of chlorophyll (Zhou et al., 2009). Exogenous application of JA relieved the adverse effect of oxidative stress on biomass production, growth, and protein content in Ni treated plants by further enhancing the activity of antioxidant enzymes (Azeem, 2018; Mir et al., 2018b). However, exogenous or endogenous biosynthesis of JA make plants tolerant to any oxidative damage (Sirhindi et al., 2016). There is information that methyl ester of JA (MeJA) affects the pools of stress antioxidant enzymes activity to combat oxidative stress (Jung, 2004). Ni stimulated the activities of SOD, APX, and CAT. SOD is the primary enzyme of Asada-Halliwell pathway that causes dismutation of superoxide radicals under elevated levels of H2O2 followed by further hydrolysis into H2O and O2 by CAT and APOX or other POD enzymes present in various cellular organelles (Keramat et al., 2010; Sirhindi et al., 2015). Higher GSH reductase and catalase activities are present in a Ni-tolerant strain of the green alga, *Scenedesmus acutus f. alternans* (Randhawa et al., 2001). Also in transgenic Arabidopsis, GSH seems to be intensely associated with increased resistance the growth inhibitory and oxidative stress induced effects of Ni. This rise in GSH concentrations was reported to be determined by serine acetyltransferase (SAT) activity in conferring tolerance to Ni-induced oxidative stress in Thlaspi Ni hyperaccumulators (Freeman et al., 2004). However, the application of Ca^{2+} has been seen to be responsible for the higher efficiency of the antioxidants for increasing tomato tolerance to the Ni stress (Asrar et al., 2014).

Drought Stress

Drought is responsible for great famines of the past. It is one of the common threats to food security. Limited supply and increasing demand of water worsened drought effects (Somerville and Briscoe, 2001). Jasmonate zip-domain (JAZ) proteins are essential regulators of proteins of JA signaling in many plants including *A. thaliana* (Vanholme et al., 2007) and rice (Ye et al., 2009). It has been reported that enhanced expression of stress responsive OsJAZ1- gene of *O. sativa* showed higher sensitivity to drought stress, while the JAZ1 mutant plants were more hyposensitive to drought stress compared to wild plants, suggesting the role of JA in combating the drought stress in plants (Fu et al., 2017). Exogenous application of MeJA (75, 150, and 225 μM) improved many characteristics of *Satureja hortensis* such as growth, water content, proline level, antioxidant activity, and essential oil percentage as well as yield. However, among different concentrations used, 75 μM was more effective, like that seen in drought tolerance in different *Brassica* species by trehalose (5 mM) treatment (Hasanuzzaman et al., 2014; Miranshahi and Sayyari, 2016). Exogenously applied 0.1 μM of MeJA to the wheat seedlings decreased the drought-induced retarded growth, lesions of membrane by increasing the level of dehydrin protein expression in them (Allagulova et al., 2020). JA treatment to cowpea plant under drought stress improves relative water content, proline, chlorophyll content, and causes stomatal closure, so as to elevate the stress pertaining to drought (Sadeghipour, 2018; Tayyab et al., 2020). Exogenous application of 10 and 150 μM JA increases the antioxidant potential of sugar beet (Ghaffari et al., 2019) and bitter melon (Alisofi et al., 2020) respectively, under drought conditions thereby imparting tolerance to them. Differential expression of Me-JA induced miRNAs was seen by Me-JA treatment in wheat under drought stress. These miRNAs could play a significant role in the activation of a particular gene, so play an important function in combating drought stress (Ma C. et al., 2019). GSH also reduces the effect of drought stress via maintaining water status, proline content, and by acting as an antioxidant (Nahar et al., 2015a). Exogenous GSH application improves growth characteristics and yield of plants under drought stress (Chen et al., 2012; Nahar et al., 2015a). Recent studies indicate that apart from increasing antioxidant activity GSH has a role in maintaining the plant mineral homeostasis (Sohag et al., 2020). However, it has also been reported that application of Ca^{2+} and H_2O_2 to plants mitigated ill effects of the drought stress (Hasanuzzaman et al., 2014; Hu et al., 2018). Ca^{2+} also regulates the water status, proline, and H_2O_2 levels in maize plants under drought stress (Naeem et al., 2018).

Flooding Stress

Flooding influences agricultural productivity all over world (Jackson and Colmer, 2005). It restricts gaseous exchange between plants and their environment, thereby resulting in lowering of oxygen, carbon dioxide levels, and increasing levels of ethylene in plants (Bailey-Serres and Voesenek, 2008). Its interference in photosynthesis and respiration in plants hinders production of ATP via oxidative phosphorylation, besides leading to generation of ROS due to hypoxic and anoxic conditions (Gibbs and Greenway, 2003; Paradiso et al., 2016). Owing to flooding stress, plants experience compound stress like energy and carbon deficiency that leads to retardation of plant growth (Armstrong, 1980; Jackson, 1985). JA upregulates the ROS and H_2O_2 detoxification system in plant cells during floods (Nanjo et al., 2011). JA is reported to have a post-flooding recovery function in soyabean by modulating the levels of nucleotidylyl transferase activity (Khan and Komatsu, 2016). Studies have shown that GSH regulates the gene expression of JA at a basal level. Ca^{2+} also has a role in regulating the cell wall integrity besides the mitigating effect of oxidative stress during

flood conditions (Porto et al., 2013). Supplementation of GSH to rice plant increased its antioxidant potential and could be an important factor to rescue plants under flooding stress (Siddiqui et al., 2020). Exogenous application of Ca^{2+} causes the root elongation and inhibits the cell death at the root tips of soyabean under flood stress (Oh et al., 2014). Moreover, external supplementation of Ca^{2+} decreased the negative effects on their physiological parameters like stomatal conductance, photosynthesis, soluble protein content, fruit size, etc., and also seem to have roles in maintaining the integrity of root cells of pepper (Ou et al., 2017). In addition, early cytosolic Ca^{2+} transients also seem to be important in circumventing the effect of flood stress among plants (Subbaiah and Sachs, 2003).

Ozone Stress

Stratospheric ozone layer depletion results in enhancement of the tropospheric ozone levels that adversely affect the terrestrial biosphere (Overmyer et al., 2000; Ainsworth et al., 2012). Ozone mediated changes at the cellular level in plants involves oxidative burst, accelerated cell senescence, and hypersensitive response kind of reactions (Vollenweider et al., 2003). Oxidative burst leads to lump and strand like protrusions on the cell wall with enhanced cellular oxidation (Günthardt-Goerg et al., 1997). ROS generation overcomes the cellular detoxification system. Its accumulation results in hypersensitive response with apparent symptoms like disruption of cellular structure, collapse of cell walls, incomplete degradation of cellular organelles, chromatin condensation, condensation of cell leftovers into apoptotic-like bodies, and nuclear degeneration leading to cell death (Vollenweider et al., 2003; Iriti and Faoro, 2007). JA plays a role in mitigating the ozone stress in plants (Tamaoki, 2008). JA insensitive mutants of Arabidopsis such as methyl-jasmonate resistant1 (jar1), coronatine insensitive1 (coi1), ozone-sensitive and jasmonate-insensitive (oji1), JA-biosynthesis defective fad3/7/8triple mutant, and the 12-oxophytodienoate reductase 3 (OPR3) mutants are extremely susceptible to ozone (Staswick et al., 1992; Feys et al., 1994; Overmyer et al., 2000; Kanna et al., 2004). O_3 tolerant cultivar of wheat is reported to have increased expression of JA as compared to the non-tolerant variety, thus rendering it to alleviate the ozone stress (Fatima et al., 2018). JA was also found to be associated with the maintenance of cellular homeostasis under ozone stress in *Brassica campestris* (Zhang et al., 2017). Change in GSH/GSSG pools is also seen as an early symptom to ozone exposure (Tausz et al., 1999). Tobacco sensitive variety (9 BelW3) upon ozone fumigation showed oxidation of GSH pool with a decrease in GR activity while the resistant variety (BelB) showed a high GSH/GSSG ratio with increased GR activity (Pasqualini et al., 1999). However, recent genome-based expression profiling of *Glycine max* GST gene (GmGST) has reported the presence of 126 putative GST gene in *G. max* and, among them, four genes, namely GmGSTU63, GmGSTF2, GmGSTU73, and GmGSTT5 are highly expressed under few abiotic stresses including ozone stress, therefore providing tolerance against adverse climatic conditions (Hasan et al., 2020). Ca^{2+} transients are also seen in ozone stress conditions (Sanders et al., 2002). But in the presence of Ca^{2+} channel blockers like lanthanum chloride decreased glutathione-*s*-transferase (GST) expression takes place which in turn affects the GSH/GSSG pool. Also, Ca^{2+} dependent differential gene expression is observed in Arabidopsis under ozone stress thereby elucidating important role of Ca^{2+} as well in combating ozone mediated stress in plants (Clayton et al., 1999; Short et al., 2012).

Temperature Stress

Extreme differential temperature exposure causes stress in plants. Temperature stress (cold/low) alters the normal functioning of plants (Dhingra, 2015; Hatfield and Prueger, 2015), JA has role in surmounting the effect of extreme temperatures in plants (Zhao et al., 2013). Role of JA, Ca^{2+}, and GSH under extreme temperature conditions are discussed below.

Cold Stress

Cold (low) temperature stress, a major threat that prevents plants from resuming full potential, results in a decrease in the crop productivity worldwide (Yadav, 2010; Dhingra, 2015). It affects plant metabolism and growth via inhibition of electron transport chain and disturbance in the activity of enzymes that participate in plant metabolism (Dhingra, 2015). Low temperature exposure of a plant leads to oxidative stress. During which, the plant's antioxidant machinery is activated to restore normal functioning of the plant. Antioxidants play key roles in cold acclimatization, low temperature stress tolerance, and maintenance of cellular redox homeostasis (Chen and Li, 2002; Khan et al., 2015). Exogenous application of MeJA to Arabidopsis (Hu et al., 2013) and loquat fruit (Cai et al., 2011b) imparted cold tolerance to them. However, it has been reported in cold tolerant *Camellia japonica* that upregulation of MYC – genes that are key regulators of JA signaling occurs in addition to an increase in the levels of precursor molecule α-linolenic acid of JA biosynthesis (Li Q. et al., 2016). The inducer of the CBF expression ICE-CBF pathway plays a core role in cold stress related response in plants. Under a normal set of conditions, ICE1 and ICE2 bind to the CANNTG sequence of the promoter region of CBF genes. These factors also bind JAZ1 and JAZ4, causing inhibition of ICE-CBF pathway. However, under cold stress conditions, the formation of more JA-Ile occurs which mediates the 26s proteasome degradation of JAZ factors that were previously bound to ICE1 and ICE2, hence activation of the ICE- CBF pathway occurs (Hu et al., 2017). It has been reported that external application of MeJA to a rubber tree eliminates the repression of JAZ proteins on ICE2 transcription factor that has an important role in the activation of CBF (C-repeat binding factor) cold signaling pathways involving genes CBF1, CBF2, COR47. So, the increase in the gene expression of CBF1, CBF2, COR47 genes tend to acclimate cold stress conditions in plants like rubber trees (Chen et al., 2019). Further, JA-related expression of genes involved in synthesis of GSH and GR occurs (Xiang and Oliver, 1998). It upregulates the antioxidant activities and protects the ultra-structure of the cell against cold stress (Li et al., 2012). Low temperatures increase the GSH level many folds which in turn alters the redox status of GSH (Wildi and Lütz, 1996; Karpinski et al., 1997). GSH accumulation was seen to be more evident in cold tolerant varieties of rice

as compared to non-tolerant (Yu et al., 2020). Cold stress can inhibit some metabolic activity of plants (Shi Y. et al., 2018). Moreover, GSH interacts with JA, which is involved in regulating plant developmental processes and signaling networks under different types of stresses (Per et al., 2018). It has also been reported that Ca^{2+} influx is required for elicitor-induced synthesis of JA (Hu et al., 2009). Thus, JA induced signaling cascade may lead to activation of nifedipine sensitive channels associated with the increase in cytosolic Ca^{2+} through release from intra-cellular stores (Sun et al., 2009). The decrease in temperature also causes significant increase in the cellular Ca^{2+} through increase in the influx of Ca^{2+} ions. Increased influx of radio labeled Ca^{2+} was seen in roots of plants in response to cold stress by the hypo-polarization of plasmalemma (Rincon and Hanson, 1986). Exogenous Ca^{2+} enhanced the tolerance potential of wheat under cold stress by regulating the levels of antioxidant machinery, photosynthetic rate, and membrane injury (Zhang et al., 2020).

Heat Stress

Constant rise in temperature due to greenhouse gases emission causes heat stress in plants. Plants are worst hit because of their sessile nature which makes them unable to shift to better place to handle the damaging effect of heat (Cassia et al., 2018). Heat stress greatly affects growth, physiological aspects, development, and yield of plant, thereby leading to generation of ROS in excess eliciting oxidative stress (Hasanuzzaman et al., 2013; Sarwar et al., 2018). It has been reported that applying JA helps to mitigate the effects of heat stress in plants via activating the oxidative defense and detoxification system (Sharma and Laxmi, 2016). Heat induced inhibition of photosynthesis is counteracted by Ca^{2+} salts that ameliorate the damage to Photosystem II as observed in tomato (Sakhonwasee and Phingkasan, 2017) and tobacco (Tan et al., 2011). Ca^{2+} ions tend to decrease the level of ROS production (Sakhonwasee and Phingkasan, 2017). Exogenous application of Ca^{2+} led to thermos-tolerance in common bean by up-regulating antioxidant enzyme activity and sugar accumulation in them (Naeem et al., 2020). GSH has also been found to protect plants under heat stress via improving photosynthetic attributes, osmolytes, and antioxidant levels in plants such as in Arabidopsis (Cheng et al., 2015). External application of GSH imparted heat tolerance in plants as seen in *Cummis sativa* (Ding X. et al., 2016). However, the involvement of GSH in mitigating heat mediated oxidative stress in plant is very well documented (Nahar et al., 2015a).

The response of exogenously applied JA, Ca^{2+}, and GSH, respectively, to different plant varieties under various kinds of abiotic stresses is given below in the **Table 1**.

POSSIBLE INTERACTION BETWEEN JASMONATES, CALCIUM, AND GLUTATHIONE

Plants have different capabilities of combating abiotic stress depending upon their antioxidant expression system (Davenport et al., 2003). Plant hormones regulate the adaptive responses that are indispensable for a plant to adapt itself to abiotic stress. JA increases antioxidant responses against abiotic stress in plants. JA effectively reduces oxidative stress by measuring the decrease in thiobarbituric reactive substance levels, increased GSH content, and scavenging of ROS via expression of enzymatic antioxidants (Maksymiec and Krupa, 2002; Chen Y. et al., 2011). Abiotic stress induces Ca^{2+} influx causing cold acclimation related necessary cellular alterations. Calcium signaling is one of the most vital signaling mechanisms that affect the JA-mediated signaling system inside plant cell via calcium channels (Fisahn et al., 2004; Beyhl et al., 2009; Lu et al., 2016). Generation and accumulation of ROS due to abiotic stresses triggers the opening of Ca^{2+} channels (Demidchik et al., 2018). Regulation and biosynthesis of JA is governed by levels of Ca^{2+} fluctuations (Wasternack and Song, 2017). However, in leaf cells of Arabidopsis JA tend to induce the increase in Ca^{2+} levels by mediating Apo- plastic calcium influx (Lu et al., 2016). It causes significant increase in Ca^{2+} into the cell due to immediate influx of Ca^{2+} ions as seen in roots of winter wheat (Erlandson and Jensén, 2006), alfa alfa (Monroy and Dhindsa, 1995), and maize (Rincon and Hanson, 1986). Drastic changes in Ca^{2+} levels of lodicle cells of panicles of rice and guard cells of Arabidopsis by exogenous application of MeJA has been reported, but transient rise in Ca^{2+} levels can also occur by other JAs in the plant cell cytosol and nucleoplasm (Qin et al., 2005; Walter et al., 2007). Ca^{2+} channel blocker like ruthenium red disrupted the Ca^{2+} transients in potato plants that also hinders the JA formation (Fisahn et al., 2004). Basal level of JA expression was increased in Arabidopsis by gain of function of two pore calcium channel 1 (TPC1) (Bonaventure et al., 2007). JA has been found to cause Ca^{2+} influx via AtCNGC2 calcium channel in epidermal cells of Arabidopsis (Lu et al., 2016). Increase in cytosolic Ca^{2+} levels result in the activation of Ca^{2+} dependent protein kinases (CDPK), calmodulins (CAM), etc., resulting in further integration of stress response pathways. This increase in the cytosolic Ca^{2+} occurs due to Ca^{2+} influx from external or release stores. It has been observed that JA also tends to induce Ca^{2+} mobilization that in turn interacts with CAM or CAM like proteins (CML) to modulate the expression of JA responsive genes like JR1 (Sun et al., 2006). Accumulation of CAM 1 type and CAM 3 type proteins was reported in tobacco plants followed by JA treatment (Yamakawa et al., 2001). CML42 of Arabidopsis is deciphered to have a crucial role in calcium mediated JA biosynthesis (Vadassery et al., 2012a). CAM gene, CAM binding protein, and CML expression also increased in response to MeJA (Bergey and Ryan, 1999; Yang and Poovaiah, 2002; Vadassery et al., 2012b). Moreover, CDPK has also seen to be upregulated by JA as they seem to trigger the formation of OPDA (Ludwig et al., 2005). Inhibitors of JA synthesis prevented the inhibitory effect induced by abiotic stress like heavy metals, on the accumulation of chlorophyll and photosynthesis (Maksymiec and Krupa, 2002). Upregulation of MeJA mediated stress defense by changing the protein profile thereby controlling the photosynthesis and antioxidant metabolism (Chen Y. et al., 2011; Maserti et al., 2011). Changes in the intracellular redox environment of a plant due to generation of ROS in response to various abiotic stresses disturbs the plants cellular physiology (Ogawa et al., 2005). ROS

TABLE 1 | Abiotic stress response in relation with JA, Ca^{2+}, and GSH among different plant species.

Stress type	Plant species	JA/GSH/Ca^{2+}	Response	Growth pattern	Cotyledon number	References
		MeJA				
High salt concentration	*Pisum sativum* (L.)	10^{-5} M	Osmoregulation, increased proline content	Annual	Dicot	Fedina and Tsonev, 1997
	Glycine max (L.)	20 and 30 μM	Increase in growth and proline content	Annual	Dicot	Yoon et al., 2009
	Arabidopsis thaliana (L.)	5 and 10 μM	Compliments lox3 mutant rescues salt stress	Annual	Dicot	Ding H. et al., 2016
	Triticum aestivum (L.)	0.1 μM	Increases cytokinin production and plant growth	Annual	Monocot	Avalbaev et al., 2016
	Solanum lycopersicum (L.)	10, 20, 30, 40, 50, and 60 μM	Increase in levels of osmo-protectants and enzymatic antioxidants	Annual	Dicot	Manan et al., 2016
	Brassica napus (L.)	100 μM	Increases relative water content, soluble sugar, photosynthesis	Annual	Dicot	Ahmadi et al., 2018
		JA				
	Pisum sativum (L.)	10^{-5} M	Decreased activity of sodium and chloride ions, increased endogenous level of proline	Annual	Dicot	Velitchkova and Fedina, 1998
	Oryza sativa (L.)	30 μM	Increases ion uptake, growth, ABA levels	Annual	Monocot	Kang et al., 2005
	Hordeum vulgare (L.)	12 μM	Induction of genes having role in imparting salt tolerance	Annual	Monocot	Walia et al., 2007
	Brassica napus (L.)	10^{-6}, 10^{-9}, and 10^{-12} M	Sugar accumulation	Annual	Dicot	Kaur et al., 2013
	Triticum aestivum (L.)	2 mM	Increase in concentration of GSH, enhanced activity of SOD, CAT, APX	Annual	Monocot	Qiu et al., 2014
		GSH				
	Oryza sativa (L.)	2 mM	Positive influence on yield contributing traits	Annual	Monocot	Wang et al., 2014
	Arabidopsis thaliana (L.)	400 μM	Abscisic acid, auxin and jasmonic acid biosynthesis	Annual	Dicot	Cheng et al., 2015
	Vigna radiata (L.)	1 mM	Activation of glyoxalase system and improved antioxidant system	Annual	Dicot	Nahar et al., 2015b
	Solanum lycopersicum (L.)	5 mM	Increased GSH biosynthesis, improved activity of SOD, CAT, POD	Annual	Dicot	Zhou et al., 2017
	Glycine max (L.)	2 mM	Improved stress tolerance and yield attributes	Annual	Dicot	Akram et al., 2017
		Ca^{2+}				
	Solanum lycopersicum (L.)	5 and 10 mM	Increased growth, physiology and fruit production	Annual	Dicot	Parvin et al., 2015
	Glycine max (L.)	6 mM	Positive effect on growth and metabolic activities.	Annual	Dicot	Yin et al., 2015
	Oryza sativa (L.)	3 and 5 mM	Elevated antioxidant enzyme levels	Annual	Monocot	Tahjib-Ul-Arif et al., 2018
	Lead	**JA**				
Heavy metal stresses	*Wolffia arrhiza* (L.)	0.1 μM	Preventing Pb accumulation by restoring plant growth and primary metabolite level	Perennial	Monocot	Piotrowska et al., 2009
	Solanum lycopersicum (L.)	0.1, 1, and 100 μM	Increase osmolytes concentration and ascorbate glutathione cycle	Annual	Dicot	Bali et al., 2018

(Continued)

TABLE 1 | Continued

Stress type	Plant species	JA/GSH/Ca^{2+}	Response	Growth pattern	Cotyledon number	References
			GSH			
	Gossypium sp. (L.)	50 μM	Stabilized ultra-structure and increased antioxidant activity	Perennial	Dicot	Khan M. et al., 2016
	Triticum aestivum (L.)	1 mM	Enhancement of enzymatic and non-enzymatic antioxidant activities and improved seedling growth	Annual	Monocot	Hasanuzzaman et al., 2018
	Cadmium	**JA**				
	Glycine max (L.)	20 μM	Increased antioxidant response	Annual	Dicot	Noriega et al., 2012
	Vicia faba (L.)	0.01 mM	Restoration of growth and pigment system	Annual	Dicot	Ahmad et al., 2017
	Brassica napus (L.)	25 μM	Osmolytes and antioxidant activity increased	Annual	Dicot	Ali et al., 2018
		MeJA				
	Oryza sativa (L.)	5 μM	GSH homeostasis, JA biosynthesis	Annual	Monocot	Singh and Shah, 2014
	Arabidopsis thaliana (L.)	0.01 μM	Suppression of genes involved in Cd uptake	Annual	Dicot	Lei et al., 2020
		GSH				
	Hordeum vulgare (L.)	20 mg/L	Improved photosynthesis	Annual	Monocot	Chen et al., 2010
	Oryza sativa (L.)	50 μM	Enhanced photosynthetic performance	Annual	Monocot	Cai et al., 2011a
	Gossypium sp. (L.)	50 μM	Reverses stressful effects, leaf ultra-morphology revived	Perennial	Monocot	Daud et al., 2016
	Populus sp. (L.)	100 μM	Increased Cd detoxifying gene transcript	Perennial	Monocot	Ding et al., 2017
		Ca^{2+}				
	Vicia faba (L.)	2%	Antioxidant enzyme up regulation	Annual	Dicot	Siddiqui et al., 2012
	Brassica juncea (L.)	50 mM	Improved photosynthesis	Annual	Dicot	Ahmad et al., 2015
	Arabidopsis thaliana (L.)	3 mM	Alleviated the inhibition of Cd on the root growth	Annual	Dicot	Li P. et al., 2016
	Sesamum indicum (L.)	50 mM	Improved growth and proline levels	Annual	Dicot	Abd-Allah et al., 2017
	Copper	**JA**				
	Oryza sativa (L.)	0.5 mM	Phytoalexin production	Annual	Monocot	Rakwal et al., 1996
	Cajanus cajan (L.)	1 μM, 1 nM,	Osmolytes and antioxidant enzyme increased	Perennial	Dicot	Poonam et al., 2013
	Triticum Aestivum (L.)	5 mM	Increased transcript of glutathione–*s*- transferase	Annual	Monocot	Li et al., 2013
		MeJA				
	Phaseolus coccineus (L.)	10^{-5} M	Promoted plant growth and development	Perennial	Dicot	Hanaka et al., 2015
		GSH				
	Triticum aestivum (L.)	2.5 mM/L	Accumulation of nitrogen, sulfur, and phosphorous	Annual	Monocot	Peng et al., 2012
	Glycine Max (L.)	0.16 and 0.32 Mm/L	Enhances amylase activity	Annual	Dicot	Chen, 2012
	Oryza Sativa (L.)	100 mg/L	Increased germination rate and vigor index	Annual	Monocot	Mostofa et al., 2015
		Ca^{2+}				

(Continued)

TABLE 1 | Continued

Stress type	Plant species	JA/GSH/Ca^{2+}	Response	Growth pattern	Cotyledon number	References
Drought	**JA**					
Water stress	*Glycine Max* (L.)	4.5 and 9 mM/L	Maintenance of membrane integrity	Annual	Dicot	Chen et al., 2008
	Vigna radiata (L.)	5 mM	Solution improved the growth of Cu-treated seedling and lowering the concentration of Polyamines putrescine and increased concentrations of spermine and spermidine in epicotyl of plants	Annual	Dicot	Shen et al., 1998
	Brassica sp. (L.)	0.5 mM	Increase in physiological, antioxidant and glyoxalase system activities	Annual	Dicot	Alam et al., 2014
	Allium cepa (L.)	25, 50, and 100 μM	Pigment and compatible solute enhancement	Annual	Monocot	Ahmad and Murali, 2015
	Beta vulgaris (L.)	5 and 10 μM	Increased germination rate	Annual	Dicot	Ghafari and Tadayon, 2019
		MeJA				
	Brassica oleracea (L.)	10 μM	Increased Net photosynthetic rate and antioxidant machinery activation	Annual	Dicot	Wu et al., 2012
	Triticum aestivum (L.)	0.25 μM	Water status and antioxidant capacity increased	Annual	Monocot	Ma et al., 2014
	Satureja hortensis (L.)	75, 150, and 225 μM	Improved many characteristics of plant like growth, water content, proline level, antioxidant activity	Annual	Dicot	Miranshahi and Sayyari, 2016
		GSH				
	Arabidopsis thaliana (L.)	400 μM	Changes at translational level of numerous hormones	Annual	Dicot	Cheng et al., 2015
	Vigna radiata (L.)	1 mM	Improved their antioxidant components under drought stress	Annual	Dicot	Nahar et al., 2015a
		Ca^{2+}				
	Zoysia japonica (L.)	5 and 10 mM	Improved photosynthesis, growth and antioxidant response	Perennial	Monocot	Xu et al., 2013
	Zea mays (L.)	5 mg/L	Improved photosynthesis, growth and soluble sugar content	Annual	Monocot	Naeem et al., 2018
	Nicotiana tabacum (L.)	10 mM/L	Stabilization of gaseous exchange and photosynthetic organelles	Annual	Dicot	Hu et al., 2018
Flooding		**JA**				
	Citrus spp. (L.)	1 mM	Increase in abscisic acid levels	Perennial	Dicot	de Ollas et al., 2013
		Ca^{2+}				
	Zea mays (L.)	0.75% (W/V)	Regulates the cell wall integrity and mitigates effect of oxidative stress during flood stress conditions	Annual	Monocot	Porto et al., 2013
	Glycine max (L.)	50 mM	Increase the root elongation and inhibited the cell death of root tip of under flood stress	Annual	Dicot	Oh et al., 2014

(Continued)

TABLE 1 | Continued

Stress type	Plant species	JA/GSH/Ca^{2+}	Response	Growth pattern	Cotyledon number	References
		JA				
Ozone stress	*Capsicum annuum* (L.)	10 mM	Regulates osmotic and antioxidant metabolism	Annual	Dicot	Yang et al., 2016
	Arabidopsis thaliana (L.)	1.4 μM	Inhibited cell death and lesion containment	Annual	Dicot	Overmyer et al., 2000
	Arabidopsis thaliana (L.) (JA insensitive mutants)	10 μM	Extremely susceptible to ozone	Annual	Dicot	Kanna et al., 2004
		GSH				
	Transgenic *Nicotiana tabacum* (L.)	Overexpression of glutathione synthetase in plastid	Ozone tolerance developed	Annual	Dicot	Wellburn et al., 1998
	Populus sp. (L.)	Overexpression of Glutathione reductase	Ozone tolerance developed	Perennial	Monocot	Foyer et al., 1995
Heat		**JA**				
Temperature stress	*Vitis* sp (L.) seedling	50 μM/L	Thermotolerance	Perennial	Dicot	Chen et al., 2006
		Ca^{2+}				
	Solanum lycopersicum (L.)	1 Mm	Operating efficiency of photosystem II increased	Annual	Dicot	Sakhonwasee and Phingkasan, 2017
	Nicotiana tabacum (L.)	20 Mm	Improved stomatal conductance and thermostablity	Annual	Dicot	Tan et al., 2011
Cold		**JA**				
	Prunus persica (L.)	0.1 Mm/L	Maintenance of fruit quality	Perennial	Dicot	Meng et al., 2009
		MeJA				
	Cucumis sativus (L.)	100 μM	Enhances chilling tolerance by regulating antioxidant enzymes	Annual	Dicot	Li et al., 2012
	Arabidopsis thaliana (L.)	30 μM	Induced freezing tolerance	Annual	Dicot	Hu et al., 2013
	Eriobotrya japonica (L.)	10 μM	Alleviates the chilling injury in the fruits of plants	Perennial	Dicot	Cai et al., 2011b
		Ca^{2+}				
	Solanum lycopersicum (L.)	27 mM	Improvement carbon fixation, electron transport, etc.	Annual	Dicot	Zhang G. et al., 2014
	Cynodon dactylon (L.)	1, 5, 10, and 20 mM	Antioxidant activation and metabolic homeostasis	Perennial	Monocot	Shi et al., 2014
		GSH				
	Eriobotrya japonica (L.)	50, 100, and 300 mg/L	Increase in membrane fluidity and decrease in lipid peroxidation	Perennial	Dicot	Wu et al., 2010

is sequestered via production of antioxidants like GSH (Noctor and Foyer, 1998). Moreover, in response to JA and heavy metals increase in GSH in plants occurs via the expression of genes transcribing the enzymes for GSH production (Schafer et al., 1997; Xiang and Oliver, 1998). JA also leads to increased activity of GCL and GS. Increase of GCL activity causes more glutathione disulfide or oxidized glutathione (GSSG) formation. GSSG in turn causes release of Ca^{2+} thereby also affecting Ca^{2+} signatures (Gómez et al., 2004; Hicks et al., 2007; Sun et al., 2009). Moreover, in response to GSH and GSSG treatment, Ca^{2+} release occurs in plants as seen in tobacco leaf. Ca^{2+} related response is linked to overall GSH supplied to the leaf. Therefore, GSSG has an effect on calcium signatures (Gómez et al., 2004). However, JA biosynthesis and signaling is in turn regulated by Ca^{2+} (Wasternack and Hause, 2013). Glutathione status is modulated by various abiotic stresses that affect the abundance of transcripts related with JA signaling, synthesis, and downstream cascade (Gómez et al., 2004; Han et al., 2013). OPR3, one of the JA biosynthetic enzyme expressions, increased with Ca^{2+} treatment (Chotikacharoensuk et al., 2006). It is also found that Ca^{2+} signaling results in increased levels of OPR3 and JA (Gust et al., 2005).

It is inferred that there must be crosstalk between the JA induced influx of apoplast and ionistol triphosphate sensitive Ca^{2+} stores as JA induced influx of extracellular Ca^{2+} concentration can be via nifedipine sensitive Ca^{2+} channel in the plasma membrane and expression of downstream genes to JA (Sun et al., 2006; Ladyzhenskaia and Korableva, 2008). It has also been demonstrated that for JA synthesis influx Ca^{2+} is necessary for elicitor induction (Hu et al., 2009). However, JA-induced signaling cascade may lead to activation of nifedipine sensitive channels to increase in cytosolic Ca^{2+} which in turn causes release of Ca^{2+} from intra-cellular stores (Sun et al., 2009). JAs also might be involved in transducing signaling pathways and upregulation of the GSH metabolic genes and encourages the synthesis of GSH which eliminates peroxides via ascorbate–glutathione cycle (Smeets et al., 2005; Rouhier et al., 2008). MeJA has found to be responsible for increased expression of JA and glutathione biosynthesis enzymes (Jung et al., 2007).

The whole cross talk or interaction between JA, Ca^{2+}, and GSH is summarized in **Figure 3**.

PHYTO-HORMONE CROSS TALK UNDER ABIOTIC STRESS

Plant hormones have critical roles in mediating the abiotic stress tolerance under unfavorable environmental conditions (Alhaithloul et al., 2020). Abiotic stresses lead to the induction of signal transduction pathways that helps a plant to adapt itself to changing environmental milieu (Dolferus, 2014; Ingole et al., 2021; Kolbert et al., 2021). It leads to the ROS generation and various phyto-hormone accumulation along with remodeling of gene expression in accordance with activation of preferable defense response. These phytohormones mediated signaling and interaction renders them ultimate entity for conferring abiotic stress tolerance in plants (Nguyen et al., 2016;

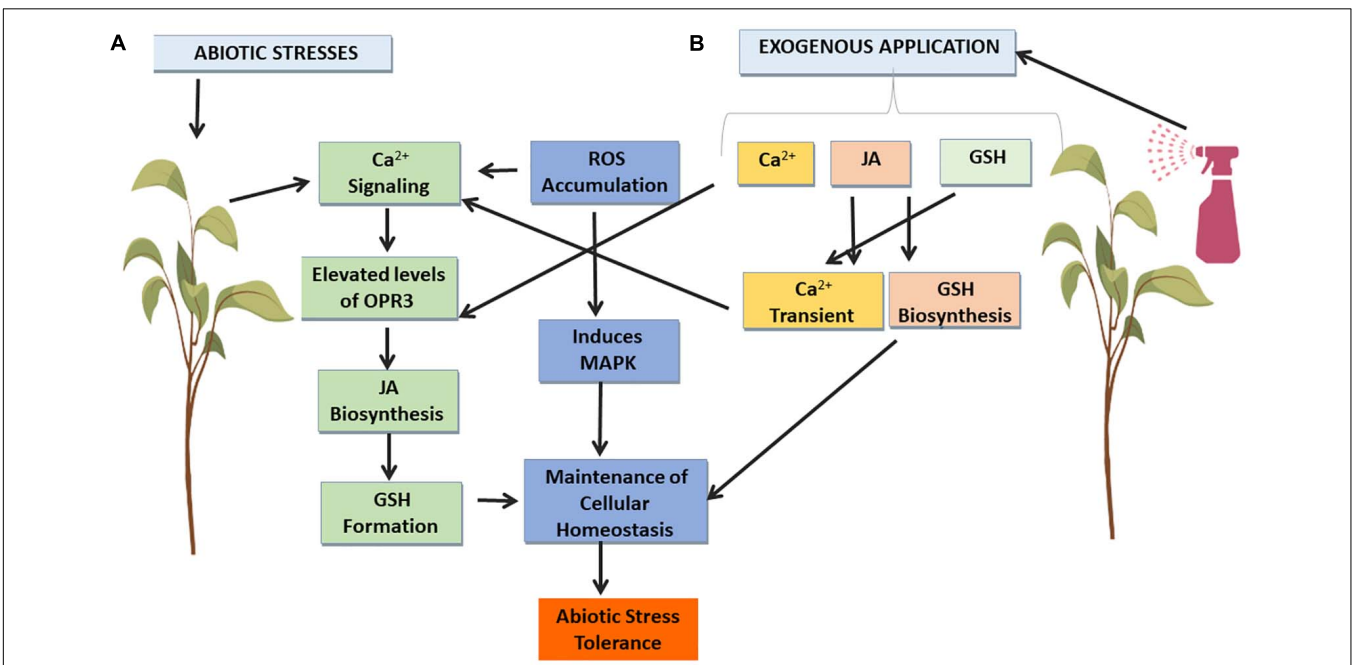

FIGURE 3 | Signaling pathways involved in abiotic stress tolerance. **(A)** Abiotic stresses causes ROS accumulation that leads to activation of Ca^{2+} signaling and MAPK pathways to combat abiotic stresses in plants by maintaining cellular homeostasis. **(B)** Exogenous application of JA, Ca^{2+} and GSH, respectively, to plants prior to or under abiotic stress mediates activation of Ca^{2+} signaling and MAPK pathways via possible crosstalk mechanisms so as to further strengthen the cellular homeostatic mechanisms of plants, thereby imparting abiotic stress tolerance.

Verma V. et al., 2016; Singh et al., 2019). Phytohormones cross talk in abiotic stress and its link with development of plant stress tolerance in accordance with JA, GSH and Ca^{2+} is discussed below.

JA Phyto-Hormone Cross Talk Under Abiotic Stress

Under multiple environmental stresses, plant hormones allocate limited resources to respond to the most serious stress and develop various signaling pathways to regulate the balance between plant growth and defense response (Tian et al., 2003; Matyssek et al., 2005; Sharma et al., 2013). Understanding the similarities and differences of plant hormone signaling may be important in agricultural production. The crosstalk between plant hormones is of vital importance in plant stress response (He et al., 2017). JA does not work independently but acts in a complex signaling network combined with other plant hormone signaling pathways (Ahmad et al., 2016; He et al., 2017; Hu et al., 2017; Wasternack and Strnad, 2018). Kazan (2015) elucidated the immense role of JA and ethylene in abiotic stress. JA and ET are known to regulate plant tolerance against abiotic stress like drought cold salinity through coordination or antagonistically (van der Fits and Memelink, 2000; Zhai et al., 2013). Ethylene response factors (ERFs) that confer roles in abiotic stress combating mechanism are induced by JA signaling apart from ethylene, thereby facilitating cross talk between them (Ramegowda and Senthil-Kumar, 2015). ERF-domain transcription factor ORA59 of *A. thaliana*, ET INSENSITIVE3 (EIN3) and its homolog EIN3-like 1 (EIL1), as well as JAZs-MYC2 are involved in the crosstalk between JA and Ethylene signaling pathways (Zhu, 2003, 2014; Zhang X. et al., 2014; Zhu and Lee, 2015). JA interacts with ABA under abiotic stress to cause a physiological response to overcome abiotic stress factors (Gomez-Cadenas et al., 2015). MYC2 and JAZ have roles in cross talk between them (Chen Q. et al., 2011). JA cross talk with ABA imparts cold stress tolerance (Hu et al., 2013). MYC2, the core regulator of the JA signaling mechanism, contributes in the ABA signaling cascade in response to drought stress (Abe et al., 2003; Liu et al., 2014). Moreover, JA and SA also have the same regulator glutaredoxin GRX480 which maintains protein redox regulation due to its ability to catalyze disulfide transitions (Meldau et al., 2012). Mitogen-activated protein kinase 4 (MAPK4) is a negative regulator of SA signaling and positive regulator of JA signaling cascade in light stress (Sharma, 2013). The C-terminus of JAZs mediates interaction between JAZs and MYC2 and between JAZs and DELLAs. So, DELLAs can completely interact with JAZs (Hou et al., 2010). In absence of gibberellic acid (GA), DELLA can interact with JAZ and mediates release of MYC2 thereby inhibiting JA biosynthesis and mediating activation MYC2 downstream gene activation (MYB21 and MYB24) (Song et al., 2011). But in the presence of GA, DELLA gets degraded, thus allowing JAZ-MYC2 interaction (Hu et al., 2013). On the contrary, JA delays GA-mediated degradation of DELLA (Yang et al., 2012). JA and auxin signaling coordinately regulate the plant growth and development. COI1, MYC2, and JAZ are the core components in the crosstalk of JA and auxin signaling pathways. In response to exogenous auxin, the activation of auxin–TIR–AUX/IAA–ARF signaling occurs, mediating JA synthesis. The endogenous JA prompts the expression of auxin synthase gene (*ASA1*) and auxin content. JA leads to formation of a complex of COI1 and JAZ leading to the degradation of JAZ, thereby activating the transcriptional activities of MYB21/MYB24 and causing flower development (Chen Q. et al., 2011). JA also interacts with Cytokinin through MYC2 transcription factor. MYC2 is reported to be a negative regulator of cytokinin response by facilitating expression of inhibitor of cytokinin signaling AHP6. JA is involved in decreased expression of PIN-FORMED 7 gene that is involved in the development of xylem and it has been reported that extra xylem formation takes place in roots of Arabidopsis under drought stress (Jang and Do Choi, 2018). So, this opposite interaction between JA and Cytokinin has a role in JA dependent stress response. It is also suggested that differential cytokinin expression under stressful conditions leads to JA – cytokinin interaction at a metabolic level (Le et al., 2012; Jang and Do Choi, 2018). So JA interacts with a different kinds of hormones to regulate the growth and development of plants such as GA, auxin, cytokinin, Ethylene, and SA (**Figure 4**). These interactions may help to optimize growth and development of plants under abiotic stress conditions.

Glutathione Phytohormone Cross Talk Under Abiotic Stress

Adverse climatic conditions lead to abiotic stress in plants. Peroxisomal or cytosolic atmosphere leads to electron absorption and subsequently causes oxidative damage via ROS generation (Hasanuzzaman et al., 2017b). ROS-mediated abiotic stress-induces apoptosis or whole plant death in many plant cultivars (Petrov et al., 2015; He et al., 2018). It imparts signals that regulate stress adaptation (Mhamdi and Van Breusegem, 2018). Plants have an antioxidant defense system comprising of non-enzymatic and enzymatic antioxidants in cell organelles, which aids in ROS removal up to a certain level (Gill and Tuteja, 2010). Among this well-defined antioxidant system, Glutathione regulates numerous metabolic functions. Glutathione peroxidase is responsible for ROS detoxification (Hasanuzzaman et al., 2017a). Glutathione increases the plant tolerance to different abiotic stresses, including salinity, drought, high temperature (HT), low temperature, and toxic metal stress (Hasanuzzaman et al., 2013). Exogenously given GSH imparts abiotic stress tolerance in plants (Nahar et al., 2015a,b). Hormonal regulation of GSH and its role in abiotic stress tolerance have been reported in many research findings. Transcriptome analysis revealed that GSH treatment leads to biosynthesis of Auxin, JA, and ABA along with activation signaling cascades pertaining to them (Cheng et al., 2015). GST overexpression in *A. thaliana* plants have signaling and regulatory roles in plant development by maintaining GSH pools (Chen et al., 2012). The increased activities of GST and GPX contributes to improved salt stress in the auxin autotrophic tobacco callus lines (Csiszár et al., 2004). Phytohormones like JA, ABA, Auxin, ethylene,

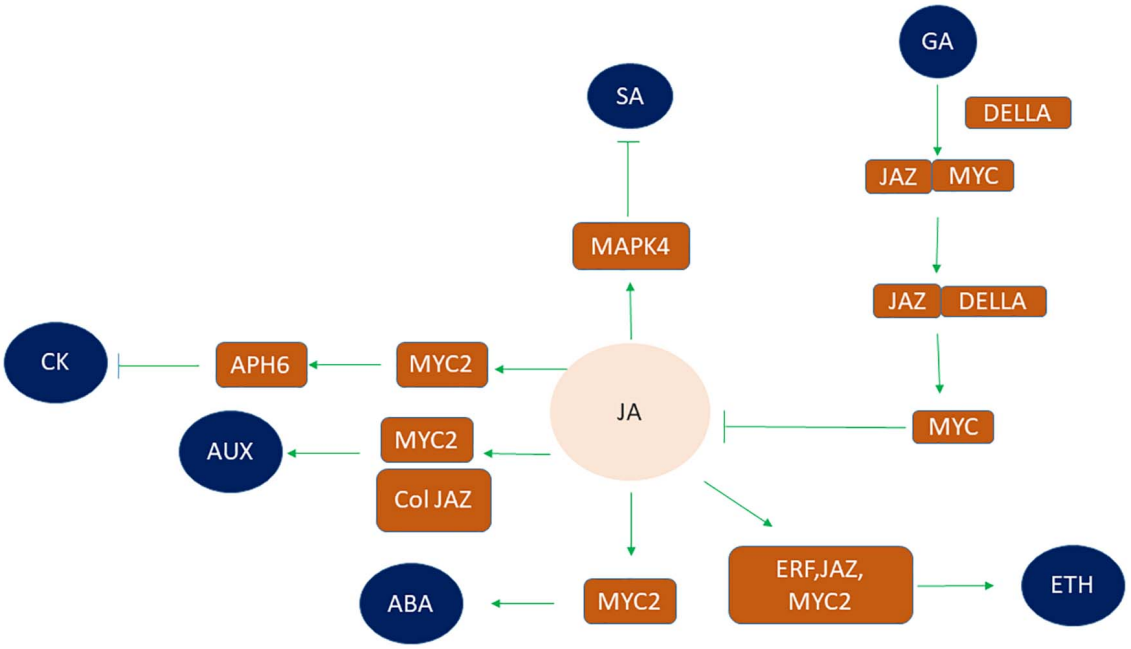

FIGURE 4 | Crosstalk between phytohormones with JA under abiotic stress conditions via different transcription factors. JA interacts with other phytohormones such as SA, ETH, ABA, GA, AUX, and CK in order to regulate plant abiotic stress. JA signaling inhibits SA by modulating mitogen activating protein kinase (MAPK4). Similarly, crosstalk of JA and ETH signaling pathway occurs through interaction of three TF's viz ERF, JAZ, and MYC2 thereby regulating plant stress response. MYC2 also participates in crosstalk of JA and ABA signaling pathway. DELLA interacts with JAZ in absence of GA to release MYC thereby inhibiting JA biosynthesis and causing activation of MYC downstream genes. However, in the presence of GA, DELLA gets degraded so allowing JAZ-MYC interaction. COL, MYC2, and JAZ are the core components in the crosstalk of JA and AUX signaling pathways. JA mediated inhibition of CK signaling by MYC2 and AHP6 transcription factors. (AUX, auxin; SA, salicylic acid; ETH, ethylene; ABA, abscisic acid; GA, gibberellic acid; CK, cytokinin).

cytokinin, and brassinosteroid induced GST expression in plants (Marrs, 1996; Moons, 2005; Deng et al., 2007). Exogenous Glutathione application brought higher levels of ABA (Chen, 2012). 2, 4-D and NAA (two synthetic auxins) and IAA induced expression of GST8 in Arabidopsis (Bianchi et al., 2002). Moreover, exogenous or endogenous auxin positively regulates the expression intensities of numerous abiotic stress-related genes along with GSH/GSSG pools and GR activity (Shi et al., 2014). JA also regulates GSH concentration and genes for GSH metabolism in Arabidopsis (Akter et al., 2010) and *Agropyron cristatum*, (Sasaki-Sekimoto et al., 2005). It functions as signaling molecule during MeJA signaling in guard cells in Arabidopsis, in addition to intracellular GSH regulating MeJA-induced movements of stomata (Koornneef and Pieterse, 2008). Exogenous SA caused changes in the levels of GSH, GR transcriptomics and activity in maize genotypes and soybean cell suspension thereby mediating abiotic stress tolerance (Knörzer et al., 1999; Kellős et al., 2008). Changes in both SA and GSH expression due to overexpression of the SA gene in rice were correlated with oxidative abiotic stress tolerance (Kusumi et al., 2006). Ethylene has been reported to regulate GSH biosynthesis positively in ozone exposed Arabidopsis leaves (Freeman et al., 2005). The inhibitor of GSH biosynthesis l-buthionine sulfoximine (BSO) effectively reduced the suppression of the JA-responsive gene PDF1.2 by SA, which suggests that SA-mediated control of the cellular redox state is an important trigger for JA signaling (Koornneef and Pieterse, 2008).

Calcium – Phytohormone Cross Talk Under Abiotic Stress

Plant hormone signaling cascades not only crosstalk with one another, but have also been reported to interact with other signaling molecules such as the Ca^{2+} and mitogen-activated protein kinase (MAPK) pathways during an abiotic stress conditions (Ludwig et al., 2005; Roychoudhury and Paul, 2012; Roychoudhury and Banerjee, 2017). The overlap between hormone-regulated gene expression profiles as adaptive responses of plants to environmental stresses suggests the presence of a complex network with widespread interactions between the different hormone signaling pathways (Suhita et al., 2003, 2004). However, phytohormones like JA and ABA induced the Ca^{2+} transients. The primary role of JA and ABA in the plasma membrane seem to be different to each other. JA aims to encounter the Ca^{2+} channels whereas ABA stimulates effector molecules in the plasma membrane like phospholipase C and D. But at the intracellular Ca^{2+} level, both signaling cascades converge. The intracellular Ca^{2+} level is regulated to a much greater extent by JA rather than by ABA. It has been reported that JA interaction with ABA-regulated stomatal closure by increasing influx of Ca^{2+} causes activation of CDPK-dependent signal pathways, contributing to the drought stress

responsiveness (Shi S. et al., 2018). Treatment of Arabidopsis leaves with MeJA or ABA results in less stomatal aperture reduction within 10 min (Munemasa et al., 2007). Though the chemical inhibitors or in ABA-deficient mutants led to inhibition of ABA biosynthesis along with suppression of the MeJA-induced Ca^{2+} oscillations in guard cells (Hossain et al., 2011). Therefore, during stomatal closure MeJA interacts with ABA leading to further Ca^{2+} signaling cascade. Ca^{2+} increase, however, favors the stomata closure by enhancing Slow Anion Channel-Associated 1 (SLAC1) and cytoskeletal rearrangement of plasma membrane (Waidyarathne and Samarasinghe, 2018). Ca^{2+} dependent ABA regulation is related to induction of enzymatic antioxidants (SOD, CAT3, APX, and GR) and non-enzymatic antioxidants [glutathione, ascorbic acid, carotenoids (Ahmad et al., 2010)]. Some drought-responsive CPKs also have some functions like, in rice, OsCPK9 regulates both drought stress tolerance and spikelet fertility through an ABA-dependent manner (Wei et al., 2014). Nevertheless, the precise role of Ca^{2+} in ABA signaling needs to be further explored (Waidyarathne and Samarasinghe, 2018). Gene expression of ethylene-induced ACC oxidase (VR-ACO1) in tissue of root of mung bean was reported due to Cytosolic Ca^{2+} transients. In fact, inhibitors and chelators of Ca^{2+} significantly inhibited the ethylene based gene expression of VR-ACO1, respectively (Jung et al., 2000). Ca^{2+} was seen to augment the conversion of ACC to ethylene in primary roots of *Zea mays* (Hasenstein and Evans, 1986). However, ethylene was found to be responsible for activation of plasma membrane Ca^{2+}-permeable channel to increase the Ca^{2+} level in suspension cells of tobacco (*Nicotiana tabacum*) (Zhao et al., 2007). So, this relationship between ethylene and Ca^{2+} seems to be an essential component in abiotic stress response (Acosta-Motos et al., 2017). It has been found that involvement of ethylene in Ca^{2+} induced adventitious rooting under salt stress (Yu et al., 2019). Ca^{2+} plays a pivotal role in the overall downward polar transport of auxin and in the absence of Ca^{2+} basipetal auxin, transport was halted. However, ca^{2+} supplementation can overcome the inhibition of this basipetal transport of auxin (Lee et al., 1983; Allan and Rubery, 1991). So, the transport of auxin plays noteworthy role in the dispersal of Ca^{2+} to developing tissues (Banuelos et al., 1987). The auxin transport pathway seems to be involved in the remodeling of root system architecture in Ca^{2+} mediated alleviation of metal toxicity like Cd toxicity (Li P. et al., 2016).

OMICS APPROACHES TO STUDY THE ROLES OF JA, Ca^{2+}, AND GSH UNDER ABIOTIC STRESS

The intricate molecular controlling systems that have roles in abiotic stress adaptation and tolerance in plants can be interpreted using an 'omics' approach (Chawla et al., 2011). The omics technologies have paved the way toward the development of well-established protocols that provide in-depth insights about the gene functionality including their phenotypic effect in defined biological frameworks. Comparative genomic analysis between the plant models like *Thellungiella halophila* and *A. thaliana* have remarkable cold, drought, and salinity tolerance. So, plants tend to modify their omics profile to withstand the fluctuating environment for their existence (Gong et al., 2005). Almost 50% of the plant genes were activated by stresses including drought and salinity. The abiotic stress responding genes can be divided into two classes based either on their response in terms of timescale or on their involvement in tolerance, some seem to respond quickly within seconds or minutes, while others respond slowly (Ramanjulu and Bartels, 2002). It has been reported that about 15% more unknown genes were expressed in the plant subjected to salt stress than in the unstressed plant suggesting that the exposure of plant to abiotic stress results in the surge in expression of genes. In response to various abiotic stresses in plants, proteomic studies has been mostly accepted to explore the protein profiles that might lead to the progression of new strategic ways to improve stress tolerance (Cushman and Bohnert, 2000). Researchers have used various omics approaches to decipher an integrated mode of plant response to different abiotic stresses (Zhu et al., 2017; Bajwa et al., 2018; Parida et al., 2018; Zhang et al., 2018). Treatment of plants with MeJA showed remarkable change in their protein profile. Nearly 194 proteins were differentially expressed in various plant physiological processes. Functional analyses revealed that carbohydrate catabolism was upregulated along with some proteins involved in JA biosynthesis pathway and stress defense (Chen Y. et al., 2011). Multi-omics analysis determined vigorous cascade of transcriptional reprogramming via TF MYC2 and MYC3 that tend to target lots of JA-responsive genes, thereby enabling expression of cohorts of genes that have distinct roles within the JA response. This depicts the complexity of the hormone-response based genome regulatory program. Further, functional importance of MYC2 and MYC3 target genes in JA responses has been validated. Mutations in six genes caused evident disturbances in JA responses, both hypersensitivity and hyposensitivity (Zander et al., 2020). Microarray experiments of wheat and barley cultivars under boron toxicity conditions suggest that genes related to jasmonate biosynthesis and GST can have roles in boron tolerance mechanisms in cereals (Öz et al., 2009). Moreover, plants have a complex antioxidant defense system to scavenge ROS under stress conditions (Hossain et al., 2009). Transgenic plants over express enzymes involved in oxidative stress protection like GPX, SOD, and GR (Tang et al., 2006). The differential expression of Medicago GST (MtGST) were upregulated whereas some of them were downregulated under abiotic stress. Two cluster groups - MtGSTU46 to MtGSTF8 and MtGSTL4 to MtGSTH5 genes were mostly increased in both drought and salinity stresses. Among them, MtGSTU8, MtGSTU17, MtGSTF8, MtGSTT2, and MtGSTZ1 members were mostly upregulated in all cases of these two abiotic stresses (Hasan et al., 2021). AtGSTU19 and AtGSTF2 favors the glutathionylation and binds to JA hormone precursor oxylipin 12-OPDA. So, GST seems to be involved in the export of 12-OPDA from the chloroplast to the site of JA synthesis (peroxisome) (Dixon and Edwards, 2009). Interestingly, greater 12-OPDA level in plants promoted less stomatal aperture and drought tolerance (Savchenko et al., 2014). This points to a potential crosstalk among 12-OPDA (JA) and GSTs as GSTs are quickly induced by 12-OPDA and JA

(Mueller et al., 2008). However, GST binding might regulate the temporal signaling of oxylipins under stress. Elevated GST expression correlates with increased stress tolerance as observed in tomato (Sun et al., 2010) and wheat (Gallé et al., 2009). Moreover, OsGSTL2 overexpression imparted a rise in tolerance level against drought, cold stress, and salinity (Kumar et al., 2013). However, the cytosolic Ca^{2+} transients have been reported under various stresses such as heat, cold, salinity, and water (Mahajan and Tuteja, 2005; Tuteja and Mahajan, 2007; Riveras et al., 2015). The promoter region analysis of the Ca^{2+} shows biased nature toward abiotic stress responsive genes. Three among four Ca^{2+} regulated promoter regions were reported to be indispensable for abiotic stress responses (Iqbal et al., 2020). About 30 CDPK genes have been reported in Arabidopsis that have role in abiotic stress responses (Hrabak et al., 2003). Arabidopsis has Ca^{2+}-binding calcineurin B-like (CBL) proteins dependent 25 SnRK3-type kinases including SALT OVERLY SENSITIVE 2 (SOS2)/CIPK24/SnRK3 which have important functions in abiotic stress response. SOS3/ScaBP8/CBL10 and SOS2 participates in activation of the plasma membrane Na+/H+ antiporter (SOS1) prerequisite for salinity tolerance (Luan, 2009). Transcriptomics has revealed that CaM binds to regulate various transcription factors called as CAMTAs generating the stress response (Reddy et al., 2011). CAMTAs comprise bZIP, MYB, WRKY, and NAC families of transcription factors (Popescu et al., 2007; Yoon et al., 2008). Several CBLs have a myristoylation site that stimulates membrane association (Ishitani et al., 2000; Kolukisaoglu et al., 2004; Cheong et al., 2007). Moreover, different combinations of CBLs and CIPKs genes have been identified in plants. Genes encoding CBLs or CIPKs are attributed only to the plant kingdom by computer analysis deciphering their function being restricted to plants (Kolukisaoglu et al., 2004). Differential expression of CBL genes indicating their role in abiotic stress response has been reported also (Kudla et al., 2010). So, different omics tools have been employed to understand plants' responses to abiotic stress conditions. It involves the integration of multiple omics. Systematic use of omics approaches such as metabolomics, transcriptomics, proteomics, and fluxome are means to connect the global data generated via phenomics has led to expansion toward stress biology for revealing the mechanisms the expression of agronomic traits. The comprehensive nature of multi-omic studies provides an entirely new avenue and future research programs that should be well planned to adapt accordingly. Different omics based tools and integrated approaches will provide glimpses of current scenarios and future perspectives to reveal the plant responses and adaptation to a specific abiotic stress.

CONCLUSION AND FUTURE PERSPECTIVE

Jasmonic acid plays a key role in plant regulatory and developmental processes. It has a potent role in alleviating abiotic stress conditions in plants. This review of literature is of the opinion that JA biosynthesis and signaling is dependent on Ca^{2+} levels, however, JA itself can modulate the Ca^{2+} transients. Ca^{2+} transients are also seen in varied abiotic stresses as an early response. In addition to this, one of the plant antioxidants GSH also has a pivotal role in abiotic stress response and tolerance. GSH tends to interact with JA and also facilitates the expression of genes involved in JA biosynthesis. Moreover, it has been seen that release of Ca^{2+} from internal plant stores is also mediated by GSH. Exogenous application of JA also results in an increase in cytosolic Ca^{2+} concentration. This all implies possible interactions between JA, Ca^{2+}, and GSH which helps in mitigating plant abiotic stress. This kind of study will help to adopt different approaches pertaining to abiotic stress tolerance. So, JA biosynthesis and signaling, calcium transients, and GSH seem to be co-related with each other. This review clearly suggests (1) Ca^{2+} signaling leads to JA formation then followed by GSH. (2) JA also facilitates the expression of GSH and vice versa. (3) JA and GSH both mediate the release of Ca^{2+} from internal plant stores. This type of interaction between the JA, Ca^{2+}, and GSH deciphers the novel mechanism of abiotic stress tolerance in plants. Detailed functional characterization of JA, Ca^{2+}, and GSH will help us to decipher the core mechanism and identification of various novel entities that could have an important role in this cross talk. It will further help us to understand plant stress biology and unravel the intricate molecular mechanisms that help plants to combat the effect of abiotic stresses which are otherwise a major threat to agricultural productivity. Therefore, it may be concluded that JA, Ca^{2+}, and GSH can enhance abiotic stress tolerance via initiating the possibly correlated signaling cascade.

AUTHOR CONTRIBUTIONS

SA and NG: conceptualization and compilation of data. SA, NG, and SQ: writing part. SA, NG, MM, MA, NA-S, and AAA: designing of figures and generation of table.

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Diethyl Aminoethyl Hexanoate Priming Ameliorates Seed Germination via Involvement in Hormonal Changes, Osmotic Adjustment, and Dehydrins Accumulation in White Clover Under Drought Stress

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Drought is a serious outcome of climate change reducing the productivity of forage species under arid and semi-arid conditions worldwide. Diethyl aminoethyl hexanoate (DA-6), a novel plant growth regulator, has proven to be involved in the amelioration of critical physiological functions in many agricultural crops under various abiotic stresses, but the role of the DA-6 in improving seed germination has never been investigated under drought stress. The present study was carried out to elucidate the impact of the DA-6 priming on seeds germination of white clover under drought stress. Results showed that seed priming with the DA-6 significantly mitigated the drought-induced reduction in germination percentage, germination vigor, germination index, seed vigor index, root length, shoot length, and fresh weight after 7 days of seed germination. The DA-6 significantly increased the endogenous indole-3-acetic acid, gibberellin, and cytokinin content with marked reduction in abscisic acid content in seedlings under drought stress. In addition, the DA-6 significantly accelerated starch catabolism by enhancing the activities of hydrolases contributing toward enhanced soluble sugars, proline content and ameliorated the antioxidant defense system to enhance the ability of reactive oxygen species scavenging under drought stress. Furthermore, exogenous DA-6 application significantly increased dehydrins accumulation and upregulated transcript levels of genes encoding dehydrins (*SK2, Y2SK, or DHNb*) during seeds germination under water deficient condition. These findings suggested that the DA-6 mediated seeds germination and drought tolerance associated with changes in endogenous phytohormones resulting in increased starch degradation, osmotic adjustment, antioxidants activity, and dehydrins accumulation during seed germination under water deficient condition.

Keywords: drought, antioxidants, osmotic adjustment, hormonal regulation, dehydrin accumulation, oxidative damage

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INTRODUCTION

Climate change in particular global warming results in significant increases in the frequency and severity of heat and drought stress worldwide. Drought has severely disrupted agricultural production and the coming drought will further extend these loses in the future (Farooq et al., 2009; Farooq et al., 2013). Seed germination is one of the most critical phases of life cycle as it provides the foundation for plant formation. However, water deficiency distracts normal physiological and metabolic activities during seeds germination leading to reduced germination rate and seedling growth (Bai et al., 2020). At this stage, carbohydrates, fats, and proteins metabolism supply energy for seeds germination and subsequent seedling growth (Jie and Kentian, 1993). On the other hand, different types of solutes including water soluble carbohydrates (i.e., sucrose, glucose, and fructose), sugar alcohols (mannitol), proteins (dehydrins), and free amino acids (proline) tend to accumulate, which play a vital function in osmotic adjustment (OA) for reducing water potential of cells during seed germination under water deficient condition (Cao et al., 2018). These solutes also perform function as antioxidants for reactive oxygen species (ROS) scavenging, membrane protection, and other defense mechanisms under drought stress (Bartels and Sunkar, 2005; Seki et al., 2007).

Hormonal regulation is one of the most important and necessary factors for seed germination. Phytohormonal metabolism, stability, and interaction such as abscisic acid (ABA), cytokinin (CTK), gibberellin (GA), and indole-3-acetic acid (IAA) are believed to be strongly associated with seeds germination and stress responses (Miransari and Smith, 2014; Llanes et al., 2016; Zhou et al., 2020). ABA is a primary stress hormone that induces seed dormancy and hinders seed germination. Moreover, ABA regulates stomatal closure to restrict transpiration and water loss in young seedlings under drought stress (Finkelstein et al., 2002). Previous studies reported that CTK could improve seed germination, early seedling morphogenesis, antioxidative defense system, and carbohydrates metabolism in plants leading to the enhanced ROS detoxification, photosynthesis, and OA under water limited condition (Nikolić et al., 2006; Merewitz et al., 2011, 2012). GA serves a plant growth regulator and facilitates in breaking seed dormancy, and IAA improves the emerging radicle growth during the process of seed germination (Vanstraelen and Benková, 2012; Shu et al., 2018). A previous study has shown that exogenous polyamines application ameliorated seed germination and growth of young seedling via alteration of endogenous phytohormones in cotton (*Gossypium hirsutum*) under drought stress (Yang et al., 2016). Moreover, seed priming with sodium chloride enhanced germination of white clover (*Trifolium repens*) seeds by regulating hormonal and carbohydrate metabolism under water stress (Cao et al., 2018).

Diethyl aminoethyl hexanoate (DA-6), a synthetic tertiary amine, is a novel plant growth regulator (PGR) that has been used extensively in many agricultural crops including cotton, pakchoi (*Brassica rapa* subsp. *chinensis*), soybean (*Glycine max*) and maize (*Zea mays*) in the last few years (Jiang et al., 2012; Qi et al., 2013; Liu et al., 2019). Previous studies have shown that the DA-6 exerted multiple advantageous effects such as improvement in seed germination, seedling establishment, photosynthetic rate, grain yield and biomass accumulation on various summer crops (Qi et al., 2013; Liu et al., 2019; Zhou et al., 2019). The DA-6 is well known for its progressive role in defense system of plants under various environmental stresses including cold stress and heavy metal toxicity (Fu et al., 2011; He et al., 2014; Li et al., 2018b). DA-6 can effectively mitigate salt stress through alleviation of oxidative damage in medicinal plants (Zhang et al., 2016). Foliar spray of DA-6 plays an important role in enhancing Cd-extraction efficiency and mitigating heavy metal stress (He et al., 2015). DA-6 application can enhance the growth of micro-algae (*Haematococcus pluvialis*) and increase the quality and quantity of lipids for biodiesel production (Ding et al., 2019). Moreover, combined application of DA-6 with ethephon has also shown its promising effects on various physiological processes such as enhanced mechanical strength of stalk vascular bundles and improved grain yield in maize plants (Xu et al., 2017). Although, numerous studies have described beneficial effects of DA-6 in physiological and metabolic mechanisms under unfavorable environmental conditions, the possible ameliorative effect of DA-6 on physiological, metabolic, and molecular mechanisms during seeds germination has not been discussed under drought stress so far.

White clover is an imperative forage legume cultivated worldwide because of its high crude protein content and excellent nitrogen-fixation potential, hence contributing toward animal nutrition and soil fertility. However, due to its shallow tap-root system and inefficient transpiration control, it is severely affected by drought stress (Annicchiarico and Piano, 2004). Therefore, understanding and amelioration of drought tolerance in white clover are indispensable to enhance legume production, forage quality and quantity, and animal performance all over the world. The aims of this current study were (1) to elucidate the impact of seeds priming with DA-6 on germination characteristics and (2) to disclose DA-6-mediated drought tolerance linking with hormone regulation, osmotic adjustment, starch metabolism, antioxidant defense, and dehydrin accumulation during seeds germination under drought stress.

MATERIALS AND METHODS

Plant Materials and Treatments

White clover seeds (cultivar "Ladino") were utilized. Seeds were sterilized with 75% ethanol and washed with deionized water for 5 min before being sown. For seeds priming, two set of treatments were used for this study. One set of seeds was drenched in deionized water as control for 3 h at 20°C while the other set of seeds was kept in deionized water for 1 h and later drenched in 2 mM DA-6 solution for 2 h at 20°C. After this, seeds priming with or without DA-6 were placed in plastic containers having three layers of filter papers under deionized water or 17% (w/v) PEG 6,000 (—0.3 Mpa) for drought stress. The plastic containers were incubated in a plant growth chamber with 700 μmol m^{-2} s^{-1} photosynthetically active radiation and average temperature of 23/19°C (day/night) for one week. Each

treatment [well-watered control (C), Control + DA-6 (C + D), PEG (P), or PEG + DA-6 (P + D)] included 6 containers (replications) with 100 seeds for each container and arranged in completely randomized design. Seedlings were sampled at 3 and 7 days after germination to examine physiological and biochemical parameters, and germination parameters, fresh weight (FW), shoot length (SL), and root length (RL) were measured at 7 days.

Measurement of Seed Germination Parameters

Germination vigor (GV) or germination percentage (GP) was calculated at 3 and 7 days of germination, respectively. GV = Number of seeds germinated on 3rd day/Total number of seeds initially sown × 100%; GP = Number of seeds germinated on 7th day/Total number of seeds initially sown × 100% (Li et al., 2007; Thabet et al., 2018). Mean germination time (MGT) and germination index (GI) were evaluated using following formula: MGT = (Ti)(Ni)/ (Ni), where (Ni) is the number of newly germinated seeds in time (Ti), and GI = (Gt)/(Tt), where (Gt) is the number of seeds germinated at "t" day after sowing, while (Tt) stands for time corresponding to (Gt) in days (Zhang et al., 2007). After 7 days of germination, seedling RL, SL, FW, and seed vigor index (VI) were determined. VI was estimated by using GV and seedlings FW, respectively (Li et al., 2014).

Determination of Endogenous Phytohormones

To measure endogenous IAA, GA and ABA, fresh seedlings (0.4 g) were mechanically ground with 1% glacial acetic acid and 3 ml of methanol plus isopropanol solution (*1:4. v/v*). The mixture was kept in a refrigerator at 4°C for 1 h in dark condition, followed by centrifugation at 8,000 g for 15 min at 4°C. The 2 ml of supernatant was collected, dried, and subsequently dissolved in methanol (300 μl). Afterward, the reaction mixture was exposed to a filtration process by passing it through (0.22 μm) poly tetra fluoroethylene filter (Müller and Munné-Bosch, 2011). Endogenous IAA, GA and ABA concentration were noticed by using Waters Acquity UPLCSCIEX Se-lex ION Triple Quad 5500 System mass spectrometer (Waters, Milford, MA, United States). Samples (5 μl) were inserted into a hoop and loaded onto an Acquity UPLC BEH C18 column (1.7 μm, 50 × 2.1 mm; Waters, Wexford, Ireland) at 40°C. The CTK content was measured by using enzyme linked immunosorbent Assay (ELISA) Kit purchased from *Beijing Fang Cheng Biological Technology Co., Ltd., Beijing, China.*

Measurement of Carbohydrate Metabolism, Proline and, Osmotic Adjustment

For the estimation of water-soluble carbohydrates (WSC), procedure was conducted according to the protocols of Fu and Dernoeden (2009). 0.5 g of seedlings were randomly sampled and dried in an electric oven. 0.05 g of dried tissue samples were kept in a centrifuge tube (10 ml) and then 80% C_2H_5OH (6 ml) was supplemented. Afterward, mixture was heated at a high temperature in a water bath (80°C) for 30 min, cooled rapidly and subsequently centrifuged at 12,000 g for 10 min. The supernatant was collected and utilized for the measurement of WSC, while the residue left was used for the analysis of starch content, respectively. The Activities of amylase enzymes were estimated by following the method described by Tárrago and Nicolás (1976) and Kishorekumar et al. (2007). 0.1 g of fresh seedlings were mechanically ground with 2 ml of deionized water at 4°C. The homogenate was centrifuged at 12,000 g for 25 min at 4°C. The obtained supernatant was utilized for determining α- and β-amylase activities. The 1 ml of 3 mM calcium chloride and 1 ml of supernatant were mixed and kept at 70°C for 5 min. The reaction solution containing 0.1 mM citrate buffer, 0.7 ml of hot enzyme extract and 2% soluble starch solution was placed in an electric oven at 30°C for 6 min and later kept at 50°C for 5 min. The α-amylase activity was measured by using a spectrophotometer (540 nm). The β-amylase activity was determined after the inactivation of α-amylase at pH 3.4. The reaction mixture comprising of 0.1 mM citrate buffer (2 ml), EDTA treated enzyme extract (0.7 ml), and 2% soluble starch solution was kept in an electric oven at 30°C for 5 min after the addition of starch. The β-amylase activity was then measured in similar way as illustrated for α-amylase. Free proline content, seedlings (0.1 g) were taken and homogenized in 35% sulphosalicylic acid (10 ml) to make a fine paste (Bates et al., 1973). Afterward, the homogenate was followed by centrifugation for 10 min and supernatant (2 ml) was mixed with acid ninhydrin solution (2 ml) and glacial acetic acid (2 ml). The reaction mixture was kept in a water bath for 1 h and the reaction was ceased by placing the mixture in ice-bath. After this, toluene (C_7H_8) was added, and the absorbance value was noted at 520 nm. To measure the osmotic potential (OP), seedlings were randomly sampled and instantly immersed in deionized water for 8 h at 4°C. Later, seedlings were thawed for 25 min at 4°C to obtain the cell sap for the estimation of the osmolarity using an osmometer (Wescor, Logan, UT, United States). The OP was converted based on the following formula: $MPa = -c \times 2.58 \times 10^{-3}$ (Blum, 1989).

Estimation of Enzymatic Antioxidants Activities and Oxidative Injury

For enzyme extraction, samples were mechanically ground with 50 μM cold phosphate buffer (4 ml, pH 7.8) comprising of 1% (*w/v*) polyvinylpyrrolidone. Afterward, the homogenate was centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected and utilized for the analysis of antioxidant enzyme activities, hydrogen peroxide (H_2O_2) content, superoxide radical ($O_2.^-$), and malondialdehyde (MDA) content. Superoxide dismutase (SOD) activity was estimated spectrophotometrically by noting the declining rate of p-nitroblue tetrazolium chloride at an absorbance value of 560 nm (Giannopolitis and Ries, 1977). Other antioxidant enzyme activities including ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) were also spectrophotometrically estimated by recording the changes in absorbance values at 290, 470, and 240 nm respectively (Chance and Maehly, 1955; Nakano and Asada, 1981). Protein content was measured using the procedure described by Bradford (Bradford, 1976). MDA content was determined following the

protocol of Dhindsa et al. (1981) with minor modifications. 0.5 ml of enzyme extract and 1 ml of reaction solution consisting of 0.5% (*w/v*) thiobarbituric acid and 20% (*w/v*) trichloroacetic acid (TCA) were supplemented and mixed. The solution was kept in a boiling water bath at 95°C for 15 min and cooled rapidly in an ice water bath. The homogenate was centrifuged at 8,000 g for 10 min. Supernatant was collected and absorbance value was recorded at 532, 600, and 450 nm. The rate of O_2^- formation was estimated using sulfanilamide ($C_6H_8N_2O_2S$) procedure (Elstner, 1976) and the absorbance value was recorded at 530 nm. H_2O_2 content was measured by following the potassium iodide method. The product of oxidation was recorded at 390 nm (Velikova et al., 2000).

Gene Expression Analysis

To detect transcript levels of genes, real-time quantitative polymerase chain reaction *(qRT-PCR)* was used. For total RNA extraction, fresh seedlings (0.1 g) were extracted by using RNeasy Mini Kit *(Qiagen)* according to the manufacturer's instructions. After this, a revert Aid First Stand cDNA Synthesis Kit *(Fermentas)* was used for the reverse transcription of RNA to cDNA. Total RNA (500 ng) was utilized for each cDNA synthesis. The cDNA was subjected to *qPCR* using primers of antioxidant enzyme genes *(FeSOD, MnSOD, Cu/ZnSOD, POD, CAT,* and *APX*; Li et al., 2016b) and dehydrin genes (*SK2, Y2SK*, and *DHNb*) (Li et al., 2016a; **Table 1**). The *PCR* conditions for all above mentioned genes were as follows: 5 min at 94°C, denaturation at 95°C for 30 s (40 repeats), annealing at 56–66°C for 30 s, and extension at 72°C for 30 s (**Table 1**). The transcript level of genes encoding antioxidant enzymes and dehydrins was calculated using the formula $2^{\Delta\Delta Ct}$ illustrated by Livak and Schmittgen (2001).

Western Blot Analysis

For western blot analysis, fresh seedlings (0.5 g) were extracted in cold 100 mM Tris-HCl buffer *(pH 8.0)* to get the soluble proteins. The obtained soluble proteins were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected and heated in a water bath for 10 min. The supernatant was centrifuged again at 12,000 g and the sediment (equivalent to 30 μg proteins) was utilized for the estimation of dehydrins. To transfer the SDS-PAGE *(12%)* to PVDF membranes, *the Bio-Rad mini protean transblotter* was used. After 2 h of transfer at 65 V and 4°C, the membranes were congested in TRIS-buffered saline for 1 h (Khedr et al., 2003; Vaseva et al., 2011). Later, the TRIS-buffered saline was withdrawn and the PVDF membranes were rinsed shortly in TTBS for 3 times each (5 min). The rinsed PVDF membranes were incubated in *rabbit anti-dehydrins dilution (1:1,000)* for 1 h. Afterward, the membranes were washed in TTBS again for 3 times (5 min) and incubated in *goat anti-rabbit lgG antibody (1: 2,000)* for 1 h. After rinsing in TTBS (20 min), the dehydrins bands were observed by using *TMB* reagent kit *(Sigma, Kawasaki, Japan)* (Close et al., 1993).

Statistical Analysis

All data was evaluated using statistix 8.1 (version, 8.1. Statistix, Tallahassee, FL, United States). Significant differences among different treatments were estimated with one-way ANOVA in combination with LSD test at the 5% probability level ($p < 0.05$).

RESULTS

Growth and Germination Parameters

The GP, GV, GI, and SVI greatly declined as the result of exposure to drought stress (**Figures 1A–C,E**). In contrast to drought-stressed seedlings, an optimal dose (2 mM) of DA-6 significantly ameliorated various germination parameters (GP, MGT, GV, GI, and SVI), however higher doses (5 and 10 mM) gradually minimized the advantageous effects of DA-6 on germination during water deficient condition (**Figures 1A–E**). Under water stress, seeds priming with 2 mM DA-6 exhibited 8.66% higher GP than seeds primed with distilled water (**Figure 1A**). Phenotypic changes showed that the DA-6 application significantly alleviated drought-induced inhibition of seeds germination (**Figure 2A**). Under normal condition, seed priming with various doses of DA-6 showed that 2 mM concentration significantly increased seedling FW, whereas no such difference was observed in SL and RL (**Figure 2B**). Under drought stress, seedling FW, SL, and RL significantly decreased in contrast to control. The DA-6 (2 mM) priming significantly mitigated the adverse effects of drought stress and resulted in higher seedling FW, RL, and SL in DA-6 primed seedlings as compared to the seedlings without DA-6 application under drought stress (**Figures 2B–D**).

TABLE 1 | Primer sequences and their corresponding GeneBank accession numbers of the analyzed genes.

Target gene	Accession No.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Tm (°C)
FeSOD	KP202173	ACACGATTTCTCAGGGTTACGAC	GCGGCCAAGACTATCAGTTCCAT	58
MnSOD	JQ321598.1	TAAGGGAACCTACCCGATAACT	CCAGGACCAAACGTCACCAAAG	66
Cu/ZnSOD	JQ321597.1	AACTGTGTACCACGAGGACTTC	AGACTAACAGGTGCTAACAACG	58
POD	JQ321606.1	CACTTGGTTTAGTTTTGTCGCC	AACACGGTCTTGTCTGCTACG	64
CAT	JQ321596.1	AACAGGACGGGAATAGCACG	ACCAGGTTCAGACACGGAGACA	58
APX	JQ321599.1	TAAAGATAGTCAACCCACCTCAACA	ACCAGTCTTGGGAAACAACGTA	58
SK2	GU443960.1	TGGAACAGGAGTAACAACAGGTGGA	TGCCAGTTGAGAAAGTTGAGGTTGT	58
Y2SK	GU443965.1	GTGCGATGGAGATGCTGTTTG	CCTAATCCAACTTCAGGTTCAGC	60
DHNb	GU443960.1	TCCAGTCATCCAGCCTGTTG	CCAGCCACAACACTTGTCA	60
β-*Actin*	JF968419	TTACAATGAATTGCGTGTTG	AGAGGACAGCCTGAATGG	58

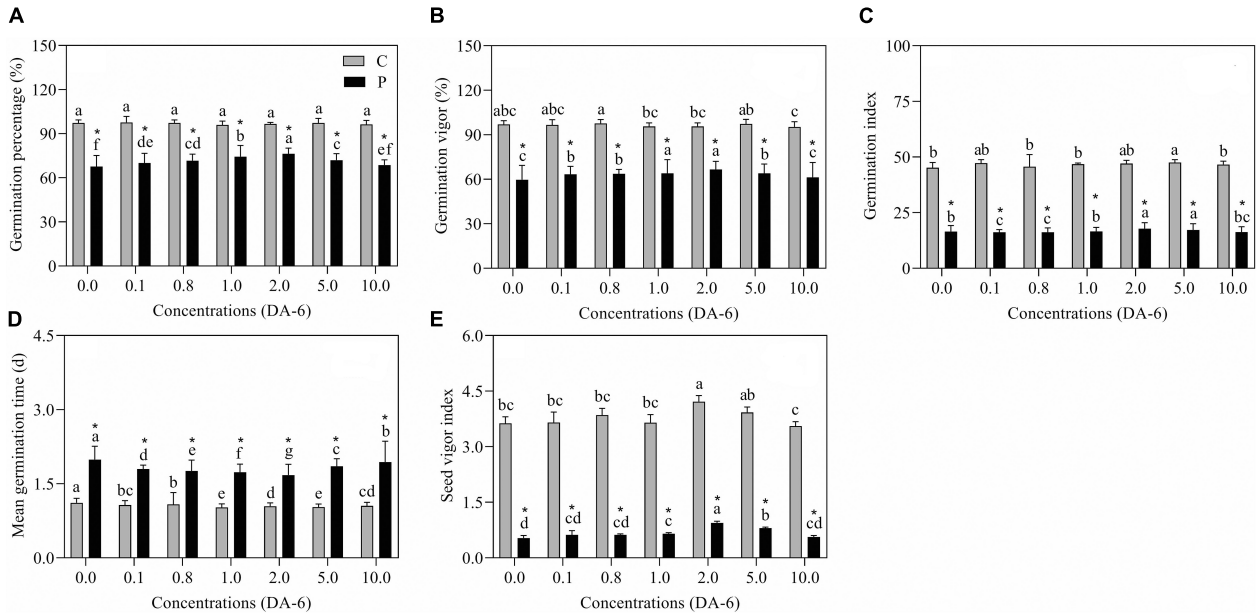

FIGURE 1 | Effect of seed priming with different concentrations of DA-6 on **(A)** germination percentage (GP), **(B)** germination vigor (GV), **(C)** germination index (GI), **(D)** mean germination time (MGT) and **(E)** seed vigor index (SVI) during 7 days of germination in white clover under well-watered condition and drought stress. Different letters represent significant differences among treatments with the application of different concentrations of DA-6 under well-watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ±SE of mean ($n = 6$), whereas *indicates significant difference between well-watered condition and drought stress under one particular concentration of DA-6.

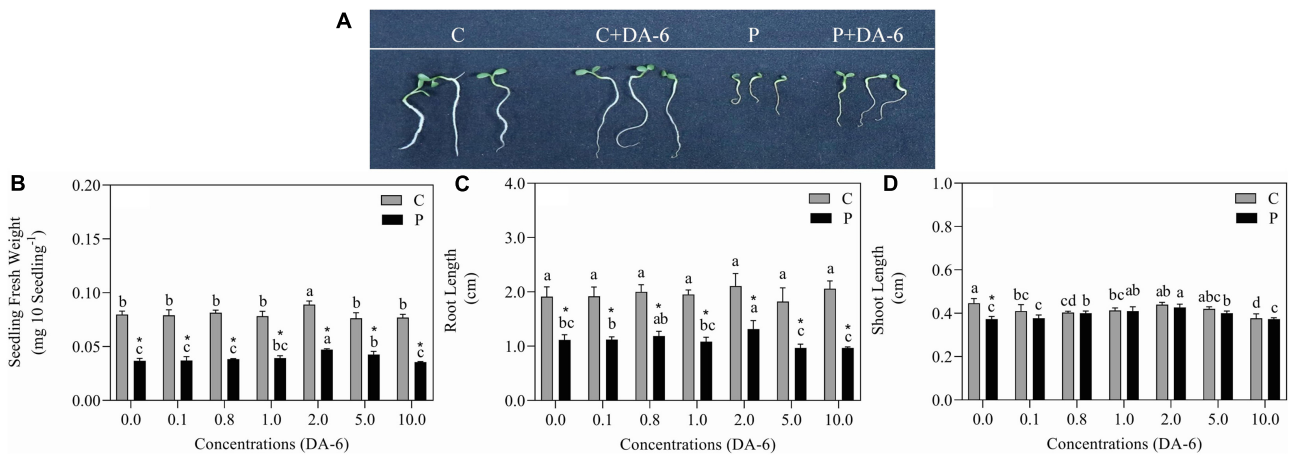

FIGURE 2 | Effect of seed priming with different concentrations of DA-6 on **(A)** phenotypic change, **(B)** seedling fresh weight (FW), **(C)** root length (RL), and **(D)** shoot length (SL) after 7 days of germination in white clover under well-watered condition and drought stress. Different letters represent significant differences among treatments with the application of different concentrations of DA-6 under well- watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$), whereas * indicates significant difference between well-watered condition and drought stress under one particular concentration of DA-6.

Effect of DA-6 on Endogenous Hormones

The content of GA, CTK, IAA, and ABA were significantly influenced by seeds priming with DA-6 in seedlings during germination (3 and 7 day) under well-watered and drought conditions (**Figures 3A–D**). The drought stress significantly enhanced IAA, GA, and ABA content when compared to control, whereas the CTK content was significantly reduced in response to drought. Seeds priming with DA-6 (2 mM) significantly enhanced IAA, GA, and CTK content under normal and drought conditions (**Figures 3A–D**). The drought stress markedly increased the endogenous ABA content in seedlings at 3 and 7 days of germination, but DA-6 priming significantly inhibited ABA accumulation under normal and water stress conditions during seeds germination (**Figure 3D**).

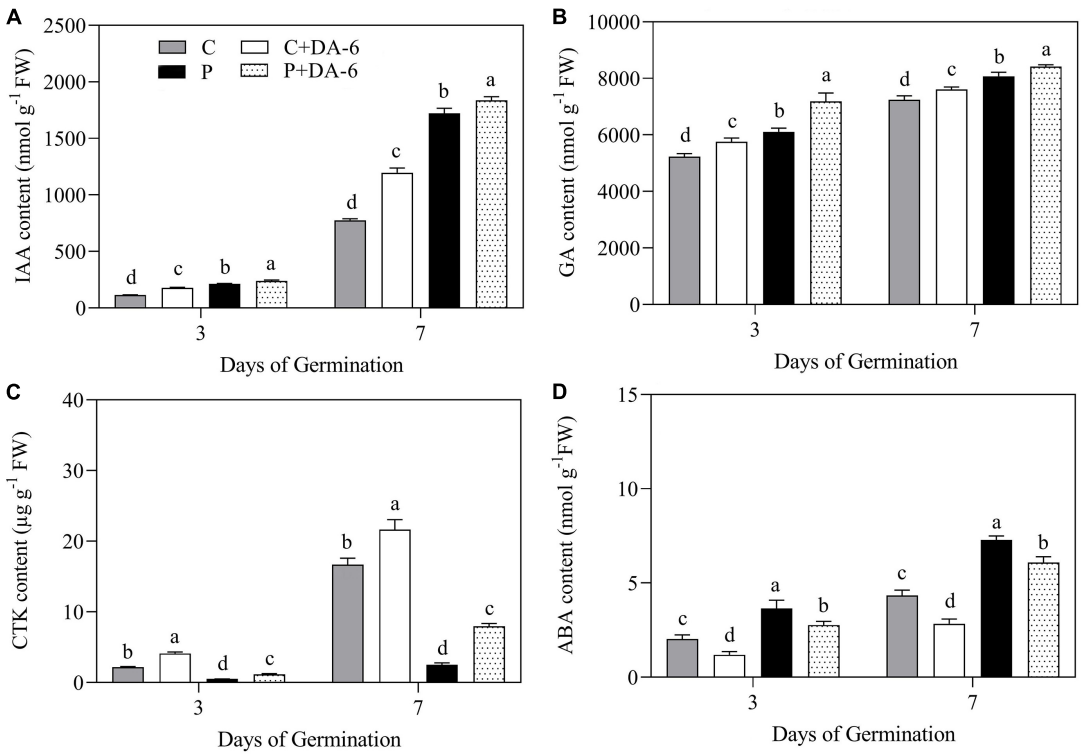

FIGURE 3 | Effects of seeds priming with water or DA-6 (2 mM) on **(A)** indole-3-acetic acid (IAA) content, **(B)** gibberellin (GA) content, **(C)** cytokinin (CTK) content, and **(D)** Abscisic acid (ABA) content after 3 and 7 day of germination in white clover under well-watered condition and drought stress. Different letters above different treatments represent significant differences at a specific time point under well-watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

Effect of DA-6 on Starch Metabolism and Osmolyte Accumulation

The exogenous DA-6 application significantly improved α-amylase activity under well-watered and drought conditions (**Figure 4A**). Moreover, seeds priming with DA-6 significantly ameliorated drought-induced declines in β-amylase and total amylase activities at 3 and 7 days of germination under water deficient condition and also significantly enhanced β-amylase and total amylase activities at 3 d of germination under normal condition (**Figures 4B,C**). Drought stress significantly restrained amylolysis, resulting in higher starch content in drought-stressed seedlings compared to seedlings grown under normal condition (**Figure 5A**). However, DA-6 primed seedlings showed significantly lower starch content than the seedlings without DA-6 priming under normal and drought conditions (**Figure 5A**). Drought stress significantly increased WSC accumulation in seedlings with and without DA-6 priming (**Figure 5B**). The DA-6 priming substantially decreased WSC content in seedlings under normal conditions, but increased WSC content under drought stress (**Figure 5B**). The DA-6 priming further enhanced the WSC content by 34.90% than untreated seedlings after 7th days of drought interval (**Figure 5B**). The proline content and OP were substantially influenced by seeds soaking with DA-6 under well-watered conditions (**Figures 5C,D**). The DA-6-primed seedlings exhibited 27.43 or 26.75% higher free proline content and 5.58 or 6.12% reduced OP than non-treated seedlings at 3 or 7 days of drought stress (**Figures 5C,D**).

Effect of DA-6 on Oxidative Damage and Antioxidant Enzymes Activity

Drought stress caused oxidative damage to seedlings, as indicated by elevated $O_2^{\bullet-}$, H_2O_2 and MDA content (**Figure 6**). At 7th days of water stress, a 42.04, 33.28 or 49.48% increase in O_2^{-}, H_2O_2, or MDA content was observed in drought-stressed seedlings without DA-6 priming as compared to that in control. However, DA-6 pretreatment significantly reduced the oxidative damage through lowering the O_2^{-}, H_2O_2 and MDA content by 17.34, 24.66 and 5% on 7th days of water stress (**Figure 6**). Except CAT, the activity of all other enzymatic antioxidants (SOD, POD, APX) significantly decreased in drought-stressed seedlings without DA-6 priming in contrast to control (**Figures 7A–D**). DA-6-pretreated seedlings exhibited significantly higher activities of all enzymatic antioxidants than non-treated seedlings on 3rd and 7th days of water stress. In contrast to drought-stressed seedlings without DA-6 priming, the drought-stressed seedlings with the DA-6 priming demonstrated elevated enzyme activities by 37.69% for SOD (**Figure 7A**), 18.83% for POD (**Figure 7B**), 15.18% for CAT (**Figure 7C**), and 17.48% for APX (**Figure 7D**), respectively at final day of stress. During germination (3 day), the DA-6 priming did not significantly affect

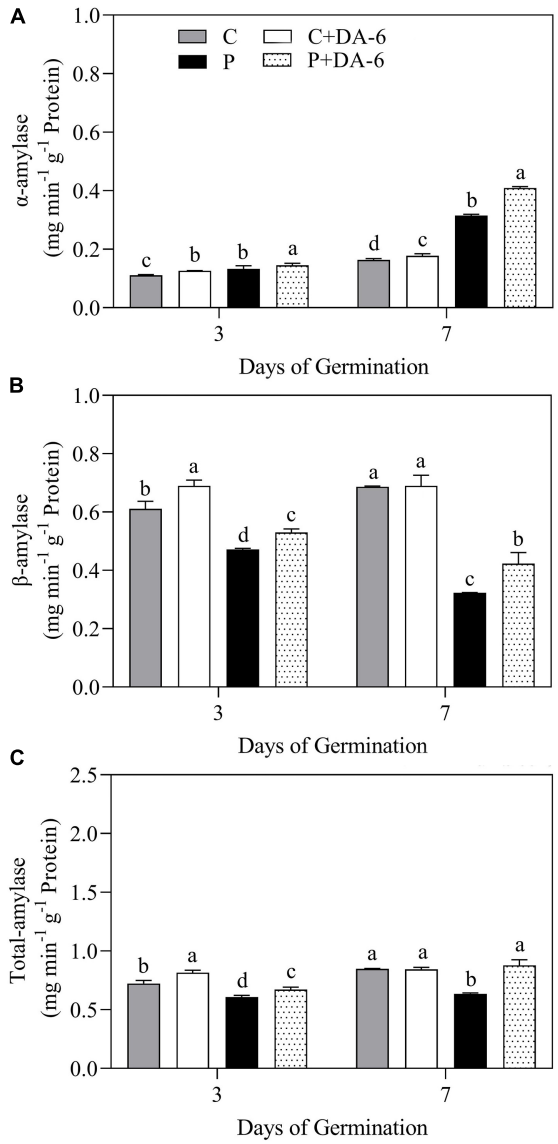

FIGURE 4 | Effect of seed priming with distilled water or DA-6 (2 mM) on **(A)** α-amylase activity, **(B)** β-amylase activity, and **(C)** total amylase activity after 3 and 7 day of germination in white clover under well-watered condition and drought stress. Different letters above different treatments represent significant differences at a specific time point under well-watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

the enzymatic antioxidants encoding genes (*FeSOD, MnSOD, Cu/ZnSOD, POD, CAT, APX*) under well-watered conditions (**Figure 7E**). Except for *MnSOD* and *Cu/ZnSOD*, the drought stress significantly increased expression levels of *FeSOD, POD, CAT* and *APX* genes (**Figure 7E**). Seeds priming with DA-6 significantly increased transcript levels of *MnSOD, Cu/ZnSOD*, *POD*, and *CAT* in seedlings under drought stress, but the DA-6 did not have significant effect on *FeSOD* expression under normal and drought conditions (**Figure 7E**). In contrast to control seedlings, the drought-stressed seedlings with DA-6 priming showed significantly higher *APX*, but the expression level of this gene was lower than drought-stressed seedlings without DA-6 priming (**Figure 7E**). The expression level of *MnSOD, Cu/ZnSOD, POD,* or *CAT* was 2.35, 4.01, 2.71, or 1.55 times higher in drought-stressed seedlings with DA-6 priming than that in drought-stressed seedlings without DA-6 priming (**Figure 7E**).

Effect of DA-6 on Dehydrins Accumulation and Expression

Seeds pretreatment with DA-6 induced dehydrins accumulation (65 KDa) during seeds germination under well-watered and drought conditions (**Figure 8A**). Under drought stress condition, seedlings primed with DA-6 showed 1.81 times higher dehydrins accumulation than the seedlings without DA-6 priming (**Figure 8A**). Under well-watered condition, the DA-6 showed no significant effect on expression levels of three dehydrin genes *SK2, Y2SK,* and *DHNb* (**Figure 8B**). The drought stress significantly up-regulated the expression levels of *Y2SK and DHNb*, while transcript level of *SK2* remained unaffected (**Figure 8B**). The seeds priming with DA-6 demonstrated considerably higher expression levels of all dehydrin genes (*SK2, Y2SK, and DHNb*) when compared with untreated seeds under drought stress. The expression level of *SK2, Y2SK,* or *DHNb* in DA-6 treated seedlings was 3.11, 32.87, or 3.72 times higher in contrast to untreated seedlings under drought stress (**Figure 8B**). **Figure 9** showed a comprehensive schematic diagram representing ameliorative effect of DA-6 in white clover seeds under drought stress.

DISCUSSION

Drought stress significantly affects the growth and development of plants via disturbance in physiological and metabolic processes, thus restricting seeds germination and the yield of various crops (Li et al., 2014; Abid et al., 2018). Germination is among the most critical physiological processes in plants commonly regulated by phytohormones and PGRs, i.e., abscisic acid (Finkelstein and Lynch, 2000), nitric oxide (Beligni and Lamattina, 2000), melatonin (Bai et al., 2020), and DA-6 (Zhou et al., 2019). As expected, drought stress significantly decreased GP, GV, GI, SVI, RL, SL, and FW after 7 days of germination in white clover seeds (**Figures 1**, **2**). These results were in accordance with the study of Li et al. (2014) about white clover seeds germination under water deficient conditions. Previous studies have shown that seed priming with phytohormones or PGRs is an inexpensive and useful technique to ameliorate germination and early growth and establishment of seedlings during adverse environmental conditions (Farooq et al., 2013; Biju et al., 2017). In this study, seed priming with DA-6 (2 mM) significantly increased seed GP and shortened MGT under drought stress. Moreover, DA-6 (2 mM) also exerted distinct beneficial effects by mitigating drought-induced decreases in GV, GI, SVI, FW, RL, and SL of white clover seedlings (**Figures 1**, **2**). These results are consistent with study of Cao et al. (2018) about exogenous NaCl pretreatment during germination in white clover seeds under drought stress. The present findings indicate that seed priming with DA-6 (2 mM) could be an effective strategy

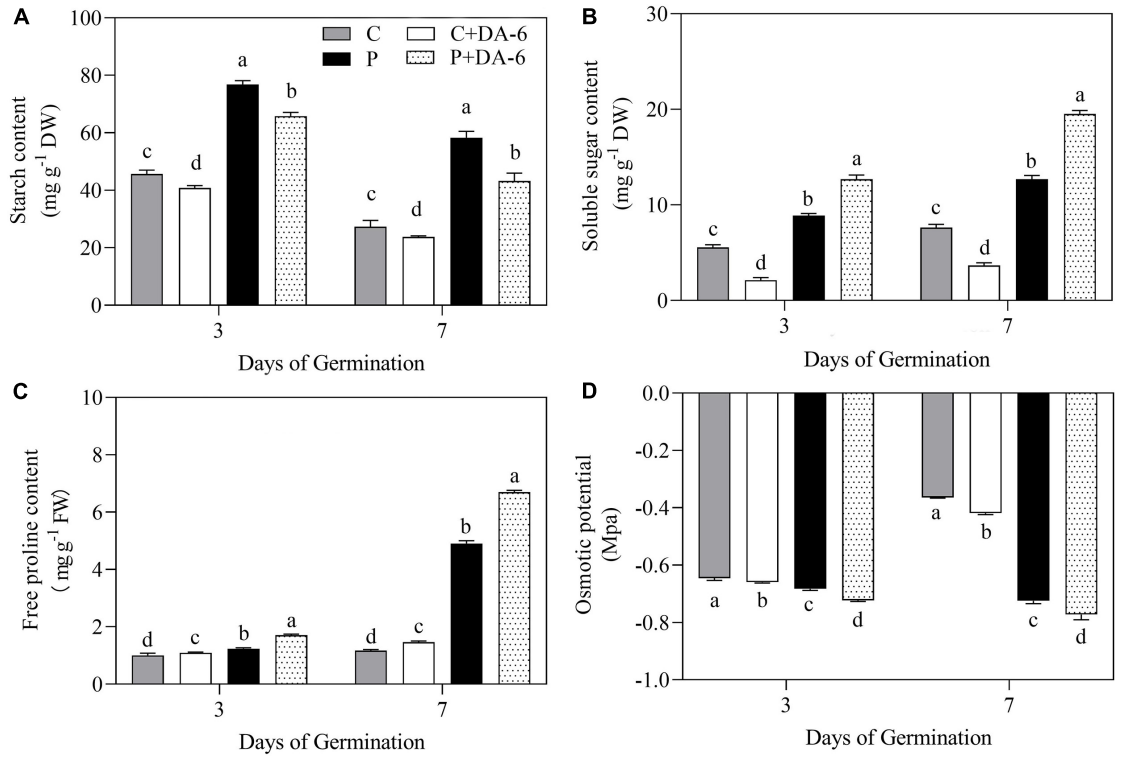

FIGURE 5 | Effect of seed priming with distilled water or DA-6 (2 mM) on **(A)** starch content, **(B)** soluble sugars content, **(C)** free proline content, and **(D)** osmotic potential after 3 and 7 days of germination in white clover under well-watered condition and drought stress. Different letters above different treatments represent significant differences at a specific time point under well- watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

to ameliorate tolerance in white clover under water deficient conditions. An earlier study found that exogenous application of DA-6 enhanced seed germination and seedling establishment by regulating fatty acids and carbohydrates metabolism in aged soyabean seeds (Zhou et al., 2019).

Various plant hormones particularly GA, IAA, CTK, and ABA perform imperative functions in seed germination (Guilfoyle et al., 2015; Liu and Hou, 2018). During seed germination, the developing embryo obtains energy from the endosperm which is composed of starch and aleurone layer (Jones and Jacobsen, 1991; Bosnes et al., 1992). The GA acts as a stimulating agent in the synthesis and production of various hydrolases including proteases, α-amylase, and β-amylase contributing to amylolysis and protein metabolism during seed germination (Appleford and Lenton, 1997; Yamaguchi, 2008). The IAA is present at radicle tip and performs vital functions in the radicle growth of emerging seedlings (Liu et al., 2007a,b). The CTK regulates a wide range of physiological processes during seed germination, especially for controlling cell division and plumule growth (Chiwocha et al., 2005; Nikolić et al., 2006; Riefler et al., 2006). Previous studies have reported that the CTK can improve seed germination by mitigating the adverse effects of heavy metals, salt, and drought stress (Khan and Ungar, 1997; Atici et al., 2005; Eisvand et al., 2010). As different from positive effects of GA, IAA, and CTK on seed germination, ABA is a primary hormone that promotes seed dormancy under drought stress (Davies and Jones, 1991). ABA exerts its negative effects on seed germination by weakening the endosperm, restricting radicle expansion, and stimulating transcriptional factors that positively regulates seed dormancy (Graeber et al., 2010; Luo et al., 2021). The current study reported that white clover seeds and seedlings demonstrated significant increase in endogenous IAA, GA, or ABA content with marked reduction in CTK content under drought stress (**Figure 3**). Drought induced increase in endogenous IAA, GA or ABA has been noticed in white clover (Cao et al., 2018). However, DA-6 seed priming significantly enhanced endogenous IAA, GA, and CTK content under water deficient condition (**Figure 3**), indicating that DA-6-regulated amelioration in germination and seedling growth related to the increases in IAA, GA, and CTK levels under water stress. Furthermore, seeds pretreatment with DA-6 significantly mitigated the drought-induced ABA accumulation, which could be one of the core reasons for alleviating drought-induced white clover seeds dormancy. Our results are consistent to a previous study where exogenous application of DA-6 improved the growth of soybean and maize seedlings by mediating photosynthesis and hormonal metabolism under normal conditions (Qi et al., 2013). These findings imply that improvement of endogenous hormonal levels (IAA, GA, CTK) by DA-6 seed priming might relate to efficient starch catabolism, osmotic adjustment and antioxidant defense system conferring enhanced tolerance in white clover under drought stress.

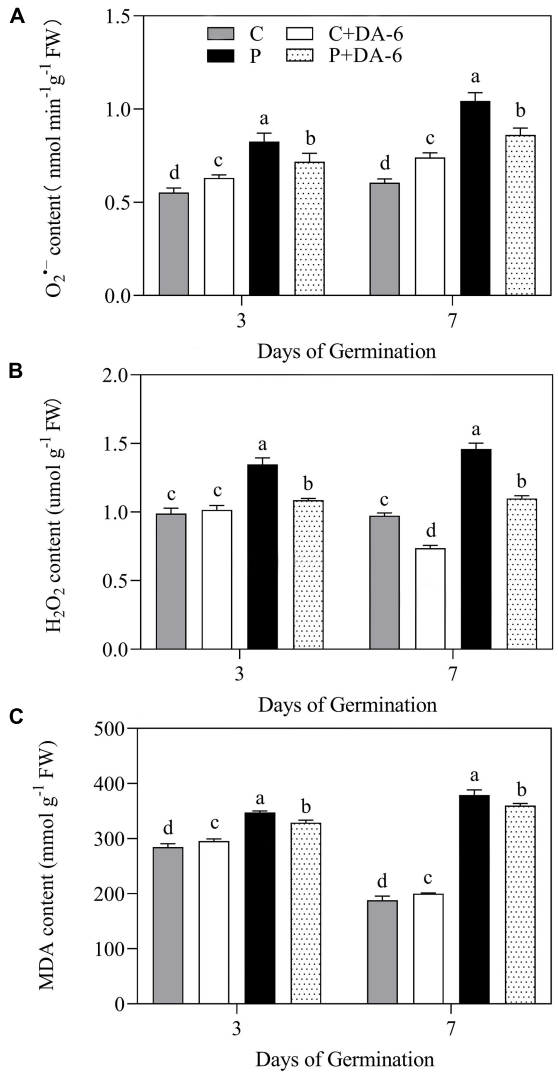

FIGURE 6 | Effect of seed priming with distilled water or DA-6 (2mM) on **(A)** O_2^- content, **(B)** H_2O_2 content, and **(C)** MDA content after 3 and 7 days of germination in white clover under well-watered condition and drought stress. Different letters above different treatments represent significant differences at a specific time point under well-watered condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

Starch catabolism governed by α-amylase and β-amylase is the chief physiological process in seed germination as it supplies soluble sugars (energy products) for seeds germination and growth (Appleford and Lenton, 1997; Yamaguchi, 2008). The metabolites of starch degradation such as soluble sugars are crucial for osmotic adjustment and energy supply when seeds are exposed to ionic and osmotic injury (Li et al., 2014). It has been reported that osmotic stresses such as salinity and water shortage diminished seeds germination due to the obstruction of starch catabolism (Kim et al., 2006; Li et al., 2014). During seed germination, the α-amylase is produced *de novo* in a special layer known as aleurone. In contrast, β-amylase exists in dry form in seeds as a precursor lacking catalytic activity, however, it is activated in consequence of peptide sequence cleavage at the C-terminal portion during the process of germination (Catusse et al., 2012). The present study demonstrated that water deficiency significantly inhibited starch degradation in seeds (**Figure 5**), but seeds primed with DA-6 considerably alleviated drought-induced decline in the process of starch catabolism through enhancing the catalytic activities of α-amylase and β-amylase under drought stress. These results inferred that DA-6-regulated stress tolerance might be associated with enhanced starch catabolism via significant improvement of hydrolases (α-amylase and β-amylase) in seeds of white clover during germination. Soluble sugars are the core constituents of cell organelles and perform important functions in osmoregulation and cellular metabolism. The current study revealed that the seeds priming with DA-6 further increased the drought-induced WSC and free proline accumulation in seedlings under water deficient conditions. Our findings are in accordance with the study of Jiang et al. (2016) about maize seedlings under saline conditions. Proline is an important osmolyte and ROS scavenger that can mitigate stress injury by lowering water potential and improving antioxidant capacity under unfavorable environmental conditions (Rathinasabapathi, 2000; Hayat et al., 2012). Our current results are parallel to the study of Zhang et al. (2016) who reported that application of DA-6 enhanced the endogenous WSC and proline content in *Cassia obtusifolia* L. resulting in improved tolerance under saline conditions. Our findings suggested that DA-6 could minimize the drought-induced damage associated with starch catabolism, WSC and proline accumulation contributing to ameliorated energy supply, OA, and osmo-protection during white clover seeds germination under water deficient condition.

Abiotic stresses disrupt the balance between production and detoxification of ROS, resulting in massive ROS accumulation conferring increased lipid peroxidation under hazardous circumstances (Li et al., 2015; Sohag et al., 2020; Luo et al., 2021). Plants contain a natural defense system comprising of enzymatic as well as non-enzymatic antioxidants to protect them from oxidative injury under unfavorable environmental conditions (Hasanuzzaman et al., 2019). SOD is the primary enzyme for the dismutation of O_2^- into H_2O_2 and molecular oxygen (O_2) (Luis et al., 2018). This liberated H_2O_2 is further detoxified by the catalytic action of POD, CAT, or ASA-GSH cycle (Fotopoulos et al., 2010; Hasanuzzaman et al., 2019). Previous studies have reported that DA-6 application improved antioxidant activities to alleviate oxidative damage in strawberry (*Fragaria* × *ananassa*) seedlings under cold stress (Fu et al., 2011), in *Cassia obtusifolia* under salt stress (Zhang et al., 2016), and in maize under low temperature stress (Zhang et al., 2020). We observed that seeds exposed to water deficient condition significantly increased O_2^- and H_2O_2 content, however, DA-6 priming greatly alleviated the drought-induced increases in O_2^- and H_2O_2 accumulation (**Figure 6**) associated with increases in the activities of antioxidant enzymes (SOD, POD, CAT, or APX) and the expression levels of genes encoding antioxidant enzymes (*FeSOD, MnSOD, Cu/ZnSOD, POD,* or *CAT*) during seed germination under water stress (**Figure 7**). These findings inferred that DA-6

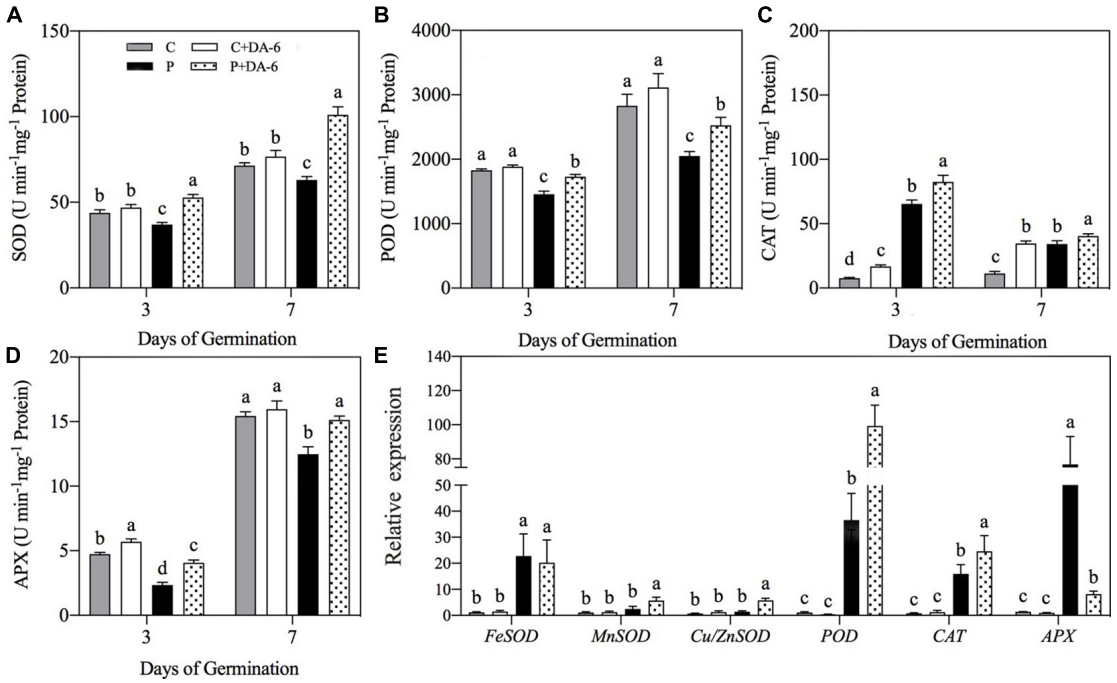

FIGURE 7 | Effect of seed priming with distilled water or DA-6 (2mM) on **(A)** superoxide dismutase (SOD), **(B)** peroxidase (POD), **(C)** catalase (CAT), and **(D)** ascorbate peroxidase (APX) activities after 3 and 7 day of germination in white clover, whereas **(E)** shows expression level of antioxidant encoding genes after 3 days under well-watered condition and drought stress. Different letters above different treatments represent significant differences at a specific time point under normal condition or drought stress ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

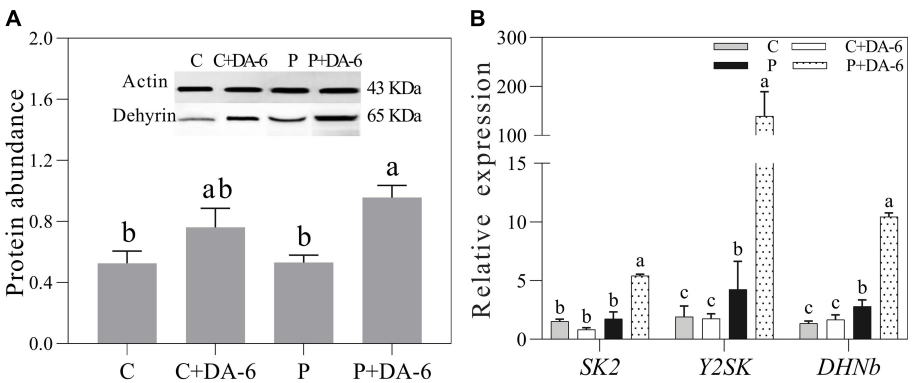

FIGURE 8 | Effect of seed priming with distilled water or DA-6 (2 mM) on **(A)** Dehydrins abundance, and **(B)** *SK2* gene, *Y2SK* gene, and *DHNb* gene expression level after 3 days of germination in white clover under well-watered condition and drought stress. Different letters above different treatments represent significant difference ($p \leq 0.05$). Vertical bars show the ± SE of mean ($n = 4$).

was influential in the elimination of ROS and mitigation of membrane lipid peroxidation through improvement of antioxidant defense under drought stress. Previous findings have shown that exogenous IAA treatment enhanced endogenous IAA level, leading to the improvement of enzymatic and non-enzymatic antioxidants activity in white clover under water deficient conditions (Li et al., 2018a). Moreover, GA application during seed germination has been found to be associated with the amelioration of antioxidants defense system in rapeseed conferring tolerance under drought stress (Li et al., 2010). The study of Eisvand et al. (2010) reported that seed priming with GA and CTK enhanced enzymatic antioxidants activities in damaged seeds of tall wheatgrass under water stress. These results indicate that DA-6 induced enhancement in antioxidants defense system might be related with enhanced endogenous IAA, GA, and CTK content, thereby reducing oxidative stress injury during seeds germination under drought stress.

DHNs are special type of proteins and also recognized as LEAP (late embryogenesis abundant proteins) that are

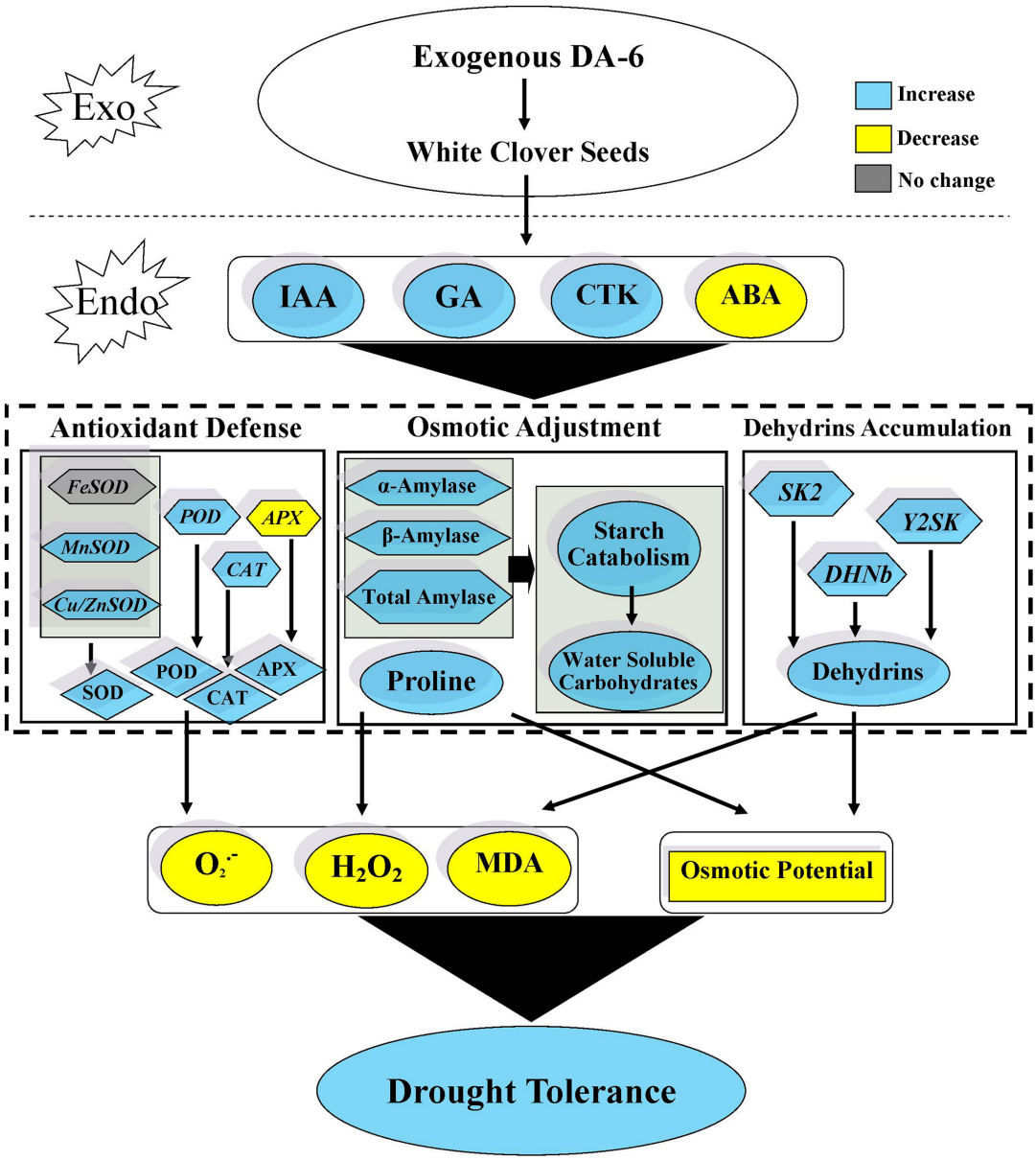

FIGURE 9 | Effect of DA-6 in white clover seed germination under drought stress.

accumulated during seed germination and also produced by plants during harsh environmental circumstances, i.e., drought, cold, salinity, and heat stress (Drira et al., 2016; Marček et al., 2016). Previous studies have revealed that DHNs are involved in defense mechanisms such as the prevention of cell dehydration, OA, ROS scavenging, and membrane structure maintenance under adverse environmental conditions (Krüger et al., 2002; Hara et al., 2005; Saibi et al., 2015). Beneficial roles of DHNs accumulation and increased transcript levels of DHN genes in regulating tolerance against abiotic stresses in different plant species have been widely reported. Specific *DHN* genes (*RAB18, XERO1 and LEA14*) could prevent seed degradation under moisture deficient condition and enhance germination in *Arabidopsis thaliana* seeds under saline condition (Hundertmark et al., 2011). The current study found that the expression levels of *Y2SK* and *DHNb* were substantially up-regulated by water stress in seedlings, while the *SK2* expression did not exhibit marked difference between the control and drought-stressed seedlings without the DA-6 priming, suggesting that different types of *DHNs* behaved differently during germination under water deficient condition (**Figure 8**). Interestingly, seed priming with DA-6 significantly up-regulated the expression levels of all *DHN* genes (*SK2, Y2SK*, and *DHNb*) and improved the DHN (65 kDa) accumulation under drought stress (**Figure 8**). Similar

results were found in the study of Cheng et al. (2018) who noticed that increased DHN (65 kDa) accumulation and *DHN* genes (*SK2, Y2K, Y2SK,* and *DHNb*) expression were related to γ-aminobutyric acid-induced salt tolerance during white clover seed germination. Previous studies have shown that exogenous application of ABA and CTK enhanced DHNs accumulation or transcript levels of *DHN* genes conferring tolerance to extreme environmental conditions in various plant species (Lee and Chen, 1993; Han and Kermode, 1996; Černý et al., 2011). Our findings imply that DA-6-induced drought tolerance is related to the DHNs accumulation and *SK2, Y2SK*, and *DHNb* expression. The DHNs accumulation might be regulated by alterations in endogenous GA, IAA, and CTK level induced by the DA-6, but it still deserves to be explored in our future study.

CONCLUSION

In summary, the findings from the present study revealed that seed priming with DA-6 (2 mM) is a simple but efficient approach to mitigate drought-induced obstruction in seed germination. Results demonstrated that DA-6 pretreatment significantly improved the endogenous phytohormones (IAA, GA, CTK) content resulting in enhanced starch catabolism via regulation of essential hydrolases contributing toward increased OA and growth under drought stress. Moreover, DA-6 enhanced the antioxidative enzymes activities (SOD, CAT, POD, and APX) and the transcript levels of antioxidants encoding genes (*MnSOD*, *Cu/ZnSOD*, *CAT*, and *POD*) which could be associated with increased endogenous hormonal (IAA, GA, CTK) levels, thus effectively mitigating the oxidative stress injury under drought conditions. In addition, DA-6 mediated increases in dehydrins accumulation and the expression levels of dehydrin encoding genes (*SK2, Y2SK, and DHNb*) might be another vital governing mechanism conferring tolerance during germination of white clover seeds under water deficient conditions. The current study will provide a better insight about the concentration dependent role of DA-6 during seed germination under water deficient condition.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ZL and YZ conceived, designed the research, and provided different chemical reagents and experimental material. MH, WG, WZ, and IK conducted the experiments. MH and WG evaluated the data. MH completed the manuscript writing. ZL, YP, MR, YZ, and MI reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Methyl Jasmonate and Sodium Nitroprusside Jointly Alleviate Cadmium Toxicity in Wheat (*Triticum aestivum* L.) Plants by Modifying Nitrogen Metabolism, Cadmium Detoxification, and AsA–GSH Cycle

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The principal intent of the investigation was to examine the influence of joint application of methyl jasmonate (MeJA, 10 μM) and a nitric oxide–donor sodium nitroprusside (SNP, 100 μM) to wheat plants grown under cadmium (Cd as $CdCl_2$, 100 μM) stress. Cd stress suppressed plant growth, chlorophylls (*Chl*), and PSII maximum efficiency (F_v/F_m), but it elevated leaf and root Cd, and contents of leaf proline, phytochelatins, malondialdehyde, and hydrogen peroxide, as well as the activity of lipoxygenase. MeJA and SNP applied jointly or singly improved the concentrations of key antioxidant biomolecules, e.g., reduced glutathione and ascorbic acid and the activities of the key oxidative defense system enzymes such as catalase, superoxide dismutase, dehydroascorbate reductase, glutathione *S*-transferase, and glutathione reductase. Exogenously applied MeJA and SNP jointly or singly also improved nitrogen metabolism by activating the activities of glutamine synthetase, glutamate synthase, and nitrate and nitrite reductases. Compared with individual application of MeJA or SNP, the combined application of both showed better effect in terms of improving plant growth and key metabolic processes and reducing tissue Cd content, suggesting a putative interactive role of both compounds in alleviating Cd toxicity in wheat plants.

Main findings: The main findings are that exogenous application of methyl jasmonate and nitric oxide–donor sodium nitroprusside alleviated the cadmium (Cd)–induced adverse effects on growth of wheat plants grown under Cd by modulating key physiological processes and up-regulating enzymatic antioxidants and the ascorbic acid–glutathione cycle–related enzymes.

Keywords: cadmium toxicity, inorganic nutrients, methyl jasmonate, wheat, oxidative stress

INTRODUCTION

Cadmium (Cd) is not required by plants for their optimum growth, so a slight increase in its levels in a growth medium causes considerable damages to several metabolic processes (Shanmugaraj et al., 2019), such as limitation of assimilation rate (Anwar et al., 2019; Zhou et al., 2020) and disturbance in plant water balance (Naeem et al., 2019). Moreover, Cd stress causes overproduction of reactive oxygen species (ROS) in plants (Gallego and Benavides, 2019). Overaccumulation of ROS can considerably damage the key membrane biomolecules, thereby causing leakage of all biological membranes (Jia et al., 2020). Plants possess a well-developed antioxidant defense system to counteract multiple stresses including Cd stress (Zaid et al., 2019). For example, one such promising mechanism is the modulation of the ascorbic acid (AsA)–glutathione (GSH) cycle enzymes (Khan et al., 2019). However, such a protective strategy does not constantly work in most plant species including wheat under Cd stress, because of being the crop highly sensitive to this metal stress (Rizwan et al., 2016).

Nitrogen (N) metabolism is a key physiological event that substantially affects growth, yield, and quality of most plants (Singh et al., 2016; Rajwade et al., 2018). Plants absorb N basically in the form of nitrate (NO_3^-) by roots and transfer it to leaves for its assimilation therein (Xuan et al., 2017). Nitrate reductase (NR) carries out the reduction of NO_3^- to NO_2^- (nitrite), and then nitrite reductase (NiR) converts NO_2^- to ammonium (NH_4^+) (Kaiser et al., 2018). The conversion of NH_4^+ to glutamate and glutamine takes place through glutamate synthase (GOGAT) and glutamine synthetase (GS) (Lea and Miflin, 2018). Thus, it is critically important to maintain optimal the activities of the N metabolism–related enzymes, i.e., NR, NiR, GS, and GOGAT, for maintaining optimum growth of plants (Liang et al., 2018). Earlier investigations have exhibited that N metabolism was suppressed in plants exposed to Cd toxicity (Balestrasse et al., 2003; Gill et al., 2012; Wani et al., 2017; Shahid et al., 2019).

Consequently, a plausible action is indispensable to allay the harmful effects of Cd on plant metabolic events. In light of several reports, it is amply clear that plant growth regulators, both natural and synthetic, can effectively regulate the metabolic phenomena taking part in growth (Sheng et al., 2016; Asgher et al., 2017). Of these regulators, methyl jasmonate (MeJA) is one of the potential internal regulators involved in regulation of an array of physiobiochemical processes taking part in plant ontogeny (Mustafa et al., 2016; Yu et al., 2019). For instance, it is believed to be actively involved in transcriptional programming for improving tolerance to multiple stresses (Faghih et al., 2017; Ho et al., 2020). The involvement of MeJA in modulating the activities of some crucial antioxidant enzymes in most plants under heavy metal stress such as arsenic stress has been found in different plants such as oilseed rape (Farooq et al., 2018) and rice (Mousavi et al., 2020; Verma et al., 2020), as well as Cd stress in *Kandelia obovata* (Chen et al., 2014) and foxtail millet (Tian et al., 2017).

Nitric oxide (NO) is another prospective biostimulator that plays a critical role in a myriad of metabolic processes in plants exposed to heavy metal stress (Corpas, 2017; Terrón-Camero et al., 2019). NO can lessen the detrimental effects of oxidative injury by upraising the antioxidant systems, which can efficiently scavenge the ROS accrued under stress situations, or it may function as a vital signal in several key molecular processes (Hasanuzzaman et al., 2018; Sadhu et al., 2019). Many studies testify that NO functions as a signal in hormonal and environmental reactions in plants (Li et al., 2020; Sharma et al., 2020). Although MeJA and NO have been tested individually on plants under various heavy metals including Cd, the joint effect and crosstalk between both metabolites on alleviation of Cd stress on plants have not been yet elucidated. So, in the current study, the premier aim was to examine the effect of combined application of these two biostimulants and their crosstalk taking part in mitigation of Cd stress in wheat plants.

MATERIALS AND METHODS

Plant Growth Under Varying Regimens

The investigation was performed under greenhouse conditions at 20–25°C and over 10°C, day and night temperatures, respectively, and with relative humidity 65–70%. Before initiation of the experiment, the wheat (*Triticum aestivum* L.) cv. "Pandas" seeds were treated with NaOCl (1% v/v solution) for sterilization. Each pot contained 5.0 L of perlite. At the beginning, 50 seeds were planted in each pot for germination, but thereafter only 35 plants were kept in each pot. During the growth season, the photoperiod was 11/13-h light–dark. Hoagland nutrient solution (NS; half strength) 0.1–1.0 L, based on plant size, was applied to each pot on alternate days during the discourse of the study. Additional details of the NS composition are given elsewhere (Steinberg et al., 2000). The pH of the NS was kept at 5.5. The layout of the experiment was a randomly completed block with three biological replications, each containing three pots. So, there were nine pots in each treatment.

Ten days after germination, different treatments were initiated. The wheat seedlings were exposed to Cd treatment (100 μM Cd) as cadmium chloride or without Cd treatment (control). These seedlings were further permitted to grow for 14 days. During this period, the wheat seedlings were sprayed every other day with 20 mL of deionized water per pot (control) or MeJA, sodium nitroprusside (SNP), or MeJA + SNP in 0.01% Tween-20 (**Figure 1A**). The control plants were well separated to avoid the spray of MeJA or SNP solution. Thereafter, the data of different parameters listed below were determined.

After determination of fresh weight, the shoot and root materials were separately kept at 75°C for 2 days in an oven and dry weights recorded.

Determination of Cd Content, Biological Accumulation Coefficient, Biological Concentration Factor, and Translocation Factor

One container (containing 35 seedlings) per replication was chosen for chemical analysis. For the quantification of shoot

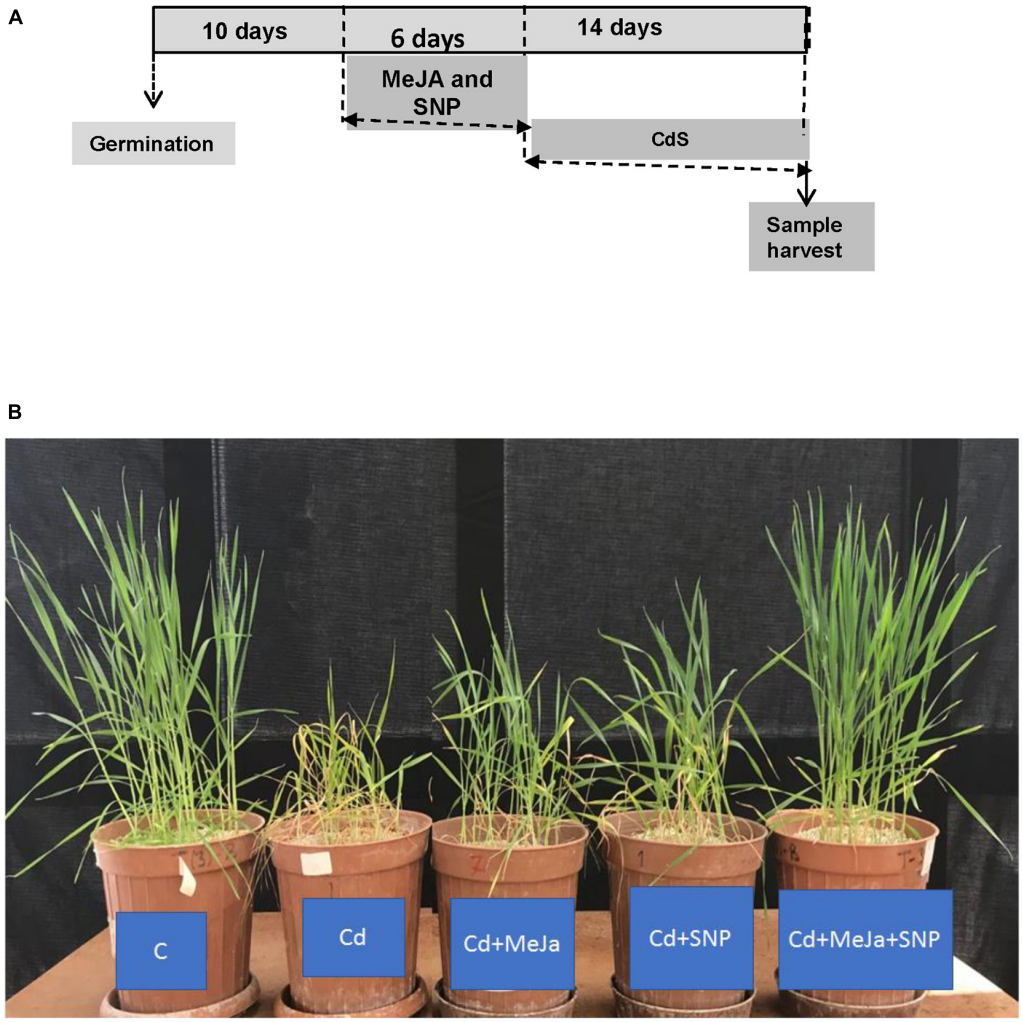

FIGURE 1 | (A) Scheme of the treatments used to study the effect of cadmium (Cd) in wheat plants. The used concentration of each chemical was 100 μM Cd, 10 μM methyl jasmonate (MeJA) and 100 μM sodium nitroprusside (SNP) as nitric oxide donor (NO) and **(B)** effects of 10 μM MeJA and MeJA plus 100 μM SNP on the growth of wheat plants subjected to Cd. Photographs were taken at the end of the experiment.

and root Cd contents, digestion of dried samples was performed in $HClO_4$:HNO_3 solution (1:5, vol/vol) and then read with an inductively coupled plasma–optical emission spectrometry. The calculation of biological concentration factor (BCF), translocation factor (TF), and biological accumulation coefficient iological accumulation coefficient (BAC) was performed following the equations described by Malik et al. (2010). BCF represents Cd concentration ratio of roots to that of a growth medium. TF shows the ratio of Cd in plant shoots to that of roots. BAC shows the ratio of Cd in shoots to that in a growth medium.

Quantification of Photosynthetic Pigments and Maximal Quantum Yield

Chlorophyll contents were quantified employing the procedure of Arnon (1949). After extraction of the leaf material in acetone (5 mL, 80%), the acetone (80%) solution was added to the extract to bring the final volume to 50 mL. The absorbance readings were taken at 480, 645, and 663 nm for carotenoids, chlorophyll a (Chl a), and chlorophyll b (Chl b), respectively.

The data for maximal quantum yield (F_v/F_m) were recorded with a portable Photosynthesis Yield Analyzer (Walz, Germany) on the leaves already subjected to dark for 30 min. The values of F_v/F_m were worked out from different parameters obtained from the equipment such as minimal fluorescence (F_0), maximal fluorescence (F_m), and maximal variable fluorescence ($F_v = F_m - F_0$) according to Maxwell and Johnson (2000).

Determination of Relative Water Content, Proline, Glycine Betaine, and Total Soluble Sugars

The protocol highlighted by Barrs and Weatherley (1962) was pursued to estimate leaf relative water content (RWC). Fresh mass (FM) was quantified by weighing the cut leaves. Thereafter, the leaves were saturated in a Petri dish filled with water for 3 h

to estimate the turgid mass (TM). Lastly, these materials were subjected to a drying oven at 80°C for 24 h to estimate dry mass (DM). The RWC was worked out using the following formula:

$$RWC(\%) = [(FM - DM)/(TM - DM)] \times 100$$

Quantification of free proline (Pro) was performed according to Bates et al. (1973). To a proportion of 0.5 g fresh leaf, an aliquot of 10 mL of 3% sulfosalicylic acid was added, and then the material was centrifuged for 10 min at 3,000 *g*. Thereafter, an aliquot (2 mL) of the filtrate was treated appropriately with glacial acetic acid and acid ninhydrin. The mixture was subjected to 100°C for 1 h. After properly cooling the treated material, toluene (4 mL) was poured into it to separate free Pro. The OD was recorded at 520 nm.

The Grieve and Grattan (1983) method was followed for the quantification of glycine betaine (GB). The procedure employing the anthrone reagent was adopted to estimate total soluble sugars. The ethanol solution (80%) was used to extract the sample (0.1 g). Thereafter, the centrifugation of the mixture was performed for 10 min at relative centrifugal force (RCF) of 5,000. A supernatant of 0.5 mL was pipetted out, and 1 mL HCl (1N) was added to it. Then, it was subjected to a water bath at 100°C. An aliquot (4 mL) of 0.2% anthrone was poured into the sample mixture, and then it was kept in a water bath for another 10 min. The OD readings were noted at 620 nm (Fong et al., 1953).

Quantification of Phytochelatins

The amount of GSH was subtracted from total non-protein thiols (NPTs) to obtain phytochelatin (PC) content. Fresh leaf tissue was macerated in sulfosalicylic acid (3%). Ellman's reaction mixture contained 0.6 mM DTNB [5,5 o-ithiobis (2-nitrobenzoic acid)] and 5 mM EDTA. The quantification of NPT was noted at 412 nm (Ellman, 1959).

Measurement of GSH and Ascorbic Acid

Fresh leaf tissue (500 mg) was homogenized in metaphosphoric acid buffer (3 mL, 5%) plus 1 mM EDTA. After centrifuging the extract for 12 min at RCF of 11,500 at 4°C, the reaction mixture was assayed for the appraisal of GSH and ascorbate.

The quantification of ascorbate was carried out according to Huang et al. (2005) by using potassium (K)–phosphate buffer (pH 7.0; 500 mM). The assay of reduced ascorbate was carried out in 0.1 M K–phosphate buffer (pH 7.0) and 0.5 U of ascorbate oxidase. The OD of all samples was noted at 265 nm.

Estimation of total AsA was performed following the extraction of each sample with 30 mM dithiothreitol. The content of dehydroascorbate (DHA) was calculated by deducting the content of reduced AsA from that of total AsA.

The assays of reduced GSH and glutathione disulfide (GSSG) were performed following Yu et al. (2003). To an aliquot of 0.4 mL of the sample extract, 0.6 mL of 500 mM K–phosphate buffer (pH 7.0) was added. The measurement of GSH was performed by the alterations in absorbance rate at 412 nm for NTB (2-nitro-5-thiobenzoic acid) produced by the reduction of DTNB. The GSSG concentration was worked out by eliminating GSH with 2-vinylpyridine (a derivatizing agent).

Measurement of Oxidative Stress–Related Parameters

Quantification of leaf hydrogen peroxide (H_2O_2) was performed following the procedure optimized by Loreto and Velikova (2001). Fresh leaf material (0.5 g) was macerated in trichloroacetic acid (TCA, 3 mL of 1%). The centrifugation of homogenate was performed at 10,000 *g* for 10 min at 48°C. Thereafter, an aliquot of 0.75 mL of the homogenate was treated with 1 M KI (1.5 mL) and K buffer (10 mM, 0.75 mL). The OD values were recorded at 410 nm.

The estimation of leaf malondialdehyde (MDA) concentration was performed employing the method depicted by Weisany et al. (2012). The extraction of fresh leaf material (each 200 mg) was performed in TCA (5 mL of 0.1% wt/vol). The extract was centrifuged (12,000 *g*) for 5 min at 4°C. Then, by adding TCA (20%) to the extract, 4 mL of thiobarbituric acid (0.5%) was added to it. The treated sample material was warmed in a water bath at 90°C for 30 min. After bringing the temperature of the treated samples down to room temperature, their OD was read at 532 and 600 nm, respectively.

Electrolyte leakage (EL) was estimated by the method described by Dionisio-Sese and Tobita (1998). After washing fresh leaf tissue with deionized water to remove any contamination on the surface, leaf discs were excised. Then, to measure the initial electrolyte conductivity (EC1), those discs were kept in vials each containing 10 mL of deionized water for a day on a rotary shaker. Finally, those materials were incubated at 120°C for 20 min to measure the second electrolyte conductivity (EC2). To calculate the EL, the following formula was used:

$$EL(\%) = (EC1/EC2) \times 100$$

Analysis of Enzymatic Activities

Leaf tissue (500 mg) was macerated using 1 mL of 100 mM ice-cold K–phosphate buffer (pH 7.0) consisting of 1% polyvinylpyrrolidone (PVP). The well-ground material was centrifuged at 12,000 *g* for 15 min at 4°C. The extracted mixture was taken for the quantification of enzyme activities.

For the quantification of superoxide dismutase (SOD, EC 1.15.1.1) activity, the procedure of Van Rossum et al. (1997) was pursued. The mixture consisting of phosphate buffer (100 mM, pH 7.4), 10 mM of methionine, 1.0 mM EDTA, 75 μM of NBT, 50 μM of riboflavin, and 100 μL of the enzyme extract was kept under fluorescent light for 15 min. The OD of the treated samples was read at 560 nm.

The catalase (CAT, EC 1.11.1.6) activity was estimated following Chance and Maehly (1955).

The quantification of glutathione reductase (GR) activity was carried out as depicted by Hossain et al. (2010). The reaction solution consisted of K–phosphate buffer (100 mM, pH 7.8), 0.2 mM NADPH, 1 mM GSSG, 1 mM EDTA, and the enzyme extract in a final volume of 1 mL. The GSSG was added to start the reaction. The decline in optical density at 340 nm because of oxidation of NADPH was noted for 1 min.

The method of Hossain et al. (1984) was adopted to estimate the monodehydroascorbate reductase (MDHAR) activity. After properly treating the sample extract systematically with different chemicals listed in the method, its OD was read for 1 min at 340 nm.

The protocol of Nakano and Asada (1981) was employed to estimate the activity of dehydroascorbate reductase (DHAR). After treating the sample solution with a series of chemicals listed in the protocol, its OD was noted at 265 nm for 1 min.

The protocol of Hossain et al. (2006) was adopted to estimate the glutathione *S*-transferase (GST) activity. The reaction mixture consisted of 1.5 mM GSH, 100 mM Tris–HCl buffer (pH 6.5), 1 mM 1-chloro-2,4-dinitrobenzene, and enzyme solution in a final volume of 0.7 mL. The changes in absorbance were noted at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

The lipoxygenase (LOX) (EC: 1.12.11.12) activity was quantified using the protocol of Axelrod et al. (1981). Enzyme extract of 0.2 mL was treated with the reaction mixture (4 mL) comprising 50 mM Na-P buffer of pH 6.5 and 400 μM linoleic acid to start the reaction. The OD of all treated samples was recorded at 234 nm.

Estimation of Total Free Amino Acids and Total Soluble Proteins

The aforementioned enzyme extract was also used to determine total amino acids following the ninhydrin method of Rosen (1957). The amount of total free amino acids was defined as μg glycine in 1 g of fresh material used. The procedure of Bradford (1976) was followed for assaying total soluble proteins in leaf tissues.

Measurement of NR and NiR Activities

For the quantification of NR and NiR activities, the extraction of fresh leaf material (1:5, wt/vol) was carried out in a cold pestle and mortar using 100 mM potassium phosphate buffer (pH 7.5) consisting of 0.5% PVP, 2 mM EDTA, and 5 mM cysteine. After properly centrifuging the mixture, the filtrate was used for appraising the activities of NR and NiR.

The assay of NR activity was performed as outlined by Debouba et al. (2006). For the quantification of maximal NR activity, 1.4 mL of the sample mixture contained 100 mM potassium phosphate buffer (pH 7.5) that comprised 0.14 mM NADH, 7 mM KNO_3, 10 mM $MgCl_2$, and the enzyme extract. To commence the reaction, NADH was reacted with the sample extract. After that, it was incubated at 27°C for 30 min, and then 0.1 mL of 0.5 M zinc acetate was added to it, and the reaction mixture was subjected for 10 min to centrifugation at 3,000 *g*. The formation of NO_2^- was quantified following the development of diazotization with 0.01% naphthylenediamine dihydrochloride and 1% sulfanilamide. Thereafter, its temperature was brought down to room temperature, and the absorbance values were noted at 540 nm. A standard calibration curve of $NaNO_2$ was prepared to estimate the amount of NO_2^- formed.

The activity of NiR was quantified as a decrease in the quantity of NO_2^- in the reaction mixture following the procedure of Debouba et al. (2006). A 2.5 mL of the reaction mixture contained 2.3 mM methyl viologen, 0.1 M potassium phosphate buffer (pH 6.8), 0.4 mM $NaNO_2$, and the enzyme extract. Sodium dithionite (4.3 mM) prepared in 0.1 M $NaHCO_3$ was added to the reaction mixture to start the reaction. Then, it was subjected to 27°C for 30 min, and the reaction was terminated by shaking and boiling for 1 min. The ions of NO_2^- left in the sample mixture were quantified at 540 nm.

Determination of Glutamine Synthetase, Glutamate Dehydrogenase, and GOGAT Activities

For the quantification of GS and glutamate dehydrogenase (GDH) activities, fresh foliage tissue (1:5, wt/vol) was triturated in 50 mM Tris–HCl buffer (pH 7.6) comprising 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, and 0.5% PVP. Then the extract was subjected to centrifugation at 20,000 *g* for 20 min, and the aliquot was taken for the quantification of the GS and NADH–GDH activities.

The activity of GS was quantified following the method of Agbaria et al. (1998). A 2.0 mL of the sample mixture consisted of 1 mM ADP, 50 mM Tris–HCl buffer (pH 7.2), 20 mM $MgCl_2$, 50 mM glutamine, the enzyme extract, 20 mM sodium arsenate, and 13 mM hydroxylamine. To initiate the reaction, hydroxylamine was added to the sample mixture. Then the treated sample was subjected to 30°C for 30 min, and the reaction lasted by adding 3 mL of the solution containing 0.2 M $FeCl_3$, 0.5 M HCl, and 0.24 M TCA. Then it was centrifuged at 3,000 *g* for 10 min, and the OD was read at 540 nm.

The assay of GDH activity was performed at 30°C by observing the oxidation of NADH at 340 nm by adopting the procedure of Groat and Vance (1981). For the determination of NADH–GDH activity, an aliquot of 2 mL of the sample mixture comprised 0.1 M Tris–HCl buffer (pH 8.0), 11 mM 2-oxoglutaric acid, the enzyme extract, 0.2 mM NADH, and 0.1 M NH_4Cl.

For the quantification of GOGAT activity, fresh foliage tissue (1:5, wt/vol) was macerated in 50 mM potassium phosphate buffer (pH 7.5) consisting of 10 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 14 mM β-mercaptoethanol, and ethylene glycol (3.58 M). Thereafter, the sample mixture was subjected for 20 min to centrifugation at 20,000 *g*, and the aliquot was taken for the quantification of NADH-GOGAT activity. The activity of NADH-GOGAT was quantified at 30°C by observing the oxidation of NADH at 340 nm following the protocol of Groat and Vance (1981); 2 mL of the sample mixture comprised 10 mM glutamine, 0.1 M potassium phosphate buffer (pH 7.5), 0.15 mM NADH, 5 mM 2-oxoglutaric acid, and the enzyme extract.

Analysis of Total N, NO_3^-, and NH_4^+ in Wheat Shoot

The plant samples were subjected for 72 h to 70°C, and then total N in the samples was appraised using the Kjeldahl method described by Muñoz-Huerta et al. (2013).

For the quantification of NO_3^- and NH_4^+, the extraction of fresh leaf material (1:10, wt/vol) was performed in redistilled water, and then the extract was heated well and filtered. The

quantification of NO_3^- was performed following the protocol of Cataldo et al. (1975). The reaction solution contained 0.2 mL of 5% salicylic acid prepared in concentrated H_2SO_4 and 0.1 mL of the filtrate. The treated sample mixture was kept for 15 min at room temperature, and then 1 mL of 4 M NaOH was mixed with it. After properly cooling the treated samples, they were read at 410 nm.

The quantification of NH_4^+ was performed using the Nessler reagent outlined by Molins-Legua et al. (2006). The reaction solution contained 0.1 mL of the Nessler reagent, 0.1 mL of the filtrate, 2.4 mL of redistilled water, and 0.01 mL of 10% K–Na tartrate. The OD was noted at 425 nm.

Statistical Analysis

The data collected for all replicates of each parameter were subjected to a software SAS version 9.1 (SAS Institute Inc., Cary, NC, United States) for calculating analysis of variance. The figures or tables presented in the article present the means of each treatment along with standard error values. The significant differences among the treatment means were calculated using the Duncan multiple-range test at the 5% significance level.

RESULTS

Phenotypic Appearance of the Wheat Plants

Figure 1B illustrates that the leaves of Cd-stressed wheat plants show clear-cut symptoms of chlorosis (yellowing). Plant height and leaf size of the plants also decreased prominently under Cd stress. The leaves of the plants supplemented with MeJa and MeJa + SNP did not show any symptoms of chlorosis and disorders mentioned earlier.

Plant Growth, Photosynthesis-Related Parameters, and Translocation and Accumulation of Cd

Cd significantly decreased the DM of different plant parts compared to the controls (**Figures 2A,B**). However, exogenously applied MeJA and SNP singly or jointly significantly mitigated the suppression in biomass. With respect to the controls, the aforementioned traits were found to be declined 29 and 41%, respectively, due to Cd, but a significant enhancement of 42 and 75% was recorded in these attributes, respectively, in the wheat plants treated with MeJA + SNP compared with those exposed to Cd toxicity only. Under non-stressed conditions, these chemicals also led to a marked rise in these attributes, attaining maximal values due to their combined supplementation. In view of these results, it could be safely inferred that SNP and MeJA actively participated in alleviation of Cd stress in the wheat plants. Compared with the controls, these parameters were increased by 19 and 29%, respectively, due to supplementation with MeJA + SNP.

The wheat seedlings exposed to high Cd regimen showed marked reductions in Chl a, Chl b, carotenoids, and photosystem II efficiency (F_v/F_m) by 44, 49, 43, and 33%, respectively

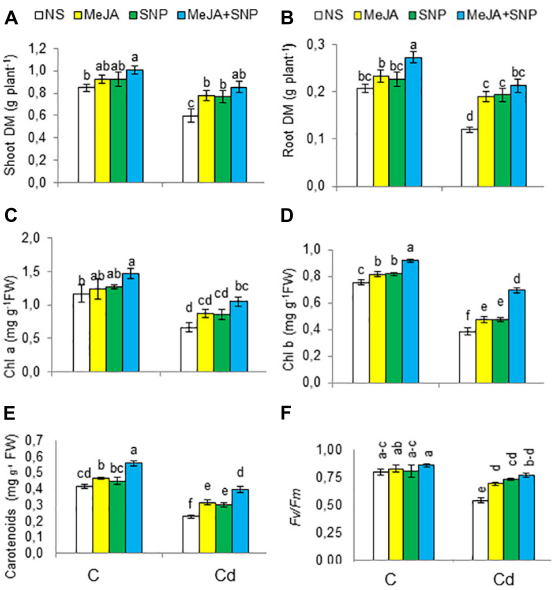

FIGURE 2 | Shoot **(A)** and root **(B)** dry weight (DW), chlorophyll a **(C)**, chlorophyll b **(D)**, carotenoids **(E)** on fresh weight (FW) basis, and chlorophyll fluorescence parameters [F_v/F_m **(F)**] in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

(**Figures 2C–F**). Externally applied MeJA substantially enhanced Chl a, Chl b, carotenoids, and photosystem II efficiency (F_v/F_m), achieving the maximal values when both substances were applied together. Compared with the controls, these parameters were found to be raised by 37, 45, 70, and 43%, respectively, due to MeJA + SNP application.

Cd was to be accumulated in both shoots and roots of the wheat plants grown under Cd toxicity (**Figures 3A,B**). The root Cd content was about 2.5-fold higher than that in the shoot. However, externally applied MeJA or SNP decreased the Cd content in roots by 27 and 31%, and in shoot by 39 and 41%, respectively, over those in the wheat seedlings exposed to Cd treatment without the supplementation of MeJA or SNP. Furthermore, the combined application (MeJA + SNP) led to a marked reducing effect on root and shoot Cd contents by 45 and 56%, respectively, over that in the Cd-stressed wheat seedlings.

The BCF, TF, and BAC of Cd from the growing medium to roots and shoots were also worked out. Cd increased BCF, TF, and BAC factors' values (**Figures 3C–E**), but MeJA or SNP led to a substantial reduction in these values. Furthermore, the combination of both chemicals led to a further reduction in BCF (48%), TF (22%), and BAC (56%), suggesting a more effective protective defense against Cd toxicity.

Modulation of RWC, Soluble Sugars, GB, and Pro Under Cd

With respect to controls, Cd reduced RWC by 27%; however, an alleviation of 20, 22, and 30% was attained due to MeJA,

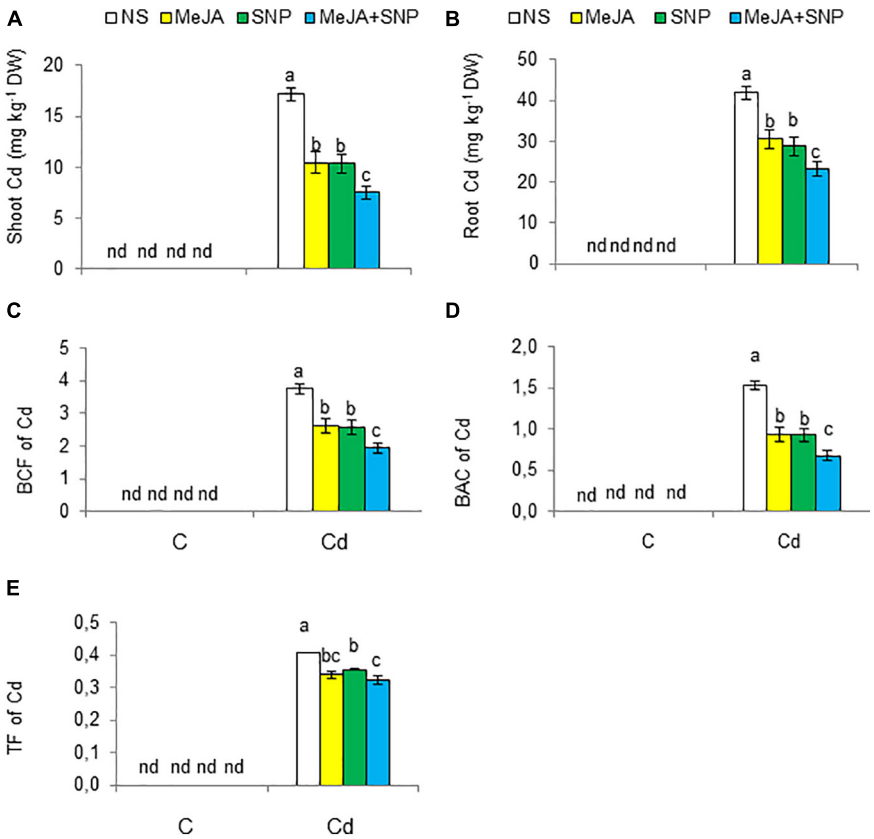

FIGURE 3 | Shoot cadmium [Cd **(A)**], root Cd **(B)** on dry weight (DW) basis, the biological concentration factor [BCF **(C)**], biological accumulation coefficient [BAC **(D)**], and translocation factor [TF **(E)**] of Cd in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test. nd, not detected.

SNP, and MeJA + SNP application, respectively, relative to the controls (**Figure 4A**).

Cd raised the contents of Pro and GB by 59 and 172%, respectively, but it reduced total soluble sugars by 42% relative to the control (**Figures 4B–D**). The supplementation of MeJA and SNP singly and in combination enhanced the accumulation of these substances under Cd stress conditions. Individually, no marked difference was observed in the effectiveness of MeJA and SNP. With reference to that in the plants treated with Cd, percent increases in Pro, GB, and sugars were 67, 33, and 50%, respectively, in the wheat seedlings treated with MeJA + SNP.

Improvement in PC Synthesis, GSH, and AsA Contents

Cd toxicity boosted PC synthesis and the activity of GST by 5.9- and 1.7-fold, respectively (**Figures 5A,B**). Furthermore, Cd toxicity inverted the reduced levels of GSH and oxidized glutathione (GSSG) contents by 28 and 66%, respectively, but reduced the ratio of GSH/GSSG **Figures 5C–E**) over those in the controls. Externally applied MeJA or SNP together with Cd treatment led to further increases in these attributes, with the exception of reduction in GSSG. The combined effect of MeJA and SNP was more pronounced on those attributes compared with their individual effect. Furthermore, it can be concluded that MeJA and SNP treatments under Cd stress increased PC and GSH contents, which might have played an essential role in detoxification of Cd.

Plants subjected to high Cd regimens exhibited a decline in AsA by 24%, but a rise in DHA content by 31% with reference to the controls (**Figures 6A,B**). Accordingly, the AsA/DHA ratio was reduced in the Cd-treated plants by 41% over the controls (**Figure 6C**). Externally supplied MeJA and SNP jointly with Cd treatment further improved AsA and AsA/DHA ratio, but a decline was observed in DHA content relative to those in the plants treated with Cd alone. Various doses did not affect the earlier-mentioned parameters under control conditions.

Cd Induces Oxidative Stress

Cd treatment considerably improved the levels of H_2O_2 and MDA, EL, and the activity of LOX by 125, 210, 233, and 112%, respectively, over the controls (**Figures 6D–G**). These oxidative stress–related traits were found to be reduced due to the supplementation of MeJA or SNP. The combination of MeJA and SNP led to maximal reductions in the aforementioned parameters

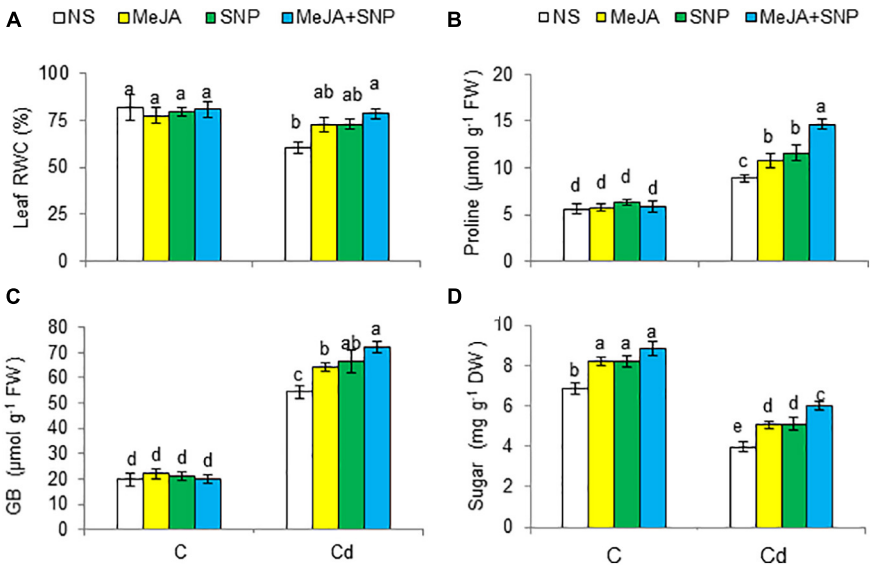

FIGURE 4 | Leaf relative water content [RWC **(A)**], proline **(B)** glycine betaine [GB **(C)**] content on fresh weight (FW) basis, and sugar content **(D)** in wheat plants grown under control (C) and cadmium (100 µM Cd) and sprayed with 10 µM methyl jasmonate (MeJA) or 100 µM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

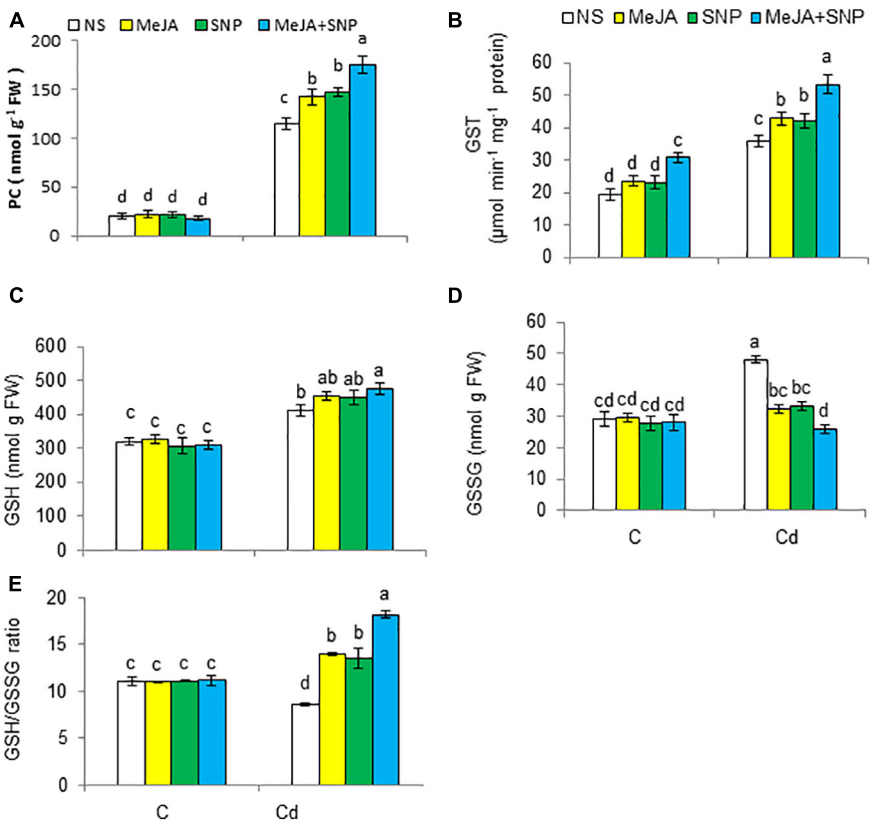

FIGURE 5 | Phytochelatin [PC **(A)**], glutathione *S*-transferase [GST **(B)**], reduced glutathione [GSH **(C)**], oxidized glutathione [GSSG **(D)**] on fresh weight (FW) basis, and GSH/GSSG **(E)** in wheat plants grown under control (C) and cadmium (100 µM Cd) and sprayed with 10 µM methyl jasmonate (MeJA) or 100 µM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

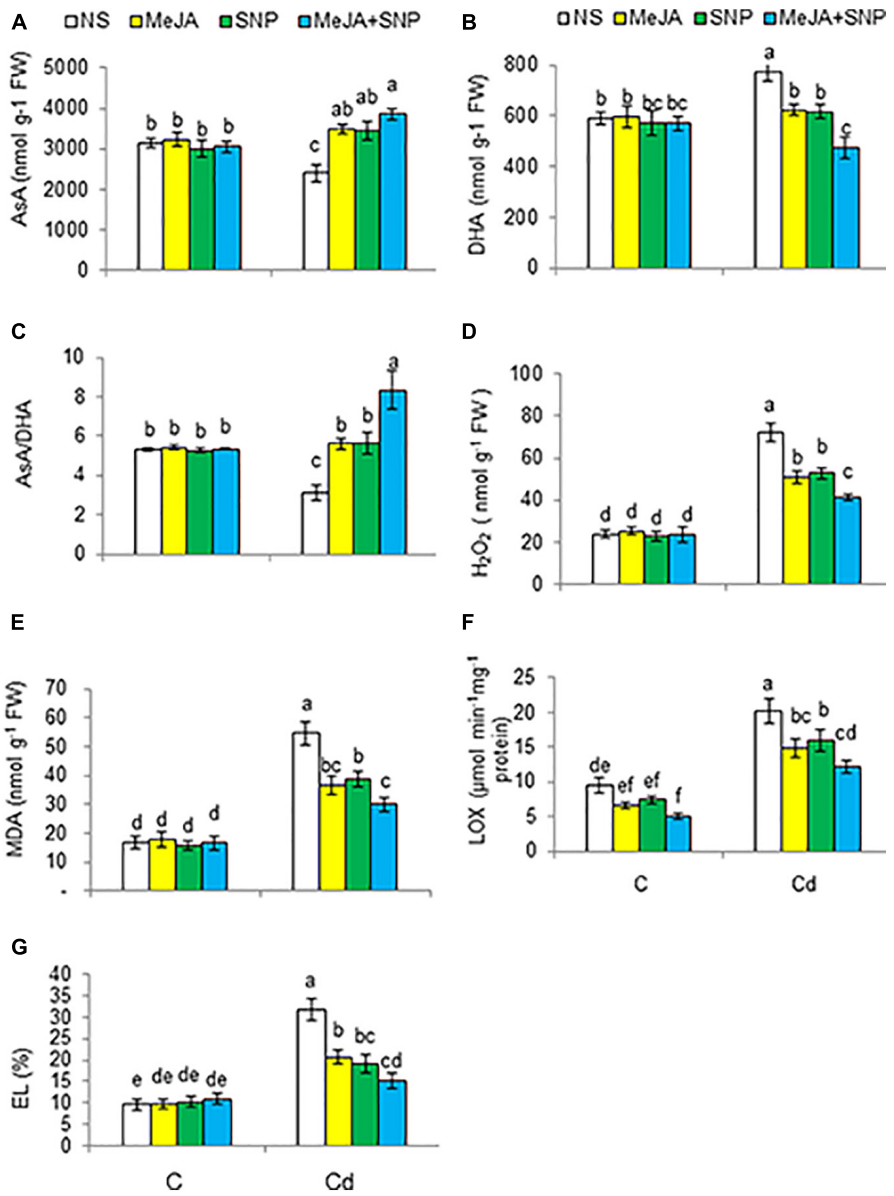

FIGURE 6 | Ascorbate [AsA **(A)**], and dehydroascorbate [DHA **(B)**] on fresh weight (FW) basis, and AsA/DHA ratio **(C)**, hydrogen peroxide [H_2O_2 **(D)**], malondialdehyde [MDA **(E)**] on FW basis, lipoxygenase [LOX **(F)**], and electrolyte leakage [EL **(G)**] in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

by 43, 46, 40, and 52%, respectively, with reference to the controls, i.e., only Cd-treated plants. Various treatments did not alter these parameters under control conditions.

Modulation of the Antioxidant System

Acknowledgments The activities of enzymes of the antioxidant defense system are presented in **Figures 7A–F**. With respect to the controls, high Cd regimens boosted the activity of SOD (35%) and that of GR (71%), but it decreased CAT (29%), MDHAR (26%), and DHAR (30%). These enzyme activities were elevated in the wheat plants sprayed with MeJA or SNP, and the combined supply of the two chemicals was more pronounced on these enzyme activities over those in the Cd-stressed wheat plants. Under the control growing conditions, the wheat plants supplemented with MeJA and SNP showed a marked elevation in the activities of CAT and SOD, but they showed insignificant alterations in the activities of GR, MDHAR, and DHAR.

Improvement in N Metabolism Under Cd Stress

High Cd regimens led to marked decreases in N metabolism–related enzymes such as NR, NiR, GS, and glutamate synthase

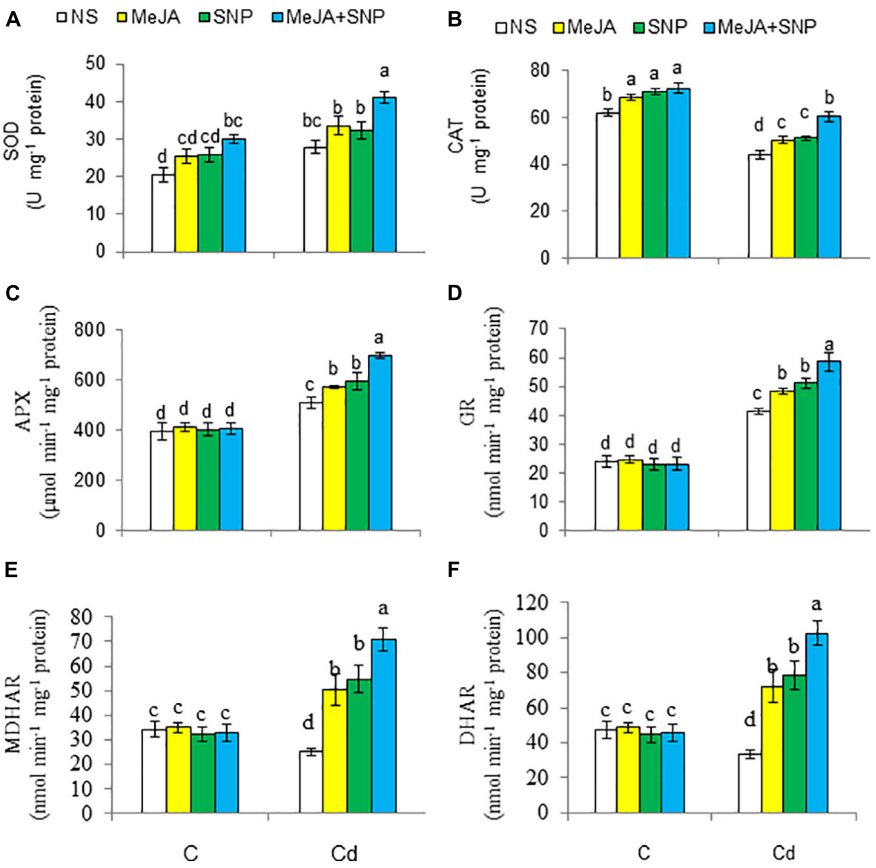

FIGURE 7 | Activities of superoxide dismutase [SOD **(A)**], catalase [CAT **(B)**], ascorbate peroxidase [APX **(C)**], glutathione reductase [GR **(D)**], monodehydroascorbate reductase [MDHAR **(E)**], and dehydroascorbate reductase [DHAR **(F)**] in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

(GOGAT) by 41, 39, 46, and 50%, respectively, but it increased the activity of GDH by 49% (**Figures 8A–E**). However, the wheat plants sprayed with MeJA or SNP alone showed a rise in the activities of NR, NiR, GS, and GOGAT and a decline in GDH. Furthermore, Cd-stressed plants sprayed with both substances together exhibited increases of 49, 44, 59, and 97% for NR, NiR, GS, and GOGAT, respectively, but a decrease in GDH by 58% over that in the Cd-stressed plants.

The contents of total N and NO_3^- decreased by 43 and 35%, respectively, but that of NH_4^+ increased by 71% in the Cd-stressed wheat plants compared with the controls (**Figures 9A–C**). The wheat plants fed with MeJA or SNP exhibited a rise in total N and NO_3^-, a decline in NH_4^+ under Cd stress, a maximal increase of 57 and 107% for N and NO_3^-, respectively, but a decrease of 56% for NH_4^+ was observed in the plants sprayed with MeJA and SNP jointly.

Relative to the controls, Cd stress enhanced total amino acid content by 53%, but reduced total protein content by 40% in the leaves of the wheat plants (**Figures 9D,E**). The application of MeJA or SNP led to a decline in total amino acids, but a rise in total proteins under Cd stress. A maximal decrease in total amino acids by 38% and an increase in total proteins by 108% were observed in the plants fed with MeJA and SNP together with reference to the controls, i.e., plants treated with Cd only.

DISCUSSION

Improvement in Wheat Plant Growth Under Cd Stress

Contaminations of heavy metals detrimentally influence the plant growth as similarly observed in other stresses (Ahanger et al., 2020). Cd-induced reduction in plant growth was found in our study, which is similar to that reported by Anjum et al. (2016) in maize, and Afzal et al. (2019) in rice. The most likely reason of reduced plant growth due to Cd could have been due to reduced uptake of water and mineral elements (Nahar et al., 2016) and reduced metabolism of sugars and photosynthetic efficiency (Zaid and Mohammad, 2018). The supply of MeJA and SNP mitigated Cd-induced reduced plant growth in our study. Especially, the joint supply of MeJA and SNP was more in effect in promoting growth of Cd-stressed wheat seedlings, as analogously recorded in mustard (Per et al., 2016) and *Mentha arvensis* (Zaid and Mohammad, 2018) in which MeJA increased plant

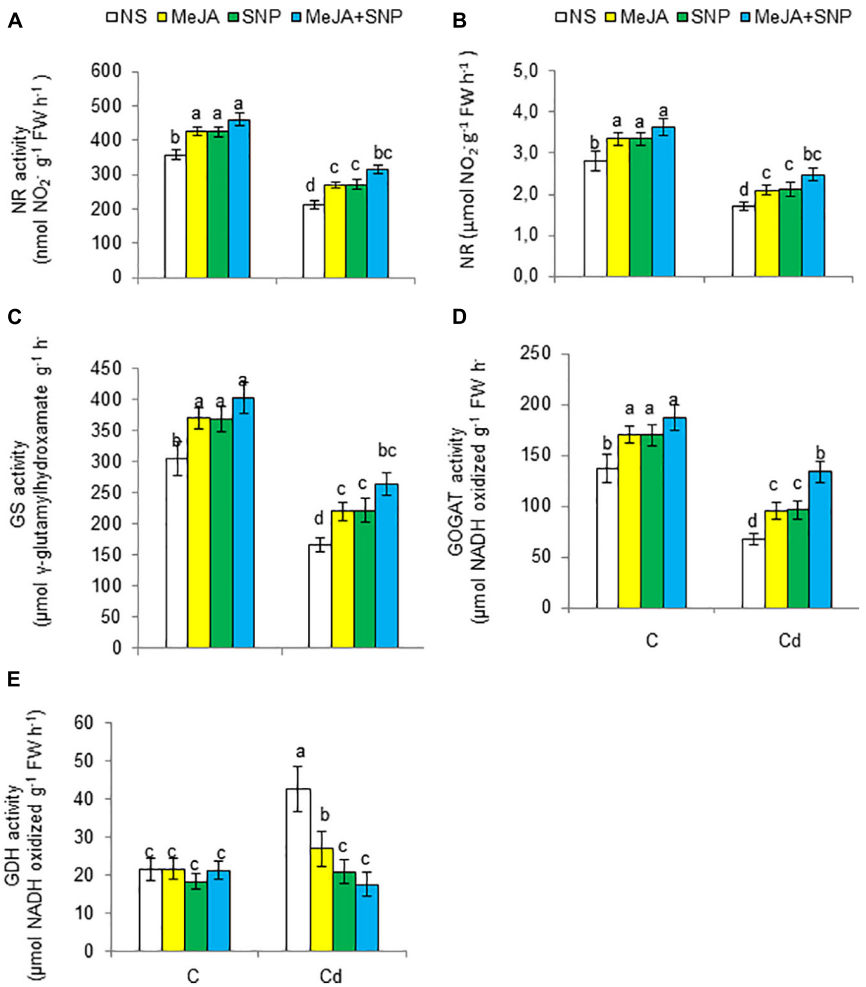

FIGURE 8 | Activities of nitrate reductase [NR **(A)**], nitrite reductase [NiR **(B)**], glutamine synthetase [GS **(C)**], glutamate synthase [GOGAT **(D)**], and glutamate dehydrogenase [GDH **(E)**] on fresh weight (FW) basis in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

biomass under Cd stress. In some other studies, SNP application was reported to be efficient in promoting growth of Cd-stressed plants, e.g., tall fescue (Zhuo et al., 2017) and *Catharanthus roseus* (Nabaei and Amooaghaie, 2020). The beneficial effect of MeJA and SNP on mitigation of Cd-induced suppression in growth of the wheat plants might have been linked with improved F_v/F_m and leaf chlorophyll contents (Per et al., 2016; Kaya et al., 2019).

Reduction in Shoot and Root Cd Contents in Wheat Under Cd

Cd is easily absorbed by plants growing in soils having toxic levels of Cd and is mostly accumulated in the roots, and relatively less quantity is transported to the shoot of plants (Nahar et al., 2016). Cell membrane and wall are the major blockers against influx of Cd to the cell (Zhang et al., 2017). The entrance of Cd to the cell can also be reduced by PCs (Uraguchi et al., 2017). In our experiment, Cd caused accumulation and a substantial elevation in Cd in the plant tissues, especially higher Cd content in the roots than that in the shoots, which in turn led to a higher decrease in the growth of roots than that in the shoot, as previously recorded in wheat (Qin et al., 2018). So, a variety of approaches have been tested to alleviate the detrimental impact of metals by inhibiting their transport to plants (Koźmińska et al., 2018). For instance, the use of externally supplied plant hormones has been focused on by researchers to relieve the damaging effects of heavy metals (Sytar et al., 2019). In the current experimentation, the potential impact of the application of MeJA and SNP singly or jointly in allaying the injurious effects of Cd stress on wheat plants was assessed. Both MeJA and SNP led to a marked reduction in Cd absorption by the roots. Furthermore, MeJA and SNP lowered the transfer of Cd from the roots to the aboveground plant parts. The joint effect of MeJA and SNP was more effective on reducing the root uptake of Cd and Cd translocation from roots to shoot with respect to its single application. Our results are in line with those reported in other studies. For example, externally supplied

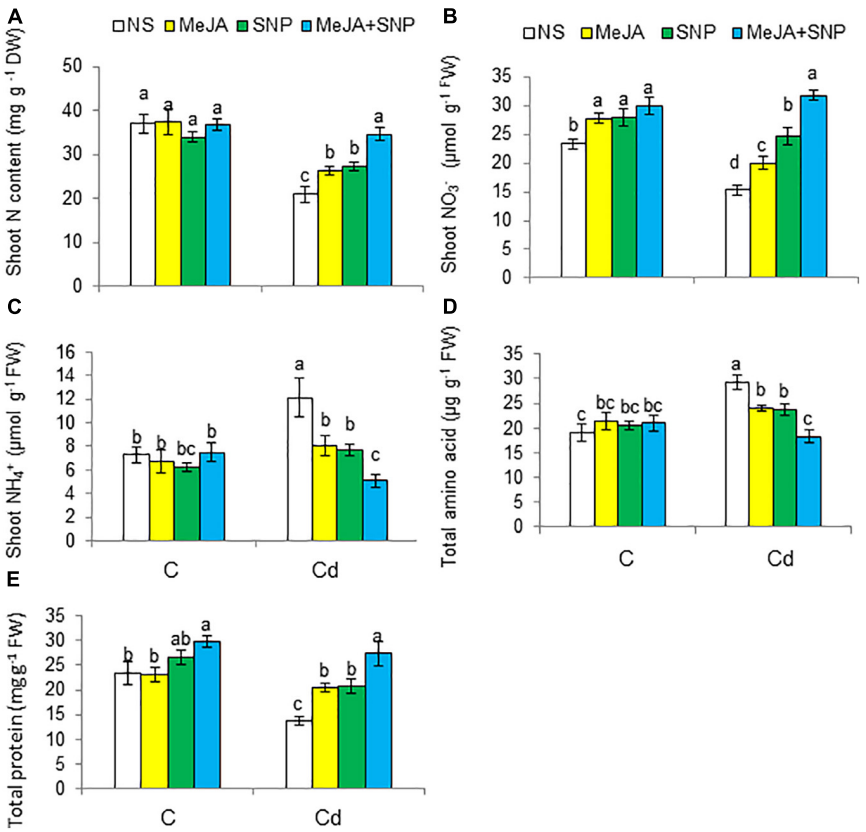

FIGURE 9 | Shoot total nitrogen [N **(A)**] on dry weight (DW) basis, shoot nitrate [NO_3^- **(B)**], shoot ammonium [NH_4^+ **(C)**], total amino acid **(D)**, and total protein **(E)** contents on fresh weight (FW) basis in wheat plants grown under control (C) and cadmium (100 μM Cd) and sprayed with 10 μM methyl jasmonate (MeJA) or 100 μM sodium nitroprusside (SNP) alone or together (mean ± SE). Mean values carrying different letters within each attribute differ significantly ($P \leq 0.05$) based on Duncan multiple-range test.

MejA decreased Cd content in foxtail millet (Tian et al., 2017) and wheat plants (Alikhani and Abbaspour, 2019). Furthermore, the reducing effect of SNP on shoot and root Cd has also been observed in *C. roseus* (Nabaei and Amooaghaie, 2020) and rice (Singh P. et al., 2020). The reduced absorption of Cd may be because of the development of a metal Cd–NO complex as suggested by Singh P. et al. (2020), but this needs to be further confirmed. Alternatively, it has been suggested that NO might defend plants against membrane injury, thereby maintaining their regular metabolic processes, as a minor portion of Cd moves to the shoot cells (Dong et al., 2016). Furthermore, externally applied MeJA and SNP reduced Cd BCF, transport factor (TF) from roots to shoots, and biological accumulation concentration (BAC); this shows the crucial controlling roles of MeJA and SNP in advancing tolerance to Cd by decreasing the Cd transportation and accumulation as similarly suggested by Nahar et al. (2016).

Photosynthesis-Related Parameters in Wheat Under Cd Stress

The exogenous application of MeJA together with SNP showed more encouraging effect on the contents of chlorophyll and carotenoids, as well as F_v/F_m under control and Cd stress. Some previous reports also show that MeJA or SNP improves photosynthesis-related parameters, e.g., in mustard (Per et al., 2016) and *M. arvensis* (Zaid and Mohammad, 2018), in which MeJA was reported to improve the aforementioned attributes. The beneficial effect of NO on these parameters was also testified in cucumber (Gong et al., 2017) and ryegrass (Chen et al., 2018) under Cd. The possible reason of the constructive role of MeJA and SNP on photosynthesis-related parameters under Cd stress could be linked to reduced chlorophyll breakdown and reduced ROS together with enhanced antioxidant enzymes' activities. Gong et al. (2017) suggested that NO strongly protects photosynthetic apparatus from Cd. Per et al. (2016) observed that MeJA enhanced the synthesis of GSH, thereby improving chlorophyll synthesis as similarly observed in our experiment. Reduced chlorophyll degradation, improved GSH contents, and the rise in the antioxidant system due to MeJA or SNP allow the plants to thrive well under Cd stress. In our study, plants treated with MeJA and SNP also exhibited lower contents of H_2O_2 and MDA, and higher chlorophyll content than those in the Cd-stressed plants. Earlier investigations have noted a beneficial effect of MeJA in wheat (Alikhani and Abbaspour, 2019) and that of NO in rice (Hsu and Kao, 2004) and tomato (Ahmad et al., 2018) under Cd stress. There seems to be no investigation

reporting the joint effects of MeJA and SNP on chlorophyll synthesis in the current literature.

Water Relations and Osmolytes in Wheat Under Cd Stress

The application of MeJA and SNP leads to higher accumulation of osmotic substances, which can improve stress tolerance by enhancing water status in the cells of plants (Arasimowicz-Jelonek et al., 2009; Sadeghipour, 2018). Like other osmotic substances, both Pro and GB participate in alleviation of a stress through osmotic adjustment in plants (Bhuiyan et al., 2019; Zhang and Yang, 2019). Furthermore, Cd stress elevated the accumulation of Pro (Zouari et al., 2016) and GB (Jan et al., 2018). In our study, MeJA and NO application increased the production of GB and Pro; this obviously shows that MeJA and NO play a protecting function in Cd tolerance. Some previous investigations also exhibit that exogenously supplied MeJA augmented Pro and GB contents as observed in *M. arvensis* (Zaid and Mohammad, 2018), and that of NO increased Pro in alfalfa (Su et al., 2018), and GB and Pro in tomato (Ahmad et al., 2018) under Cd stress. Furthermore, our observations suggest that MeJA- and NO-induced enhanced generation of Pro could have been due to the modulation of Pro metabolism, as suggested for the effect of NO by Ahmad et al. (2018) under Cd stress. Increased Pro and GB in MeJA- or SNP-applied plants led to improvement in leaf RWC, possibly due to improvement in hydraulic conductivity, as suggested by Ahmad et al. (2018), who reported that NO improved Pro, which in turn enhanced the hydraulic conductivity in tomato. Moreover, Cd stress is believed to inhibit water uptake (Naeem et al., 2019) due to reduced root hydraulic conductance, which may lead to a substantial decrease in cellular turgor, thereby leading to reduced RWC.

Upregulation of Cd Detoxification Process in Wheat

The metabolites, such as GSH, PCs, and GST activity are straightly involved in the detoxification of Cd (Li et al., 2018). Metals can be bound to the thiol (-SH) group of GSH (Baig et al., 2019), and in this way, GSH can sequester a metal and transfer it to the vacuole as a precursor of PCs (Ramakrishna and Gill, 2018). Similarly, PCs can efficiently bind to Cd as a chelating agent (Rahimzadeh et al., 2017). Heavy metal stress increases PC contents, which are believed to have a potential role in the detoxification of Cd (Uraguchi et al., 2017). This shows that PCs and GSH together detoxify Cd in plants. Plants under Cd stress showed a rise in GSH, which may detoxify Cd, and it is converted to GSSG; this could have been the reason of GSSG being higher in the Cd-treated wheat plants than that in the controls. High GSSG together with decreased GSH/GSSG ratio indicates an oxidative injury induced by Cd (Singh S. et al., 2020). The application of SNP and MeJA inverted the GSH/GSSG ratio and GSH content by rising the GSH concentration and GSH/GSSG ratio; our findings are also in line with those of an earlier report of Per et al. (2016) wherein MeJA increased GSH in mustard under Cd stress. Nahar et al. (2016) also testified restoration effect of SNP on GSH and GSH/GSSG ratio in Cd-treated mung bean. The PC synthesis increased in Cd-stressed wheat seedlings, and MeJA and SNP further increased the PC concentration in the Cd-fed plants with reference to the plants treated with Cd only. This obviously shows that MeJA and SNP alone or in combination participate in activating the PC biosynthesis, which in turn leads to the chelation of Cd. Hu et al. (2019) also reported that NO synthesis improved chelation of Cd in *Sedum alfredii*. Moreover, Alikhani and Abbaspour (2019) reported the MeJA showed a beneficial effect on the chelation of Cd in wheat. The combined effect of MeJA and SNP was more pronounced in terms of increasing the PC content. There seems to be no study in the literature on the combined effect of these two metabolites on PC synthesis in Cd-stressed plants.

Reversal of Oxidative Injuries Under Cd Stress

The application of MeJA and SNP substantially suppressed the contents of H_2O_2 and MDA, the activity of LOX, and EL in the wheat plants under Cd stress. Previous reports also exhibit that Cd elevated the aforementioned oxidative stress parameters, particularly in tomato (Alyemeni et al., 2018) and *Glycine max* (Molina et al., 2020) plants. Overaccumulation of H_2O_2 can damage crucial processes of cells including photosynthesis by decreasing the strength of organelle ultrastructure's (Smirnoff and Arnaud, 2019). Furthermore, high H_2O_2 accumulation in plants under a stress results in enhanced injury to lipids and proteins, which in turn affects their integrity and effectiveness (Palma et al., 2019); this was also obvious in our investigation in terms of enhanced MDA and EL. Cd-induced ROS accumulation leads to the generation of LOX (Kaya et al., 2019), which is known as an indicator of considerable injury to lipids. In our study, externally applied MeJA and SNP decreased the LOX activity in Cd-stressed wheat plants. NO has been shown to reduce LOX activity under Cd stress (Nahar et al., 2016). Reduced membrane leakage due to the application of MeJA and SNP might have been due to improved antioxidant activity.

Enhancement in Antioxidants in Wheat Under Cd Stress

The supply of MeJA and SNP-induced reversal of oxidative injury under Cd stress might have been due to upregulation of the antioxidant system. Accordingly, MeJA and SNP supplementation substantially elevated the activities of antioxidant enzymes such as CAT, SOD, and the AsA–GSH cycle–related enzymes under Cd treatment. Various antioxidant enzymes perform specific functions in different parts of the cell (Caverzan et al., 2016). SOD is pervasive in the scavenging of superoxide, but CAT eliminates H_2O_2 in the cytosol (Azarabadi et al., 2017). Furthermore, AsA–GSH cycle enzymes and components of the redox system, AsA and GSH, can scavenge H_2O_2, thereby sustaining electron transport (Caverzan et al., 2019). Previous results have reported that application of MeJA augmented the activities of SOD and GR in mustard (Per et al., 2016) and *Arabidopsis* (Lei et al., 2019) under Cd stress. The

MeJA-induced enhanced activities of the antioxidant enzymes might have been due to the interaction of MeJA with H_2O_2. This has also been suggested by Zaid and Mohammad (2018). MeJA and SNP enhanced the GR activity, which can promptly remove H_2O_2 through the AsA–GSH cycle system, thereby resulting in allaying Cd-induced oxidative injury in the wheat seedlings. Earlier investigations have suggested that MeJA decreases oxidative stress due to Cd toxicity by elevating the GSH levels and antioxidant enzyme activities, thus reducing the levels of MDA and H_2O_2 in plants under high Cd regimens (Zaid and Mohammad, 2018; Lei et al., 2019), similar to that found in the current research. Our results also indicate that NO elevated the SOD, CAT, and AsA–GSH cycle–related enzymes' activities, as earlier recorded in wheat (Zhao et al., 2016) and tomato (Ahmad et al., 2018), but no studies reporting the joint effect of MeJA and SNP are available. The enhanced ASA-GSH cycle–related enzymes' activities due to MeJA and SNP application may have provided higher defense to cellular organelles and metabolic processes against the harmful effects caused by Cd stress.

Cd suppressed AsA levels and enhanced those of DHA in the wheat seedlings, as earlier observed in *Brassica napus* (Maresca et al., 2017). However, addition of MeJA and SNP with Cd reduced DHA content and elevated that of AsA by enhancing the MDHAR and DHAR activities, which elevated AsA/DHA ratio under Cd stress. Nahar et al. (2016) also observed increased DHAR activity in mung bean treated with NO under Cd stress.

Upregulation of N Metabolism in Wheat Under Cd Stress

N metabolism can be disturbed by high Cd regimens (Khan et al., 2015; Ahanger et al., 2020). For example, Cd stress reduced NR activity and total N and NO_3^- contents, but it increased NH_4^+ content in the wheat plants. Analogous findings can be seen elsewhere, showing that Cd reduced the NR activity and N content in plants (Khan et al., 2015; Ahanger et al., 2020). Plants use NO_3^- as an N source (Liu et al., 2018); NR acts as a main catalytic enzyme in the reduction of NO_3 to NO_2 in plant cells (Yoneyama and Suzuki, 2019). Then, NO_2 is converted to NH_4^+ by NiR activity (Balotf et al., 2016). Decreased NO_3 content due to Cd in the wheat plants might be linked with reduced transpiration due to Cd stress; this may have led to decreased NO_3 transport from the root to the above parts of plants through xylem (Singh and Prasad, 2017). Alternatively, overaccumulation of ROS due to Cd led to enhanced cell membrane permeability, thereby reducing the absorption of NO_3^- by the root cells (Ramakrishna and Gill, 2018). Moreover, reduced absorption of NO_3 and NR activity, as observed in the wheat plants, has also been observed by Singh and Prasad (2017) in Cd-stressed tomato plants. However, augmentation of NH_4^+ content in the Cd-stressed wheat plants may have occurred because of limitation in the assimilation of ammonia (Khan et al., 2016; Yang et al., 2019). Overaccumulation of NH_4^+ is harmful to the plant cells (Wang et al., 2019), and so plants possess a strategy through the GS/GOGAT pathway or GDH pathway, which are other paths for assimilating NH_4^+ so as to lower the harmful effects due to the accumulation of ammonia, particularly when the activity of GS is diminished (Aly et al., 2018). NH_4^+ is transformed into an organic substance mainly by the GS/GOGAT pathway (Ashraf et al., 2018). The findings of our experimentation exhibited that reduced GS and GOGAT activities in the wheat plants under Cd stress could be linked to the disruption in NH_4^+ assimilation, which is evident by reduced N and protein contents and increased NH_4^+ content. However, Cd-induced increased GDH activity might have reduced the activities of GS and GOGAT. Enhanced GDH activity could not be high enough to maintain NH_4^+ assimilation; this was evident as enhanced NH_4^+ content along with reduced growth occurred in the Cd-stressed wheat plants. However, enhanced GDH activity is believed to participate in lessening NH_4^+ accumulation and glutamate production for the synthesis of defensive substances (Xiaochuang et al., 2020).

The exogenous supply of MeJA and SNP markedly augmented the NR activity, total N, and NO_3 and NO_2 contents, as well as reduced the NH_4^+ content due to rapid use of NH_4^+ to produce amino acids via enhanced GS and GOGAT activities. This led to enhanced utilization of N in chlorophyll synthesis, thereby increasing plant growth in the wheat plants under Cd stress. NR regulates the rate-restricting reaction in N metabolism, which in turn mediates the crucial physiological processes, e.g., amino acids and secondary metabolites containing N (Ahanger et al., 2017; Chamizo-Ampudia et al., 2017). Similarly, Zaid and Mohammad (2018) noted that MeJA increased the NR activity and N content. Increased uptake and assimilation of NO_3^- lead to increased conversion of available N to amino acids (Hachiya and Sakakibara, 2017). Furthermore, elevated NR activity leads to enhanced N assimilation (Alt et al., 2017), which in turn increases stress tolerance by probably increasing the protein synthesis. Furthermore, application of MeJA and SNP reduced the GDH activity in the Cd-stressed wheat plants, suggesting that this enzyme enhances the main pathway of NH_4^+ assimilation by regulating the GS/GOGAT cycle under Cd stress. Moreover, MeJA and SNP-induced mitigation of Cd toxicity is most likely due to enhanced content of proteins.

CONCLUSION

Cd impaired growth, water relations, N metabolism, and AsA–GSH cycle. The combined application of MeJA and SNP showed promising results in terms of improving plant growth and physiology and N metabolism, upregulating the AsA–GSH cycle–related enzymes' activities, and reducing Cd content in wheat plants, suggesting a possible interactive role of the two compounds in alleviating Cd-toxicity in wheat plants. This work further suggests an ecofriendly approach for mitigation of heavy metal toxicity in food cereals.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CK, MA, and PA designed the experimentation. CK performed the experiments and generated the data. MA, HD, and AN analyzed the data. CK and MA jointly wrote up the manuscript. MA and PA thoroughly edited the entire manuscript. All authors contributed to the article and approved the submitted version.

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Crucial Cell Signaling Compounds Crosstalk and Integrative Multi-Omics Techniques for Salinity Stress Tolerance in Plants

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In the era of rapid climate change, abiotic stresses are the primary cause for yield gap in major agricultural crops. Among them, salinity is considered a calamitous stress due to its global distribution and consequences. Salinity affects plant processes and growth by imposing osmotic stress and destroys ionic and redox signaling. It also affects phytohormone homeostasis, which leads to oxidative stress and eventually imbalances metabolic activity. In this situation, signaling compound crosstalk such as gasotransmitters [nitric oxide (NO), hydrogen sulfide (H_2S), hydrogen peroxide (H_2O_2), calcium (Ca), reactive oxygen species (ROS)] and plant growth regulators (auxin, ethylene, abscisic acid, and salicylic acid) have a decisive role in regulating plant stress signaling and administer unfavorable circumstances including salinity stress. Moreover, recent significant progress in omics techniques (transcriptomics, genomics, proteomics, and metabolomics) have helped to reinforce the deep understanding of molecular insight in multiple stress tolerance. Currently, there is very little information on gasotransmitters and plant growth regulator crosstalk and inadequacy of information regarding the integration of multi-omics technology during salinity stress. Therefore, there is an urgent need to understand the crucial cell signaling crosstalk mechanisms and integrative multi-omics techniques to provide a more direct approach for salinity stress tolerance. To address the above-mentioned words, this review covers the common mechanisms

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of signaling compounds and role of different signaling crosstalk under salinity stress tolerance. Thereafter, we mention the integration of different omics technology and compile recent information with respect to salinity stress tolerance.

Keywords: antioxidant defense, crosstalk, homeostasis, omics approaches, signaling network, plant growth regulators, salinity stress tolerance

INTRODUCTION

Soil is an indispensable component of the environment and a fundamental prerequisite for nourishing optimistic plant growth and development. Along with its supporting role, soil provides essential nutrient and mineral elements for the vigorous and productive growth of plants. Despite this, the extensive use of chemical fertilizers, excess irrigation, farm mechanization, and other anthropogenic activities act as stress factors for soil natural properties and lead to soil salinity (Pessarakli and Szabolcs, 1999). Soil salinity is one of the global indispensable stress factors, affecting plant growth drastically in both irrigated and rain-fed areas (Hussain et al., 2019; Sabagh et al., 2019; Liu L. et al., 2020). According to the Land and Plant Nutrition Management Service report, approximately 6% (>45 Mha) of the world's cultivated areas and about one-third of irrigated land on earth is affected by the salinity stress (Carillo et al., 2011; Deinlein et al., 2014; Parihar et al., 2015). Soil salinity situations emerge when glut salts leach and accrue in soil and at the same time, there is no room to flush out the accumulated salts to a well-managed drainage system (Qadir et al., 2008). At the same time, salt concentration swiftly reaches levels that are injurious to salt-sensitive species and beyond this, salt concentration levels can also affect salt-tolerant species. The initial stage of salinity stress is osmotic stress and perused by ion toxicity, which mainly targets uptake and transport of essential ions in plant roots (Serrano and Rodriguez-Navarro, 2001). Salinity stress induces considerable changes in physiological, biochemical, and molecular processes, depending on the extent and severity of the stress, types of genotypes, and crop stages which ultimately leads to huge yield penalty in important agricultural crops (Zeng et al., 2001; Thitisaksakul et al., 2015; Negrão et al., 2017). Osmotic stress culminates in loss of water absorption capacity of the root system, water potential of leaves, membrane damage, nutrients inequity, reduced photosynthetic and metabolic processes, and abatement of the antioxidant defense of plants (Amirjani, 2010; Yan et al., 2013; Parihar et al., 2015). Severe toxicity leads to alterations in crucial plant processes and destroys root functions via modifying redox potential, ion homeostasis, hormonal balance, transpiration, and generates a high amount of ROS [singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide (H_2O_2)], which damages the cellular membrane integrity and macromolecular structure (carbohydrate, proteins, lipids, and DNA) (Läuchli and Grattan, 2007; Farkhondeh et al., 2012). The consequences of salinity on root and plant functions, fundamental processes, and at molecular levels are illustrated in **Figure 1**.

Hence, salt stress adaptivity or tolerance responses are very crucial to develop stress-tolerant varieties under unfavorable situations. As a consequence, plants activate some exclusive physiological, biochemical, and molecular mechanisms in order to survive under stress conditions, which involve the activation of antioxidant enzymes, compartmentalization, uptake and transport of ions, accumulation of osmoprotectants and compatible solutes, ion homeostasis, and secondary metabolites (Gupta and Huang, 2014; Singhal et al., 2017). Nevertheless, the natural capacity of plants enable them to achieve the desired food potential under these circumstances. Therefore, researchers are continuously working on finding strategies and mechanisms to boost their final potential. In this regard, identifying differentially expressed genes and gene products and transgenic approaches that are associated with stress tolerance are promising approaches to develop smart crops (Yang and Guo, 2018). Even if, due to the complex nature and effects of abiotic stresses on plant processes, these strategies are also completely efficient to achieve the goal of food security under climate change. Therefore, recently scientists have been working on finding and elucidating sophisticated signaling and molecular approaches to develop multiple stress-tolerant crops.

At this point, actuating the signal-transduction cascade for versatile climate plant responses includes various adjustments that are produced in an exceptionally well-coordinated way for exhibiting great opportunities to provide stress tolerance. The promoter-reporter approach has also been exceptionally helpful for identifying genes involved in osmotic stress (Ishitani et al., 1997), and has significantly improved salt-stress monitoring in higher plants. Ionic homeostasis under salt stress is mediated by the SOS (salt overlay sensitive) pathway in a Ca^{+2}-dependent manner, which transduces the salt stress signal in a regulated pattern with the SOS3-SOS2 protein kinase complex at the cell membrane that adds an Na^+ ion into the cell and balances ion homeostasis (Ji et al., 2013; Gupta and Huang, 2014). Interestingly, several other signaling compounds such as nitric oxide (NO), hydrogen sulfide (H_2S), H_2O_2, Ca, ROS, and plant growth regulators salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) have crucial roles during cell signaling and crosstalk as they provide tolerance to multiple stresses (Chauhan et al., 2017; Noctor et al., 2018; Pei et al., 2018). Moreover, germplasm resources and integrated "omics-assisted" approaches such as phenomics, ionomics, transcriptomics, proteomics, genomics, miRNAomics, lipidomics, and metabolomics are prominently used for developing salt tolerance in crop species (Ho et al., 2020). Correspondingly, epigenetics and next generation phenotyping also provide efficient platforms in context to the production

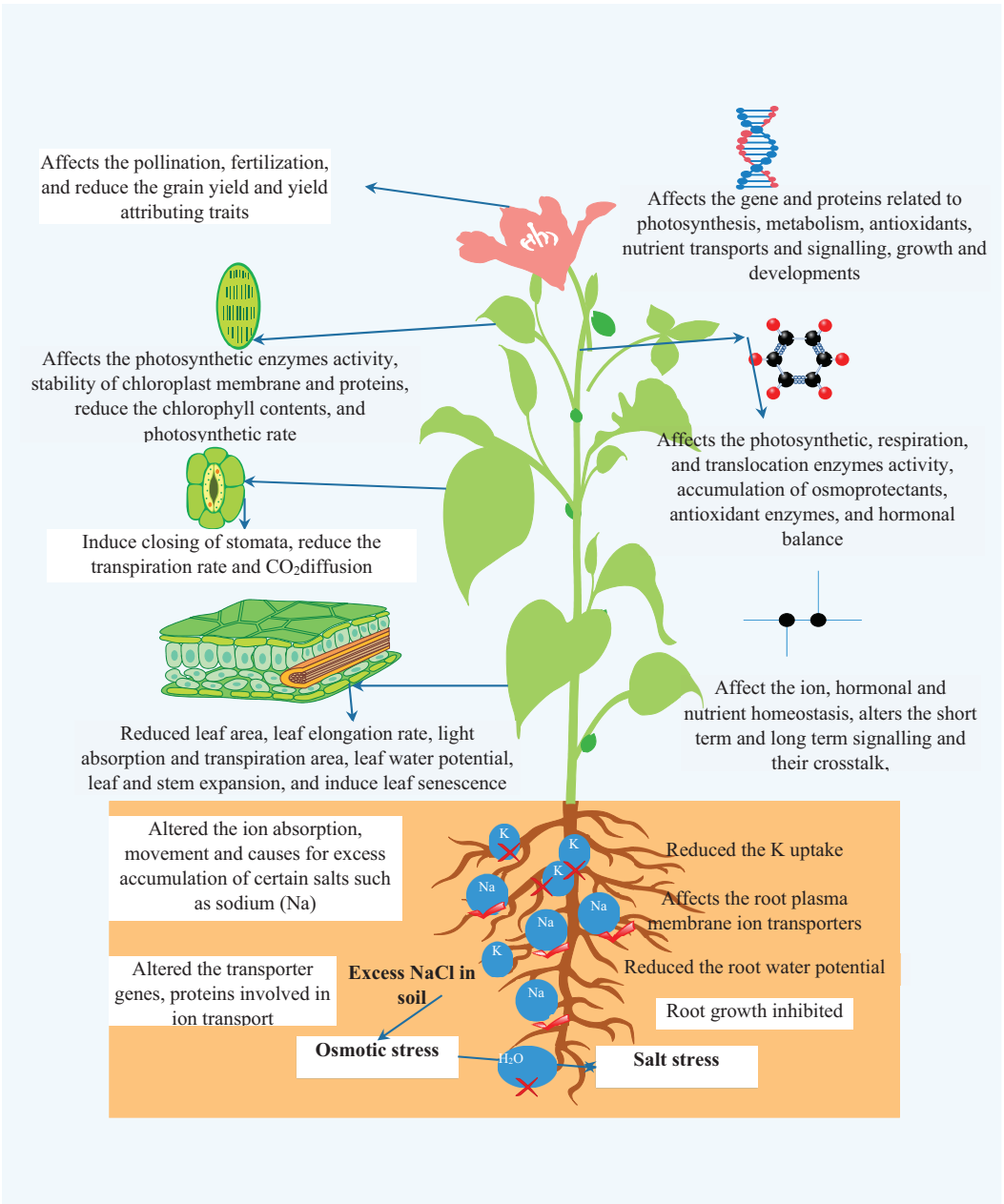

FIGURE 1 | The effect of salinity stress on root growth, ionic homeostasis, physiological, biochemical, and molecular processes.

of salt stress-tolerant species (Jha et al., 2019). All integrated omics-assisted approaches have contributed extraordinarily to understanding the outcomes of salinity stress and the alterations which plants adopt for survival and mitigation under unfavorable conditions (Mehta et al., 2019; Pan et al., 2020; Rasel et al., 2020). It is important to keep in mind that, in the present perspective, we point out the recent advances in the crosstalk of important signaling compounds and their role under salinity stress tolerance. Further, we address the recent advances in integrative multi-omics approaches, which are crucial to provide salinity tolerance and a future platform to develop promising salt-tolerant varieties for salt stress conditions.

PLANT SIGNALING COMPOUNDS AND CROSSTALK MECHANISMS UNDER STRESS REGULATIONS

Plants under stressed conditions such as abiotic (heat, cold, salinity, heavy metal) and biotic stresses must be acknowledged and the innate immune system must be activated for survival

and better fitness. The survival of plants under stressful conditions depends on compact signaling networks and their crosstalk (Tena et al., 2011; Smékalová et al., 2014). Signaling pathway activities are activated after the sensation of a signal produced by a specified receptor that triggers the urging of secondary signals and protein phosphorylation cascades like MAPK signaling. Secondary messengers such as ROS, Ca^{2+}, NO, H_2S, H_2O_2, phospholipids, and PGRs act as secondary signals during signaling cascades (DeFalco et al., 2010; Suzuki et al., 2012). These signals are involved in signaling pathways through multiple ways and administer fundamental processes such as cell division and growth, differentiation, and programmed cell death under normal as well as abiotic and biotic interactions (Müller et al., 2010; Tena et al., 2011; Sasabe and Machida, 2012).

Salt stress changes to membrane structures induce metabolic stress, form ROS, and prevent photosynthesis leading to nutrient deficiency (Hasegawa et al., 2000; Tuteja, 2007). The growth responses to salinity include two distinct stages (Munns, 1993). Hormonal signals from the root's delay development, and then switch off the signal when the plant is mature. Growth reduction can be attributed to a salt-specific effect, which often takes a while (varies between weeks to years) to create. The second step is a result of decreased water availability and accumulation of salt in transpiring leaves, adding to thresholds that surpass the capacity of a cell to sequester salts into vacuoles (Munns, 1993, 2005; Läuchli and Grattan, 2007). Na^+ reaches cells quite quickly just after the occurrence of salt stress. The increased sodium in this water might influence its salinity adaptation. Therefore, the crucial mechanism of mitogen activates protein kinase and Ca signaling under stress conditions are discussed in the next section.

Mitogen-Activated Protein Kinase Signaling (MAPK)

The function of MAPK relies on post translational phosphorylation signaling, established by a serine/threonine kinase, i.e., mitogen-activated protein kinase kinase kinase (MAPKKK or MAP3K) that reversibly phosphorylates MAPKK (a dual-specificity kinase), then that phosphorylates MAPKs (Keshet and Seger, 2010; Smékalová et al., 2014). MAPKs are involved in phosphorylation of transcription factors, cytoskeleton-associated protein, and protein kinase in plants (Nakagami et al., 2005). Recently, various researchers established that root growth initiation was due to the activity of Ca^{2+} channels and production of auxin which boost the accumulation of NO. This NO is culpable for the modulation in Ca^{2+} channel movement and MAPK cascade enzyme activities. ROS molecules, which form as metabolic by-products under stressed conditions, can also induce the activation of MAPKs (Nakagami et al., 2005).

Plant hormones act as an impressive signaling molecule under both normal and stress conditions. MAPK cascades also respond to various hormone signaling like auxin, SA, JA, brassinosteroids (BRs), strigolactones, ABA, and ET. These signaling molecules accomplish a distinct signaling network, which crosstalk to each other and respond under normal and stress conditions (Devoto and Turner, 2003; Depuydt and Hardtke, 2011; Chini et al., 2016). Treatment with natural and synthetic auxin triggers the prompt actuation of MAPKs in the roots of Arabidopsis (Mockaitis and Howell, 2000). ABA signaling has a massive role in the plant growth process that deals with the turgor and stomatal activity of plant cells. MPK4, MPK9, MPK12, and MPK15 proteins exist in guard cells (Zhao et al., 2008), which exhibit the decisive role in ABA signaling and are possibly associated with the activation of the ABA-dependent anion channel (Jammes et al., 2009). Under stress conditions, ABA induces the production of H_2O_2 and the expression of catalase (CAT) isoform CAT1. This expression is mediated by Arabidopsis MAP2K, in response to H_2O_2 (Xing et al., 2008). H_2S is considered an endogenous gaseous transmitter that exhibits a specific role in the germination of seed, root growth, stomatal activity, photosynthesis, and abscission of plant organs under normal as well as stressed conditions (Corpas and Palma, 2020). H_2S interacts with other signal molecules such as ABA, ethylene, auxin, Ca^{2+}, CO, and NO, and controls post transitional modification of proteins (Hancock and Whiteman, 2016; Xuan et al., 2020). ABA induced H_2S accumulation via activating SnRK2.6 activities at Cys131 and Cys137 by *S*-sulfhydration of SnRK2.6 that enhances the interaction of SnRK2.6 with ABA responsive element-binding factor ABF2 (Chen S. et al., 2020). NO is involved in ABA and ethylene crosstalk (Domingos et al., 2015). Indeed, NO was produced during the initial phase of seed germination and promoted seedling growth by inducing ABA 8′-hydroxylase gene expression and ethylene production. Ethylene protects the Brassicaceae seed from the inhibitory effect of ABA by stimulating weakening and rupturing seed testa and endosperm (Arc et al., 2013). NO donors inhibit ethylene biosynthesis and prevent the dormancy of seeds and stimulate germination in apples (Gniazdowska et al., 2007). Breaking of apple seed dormancy by NO encourages ROS production, which stimulates ethylene accumulation due to an increase in ACS and ACO activity (Gniazdowska et al., 2010). EREBPs, which are described as transcriptional factors induced by NO, and ethylene stimulate EREBP-3 just before the rupturing of endosperm during tobacco seed germination, which is inhibited by ABA (Leubner-Metzger et al., 1998). H_2S effectively alleviated ethylene-mediated fruit softening in Kiwi fruits and enhanced the ascorbic acid, starch, sugar protein, and titratable acidity (Li T. T. et al., 2017). Combined treatment of H_2S-ET inhibited ET synthesis and its related genes such as ACS6, ACO1, ACO4, ERF1, and ETR4, thus suppressed ET induced petiole abscission in tomatoes (Liu D. et al., 2020). Treatment with a higher level of NaHS inhibited primary root growth, initiated by ROS and NO accumulation and activation of the MPK6 gene (Zhang et al., 2017), which denoted that ROS-MPK6-NO cascading intermediates have repressive impacts of high concentration of H_2S on root activity (Zhang et al., 2017). H_2S strengthens the plant capacity to heat and aluminum (Al) tolerance by reducing oxidative damage after interaction with NO (Sun et al., 2016). Both H_2S and NO interactions improved the survival rate of plants under heat stress conditions, due to a decrease in malondialdehyde (MDA) accumulation

and enhanced antioxidant capacity in maize and strawberry (Uchida et al., 2002; Christou et al., 2014; Li J. et al., 2014; Li Z. G. et al., 2014).

Calcium (Ca^{2+}) Signaling

Under salinity stress, plants depict two forms at the same time including osmotic and ionic stresses. "Cell apoptosis versus adaptation" is dependent on the timing of two cellular responses: the first prompted by Ca, and the second prompted by oxidative outburst in the apoplast. A delay in the formation and dissipation of a salinity-triggered Ca-dependent signal coupled with ROS activates JA signaling, leading to the death of the cells. In contrast to the same molecular signal, calcium will, when properly timed, activate various adaptive processes including sequestration and extrusion of sodium, and also through ABA signaling. With respect to the perception of external inputs, calcium transients, from a number of extracellular compartments, become cytosolic through transient Ca^{2+}-dependent Ca^{2+} channels. The "Ca^{2+}-signature/Ca^{2+}-spiking" concept became common when it was defined by Webb et al. (1996). The pattern of calcium signaling is determined by the type and amplitude of the stimuli. The calcium level in the body has an enormous impact on the success level of life forms. Calcium-binding proteins, functioning as calcium receptors, relay the information to be conveyed from Ca signals. An unprecedented rate of sensitivity is accomplished by a group of calcium binding modules, that include 'calmodulin' (CaM), 'calmodulin-like protein (CML) family,' 'Ca^{2+}-dependent protein kinases' (CDPK), Ca^{2+}-binding proteins serving as "Ca^{2+} sensors," 'calcineurin B-like proteins (CBLs),' "Ca^{2+}-decoders," and 'CBL-interacting protein kinases (CIPKs)' which all together transmit the information embedded within calcium signatures. CaM is conserved regardless of species, while CML, CDPK, and CBL are unique to plants and some prokaryotes (Day et al., 2002; Harper and Harmon, 2005; Batistič and Kudla, 2009). Single-cell systems, including pollen germination, provide an excellent model to unveil the coding mechanism and determinants of "Ca^{2+}-signature." Induction of calcium transients mainly occurs at the organ level *via* a single spike. The induction of "Ca^{2+}-signature" is in accordance with (i) Ca^{2+} in various plasma membrane (PM) and endomembrane (EM) flux channels, (ii) cytosolic Ca^{2+} rallying (in and out) induced by Ca^{2+} influx and efflux transporters, respectively (McAinsh and Pittman, 2009; Kudla et al., 2010). Therefore, plant signaling is very complex in nature and numerous signaling compounds regulate the plant processes under normal and stress conditions.

SIGNALING COMPOUNDS CROSSTALK DURING SALT STRESS TOLERANCE

Salt tolerance is very complex in nature and affects various processes in plants. In this regard, signaling compounds such as NO, H_2S, H_2O_2, ROS, and plant growth regulators crosstalk with each other and coordinate numerous plant functions and processes, which are associated with salinity tolerance. The crosstalk of various signaling compounds for salinity tolerance are discussed, followed, and represented in **Table 1** and **Figure 2**.

NO Crosstalk

Nitric oxide is the primary gasotransmitter, administering numerous physiological and signaling functions, and also promotes salinity tolerance in plants (Xie et al., 2008). In recent years, it was confirmed that NO crosstalk with other signaling compounds and phytohormone signaling pathways helps in alleviating salinity stress (Tanou et al., 2009; Poór and Tari, 2011; Zhao et al., 2018). Auxin (AUX), ET, and ABA are imperative plant hormones that move from salt-treated roots to leaves that induce synthesis of NO or are transported throughout the plant (Molassiotis et al., 2010). Further, an increase in antioxidant activity and a decrease in thiobarbituric acid, which is reactive material content, is associated with NO-induced alleviation of oxidative damage in saline areas (Xu J. et al., 2011). In cotton, supplying exogenously NO (using SNP sodium nitroprusside for the NO donor) reduces the salt-induced senescence in leaves through downregulating the manifestation of ABA biosynthesis genes such as *NCED 9* (9-*cis*-epoxycarotenoid- dioxygenase) and *NCED 2* (Kong et al., 2016). In Arabidopsis, a callus treated with 100 mM of NaCl stimulated NO accumulation that added to ET emission, and resulted in induction of H^+-ATPase gene expression in the plasma membrane (PM) (Wang et al., 2009). However, an antagonistic relationship between NO and ET in a suspension culture of tomato cells treated with 100 and 200 mM of NaCl was reported and suggested that an increase in ET synthesis encourages ROS production that is associated with high dead cell ratio, whereas production of NO curtails the dead cell ratio (Poór and Tari, 2011). In the cell suspension culture and segments of apical root, both lack NO and ET-generated (Na^+/K^+) ionic imbalance, respectively, that leads to an increase in susceptibility toward salinity stress (Poór et al., 2011). Application of SA and SNP, in combination, reduces NaCl-induced toxicity by supplementing the accumulation of proline and stimulation of GPX (glutathione peroxidase), CAT (catalase), and APX (ascorbate peroxidase) in soybean seedlings (Simaei et al., 2011). SA interaction along with signaling flow of NO alters the photosynthetic capacity along with diminished accumulation of H_2O_2, which enhances the influx of H^+-ATPase into PM. The collaborative effect of both SA and NO promotes the absorption of Ca^{2+}/Mg^{2+} with decreased Na^+ uptake in saline conditions (Dong et al., 2015).

Among metabolites, sulfur is the main constituent present in reduced glutathione (GSH), methionine, coenzyme A, cysteine (Cys), iron–sulfur (Fe–S), thioredoxin, and sulfo-lipid systems associated with regulating the physiological process in salt stress environments (Khan et al., 2013). Further, NO enhances S-assimilation which is linked with ET synthesis through cysteine production. Sulfur and NO interact to regulate ABA and ET level in the guard cell and regulate photosynthetic and stomatal activities under salt conditions (Fatma et al., 2016). NO acts as a crucial regulatory signal, which activates various biochemical activities and their interaction with the sulfhydryl and nitro class during nitration enhances tolerance against salinity (Leterrier et al., 2011). NO along with other signaling compounds like

H_2S helps in building tolerance toward salinity stress in plants. It is observed that exogenous application of NO under saline conditions alters proline (Pro) metabolism and enhances the ratio of free proline accumulation that maintains the turgor potential and protects cucumber seedlings from salinity (Fan et al., 2012). In mustard, $CaCl_2$ and/or SNP application alleviates salt stress by influencing antioxidant enzyme activities along with promoting glycinebetaine (gb) and proline (pro) accumulation, which is associated with a decrease in H_2O_2, TBARS (thiobarbituric acid reactive substances), and electrolyte leakage (Khan et al., 2012). In *Lactuca sativa,* application of NaCl triggers osmotic, oxidative, and ionic stress that arise into hormonal imbalances and reduced growth of the plant. Exogenous NO application results in reduction of Na^+ accumulation, balancing the concentration of mineral nutrient, which is associated with balanced photosynthetic rate along with the established growth (Campos et al., 2019). Through NO signaling, phytohormone balance leads to osmotic regulation and also activates the antioxidant system with subsequent increase in tolerance level against salinity. The corm of *Crocus sativus* treated with NO shows more growth under salt-stress and promotes biosynthesis of the secondary metabolites, deposition of compatible solutes, and accelerates antioxidative enzyme activity, whereas treatment with SA did not boost plant growth during salinity (Babaei et al., 2021). Under saline conditions, NO-releasing substances and melatonin application counteracted inhibition of NaCl-treated seedling growth in addition to redox and ion homeostasis which is proved by retardation of ROS overproduction, Na+/K+ ratio, and reduction in the synthesis of TBARS. Consequently, increased level of NO augments addition of melatonin in seedling roots under salt stress (Zhao et al., 2018). Therefore, NO acts as an important signaling network with different signaling factors in plant systems under salinity stress.

H_2S Crosstalk

Hydrogen sulfide is a signaling molecule, which plays a major role in adventitious rooting, postharvest senescence, and seed germination (Deng et al., 2020), and provides a protective response toward multiple abiotic and biotic stresses (Corpas, 2019). Salt tolerance is enhanced through H_2S by increased soluble protein content and chlorophyll under saline conditions but also inhibits ROS accumulation (Mostofa et al., 2015a). H_2S donors which are identified/synthesized include CaS_2, morpholin-4-ium 4-methoxyphenyl (morpholino)

TABLE 1 | The crosstalk of crucial signaling compounds under salinity stress and their salt tolerance mechanism in different crops.

Crosstalk	Crop	Salt tolerance mechanism	References
GST-NO	*Glycine max* L.	NO induces the GST1 and GST4 isoenzymes and transcript levels in ABA-dependent and independent pathways	Dinler et al. (2014)
NO-$CaCl_2$	*Brassica juncea* L. cv. Varuna	Enhances the antioxidant enzymes activities (SOD, CAT, APX, GR, and POX), osmoprotectant (proline and glycinebetaine), and nutrient homeostasis (increase leaf K^+, Ca, and decrease Na^+). Combined application reduces oxidative stress by decreasing H_2O_2 content and lipid peroxidation	Khan et al. (2012)
H_2O_2-NO	*Oryza sativa* L. cv. Nipponbare	Improves antioxidant enzymes activity, and induces the expression of sucrose phosphate synthase (SPS), Δ′-pyrroline-5-carboxylate synthase, and HSP26	Uchida et al. (2002)
H_2O_2-NO	*P. euphratica* and *P. popularis*	Improves antioxidant defense by activating antioxidant enzymes, reduces oxidative stress, and maintains redox and nutrient homeostasis	Sun et al. (2010)
H_2S-NO	*Medicago sativa* L., Victoria	Induction of APX1, APX2, Mn-SOD, Fe-SOD, Cu/Zn-SOD isoforms transcripts level, and re-establishment of ion homeostasis	Wang et al. (2012)
H_2S-H_2O_2	*Arabidopsis thaliana*	Promotes the expression and phosphorylation of PM H^+-ATPase and Na^+-H^+ antiporter protein, and regulates the activity of G6PDH and PM NADPH oxidase in roots	Khan et al. (2020)
H_2S-H_2O_2	*Vicia faba*	Increased L/D cysteine desulfhydrase activity and induction of stomata closing	Ma et al. (2019)
H_2O_2-NO	*Citrus aurantium* L.	Prevents the modification in accumulation levels of crucial enzymes in the Calvin-Benson cycle, switches 'on' the antioxidant immunity system, prevents protein carbonylation, protects plant metabolism by regulating the enzymes in mitochondria, and protein reprogramming by prevention of NaCl responsive proteins	Tanou et al. (2009)
H_2O_2-NO-Ca	*Bruguiera gymnorrhiza* and *Kandelia candel*	Maintains ion flux and K^+/Na^+ ion homeostasis	Lu et al. (2013b)
NO-H_2S	*Capsicum annuum* L	Improves total, shoot, and root biomass, decreases oxidative stress by reducing H_2O_2 production, prevents electrolyte leakage and MDA content, promotes CAT and SOD antioxidant activity, and maintains ion homeostasis	Kaya et al. (2020)
Ca-ROS	*Arabidopsis thaliana* and Halophytes	Promotes cytosolic ion balance and downstream signaling in activation of antioxidant enzymes	Kurusu et al. (2015)
H_2O_2-NO-Ca	*Chenopodium quinoa*	Induces amylase activity, seed reserve hydrolysis, accumulation of water-soluble sugar, and enhances protein and amino acid contents in seedlings	Hajihashemi et al. (2020)
Ca-H_2S	*Vigna radiate*	Promotes ion homeostasis, improves transport of nutrients, reduces oxidative damage, and induces antioxidants defense and proline metabolism	Khan et al. (2020)

Ψ *HSP, heat shock protein; G6PDH, glucose-6-phosphate dehydrogenase; ROS, reactive oxygen species; CAT, catalase; SOD, sodium dismutase; MDA, malondialdehyde; APX, ascorbate peroxidase; POX, peroxidase.*

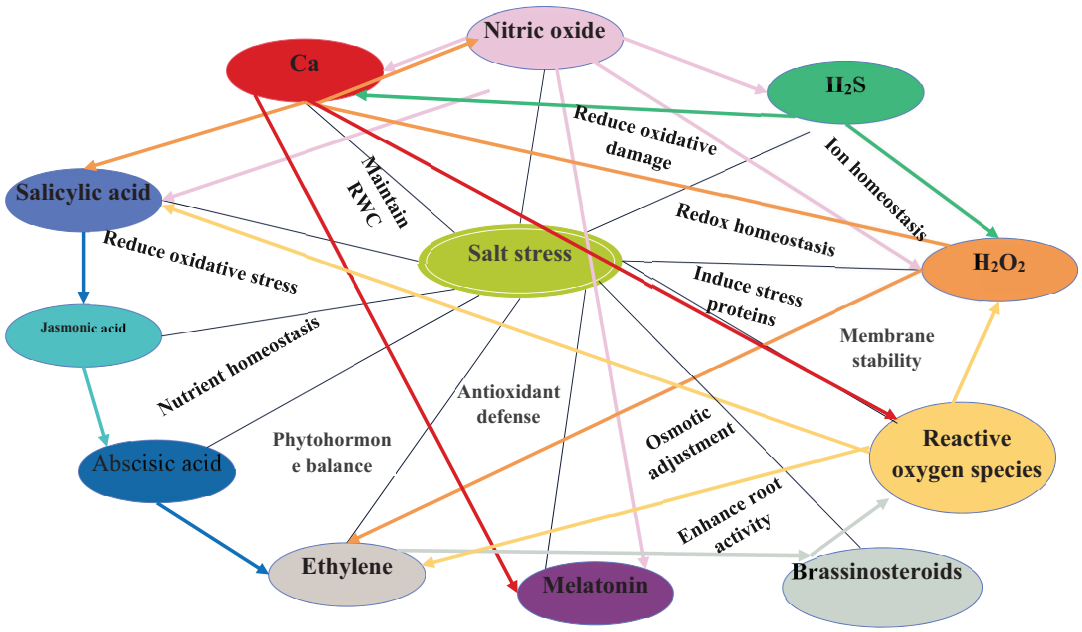

FIGURE 2 | The crosstalk of signaling compounds and function regulated under salinity stress.

phosphinodithioate (GYY4137), sodium hydrosulfide (NaHS), NOSH-aspirin, dialkyldithiophosphate (ZDDP), AP39, and diallyl trisulfide (DATS). NOSH-aspirin releases two gasotransmitters simultaneously, H_2S and NO (Kodela et al., 2012).

In plants, ROS levels are regulated by two ways, by scavenging excess ROS through antioxidant substances (like glutathione and ascorbate) and antioxidant enzymes linked with the AsA–GSH (ascorbate–glutathione) cycle. Accumulation of ROS is reduced by external application of H_2S because H_2S promotes antioxidant enzyme activities like SOD and CAT in Chinese cabbage (Zhang et al., 2015). From this we can assume that activity of antioxidant enzymes may be controlled by H_2S through their protein expressions, thus decreasing accumulation of ROS due to Al toxicity. It can also maintain membrane integrity and ROS homeostasis by controlling the antioxidant mechanism (AsA-GSH cycle and enzymes), therefore enhancing the tolerance level in plants toward salinity stress. In recent studies, by maintaining Na^+/K^+ homeostasis, H_2S helps in enhancing plant salt tolerance level. Under salinity conditions, the content of cellular Na^+ mainly increased whereas K^+ content reduced in rice, which shows a hike in Na^+/K^+ ratio in leaves and roots of rice. Therefore, exogenous H_2S application maintains Na^+/K^+ homeostasis in saline conditions in rice (Mostofa et al., 2015b).

Along with NO as a molecule for signaling, H_2S is also included in stress responses as they exist in a synergistic relationship (Hancock and Whiteman, 2016). The upstream and downstream relationship between H_2S and NO focuses on two factors: NO involvement in the stress tolerance enhances H_2S in plants and NO-induced stress tolerance where H_2S acts as a downstream signal molecule. Nitrosothiol is a new compound produced when H_2S reacts with NO and also results in a decrease of both the compound levels (Huang et al., 2020). In Arabidopsis roots, treatment with 100 mM of NaCl causes electrolyte discharge and also disturbs the Na^+/K^+ ratio but post treatment with NaHS promoted tolerance toward salt in roots (Li et al., 2016). Production of H_2S in alfalfa plants boosted the tolerance level against salinity in the germination stage of seeds which may be induced through oxidative damage (Wang et al., 2012). H_2S and NO modify the activity of many antioxidant enzymes like CAT, APX, and SOD to scavenge ROS which deceases their accumulation, thus helping in enhancing the tolerance level of plants toward higher salinity conditions (da-Silva et al., 2018).

Hydrogen sulfide interaction with various phytohormones such as ET, MT, ABA, and SA plays a fundamental role in response to abiotic stress (Huang et al., 2020). Gene expression related to ABA metabolism is increased by the exogenous H_2S along with an upregulation of ABA receptor expression levels in roots of wheat during stress conditions, which indicates that the stress tolerance of wheat is promoted by H_2S through the involvement of the ABA signaling pathway (Ma et al., 2016). When production of NO is restricted, ET is reduced which enhances the level of H_2S. A study on *Vicia faba* L. showed that H_2S biosynthesis inhibitors do not block the stomatal closure which is ET-induced with NO accumulation (Liu et al., 2016). Through the activation of endogenously synthesized H_2S, SA enhances the tolerance level of plants toward several other abiotic stresses. Crosstalk of NO and H_2S plays a crucial role in promoting the tolerance level against abiotic stresses. H_2S not only regulates the uptake along with transport of metal ions and maintains Na^+/K^+ homeostasis during salinity but also shows an interaction with different signaling molecules such as

Cys, phytohormones, and NO for enhancing the plant tolerance toward salinity stress.

H_2O_2 Crosstalk

Hydrogen peroxide is synthesized in a form of ROS and as a result of oxidative stress that cause damage due to excess accumulation of ROS under different stress conditions, which results in the death of cells (Fotopoulos et al., 2006). H_2O_2 production is induced in plants following exposure to a different range of environmental stimuli. Further, it was found that H_2O_2 acts as a signal to determine different physiological, biochemical, and molecular responses within plants and cells and their crosstalk in between other signaling pathways (Neill et al., 2002). NO and H_2O_2 signaling pathways are coordinated and tightly linked to different plant responses toward the environmental stimuli (Molassiotis and Fotopoulos, 2011). Alteration in production of both NO and H_2O_2 is considered under these plant responses toward salt stress (Zhang et al., 2006). Further, it was reported that expression of the *AtNOA1* gene in Arabidopsis was suppressed by NaCl treatment which leads to a reduced NO level (Zhao M. G. et al., 2007). In contrast, expression of *OsNOA1*, the rice homolog of *AtNOA1,* was moderately enhanced due to salinity (Qiao et al., 2009). By pre-treating wheat seeds with H_2O_2, the salt tolerance level of the seedlings was improved (Wahid et al., 2007). Apart from the biosynthetic level, H_2O_2 and NO crosstalk are also included in protein activity and regulation of gene expression (Qiao et al., 2014). In Bermuda grass, NO and H_2O_2 regulate induction of CAT and SOD activity through ABA (Lu et al., 2009). From further studies, it has been observed that both ABA-dependent NO and H_2O_2 influenced the activity of antioxidant enzymes and transcription under salt tolerance (Zhang et al., 2009). Consequently, SA enhances endogenous H_2O_2 levels significantly through regulating activation of SOD. Therefore, SA and H_2O_2 work together in a self-amplifying process (Rao et al., 1997). The high concentration of H_2O_2 causes oxidative/nitrosative stress, while in low concentrations H_2O_2 acts as a signaling molecule to regulate stress responses.

Ca Crosstalk

Calcium is an important signaling molecule, and secondary messenger crosstalk with several other signaling compounds help in the mitigation of salinity stress. Several studies have shown that flavonoids and phenylalanine ammonia lyase (PAL) activity are elevated after adding calcium chloride or an ionophore to the nutrient medium of a plant species (Paranhos, 2014). Ca^{2+}-mediated modulation for the development of the specialized metabolites is collectively induced by JA (Lee-Parsons and Ertürk, 2005), ABA (Vighi et al., 2019), and SA (Guo H. et al., 2015). The effects of MT and Ca, and how they affect the development of phenolic compounds (PCs), were investigated in the plant *Dracaena kotschy*i under stress conditions of salt water and compared with control. Vafadar et al. (2020b) reported that external NaCl application (mimicking salinity stress) reduces dry biomass of shoots but elevates H_2O_2 content, electrolyte leakage (EL) level, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, and upregulates gene expression of PAL, RAS, and TAL enzymes. Vafadar et al. (2020a) found that pre-treatment of *D. kotschyi* with a Mel biosynthetic pathway inhibitor has no effect on Ca^{2+}-mediated production of PCs in salt-affected plants. On the contrary, pre-treatment of *D. kotschyi* with a plasma membrane channel blocker, Ca^{2+} chelator, and calmodulin (CaM) antagonist resulted in impairment of Mel effects under salinity stress. This suggests that biosynthetic triggering of phenolics is attributed to Mel application only when influx of Ca^{2+} (carry out Ca^{2+}/CaM signaling) ions are there (Vafadar et al., 2020a,b,c).

Under stress conditions, H_2O_2 and NO interact with Ca^{2+} ions forming an intricate signaling web to withstand salinity (Hasanuzzaman et al., 2018). Under salt stress conditions, it was observed that pre-treated quinoa seeds with an NO donor (sodium nitroprusside; SNP), a reactive oxygen species (H_2O_2), and $CaCl_2$ showed a significant positive linear correlation with germination rate (GR) and germination index (GI), whereas a reversed linear correlation occurred between them with mean germination time (MGT) (Hajihashemi et al., 2020). The pretreatment enabled seed germination and rapid seedling establishment in the salt-affected soil. Pre-treating with NaCl completely prevented the decrease in the activities of alpha amylase and beta amylase. Salinity stress decreases seed germination by inhibiting the main enzymes, α- and β-amylase, which hydrolyze starch during germination, and pretreatment lowered this negative effect of salinity on these enzymes. Studies indicate that exposure to molecules, such as H_2O_2 or $CaCl_2$, may reduce the adverse effect of environmental stress on amylase activity and restore normal germination (Zheng et al., 2009; Li et al., 2013; Li Z. et al., 2017; Bouallègue et al., 2017). The most significant finding from the study conducted by Hajihashemi et al. (2020) was that the presence of NO, H_2O_2, and Ca^{2+} resulted in enhanced amylase activity. The rise in starch degradation increases germination, and more seeds sprout (Li et al., 2013). This connection could alleviate the negative impact of salt stress on quinoa germination.

Polyamines (PAs), like putrescine, spermidine, and spermine, are well regarded besides their substantial plant developmental processes and adaptation toward environmental cues (Pathak et al., 2014). It has been established that PAs play a vital role in a variety of cellular pathways including programmed cell death. Expression levels of PA biosynthesis-related genes were shown to be modulated under stress (Gupta et al., 2013; Shi and Chan, 2014). The modulatory response of arginine decarboxylase (ADC) (regulatory enzyme of PA biosynthesis) to salinity is a key regulator of the adaptive response in plants (Liu et al., 2006). Transcriptomics of PA biosynthetic genes in different varieties of rice revealed that the ADC gene is induced under salinity stress (Do et al., 2014). Studies indicated that perhaps the PA metabolic pathway is in intricate crosstalk with other signaling pathways including ABA, H_2O_2, and gamma-aminobutyrate (GABA) (Marco et al., 2011; Seifikalhor et al., 2019). Within that direction, Kalhor et al. (2018) have demonstrated that GABA helps increase salinity tolerance in lettuce. PA metabolism further induces NO output, which has been linked to several other stress intermediaries like Ca^{2+} ions and protein kinases. Under salinity conditions, PAs can engage ion channel proteins thereby affecting their conductivity

(Zhao F. et al., 2007; Velarde-Buendía et al., 2012). Garufi et al. (2007) proposed that PAs perform through regulating the activity of multiple ion channels indirectly by enhancing interactions with "14–3–3 proteins" (a family of highly conserved regulatory molecules). In stressful conditions, it is possible that high levels of cytoplasmic Ca^{2+} can be deleterious to standard cellular metabolism. Since active Ca^{2+} efflux networks play a pivotal role in sustaining cell Ca^{2+} contents, PAs activate Ca^{2+} efflux mechanisms, such as the PM channel and the membrane Ca^{2+}-ATPase, while maintaining steady plasma Ca^{2+} levels (Pottosin et al., 2012, 2014; Pottosin and Shabala, 2014). In this regard, PAs metabolism is related to Ca^{2+} signaling indirectly, even though underlying regulatory mechanisms remain unclear.

Several studies indicate the control of NO metabolism during salt tolerance (Wimalasekera et al., 2011; Ahmad et al., 2016). As a major NO production pathway, NO synthase (NOS) enzyme leads to most of the NO production in animals (Santolini et al., 2017) and plants (Moreau et al., 2008). When realizing that NO is a molecule linked to PAs via the common precursor of 1-arginine, it could be probable that PAs like spermidine and spermine produce NO in plants. The role of NO in signaling may be influenced by mobilization of intracellular calcium or interaction with calcium channels which ultimately elicit Ca^{2+} signaling (Courtois et al., 2008). These underlying mechanisms of Ca^{2+} and NO signaling affect each other. NO synthesis mediated by NOS operates via Ca^{2+} and CaM signaling (Corpas et al., 2004, 2006). Lamotte et al. (2006) found that NO plays a massive part in the activation of plasma membrane channels and the subsequent release of Ca^{2+} under salt stress recovery.

ROS Crosstalk

The 'salt overly sensitive' (SOS) pathway could potentially play an important role in the membrane conductance of the root epidermal cell of plants to the extracellular acidic environment, thereby helping to detoxify this ion in the root epidermal cells and thereby extruding this ion form the root epidermal cells (Quintero et al., 2002; Martínez-Atienza et al., 2007). The "salt overly sensitive" (SOS) pathway forms a critical pillar for preserving ion homeostasis when exposed to salinity stress (Ji et al., 2013). Nevertheless, during salinity, sustained accumulation and scavenging of ROS serve mostly as distress indicators, whereas redox homeostasis as well as antioxidant signaling at the cellular levels are engaged in stress sensing and tolerance. However, high levels of ROS accumulation can impair essential plant metabolic processes and development (Gill and Tuteja, 2010; Bose et al., 2014; Jajic et al., 2015). Electrons released by oxidants serve as a signaling cue in the cell to alert the plant to stress adaptation (Mittler et al., 2011). Salinity stress also causes ROS-mediated damage to lipids and proteins, and contributes to programmed cell death (Poór et al., 2012). Yet, ROS and calcium (Ca^{2+}) are considered to be effective intracellular signals (Gilroy et al., 2014). In response to a high salinity environment, cytosolic calcium increases, which stimulates calcium sensors and calcium signaling pathways (Boudsocq and Sheen, 2009). However, plants also employ ion transport control (e.g., sodium and potassium accumulation), compatible solute aggregation, and expression of genes under salt stress (Kurusu et al., 2015).

Reactive oxygen species are continually formed in plant organelles as inevitable byproducts of metabolic activities (Apel and Hirt, 2004; Abogadallah, 2010). Although, the increased oxidative stress that would lead to cell death through apoptosis is preventable and can be counteracted with antioxidants, it would also be a point of differentiation. It is clearly right, as many plants often undergo necroptosis, including those that do not typically reside in a stressful climate (Coll et al., 2011). In comparison, singlet oxygen is utilized as a substrate of lipoxygenases activating a metabolic cascade that will produce a further essential stress signal, JA (Farmer and Mueller, 2013). Furthermore, ABA synthesis is also triggered by ROS (Xiong and Zhu, 2003). Plant species under salinity or drought stress will close their stomata and thus reduce moisture loss and their CO_2 influx (Hsu and Kao, 2003). Subsequently, carbon reduction and photosynthetic NADPH utilization by the Calvin cycle decreases, resulting in the development of electron holes in photosystem I that leads to electron leakage to O_2 (Türkan and Demiral, 2009).

Peroxisomal glycolate oxidase is a major source of ROS that is involved both in basal conditions and stress conditions (Mittler, 2002). ROS quenching can also be accomplished by other signaling molecules such as NO. NO nullifies Fenton-type oxidative stress by scrounging superoxide thus inhibiting the development of oxidizing agents that leads to recovery of redox homeostasis (Lamattina et al., 2003). As an alternate regulator of NO and GSH, H_2S, recently identified as a signaling molecule in crops, enhances GSH concentrations, affects enzymatic activity, and communicates with NO (Paul and Snyder, 2012; Lisjak et al., 2013). The NO formed by the phytohormone will serve as a crosstalk intermediary between the two signaling pathways. This signal attenuation is comprehensive, since even ROS derived from NADPH oxidase activity in the plasma membrane are vital to trigger ABA signaling (Kwak et al., 2003).

Reactive oxygen species aggregation throughout stress conditions often depends on the capabilities of ROS recycling through the scavenging system. The buildup of different reactive oxygen forms gradually contributes to the adaptability to stress factors and culminates in apoptosis. SA administered through the root system resulted in a raised H_2O_2 concentration in young leaf and root tissue which caused plant death (Gémes et al., 2011). Szepesi et al. (2009) observed that even under salinity, plants pre-treated with SA contained even more Na^+ than controls. The leaves did not exhibit any signs of salt damage, and rather, their photo- and membrane-integrity remained intact. As intracellular ROS is hugely involved in salt responses and active oxygen radical production within the cell is sometimes correlated with abiotic stress, it was of concern whether intracellular ROS and NO could make a significant contribution to the loss of vitality of distressed leaf protoplasts.

Reactive oxygen species-activated calcium-permeable channels including "respiratory burst oxidase homolog" (Rboh) are being hypothesized to entail an optimistic feedback loop that activates calcium ion and active radical signals in root cells (Takeda et al., 2008). RbohC/RHD2 has an affect on the production of ROS and controls Arabidopsis root hair elongation

in a Ca^{2+}-dependent fashion (Takeda et al., 2008; Monshausen et al., 2009). Salinity-induced (Ca^{2+}) cyt is enhanced through hyperpolarization- and depolarization-activated PM Ca^{2+}-permeable channels (Tracy et al., 2008). Salinity-induced escalation of cytosolic Ca^{2+} entry plays an important role in ROS signaling and saline tolerance (Shabala and Newman, 2000; Kader and Lindberg, 2010). Polyamines induced by salinity can produce oxygen-derived free radicals as substrates for apoplastic ROS enzymes (Kärkönen and Kuchitsu, 2015). Both OH and polyamines may significantly change the cationic and anionic conducting pathways and affect Ca^{2+} signaling in plants (Pottosin et al., 2014). It can unwrap a novel feature of ROS development during salinity adaptation and/or the acclimation process. A notable research paper demonstrates that perhaps the recruitment of Ca^{2+}/CaM-like proteins (CDPKs) are far more pronounced in halophytes compared to glycophytes which experiences salt stress (Xu P. et al., 2013). Such calcium-binding proteins can play a pivotal role as critical amplifiers of initial calcium influx under salt stress. Ca^{2+} signaling is also very important in seawater stress's impact on signaling mechanisms. It is suggested that TPC1 is engaged in the dissemination of salt stress cues and seems also to participate in the plant defense response (Choi et al., 2014). Inflammation triggered by Ca^{2+}-ROS pulses concerning TPC1 can be one of the plant stresses tolerating mechanisms (Choi et al., 2014; Gilroy et al., 2014). Furthermore, NO has been demonstrated to have potent antioxidant activity; preventing and minimizing lipid peroxidation and protein oxidation (Fancy et al., 2017). Saline conditions enhance NO output that minimizes salinity-induced damages (Fatma et al., 2016; Da Silva et al., 2017). At about the same time, numerous sources claim a preventive action for NO in salt-stress tolerance due to upregulating antioxidant activity in various native plants (Zheng et al., 2009; Sheokand et al., 2010).

Plant Growth Regulators Crosstalk

The productivity of agricultural crops has continuously and adversely been affected by soil salinity. A plant's ability to tolerate salinity stress can be augmented by application of signaling molecules such as H_2S, NO, and H_2O_2. For example, exogenous application of NO decreases the accumulation of Na^+ and stabilizes the concentration of mineral nutrients, and thereby results in a balanced photosynthetic rate and re-establishment of vegetative growth in lettuce (*Lactuca sativa*). Osmotic regulation, activation of the antioxidant system, and balanced phytohormones help to increase plant tolerance against salinity stress (Campos et al., 2019). Salinity stress adversely affects plant growth through upregulation of proline, MDA, and ABA content while downregulating K^+/Na^+ ratio and electrolyte leakage. Exogenous application of sodium nitroprusside (SNP) and 24-epibrassinolide (EBL) in combined form increases the endogenous level of ABA in Indian mustard (*Brassica juncea* var, Varuna) through proline, nitrogen, and ABA metabolism (Gupta et al., 2017). Soil salinity has a detrimental effect on agricultural crops through water deficiency and modified K^+/Na^+. This process leads to altered cellular redox pathways by producing reactive oxygen species such as H_2O_2, superoxide (O_2^-), and hydroxyl radicles ($^{\bullet}OH$). These free radicals pose a severe toxic impact on a molecular, biochemical, physiological, and cellular level via the lipid peroxidation pathway and lead to protein and nucleic acid destruction, and thereby negatively affect several vital pathways such as gaseous exchange, plant growth and development, and proline and nitrogen metabolism (Siddiqui et al., 2012; Gupta et al., 2017). The crosstalk of PGRs with other signaling compounds under salinity stress tolerance are illustrated in **Table 2**. NO metabolism regulates several biochemical pathways of ABA homeostasis in plants such as seed germination, dormancy, leaf senescence, stomatal movement, and fruit ripening in normal and stress conditions. The signaling molecule NO induces post translational modifications such as tyrosine nitration and sulfur-nitrosylation of proteins which regulate ABA signaling pathways. NO modulates antioxidant systems such as SODs and the catalase and ascorbate GSH cycle, and also affect ABA-induced reactive oxygen species production (Prakash et al., 2019). Phytohormones play a critical role in plants to adapt them to an unfavorable environment such as salinity via modulating physiological responses. Both phytohormone-ethylene and GAs play crucial roles to mitigate salinity stress by activating defense regulatory genes or increasing plant growth. However, both ethylene and GA are interconnected with each other; GA is well known to increase ethylene synthesis while its signaling is also dependent on ethylene (Iqbal et al., 2012). Transcript-based meta-analysis studies have illustrated that both ethylene and GA metabolism-related genes are expressed in plants under salinity stress. The precursor ACC may be synthesized unanimously for ethylene and GA synthesis. Exogenous application of ethephon and GA_3 reduces the adverse effect of salinity on seed germination of *Amaranthus caudatus* (Bialecka and Kepczynski, 2009). Ethephon showed a more stimulatory effect on seed germination than GA_3 under soil salinity. Foo et al. (2006) studied the interaction effect of ethylene and GA synthesis in pea and revealed that ethylene synthesis was negatively controlled by phytohormones and also tended to reduce GA production. Similarly, ethylene and GA have a positive effect on hypocotyl elongation in Arabidopsis (De Grauwe et al., 2007). It has been reported that GA alone is ineffective but acts synergistically with ethylene and promotes the number of penetrating roots and growth rate of emerged roots (Steffens et al., 2006). Their effect is not additive in nature, but both are synergistic with each other.

Seed germination is affected by both ethylene and NO under salinity in plants. Exogenous application of ACC (a precursor of ethylene biosynthesis) or SNP (an NO donor) inhibits the negative impact of salinity on seed germination of Arabidopsis (Lin et al., 2013). However, the stimulatory effect of both ACC and SNP was reduced by the inhibitor of ethylene biosynthesis, i.e., aminoisobutyric acid (AIB) or NO scavenger compound, i.e., 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (cPTIO) and indicated the interaction effect of both NO and ethylene on seed germination under salinity. Moreover, NO production was increased by ACC and overexpression of the *ACS2* gene was noticed by SNP which is directly involved in ethylene biosynthesis. Thus, this indicates the importance of both in

their production under salinity stress. Interestingly, exogenous application of ACC increased seed germination under oxidative stress induced by H_2O_2. However, NO-treated wild-type Arabidopsis plants were less affected and no effect was observed on ethylene-insensitive mutant seeds.

OMICS TECHNOLOGY IN SALINITY STRESS TOLERANCE

The major influences on plant growth limitation in salt-affected soil are due to osmotic stress and ion toxicity (Munns and Tester, 2008; Bargaz et al., 2016). Plants adapt distinct strategies (molecular, biochemical, and physiological adjustments) to modulate metabolic pathways and at the same time, to combat cellular salt levels via regulating water and ion homeostasis. In this regard, the modern integrative "omics" approach in plant biology has taken momentum over the last two decades in the research area powered by advances in platforms for nucleic acid sequence, peptide sequencing platforms, mass spectrometry (MS), efficient computational skills, and data analysis methodologies. This integrated "omics" system offers a snapshot of cells, tissues or organisms' developments, functions, and relationships by characterizing and quantifying all their biomolecules using a high-performance approach (Soda et al., 2015; Mosa et al., 2017; Parida et al., 2018). In recent years, omics technologies have shown promising results and been tested in numerous crops, which are highlighted in **Table 3**. Among them the application of some omics approaches under salinity stress tolerance are represented in **Figure 3** and discussed below.

Genomics

It is very crucial to understand the plant genomic response toward environmental stress. Plants are acutely complex and consist of a large number of genes in the reaction to salinity. It has been difficult to absolutely understand how plants react to salinity because of their multi-genetic nature. Genomics has made considerable strides over the past decade and has played a critical role in delivering the information needed to promote crop production. Genomics is a branch of "omics" that studies a certain genome and discloses useful knowledge on the organism's biology (Gilliham et al., 2017). By genomics, the genes involved in salinity stress response have been identified and characterized; signaling pathways have been mapped and certainly this information can be used for salinity tolerance of existing plants. It is significant to mention that genomics as a tool primarily improves and does not replace current technologies. Several high-throughput approaches, such as forward genetics, candidate gene approach, serial analysis of gene expression (SAGE), expression sequence tag (EST), next generation sequencing (NGS), high resolution melting (HRM), targeting-induced local lesion in genomes (TILLING), RNA interference (RNAi), and genome wide association study (GWAS), etc. have enabled us to not only understand salinity tolerance in plants but have also opened the path for developing plants under salinity stress. Using forward genetics, fundamental genes (*SOS1*, *SOS2*, *SOS3*) of the salt

TABLE 2 | The crosstalk of plant growth regulators with crucial signaling compounds under salinity stress and their salt tolerance mechanism in different crops.

Crosstalk	Crop	Tolerance mechanisms	References
NO-Melatonin	*Brassica napus* L. zhongshuang 11	Promotes seedlings root growth, maintains redox balance, lowers the Na^+/K^+ ratio, and modulates the antioxidant defense genes, *NHX1*, and (*SOS2*) transcripts	Zhao et al. (2018)
NO-Salicylic acid (SA)	*P. sativum* L. (var. Shubhra IM-9101)	Improves seedlings radicle length, reduces oxidative stress by decreasing superoxide radicles and H_2O_2, induces the isoform transcript of SOD, POX, APX, and enhances osmolytes accumulation	Yadu et al. (2017)
NO-SA	*Capsicum annuum* L.	Regulates the enzymes of AsA-GSH cycle enzymes, lowers the Na^+/K^+ ratio and electrolyte leakage, reduces H_2O_2, MDA, and proline contents	Kaya et al. (2020)
SA-H_2O_2-Ca	vena nuda cv. North China No. 1	Improves shoot and root dry weight, improves SOD, CAT, GSH, and ascorbic acid, and reduces MDA contents	Xu et al. (2008)
24-Epibrassinolide-SNP	*Brassica juncea* L. cv. Varuna	Improves length and biomass of root and shoot, enhances leaf area, chlorophyll, and carotenoid contents, 51% decline in electrolyte leakage and 37% in lipid peroxidation, improves stomatal opening by enhancing length and width of stomatal aperture, maintains ion homeostasis, and lowers ABA content	Gupta et al. (2017)
SA-ROS-NO	*Solanum lycopersicum* cv. Rio Fuego	Reduces the production of ROS, improves cell viability, and readjusts polyamines	Gémes et al. (2011)
NO-Phytohormones	*Lactuca sativa*	Decreases Na+ accumulation, stabilizes mineral nutrient concentration, improves photosynthesis rate, activates the antioxidant system, adjusts osmotic and hormone balance	Campos et al. (2019)
ABA-JA	*Nicotiana tabacum* NC89	Improves photosynthetic efficiency, reduces photo damage, induces stomatal closure, and improves antioxidant defense genes	Yang et al. (2018)
Melatonin-Ca	*Dracocephalum kotschyi* Boiss.	Improves relative water, proline contents, and ion homeostasis, enhances antioxidant enzymes activities, and induce systematic salt tolerance via influencing other signaling compounds	Vafadar et al. (2020c)
Ethylene-H_2O_2	*Solanum lycopersicum* cv. Yuanbao	Improves seedling biomass, chlorophyll content, and photosynthetic rate, enhances brassinosteroids synthesis, and reduces oxidative damage by enhancing antioxidant enzymes	Zhu et al. (2016)

TABLE 3 | The crucial genomics, transcriptomics, metabolomics, and proteomics approaches used in different crops.

Omics approach	Crop	Technology	References
Genomics	Arabidopsis	BAC (bacterial artificial chromosome) by BAC	Kaul et al. (2000)
	Rice	Whole-genome sequencing analysis Genome-wide meta-analysis	Subudhi et al. (2020), Mansuri et al. (2020)
	Barley	BAC by BAC Hierarchical shotgun sequencing Roche/454 pyrosequencing Genome-wide association study	Schulte et al. (2009), Wicker et al. (2009), Mwando et al. (2020)
	Maize	BAC by BAC	Pennisi (2008), Schnable et al. (2009)
	Poplar	Whole-genome shotgun sequencing (WGS)	Tuskan et al. (2006)
	Grape	WGS	Jaillon et al. (2007)
	Papaya	WGS	Ming et al. (2008)
	Sorghum	WGS	Paterson et al. (2009)
	Soybean	WGS	Schmutz et al. (2010)
	Apple	Genome-wide duplication (GWD)	Velasco et al. (2010).
	Potato	WGS	Potato Genome Sequencing Consortium (2011)
	Mango	PAC biosequencing	Singh et al. (2014)
	Peach	WGS	Ahmad et al. (2011)
	Banana	WGS	D'Hont et al., 2012
	Tomato	WGS	https://www.wur.nl/en/show/Sequencing-of-the-tomato-genome.htm
	Cucumber	WGS	Huang et al. (2009).
	Wild strawberries	Roche/454, Illumina/Solexa and Life Technologies/SOLiD platforms, next-generation sequencing (NGS)	Shulaev et al. (2011)
	Musk melon	NGS	Garcia-Mas et al. (2012)
	Watermelon	NGS	Garcia-Mas et al. (2012)
Transcriptomics	Rice	Micro array SAGE (serial analysis of gene expression)	Li et al. (2006), Matsumura et al. (1999)
	Wheat	DNA array	Jauregui et al. (2015)
	Maize	RNA sequencing	Zhao et al. (2019).
	Barley	RT-PCR (real time polymerase chain reaction)	Svensson et al. (2006)
	Arabidopsis	SAGE	Ekman et al. (2003)
	Cassava	ESTs (expression sequence tags)	Suarez et al. (2000)
	Peas	RNA display analysis	Lapopin et al. (1999)
Proteomics	Rice	2D-GE (2-dimension gel electrophoresis) Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)	Zi et al. (2012), Xu et al. (2017), and Lakra et al. (2019)
	Maize	2D-GE iTRAQ	Amara et al. (2012), Luo et al. (2018), and Chen et al. (2019)
	Wheat	2D-PAGE (*Polyacrylamide gel electrophoresis)* MS/MS (mass spectrometry)	Amiour et al. (2002), Singh et al. (2017)
	Barley		Süle et al. (2004)
	Soyabean	Mass spectrometry	Galant et al. (2012)
	Tomato	2D-PAGE	Afroz et al. (2009)
	Sugar beet	Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	Hajheidari et al. (2005)
	Grape berry	2D-PAGE	Di Carli et al. (2011)
	Peanut	LC-Q-TOF (MS/MS)	Chassaigne et al. (2007)
	Apple	2D electrophoresis and IgE-reactivity. *Electrophoresis*	Herndl et al. (2007)
	Pear		Pedreschi et al. (2008)
	Peach		Zhang L. et al. (2011)
	Populus	Shotgun MS/MS profiling	Kalluri et al. (2009)
	Stone fruit	2D PAGE	Abdi et al. (2002)
Metabolomics	Rice	GC-MS, NMR (nuclear magnetic resonance)	Gayen et al. (2019), Ma et al. (2018)

(Continued)

TABLE 3 | Continued

Omics approach	Crop	Technology	References
	Maize	HNMR, GC-MS	Gavaghan et al. (2011), Zörb et al. (2013)
	Wheat	GC-MS, HPLC, GC-TOF/MS	Guo R. et al. (2015), Borrelli et al. (2018), and Che-Othman et al. (2019)
	Barley	TIC (total ion chromatogram)	Shelden et al. (2016)
	Arabidopsis	RHPLC (high performance liquid chromatography)	Arrivault et al. (2009)
	Tobacco	NMR	Zhang J. et al. (2011)
	Tomato	UHPLC-ESI/QTOF-MS	Rouphael et al. (2018)
	Nitraria	GC-TOF/MS	Ni et al. (2015)
	Lotus	GC-TOF/MS (gas chromatography- time-of-flight mass spectrometry)	Sanchez et al. (2011)
Ionomics	Alfalfa Halophytes Lotus Barley	High-throughput sequencing, element-specific profiling, mass spectrometry, deletion mapping, X-ray fluorescence, neutron activation analysis, DNA microarray, and bulk segregant analysis	Baxter (2009), Becker and Becker (2010), Sanchez et al. (2011), Wu et al. (2013), Huang and Salt (2016), and Arshad et al. (2017)
Phenomics	Rice	PHENOPSIS WIWAM	Granier et al. (2006), Humplík et al. (2015), and Meng et al. (2017)

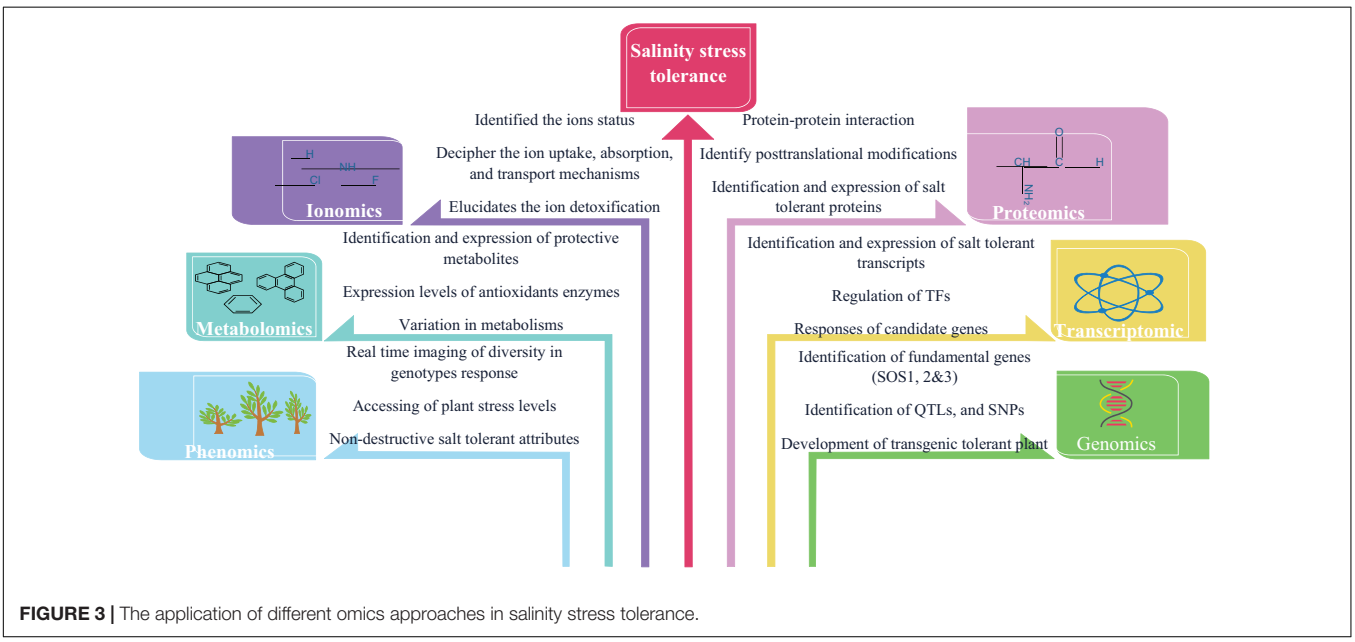

FIGURE 3 | The application of different omics approaches in salinity stress tolerance.

tolerance pathway have been identified (Zhu et al., 1998) and this knowledge of candidate gene approach has been pursued to efficiently identify the SOS gene orthologs in rice (Martínez-Atienza et al., 2007), Poplar (Tang et al., 2010), and tomato (Olías et al., 2009). Eleven single nucleotide polymorphisms (SNPs) were identified in the coding region of five salt-tolerant rice genotypes by exploring the advance method of TILLING, i.e., Eco-TILLING (Negrão et al., 2013). Because of its ability to boost the resolution of quantitative trait loci (QTL) detection without spending additional efforts in population development, GWAS receives greater attention. Recently, three novel QTLs were identified on chromosomes 4, 6, and 7, which are associated with salt tolerance in rice, through the use of molecular breeding approach GWAS (Kumar et al., 2015). Salinity tolerance may also be correlated with six genomic regions in soybean confirmed through SoySNP50K BeadChip iSelect (Huang et al., 2018). Consequently, nine SNP-rich regions were identified in 215 accessions of Asian cotton using the same GWAS approach as those related to plant parameters in salt stress conditions (Dilnur et al., 2019).

The use of RNAi technology in salt tolerances shows the positive control of tocopherol cyclase (Ouyang et al., 2011). New technologies of genomics like zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALENs), CRISPR-Cas9 (Pennisi, 2013), and Speed Breeding (Li et al., 2018) provides the opportunity to generate precisely engineered crops for salinity tolerance. The latest study of genotyping-by-sequencing in cowpea has been used to discover the effects of salinity tolerance on seed germination and seedling growth (Ravelombola et al., 2018). Nine haplotypes, two salt-tolerant

and seven salt-sensitive, were addressed by a new genome sequencing experiment of 31 landraces and 22 wild soybeans (Guan et al., 2014).

Transcriptomics

Soil salinization is recognized as a major problem for agricultural production and sustainability at a global level. The mechanisms of salinity tolerance are well known to be complicated and governed by polygenic traits (Munns and Tester, 2008). Therefore, improvements in understanding other "omics" beyond genomics have helped assign functional roles to candidate gene(s)/QTL(s) that relate to multiple abiotic stresses including salinity stress in crop plants (Salt et al., 2008). Another branch of "omics" technology is transcriptomics which deals with the RNA expression profile of organisms at temporal and spatial bases (Duque et al., 2013; El-Metwally et al., 2014; Shen et al., 2019). Unlike genomics, the transcriptome is highly complex and dynamic, and changes depending on diverse factors (El-Metwally et al., 2014). Recent shifting of RNA sequencing (RNA-seq) high-performance technology from the microarray accelerated the response of the candidate gene to stress (Liu et al., 2014; Vu et al., 2015; Conesa et al., 2016). The recently developed transcriptomics measure the abundance of transcripts of thousands of genes in parallel. RNA profiling is currently being carried out by means of RNA sequencing, microarray platforms, digital gene expression profiling, and SAGE (Molina et al., 2011; Raney, 2012; Duque et al., 2013; Xu Y. et al., 2013; Leisner et al., 2017; Li P. et al., 2017; Kreszies et al., 2019). This technology improves the ability, in salt stress, to identify transcripts/genes that are essential in controlling transcription and translation machinery (Sahi et al., 2006; Jamil et al., 2011).

To understand the transcriptomic changes during salt stress, RNA sequencing (RNA-Seq) has become the most used method for identification of novel genes and their expression pathways (Hrdlickova et al., 2017). Transcriptome profiling has been extensively and successfully used to analyze salt stress response mechanisms of plants. It is an effective method to find common sets of genes that are differentially expressed between stress-tolerant and sensitive genotypes with diverse genetic backgrounds (Peng et al., 2014). Comparing the difference in transcriptional levels between tolerant and sensitive genotypes under stress conditions, the genes related to stress tolerance can be isolated. A transcriptomics study also revealed different up- and downregulated transcriptional factors such as MYB, MYB-related, AP2-EREBP, NAC, and WRKY (Chen F. et al., 2020). This transcriptional profiling study gives a better insight into the understanding of the key components in the plant salt tolerance network which is important for developing more salt-tolerant plants. NGS and SAGE techniques were employed together by Molina et al. (2008, 2011) to classify the entire chickpea salt transcriptome. Likewise, for *Arabidopsis thaliana* responses to salt stress, Rasmussen et al. (2013) used large-scale microarray analysis. The comprehensive genome-wide study of common beans was used to recover a total of 155 bHLH (helix loop helix) genes related to salt stress response (Kavas et al., 2016). A research was performed with Solexa/Illumina to investigate the transcriptome expression profiles for Poplar (*Populus simonii* X *Populus nigra*) under salinity stress (Chen et al., 2012). Similarly, differential expression levels were checked in salinity stress for WRKY-TF genes (Garg and Singla, 2016). Most currently, miR156 working in reaction to salinity stress in alfalfa was shown by Arshad et al. (2017).

Proteomics

Salt stress is predicted to cause salinization of arable lands within the next 25 years, which may result in 30% land loss and up to 50% by the year 2050. Salinity stress causes different genes to be expressed and the result is reflected in the protein profiles. It could thus be essential to collect whole proteins created by various abiotic stresses, including salt stress, to enhance our knowledge of protein networks associated with salt-interacting pathways (Ji et al., 2016). Recently developed "omics" technologies are being designed in plant sciences to determine key proteins or metabolites that are novel, covering metabolomics, proteomics, and genomics responsible for plants stress tolerance and also biomolecules that regulate the genes. These omics studies give us a better insight into the agents affecting plant growth and development. Proteomics deals unshakably with the identification of proteins, expression profile, post-translational modifications (PTMs), and protein–protein interactions underneath stress and non-stress conditions. Proteomics studies offer a new replacement approach to obtain proteins and pathways related to crop physiological and stress responses. Thus, determining plants at proteomic levels might facilitate the finding of pathways concerned in stress tolerance.

Plant responses to salt stress through the proteomics approach have been studied in both glycophytes and halophytes. Plant scientists have worked with model plants under saline stress at proteomic levels, Razavizadeh et al. (2009) in *Nicotiana tabacum*, Chen et al. (2012) in *Populus cathayana*, Chattopadhyay et al. (2011) in grass pea, and Xu C. et al. (2011) in *Agrostis stolonifera*. Moreover, agricultural plants have also been examined under saline stress in different analyses, e.g., durum wheat (Peng et al., 2009; Jacoby et al., 2010), canola (Bandehagh et al., 2011), sugarbeet (Wakeel et al., 2011), soybean (Sobhanian et al., 2010), peanut (Jain et al., 2006), S. bicolor (Swami et al., 2011; Ngara et al., 2012), maize (Zörb et al., 2010), tomato (Chen et al., 2009; Manaa et al., 2011), potato (Aghaei et al., 2008), and cucumber (Du et al., 2010), etc.

Plant roots exhibit the foremost negative symptoms of salt stress because the few genes that are responsive to salinity stress are induced more in roots than in shoots as evident from the findings of different workers in soybean, rice, wheat, maize, and potato (Hasanuzzaman et al., 2013). A proteomics study in soybean was carried out under salt stress with the use of different tissues. They recognized that 50S ribosome protein was downregulated in leaves and that it was thought to participate in the biosynthesis of soybean protein and cause a decrease in plant growth. A phosphoproteome study of the roots of rice on exposure to NaCl (150 mM) for a few hours by using Pro-Q Diamond stain revealed that 20 proteins were upregulated and 18 downregulated. They positively identified 17 of the 20 upregulated proteins and 11 of the 18 downregulated ones. Proteins related to GST,

Hsp70, and mannose binding rice lectin were upregulated, while protein kinase, ATP synthase beta-chain, GALP hydrogenase were downregulated. They believed that phosphorylated proteins could be identified using Pro-Q Diamond stain under saline conditions. Of all proteins, 17 overexpressed proteins were responsive to salinity, however, some other proteins identified did not express in any of the proteomic reports on rice on exposure to salinity (Chitteti and Peng, 2007).

Metabolomics

Higher plants have an excellent capacity to synthesize a broad variety of different molecules and play an important role in chemical defenses against biotic and abiotic stress. The synthesis and accumulation of all small molecule's metabolites (less than 1.5 kDa) is an evolved, conserved, and ubiquitous process that shows immense variety in chemical structure and function known as metabolome, analogous to transcriptome, and proteome. Metabolism is an effective plant physiology method that is closer to phenotype than genes and proteins in response to abiotic stresses and metabolites, quite accurately representing the overall effects of genetic expressions and complex regulatory procedures (Scherling et al., 2010; Arbona et al., 2013; Ramalingam et al., 2015). Metabolites include a number of organic acids, hormones, amino acids, ketones, vitamins, and steroids. In this regard, metabolomics (i.e., the study of the metabolome, or the set of metabolites found in a given plant tissue or organ) play an essential role in procuring metabolic fingerprints or metabolic profiles based on the physiochemical properties of each metabolite using different test instruments and separation technologies (Jogaiah et al., 2013).

Relative to proteome and transcriptomics, this method generates more reliable information (Dos Santos et al., 2017). The illustration of stress tolerance mechanisms and metabolite profiling in plants has been improved by progress in mass spectrometry liquid chromatography or gas chromatography (LC-MS and GC-MS), high performance liquid chromatography nuclear magnetic resonance (NMR), direct injection mass spectrometry (DIMS), and other metabolomics techniques (Wolfender et al., 2013; Parida et al., 2018). Different researchers have noticed that variations in metabolites involving carbohydrate metabolism, tri carboxylic and glycolytic acid, amino acid biosynthesis, and at other protective antioxidant levels under stress are easily analyzed using metabolomics (Kumari et al., 2015; Jiao et al., 2018). A study of Lu et al. (2013a) revealed that a comparative study between soybean wild-type W05 and cultivated soybean C08 indicated abundance at a metabolic level of several compounds in a wild form, such as disaccharides, sugar alcohols, and acetylated amino acids. The increase of tocopherol in maize shoots and the sharp decrease in ascorbic acid levels after salt stress were reported by AbdElgawad et al. (2016). Wang et al. (2015) also stated that in *Kosteletzkya virginica* seedlings, proline levels increased when introduced to high salinity. In a study by Shen et al. (2016), a decrease in the levels of glycolysis pathway-associated sugars occurred in barley in response to salt stress. A review of metabolomics comparison reported by Jiao et al. (2018) in common wild-type soybean W1 and W2 salinity-tolerant wild-type soybean revealed increased accumulation of various organic acids, TCA cycle metabolites, and various amino acids, which in turn gave W2 greater tolerance to salinity than W1. The study of metabolome data from foxtail millet roots showed in this research that 17 associated genes of flavonoid biosynthesis were significantly raised 2- to 11-fold under salinity in Yugu 2 (Pan et al., 2020). Salinity stress-specific metabolites could therefore serve as biomarkers to evaluate a salt-tolerant and sensitive genotype.

Ionomics

The "ionome" is said to be the mineral and elemental collection of an organism (Salt et al., 2008). A landmark in ionomics research was identified in *A. thaliana* (Hirschi, 2003; Rea, 2003) where more than a thousand plants were analyzed for ionomic mutants. Ionomic circuits in crops are orchestrated and require critical reviews for high efficiency elemental profiling (Salt et al., 2008; Baxter, 2009). High-throughput sequencing, element-specific profiling, mass spectrometry, deletion mapping, X-ray fluorescence, neutron activation analysis, DNA microarray, bulk segregant analysis, and various reverse genetic tools confirmed the involvement of multiple regulators that regulate the ionome (Baxter, 2009; Becker and Becker, 2010; Sanchez et al., 2011; Wu et al., 2013; Huang and Salt, 2016). This has provided another possible avenue for exploration for plant-based genetic engineering for stress tolerance. Ionomics could lead to better management of root mineral nutrients status in plants (Shelden and Roessner, 2013). Ionomics has been deciphering the key elucidation toward ion homeostasis and ion detoxification in response to salinity stress in crops (Sanchez et al., 2011; Wu et al., 2013). The research findings indicate altered expression of calcium, magnesium, manganese, iron, and zinc within plants manifested with salinity stress. Phytogeographically plants reacted differently with increasing salinity. Research showed that a higher expression of salinity-responsive miR156 in alfalfa plants results in the accumulation of lower levels of Na^+ (Arshad et al., 2017).

To adjust high salinity, plants both manage an ion uptake and distribution system (Sanchez et al., 2008). Since normal plant cells require high amounts of K1 and Na1, this ratio should be high. Low retention of K1 results in high K1 levels in the cytosol at higher Na1 concentrations that get depolarized through membrane currents and causes K1 efflux through outward-rectifying potassium (KOR) channels (Shabala and Mackay, 2011; Bose et al., 2014). Entry of Na1 into the cytosol occurs either via selective transporters or via cation channels, in a saltier environment (Sanchez et al., 2011). During salt stress, Ca^{2+} alters Na^+ influx through extracellular channels and then Na1, K1, and Ca1 remain in balance through SOS pathways (Mahajan and Tuteja, 2005). Membrane transporters maintain appropriate levels of ions such as sodium (Na), potassium (K), hydrogen (H), and others (Shi et al., 2002; Maathuis, 2006; Sanadhya et al., 2015). To expel Na1 from the cell, SOS1 controls SOS3 along with a Ca21 sensor pathway (Khan, 2011). Through Ca21 messages,

SOS3 experiences the extracellular salt environment. SOS2 activates the SOS system.

Na1 transport proteins keep Na1 concentrations low in cell cytosol. In soil, HKT1 contributes to the import of anions into plant roots, and the uptake of cations. The electrochemical potential results in accumulation of Na^+ ions in the leaves in hyper-salinity environments (Su et al., 2003). The H1-ATPase complex consists of 11 heteromeric subunits (Shabala et al., 2014). By moving protons across the tonoplast of the endoplasmic reticulum (ER), V-type H1-ATPase produces the proton motive force which allows invisible influx of Na^+ into the vesicle and thus lowers Na^+ toxicity in the cell's cytoplasm. Therefore, Na^+ accumulation inside the vesicle of the cell emerged as an effective framework for osmotic pressure regulation in plant cells (Du et al., 2010). Halophytes have developed salt glands that remove surplus salts from metabolically active tissues (Agarie et al., 2007; Flowers and Colmer, 2008; Shabala et al., 2014).

Phenomics

With the increasing output of genomics data, phenotyping ability does not offer a crucial benefit in regard to the understanding of phenotypically affected genetic variants which are significantly affected by the environment (Furbank and Tester, 2011). Multi-laboratory automated phenotyping systems are in development. Of the next-generation techniques, phenotyping techniques provided multiple advantages over traditional tests including non-destructive testing, automating data, and spectral imaging (Berger et al., 2012; Campbell et al., 2015; Al-Tamimi et al., 2016; Negrão et al., 2017). The technique for capturing molecular phenotypes at "high levels of detail, at unprecedented times and spatial scales" is gaining attention (Negrão et al., 2017). Some findings have been reported on the color of leaves, as well as stomatal responses to a variety of stresses applied to growing progeny (Berger et al., 2012; Hairmansis et al., 2014; Campbell et al., 2015; Awlia et al., 2016). Relative growth rate dynamics of chickpea plants under high salinity stress are notable and worth working on (Atieno et al., 2017). High-throughput phenotyping would be useful in assessing the impact of salt stress on photosynthesis, transpiration, ionic relationships, plant senescence, and on yield. Automatic and digital imaging of plants can further increase understanding of diversity of response of genotype to salinity. Besides, active vision cell technology, deep learning, and other modern phenotyping techniques may be used for defining, quantifying, and predicting plant salinity response with enhanced precision.

Salinity stress can affect seedling growth and seed germination. During vegetative growth, it decreases germination percentage, leaf area, total chlorophyll content, total biomass, and root and shoot length. Visible imaging is used for research, while RGB is used to classify the chlorophyll content of various samples (Mishra et al., 2016a,b). Next-generation phenotyping assays are used to determine salt tolerance, for example, "PHENOPSIS" (Granier et al., 2006) and "WIWAM"[1] in rice and other field crops (Humplík et al., 2015; Meng et al., 2017).

[1]https://www.wiwam.be/

CONCLUSION AND FUTURE PERSPECTIVES

The regulation of plant growth and development processes under salinity stress is very complex. Its effect varies with the type of crop species, their growth habit, growth stages, and with environmental conditions. It affects germination to vegetative stage and up to maturity stage from very low to very high levels depending upon the mechanisms adapted or acclimatized by plants. At present, research on salinity stress tolerance is mainly based on the physio-morphological, biochemical, and molecular levels. The most promising ways to enhance salinity stress tolerance will be (1) screening of stress-tolerant genotypes, (2) a deep understanding of the effects and mechanisms of salinity stress in plants, and (3) identifying new genes, proteins, alleles, and transcription factors in respect to higher salinity tolerance. This study aims to understand the signaling mechanisms under salinity stress and we focused on the important signaling components MAPK and Ca under stress conditions. Thereafter, we addressed the crosstalk of important signaling compounds and plant growth regulators and cleared some ideas regarding their function and regulations under salinity stress. Then we discussed the role of recent advanced technology "omics" (genomics, proteomics, transcriptomics, and metabolomics) and how these technologies have helped in recent developments in salinity stress tolerance.

To address the challenges identified by existing research and studies, we came to following conclusions, which can be considered for future research in salinity stress tolerance.

(1) Plant stress tolerance is very complex, therefore existing strategies such as physiological, biochemical, soil, agronomical, and molecular approaches should be integrated to achieve salinity stress tolerance.

(2) Gasotransmitters and plant growth regulators have a crucial role in cell signaling, which needs to be focused on more to help understand the complexity of signaling pathways.

(3) "Omics" technologies are very promising to develop smart crops under environmental fluctuations. Integration of omics technology is a good choice for stress crop improvement programs.

Therefore, this study comprised recent progress in signaling mechanisms, crosstalk mechanisms of signaling compounds, and omics technology for salinity stress tolerance. These developments give a novel insight into understanding the signaling mechanisms and crosstalk under salinity stress tolerance and development of salinity stress tolerance genotypes by applying omics approaches.

AUTHOR CONTRIBUTIONS

All authors have prepared the draft of the manuscript. And also contributed during writing the manuscript, advised scientific suggestion as well as revised/edited the manuscript. All authors contributed to the article and approved the submitted version.

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Understanding the Role of Gibberellic Acid and Paclobutrazol in Terminal Heat Stress Tolerance in Wheat

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Understanding the physiological mechanism of tolerance under stress conditions is an imperative aspect of the crop improvement programme. The role of plant hormones is well-established in abiotic stress tolerance. However, the information on the role of gibberellic acid (GA) in abiotic stress tolerance in late sown wheat is still not thoroughly explored. Thus, we aimed to investigate the role of endogenous GA_3 level in stress tolerance in contrasting wheat cultivars, *viz*., temperature-tolerant (HD 2643 and DBW 14) and susceptible (HD 2189 and HD 2833) cultivars under timely and late sown conditions. We created the variation in endogenous GA_3 level by exogenous spray of GA_3 and its biosynthesis inhibitor paclobutrazol (PBZ). Tolerant genotypes had higher antioxidant enzyme activity, membrane stability, and photosynthesis rate, lower lipid peroxidase activity, and better growth and yield traits under late sown conditions attributed to H_2O_2 content. Application of PBZ escalated antioxidant enzymes activity and photosynthesis rate, and reduced the lipid peroxidation and ion leakage in stress, leading to improved thermotolerance. GA_3 had a non-significant effect on antioxidant enzyme activity, lipid peroxidation, and membrane stability. However, GA_3 application increased the test weight in HD 2643 and HD 2833 under timely and late sown conditions. GA_3 upregulated GA biosynthesis and degradation pathway genes, and PBZ downregulated kaurene oxidase and GA_2ox gene expression. GA_3 also upregulated the expression of the cell expansins gene under both timely and late sown conditions. Exogenous GA_3 did not increase thermotolerance but positively affected test weight and cell expansins gene expression. No direct relationship existed between endogenous GA_3 content and stress tolerance traits, indicating that PBZ could have conferred thermotolerance through an alternative mechanism instead of inhibiting GA_3biosynthesis.

Keywords: gibberellic acid, wheat, terminal heat tolerance, reactive oxygen species, cell expansin

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INTRODUCTION

Sustainable food production is always at increased risk due to abiotic stresses. The high-temperature stress, especially at the reproductive stage, i.e., terminal heat stress, poses a threat to global crop production. Zhao et al. (2017) estimated that a unit-degree Celsius increase in global mean temperature would, on average, reduce global yields of wheat by 6.0%, rice by 3.2%, maize

by 7.4%, and soybean by 3.1%. Wheat is the second-largest source of global human calorie intake. Terminal heat stress in wheat propounds a challenge to the scientific community in the present and future climate change scenarios. The abnormal delay in sowing wheat in the Indian rice-wheat belt exposes it to unsuited photothermal regimes at all phenophases. A crop simulation model-based study by Dubey et al. (2020) predicted terminal heat stress would reduce Indian wheat yield by 18.1, 16.1, and 11.1% in the present, 2020, and 2050 scenarios, respectively. Terminal heat stress at the time of anthesis and grain filling stage causes flower abortion, reduced pollen viability, availability and translocation of photosynthates to the developing kernel, and starch synthesis and its deposition within the kernel, thus resulting in lower grain number, grain weight, and grain quality (Farooq et al., 2011; Reynolds et al., 2012; Nagar et al., 2015).

High-temperature stress increases the availability of free electrons in chloroplast and mitochondria, leading to the generation of Reactive oxygen species (ROS) (O_2^-, H_2O_2, OH^-, • OH) (Foyer et al., 1997; Caverzan et al., 2016). These ROS cause damage to lipids, nucleic acid, and protein, leading to a decrease in the overall efficiency of the physiological processes of the plant (Mathur and Jajoo, 2014). When under stress, the degradation of chlorophyll pigments causes a significant reduction in photosynthesis and reversible effects on decrease in CO_2 solubility, enzymatic activities of rubisco and rubisco activase, and structural damage to thylakoid membranes and oxygen-evolving complex of photosystem II (Blum and Ebercon, 1981; Harding et al., 1990; Farooq et al., 2011).

In response to high-temperature stress, plants have evolved a highly responsive mechanism of temperature sensing and signaling. An increase in temperature is perceived by membrane-bound receptors directly and in terms of change in membrane fluidity as membrane lipids reach their melting points. Calcium channels become active and initiate the influx of Ca^{2+} ion. An increase in concentration of Ca^{2+} ion, misfolded/unfolded protein, ROS, and stress-induced histone modification causes activation of signal transduction and transcription factor. In response to high-temperature stress, plants activate stress-adaptive physiological responses, i.e., increase in the level of stress proteins (heat shock transcription factors, heat shock protein, and drought response element-binding protein) and production of antioxidant enzymes and ROS scavengers. Further, the change in the fatty acid composition of membrane lipids, accumulation of compatible solutes and increase in levels of stress protecting hormone also provides stress tolerance (Wahid et al., 2007; Sajid et al., 2018).

Plant hormones play a pivotal role in the regulation of plant growth and development. However, their role becomes more crucial under stress because they also act as stress signaling molecules, response determiners, and regulators. Thus, phytohormones are considered the most critical endogenous substances because they regulate many physiological and developmental processes. They also have a role in developing stress tolerance by affecting/regulating them directly and indirectly. The role of phytohormones like abscisic acid, cytokinins, auxins, and salicylic acid is well-established under abiotic stress (Wahid et al., 2007). Gibberellic acid (GA) is a crucial plant hormone that is essential for plants throughout their life cycle. It regulates process such as seed germination, leaf expansion, stem elongation, flower and trichome initiation, source–sink relationship and flower, fruit and seed development (Yamaguchi, 2008; Iqbal et al., 2011). Many workers have reported reduced GA levels and signaling under stress (Ahmad, 2010; Yang et al., 2013; Abdulaziz et al., 2020).

We encountered enough evidence of exogenous application of GA_3 on various plant growth and physiological processes under salinity (Maggio et al., 2010; Forghani et al., 2020), drought (Kaya et al., 2006; Moumita Al Mahmud et al., 2019), and cold and osmotic stresses (Skirycz et al., 2011; Claeys et al., 2012). GA modulated the oxidative stress processes and antioxidant enzyme activity, consequently suppressing the negative effect of abiotic stress (Khan et al., 2012). In addition to the antioxidant defense system, exogenous GA upregulated the glyoxalase system, which assisted the survival of wheat seedlings under drought stress (Moumita Al Mahmud et al., 2019). Unexpectedly, few reports are available to witness the vital roles of GAs in high-temperature stress response and adaptation, especially at the reproductive stage. Paclobutrazol (PBZ), a triazole derivative, inhibits GA biosynthesis in the plant by inhibiting kaurene oxidase and blocking oxidation of kaurene to kaurenoic acid. PBZ can increase cold, heat, drought, and salt resistance in various plants. The triazole-mediated stress protection is due to changes in hormones such as an increase in cytokinins, a transient rise in ABA, and a decrease in ethylene (Soumya et al., 2017). It helps in plant stress adoption by maintaining relative water content and electrolyte leakage, and protecting the photosynthetic machinery by enhancing the levels of osmolytes, antioxidant activities, and endogenous hormones; thereby enhancing photosynthetic activity contributing to plant growth and yield (Pinhero and Fletcher, 1994; Desta and Amare, 2021). Here, we present a comparative study to understand the mechanism of PBZ- and GA_3-mediated stress tolerance in wheat. At the reproductive stage, contrasting spring wheat cultivars, i.e., heat stress-sensitive and tolerant plants, were exposed to terminal heat stress by changing the sowing date. We applied GA_3 and PBZ through foliar application in the same experiment set to create the variation in endogenous GA level. We studied that, whether the level of endogenous GA regulated the trait determining the antioxidant system, photosynthesis, stress tolerance ability and finally yield attributes in late sown wheat. The knowledge gained through this study would provide a better insight into the role and mechanism of GA_3-mediated heat stress tolerance ability at a reproductive stage, which may play a crucial role in crop improvement for abiotic stress tolerance through breeding and biotechnological approaches.

MATERIALS AND METHODS

Plant Growth Condition and Stress Treatment

Four wheat genotypes—*viz*., heat-tolerant HD 2643 and DBW 14, and heat-susceptible HD 2189 and HD 2833—were selected based on initial screening of 40 wheat genotypes for heat stress

tolerance and susceptibility under late sown condition (Nagar et al., 2015). Genotypes were grown under open field conditions at the Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi, India during Nov-April, 2014–15. The sowing was done on 18th Nov (Timely Sown; TS) and 5th Jan (Late Sown; LS) to expose plants to different temperature regimes (**Supplementary Table 1**). Pots were filled with 15-kg mixture of clay loam soil and farmyard manure (3:1). Farmyard manure (decomposed mixture of dung and urine of farm animals) is a good source of organic carbon, and it also contains 0.5% N, 0.2% P_2O_5, and 0.5% K_2O. Fertilizers, i.e., urea, single super phosphate, and muriate of potash were applied, respectively, at sowing (N:P:K in the dose of 60:60:60 kg ha^{-1}). The remaining 60 kg, N ha^{-1} was applied in the form of urea at 25 days after sowing. During the whole crop growing season, plants were maintained under well-watered and pest-free conditions. On average, LS wheat faced 3.82°C higher temperature during booting to anthesis and 5.96°C higher temperature from 50% anthesis to maturity compared with TS plants (**Supplementary Table 2**). At 5 days after booting and 50% anthesis, the PBZ (50 ppm) and gibberellic acid (100 ppm)—concentrations were finalized based on previous studies and preliminary work in our lab—applied. The mock solution was sprayed on the control plant. At 10 days after anthesis, the physiological study and enzyme activity in flag leaf was estimated. Plants were tagged at the time of booting and 50% anthesis to maintain uniformity in spray and sampling plants.

GA_3 extraction and gene expression studies in developing grains of the wheat spike were conducted. Main spikes were identified and tagged on 50% anthesis.

Growth and Yield

At the time of crop maturity, data related to total dry weight, plant height, number of tillers per plant, grain weight per ear, grain number per ear, test weight, and grain weight per plant were recorded. Test weight is a good indicator of grain quality and was measured as the average weight of 1,000 seeds. Harvest index was also calculated, and the ratio of the economic yield to biological yield was expressed as a percentage.

Membrane Stability Index (MSI)

MSI was measured as a function of electrolyte leakage by Sairam et al. (1997). Initial conductivity (C_1) at 40°C and final conductivity (C_2) at 100°C from leaf leachate were recorded on a conductivity bridge. MSI was calculated as: $MSI = [1 - (C_1/C_2)] \times 100$.

H_2O_2 Content

Leaf samples were homogenized with Liq. N_2, followed by the addition of 5 ml cooled acetone. The mixture was centrifuged at 12,000 rpm, 4°C for 20 min and the supernatant was collected. In supernatant, 2 ml titanium reagent (Teranishi et al., 1974) and 2.5 ml liquid ammonia solution were added to precipitate the titanium-hydro peroxide complex. The reaction mixture was centrifuged at 10,000 g for 10 min at 4°C. The precipitate was dissolved in 5 ml of 2 M H_2SO_4 and read at 415 nm in UV-visible spectrophotometer against reagent blank, and the concentration of H_2O_2 was expressed as μmol $H_2O_2 g^{-1}$ DW.

Lipid Peroxidation

Standard protocol of Heath and Packer (1968) was used, which measured the formation of thiobarbituric acid reactive substances. Leaf sample was homogenized in 0.1% TCA and centrifuged at 10,000 g for 15 min. Take one ml supernatant and add 4.0 mL of 0.5% thiobarbituric acid prepared in 20% Trichloroacetic acid. The mixture was heated at 95°C for 30 min followed by cooling in an ice bath. After centrifugation at 10,000 g for 10 min, the specific and non-specific absorbance of the supernatant was recorded at 532 and 600 nm, respectively. The concentration of malonaldehyde was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Antioxidant Enzymes Estimation

Extraction of enzymes superoxide dismutase (SOD), catalase, and glutathione reductase (GR) was done by grinding leaf samples in liquid N_2, followed by adding extraction buffer consisting of 100 mM phosphate buffer, which contained 0.5 mM EDTA, pH 7.5. For ascorbate peroxidase (APX), the ascorbic acid was added to the extraction buffer in the final concentration of 1 mM, and pH was further adjusted to 7.5. Leaf extracts were centrifuged at 12,000 rpm at 4°C for 20 min, and the supernatant was collected for enzyme estimation. SOD activity was estimated in terms of decrease in absorbance of formazone at 540 nm made by nitro-blue tetrazolium with O^{2-} radical due to SOD (Dhindsa et al., 1981). The method by Aebi (1984) was used to measure catalase activity. Breakdown of H_2O_2 by catalase enzyme was recorded in terms of decreased absorbance at 240 nm after adding H_2O_2. The enzyme activity (μmol H_2O_2 reduced min^{-1} mg^{-1} protein) was calculated using 36.5 $M^{-1}cm^{-1}$ as the extinction coefficient of H_2O_2. GR activity was assayed as per method by Smith et al. (1988). The formation of reduced glutathione from oxidized glutathione in the presence of Nicotinamide adenine dinucleotide phosphate (NADH) was assayed in terms of the colored complex formed by reduced glutathione 5,5-dithiobis-2-nitrobenzoic acid (DTNB). An increase in absorbance at 412 nm was recorded, and enzyme activity (μmol oxidized glutathione formed min^{-1} mg^{-1} protein) was calculated using 6.2 $mM^{-1}cm^{-1}$ as extinction coefficient of oxidized glutathione. Ascorbate peroxidase (APOX) activity was assayed by method given by Nakano and Asada (1981). Ascorbic acid reduced hydrogen peroxide to water in the presence of 0.1 M phosphate buffer. A decrease in ascorbic acid was recorded at 240 nm. The enzyme activity (μmol H_2O_2 reduced min^{-1} mg^{-1} protein) was calculated using 2.8 $mM^{-1}cm^{-1}$ as extinction coefficient of H_2O_2.

Photosynthesis Rate

The portable Infrared Gas Analyzer (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA) under standard operating condition (temperature: 30–35°C, relative humidity: 50–60%, CO_2 concentration: 350 ± 50 μmol mol^{-1}, and photosynthetic photon flux density: 1,200 μmol $m^{-2}s^{-1}$) was used to measure photosynthetic rate, Fv/Fm

ratio, transpiration rate, and stomatal conductance on fully expanded flag leaf of each plant. These measurements were made between 10:15 and 11:45 a.m. at 10 days after athesis (DAA).

Gibberellic Acid Extraction

Developing grains were ground into a fine powder and placed into screw cap tubes filled with 30 ml methanol 70% (v/v) and kept overnight at 4°C. The extract was centrifuged, and methanol was evaporated under a vacuum from the supernatant. The pH of the aqueous phase was adjusted to 8.5 and then partitioned with ethyl acetate. After removal of the ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5. The solution was partitioned with diethyl ether, and then passed through sodium sulfate. Diethyl ether was evaporated under vacuum, and dry residue containing GA_3 was dissolved in 2.0 ml of absolute methanol. The GA_3 analysis was performed using high performance liquid chromatography (HPLC) (Waters) equipped with reversed-phase column Crestpak C18 (150 × 4.6 mm i.d.; 5 μm) maintained at 30 ± 1°C. The mobile phase of acetonitrile-water (30:70%; v/v) was used with pH-4.5 and a flow rate of 1 ml/min. An injection volume of 10 μl was used for each analysis, and the wavelength used for analysis was 208 nm.

Analysis of Gene Expression by RT-PCR and Real-Time PCR

Total RNA was extracted from developing seed (cell expansin gene) and leaf tissue (GA_3 biosynthesis gene) using RNA easy kit (Qiagen Inc., Chatsworth, CA USA) according to the instructions of the manufacturer. DNase treatment was done to remove the DNA contamination (Ambion TURBO DNase, Thermo Fisher Scientific Waltham, MA, USA). RNA was reverse transcribed in a 20 μl reaction using cDNA Reverse Transcription Kit [SuperScript® III First-Strand Synthesis System (Invitrogen™, Carlsbad, CA, USA) according to the manual provided by the manufacturer. The details of NCBI gene accession number for specific gene and primers used in this study are given as **Supplementary Table 3**. For RT-PCR expression of GA biosynthesis pathway genes [ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase, (EKS), ent-kaurene oxidase (KO), ent-kaurenoic acid oxidase (KAO), 20-oxoglutarate-dependent dioxygenase (GA20ox), 3 oxidase (GA3ox), 2 oxidase (GA2ox)], complementary DNA (cDNA) was normalized by using wheat actin as a control. Normalized quantity of cDNA was used for amplification (27–28 cycles standardized for genes). Specific genes were amplified using Invitrogen two-step RT-PCR Kit (Invitrogen™, Carlsbad, CA, USA) with gene-specific and degenerate forward and reverse primers. A real-time expression study was conducted by real-time quantitative PCR (RT-qPCR) with Power SYBR® Green Master Mix (Thermo fisher scientific, USA) in Stratagene Mx3005P (Agilent Technologies, USA). The relative expression levels in data were determined based on the 2–ΔΔCT method (Livak and Schmittgen, 2001) using wheat actin as an internal control.

Statistical Analysis

The results for physiological, biochemical, and growth and yield traits in cultivars with varied heat-tolerance and GA-sensitivity were expressed as means with standard error (SE). All the statistical data analyses were performed using three biological replicates of physiological and biochemical traits for each treatment, while five biological replicates were taken for growth and yield traits. The three factorial Analysis of variance (ANOVA) for completely randomized design (CRD) consisting of genotype, stress, and spray as fixed factors was performed using the "aov" function available in the "stats" package of statistical software "R" version 4.04. The *P*-value of main and interaction effects for all the response variables is provided in **Supplementary Table 4**. LSD method (using LSD.test function in "agricolae" package of R) was used to analyse the differences between the means of the levels of a factor at $P < 0.05$.

RESULTS

Endogenous GA3 Content

It was observed that, in LS plants, average GA_3 content decreased by 33% compared to TS plants (**Figure 1**). GA_3 content was highest in HD 2643 and HD 2189 in stressed and non-stressed conditions, respectively. PBZ significantly ($P < 0.05$) decreased GA_3 content by 32 and 18% in all cultivars under TS and LS conditions, respectively. Exogenous application of GA_3 significantly ($P < 0.05$) increased GA_3 content by 13.7 and 36% in all cultivars under TS and LS conditions, respectively.

Growth and Yield Traits

In LS plants, the main effect was a significant decrease ($P < 0.0001$) in total dry weight, plant height, number of tillers per plant, grain weight and number per ear, test weight, grain weight per plant, and harvest index in all cultivars (**Table 1**, **Supplementary Table 4**). The ANOVA showed that contrasting varieties as a main effect is significant at $P < 0.001$ except ear per panicle and tiller per plant (**Supplementary Table 4**). Under the LS condition, tolerant cultivars had an average decrease of 21, 12, 12, 13, and 15% in total dry weight, grain weight per ear, grain number per ear, grain weight per plant, and harvest index; whereas, in susceptible cultivars, the decrease was by 26.6, 16, 17, 23, 25%, respectively. These results were statistically different at $P < 0.05$, where the average values of grain weight per ear ranged from 2.3 to 3.2 g for different cultivars under TS conditions, while it varied from 1.58 to 2.37 g under stressed conditions.

An exogenous spray of GA_3 and PBZ had a statistically non-significant ($P < 0.05$) effect on total biomass, the number of tillers per plant, and the height of the plant in all cultivars under both sowing conditions. However, the harvest index increased significantly only in TS HD 2833. Application of GA_3 and PBZ showed varietal variation for grain number per ear under TS condition. In stress plants, however, improvement in grain number per ear was recorded with the application of growth regulators. The application of GA_3 enhanced grain weight per ear, grain number per ear, test weight, and grain yield per plant by

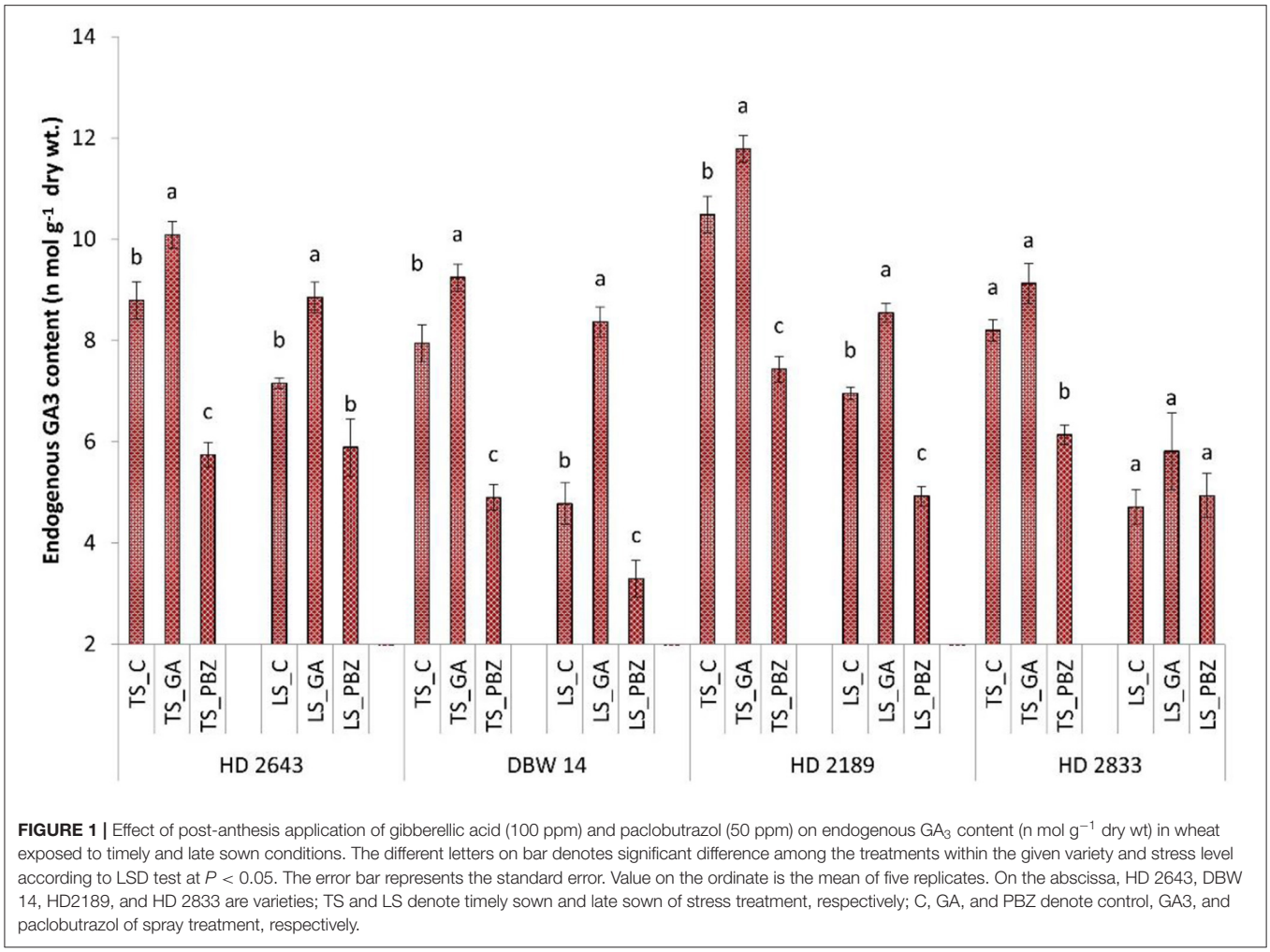

FIGURE 1 | Effect of post-anthesis application of gibberellic acid (100 ppm) and paclobutrazol (50 ppm) on endogenous GA_3 content (n mol g^{-1} dry wt) in wheat exposed to timely and late sown conditions. The different letters on bar denotes significant difference among the treatments within the given variety and stress level according to LSD test at $P < 0.05$. The error bar represents the standard error. Value on the ordinate is the mean of five replicates. On the abscissa, HD 2643, DBW 14, HD2189, and HD 2833 are varieties; TS and LS denote timely sown and late sown of stress treatment, respectively; C, GA, and PBZ denote control, GA3, and paclobutrazol of spray treatment, respectively.

13.4, 3.8, 3.6, and 13.6% in the tolerant cultivars, and by 22.2, 8.2, 10, and 16.5% in susceptible cultivars under stress, respectively. Application of GA_3 and PBZ significantly ($P < 0.05$) increased test weight and grain yield per plant in all cultivars under LS condition, except in a few cases [DBW 14 showed non-significant (NS) effect of GA_3 in the case of test weight and PBZ on grain yield]. In LS plants, application of PBZ enhanced grain weight per ear, grain number per ear, test weight, and grain yield per plant by 21, 6.3, 10.3, and 26.5% in the tolerant cultivars, and by 25.6, 6.8, 12.9, and 16.9% in the susceptible cultivars under stress, respectively.

Photosynthesis Rate and Related Traits

The photosynthesis rate and associated traits showed statistically significant difference ($P < 0.001$) in variety, stress, and spray as a main effect (**Supplementary Table 4**). Under stress, a decrease of 42 and 45% was recorded in mean photosynthesis (values averaged over varieties of contrasting group) in tolerant and susceptible cultivars, respectively. Photosynthesis rate was lowest in HD 2189 and highest in DBW 14 under both environmental conditions. PBZ augmented photosynthesis rate by 6% (NS at $P < 0.05$) and 22% (significant at $P < 0.05$) in TS and LS plants of tolerant cultivars and by 9% (significant at $P < 0.05$) and 13% (significant at $P < 0.05$) in susceptible cultivars, respectively. An increase of 5.1/6.6% (NS at $P < 0.05$) in photosynthesis rate on GA_3 application was observed in TS/LS tolerant cultivars. In comparison, there was a 2.6% (NS at $P < 0.05$)/13% (significant at $P < 0.05$) increase in susceptible cultivars (**Table 2**). Stress conditions reduced photochemical efficiency by 20% (significant at $P < 0.05$), with a more considerable reduction in susceptible cultivars (26.4% significant at $P < 0.05$) than in tolerant cultivars (13.7% significant at $P < 0.05$). PBZ application led to a significant increase in photochemical efficiency under most of the treatments. On the contrary, GA_3 application significantly ($P < 0.05$) increased photochemical efficiency under few treatments such as TS HD 2643 and HD 2189 and LS HD 2189.

The mean value of stomatal conductance and transpiration rate in TS plants was 3.28 cm s^{-1} and 0.28 mmol cm^{-2} s^{-1}, respectively. The imposition of stress conditions increased stomatal conductance (23% significant at $P < 0.001$) and transpiration rate (29% significant at $P < 0.001$). Among the cultivars, HD 2833 recorded the least stomatal conductance, and maximum was recorded by DBW 14 and HD 2643. GA_3

TABLE 1 | Effect of post-anthesis application of gibberellic acid (100 ppm) and paclobutrazol (50 ppm) on plant height (cm), tiller number per plant, grain number/ear, grain weight per ear (g), test weight, grain yield (g/plant), and total dry weight (g/plant) in wheat exposed to timely and late sown conditions.

Variety	Stress _Spray	Plant height (cm)	Tiller number	Grain number/ ear	Grain weight/ ear (g)	Test weight (g)	Grain yield (g)	Total dry matter (g)	Harvest index (%)
HD 2643	TS_C	118.33 ± 3.48^{a}	7.33 ± 0.33^{a}	62.33 ± 0.33^{a}	2.79 ± 0.07^{b}	42.33 ± 0.7^{b}	18.05 ± 0.47^{b}	40.33 ± 0.88^{a}	55.86 ± 1.06^{a}
	TS_GA	118.33 ± 0.88^{a}	6.67 ± 0.33^{a}	64 ± 0.58^{a}	2.82 ± 0.06^{b}	45.1 ± 0.4^{a}	18.79 ± 0.42^{b}	41.33 ± 2.03^{a}	56.17 ± 0.67^{a}
	TS_PBZ	116 ± 3.79^{a}	7.33 ± 0.33^{a}	63.67 ± 0.88^{a}	3.25 ± 0.1^{a}	45.99 ± 0.46^{a}	20.92 ± 0.42^{a}	40.07 ± 0.52^{a}	56.56 ± 0.6^{a}
	LS_C	105 ± 2.08^{a}	7 ± 0.58^{a}	54.33 ± 0.88^{b}	2.04 ± 0.04^{b}	37.02 ± 0.32^{b}	15.1 ± 0.45^{c}	32 ± 0.84^{a}	45.98 ± 1.51^{a}
	LS_GA	106 ± 4.16^{a}	6.67 ± 0.33^{a}	56.7 ± 0.88^{a}	2.33 ± 0.06^{a}	39.8 ± 0.38^{a}	18.23 ± 0.55^{b}	33.5 ± 0.81^{a}	46.82 ± 0.79^{a}
	LS_PBZ	105 ± 3.06^{a}	7.33 ± 0.33^{a}	57.9 ± 0.58^{ab}	2.43 ± 0.05^{a}	41.6 ± 0.35^{a}	19.33 ± 0.58^{a}	35.53 ± 1.34^{a}	47.51 ± 1.5^{a}
DBW 14	TS_C	97 ± 4.04^{a}	8.67 ± 0.33^{a}	57 ± 0.58^{b}	3.21 ± 0.06^{b}	40.61 ± 0.64^{c}	20.74 ± 0.35^{b}	38.58 ± 0.63^{a}	56.74 ± 0.56^{a}
	TS_GA	103.33 ± 4.26^{a}	8.33 ± 0.88^{a}	62 ± 0.58^{a}	3.45 ± 0.08^{a}	42.84 ± 0.2^{b}	23.6 ± 0.7^{a}	37.83 ± 0.35^{a}	57.31 ± 0.86^{a}
	TS_PBZ	101.33 ± 6.94^{a}	8.67 ± 0.88^{a}	57.67 ± 0.33^{b}	3.52 ± 0.05^{a}	45.16 ± 0.62^{a}	24.37 ± 0.34^{ab}	39.43 ± 0.69^{a}	58.04 ± 0.92^{a}
	LS_C	89 ± 2.65^{a}	7.67 ± 0.33^{a}	50.33 ± 0.88^{b}	2.17 ± 0.13^{b}	38.55 ± 0.74^{b}	18.13 ± 0.48^{b}	30.24 ± 0.76^{a}	48.97 ± 1.75^{a}
	LS_GA	88.33 ± 2.33^{a}	7 ± 1^{a}	52 ± 0.58^{ab}	2.25 ± 0.1^{ab}	38.4 ± 0.48^{b}	19.29 ± 1.05^{b}	30.45 ± 0.99^{a}	51.72 ± 1.42^{a}
	LS_PBZ	86.33 ± 1.45^{a}	7 ± 0.58^{a}	53.33 ± 0.88^{a}	2.61 ± 0.09^{a}	41.72 ± 0.54^{a}	22.5 ± 0.7^{a}	32.48 ± 1.14^{a}	51.57 ± 1.65^{a}
HD 2189	TS_C	86.33 ± 5.04^{a}	7.33 ± 0.33^{a}	61 ± 0.58^{ab}	2.48 ± 0.02^{b}	41.37 ± 0.49^{b}	19.37 ± 0.27^{c}	32.22 ± 1.26^{a}	46.22 ± 1.22^{a}
	TS_GA	88 ± 4.93^{a}	7.33 ± 0.88^{a}	62.33 ± 0.58^{a}	2.56 ± 0.07^{ab}	42.96 ± 0.65^{ab}	21.57 ± 0.62^{b}	31.96 ± 1.09^{a}	47.04 ± 0.99^{a}
	TS_PBZ	86.33 ± 1.45^{a}	6.33 ± 0.33^{a}	58.67 ± 1.2^{b}	2.72 ± 0.07^{a}	43.4 ± 0.4^{a}	23.93 ± 0.17^{a}	32.9 ± 1.92^{a}	46.49 ± 2.38^{a}
	LS_C	75.5 ± 0.87^{a}	6.33 ± 0.33^{a}	50.8 ± 0.88^{a}	2.04 ± 0.05^{c}	34.38 ± 0.57^{c}	15.03 ± 0.46^{b}	23.9 ± 0.82^{a}	33.83 ± 2.69^{a}
	LS_GA	76.33 ± 0.88^{a}	6.33 ± 0.67^{a}	54.33 ± 0.88^{a}	2.51 ± 0.05^{b}	37.89 ± 0.06^{b}	17.99 ± 0.69^{a}	22.15 ± 1.21^{a}	34.32 ± 1.94^{a}
	LS_PBZ	73 ± 1.53^{a}	7 ± 0.58^{a}	55.7 ± 0.88^{a}	2.7 ± 0.05^{a}	39.07 ± 0.63^{a}	18.4 ± 0.1^{a}	25.66 ± 1.27^{a}	35.43 ± 1.27^{a}
HD 2833	TS_C	78.33 ± 2.4^{a}	7.67 ± 0.33^{a}	59.67 ± 0.33^{a}	2.34 ± 0.04^{b}	38.71 ± 0.52^{c}	16.03 ± 0.43^{b}	31.8 ± 0.98^{a}	50.66 ± 0.29^{b}
	TS_GA	80.67 ± 2.33^{a}	7.67 ± 0.67^{a}	61.33 ± 0.58^{a}	2.52 ± 0.05^{a}	41.76 ± 0.77^{b}	17.99 ± 0.16^{a}	33.35 ± 0.78^{a}	56.29 ± 1.07^{a}
	TS_PBZ	78.33 ± 4.37^{a}	7.33 ± 0.33^{a}	60 ± 0.58^{a}	2.66 ± 0.04^{a}	44.46 ± 0.83^{a}	18.4 ± 0.31^{a}	33.49 ± 1.69^{a}	55.51 ± 0.67^{a}
	LS_C	67 ± 1.53^{a}	6.67 ± 0.33^{a}	49.33 ± 0.67^{b}	1.58 ± 0.06^{b}	34.02 ± 0.97^{b}	12.23 ± 0.42^{c}	23.1 ± 1.22^{a}	39.4 ± 1.7^{a}
	LS_GA	67.33 ± 2.33^{a}	6.67 ± 0.33^{a}	54 ± 0.58^{a}	1.92 ± 0.02^{a}	37.8 ± 1.12^{a}	13.88 ± 0.56^{b}	22.16 ± 0.87^{a}	40.35 ± 2.55^{a}
	LS_PBZ	67 ± 1.73^{a}	6.33 ± 0.67^{a}	51.33 ± 1.45^{ab}	1.88 ± 0.08^{a}	38.17 ± 0.35^{a}	16.17 ± 0.37^{a}	24.12 ± 1.05^{a}	40.38 ± 0.93^{a}

The different letters (as superscript) in a column denotes significant difference among the treatments within the given variety and stress level according to LSD test at $P < 0.05$. The value follows ± represent the standard error ($n = 3$).
TS, Timely sown; LS, Late Sown; C, Control; GA, GA3 spray; PBZ, Paclobutrazol spray.

had a non-significant ($P < 0.05$) effect on increased stomatal conductance except in HD 2643 under the stressed condition. There was a significant increase in stomatal conductance and transpiration rate on PBZ application under non-stressed conditions. However, stressed conditions could significantly ($P < 0.05$) affect stomatal conductance and transpiration rate in the cultivars HD2643 and HD2833.

Membrane Stability Index and Lipid Peroxidation

The mean Membrane stability index (MSI) value varied from ∼84 to 91% under control conditions, and it ranged from 72 to 79% under stress conditions (**Figure 2**). Heat stress as a main effect caused a significant ($P < 0.001$) reduction in MSI. The PBZ application resulted in a significant ($P < 0.05$) increase between 6 and 10% in MSI under a stressed environment. GA_3 had a non-significant ($P < 0.05$) impact on the improvement of MSI under a non-stressed environment. However, it significantly ($P < 0.05$) increased MSI in cultivars DBW 14 under stress. The mean value of Lipid peroxidation (LPD) in plants under non-stressed conditions ranged from 500 to 770 nmol g^{-1} dry wt while it increased to 1,100–1,800 nmol g^{-1} dry wt under stressed conditions. It was about 100–150% higher than control plants under late sown conditions. In the PBZ application, the LPD content was significantly reduced between 10 and 24%. The effect of PBZ spray was most evident in DBW 14 (20%). GA_3 application resulted in a non-significant ($P < 0.05$) reduction in LPD under the stressed condition.

Antioxidant Enzymes Activity and H_2O_2 Content

The main effect of variety, stress, and spray showed significant ($P < 0.001$) impact on antioxidant enzymes system and H_2O_2 content (**Supplementary Table 4**). In LS plants of tolerant cultivars, mean value of SOD, APOX, CAT, and GR activity were increased by 121, 218, 201, and 62%, and an increase of 51, 121, 136, and 33% was recorded in susceptible cultivars, respectively (**Table 3**). Application of GA_3 had a non-significant effect on SOD, APOX, CAT, and GR enzyme activity in LS plants of all the cultivars except SOD activity in HD 2643 at $P < 0.05$. However, PBZ significantly boosted the antioxidant defense mechanism in stressed plants. Under stress, PBZ increased mean value of SOD, APOX, CAT, and GR activity by 25.25, 16.8,

TABLE 2 | Effect of post-anthesis application of gibberellic acid (100 ppm) and paclobutrazol (50 ppm) on photosynthesis rate (μmoles CO_2 m^{-2} s^{-1}), Fv/Fm ratio, transpiration rate (mmol cm^{-2} s^{-1}), and stomatal Conductance (cm s^{-1}) in wheat exposed to timely and late sown conditions.

Variety	Stress_Spray	Photosynthesis rate (μmoles CO2 m^{-2} s^{-1})	Fv/Fm ratio	Transpiration rate (mmol cm^{-2} s^{-1})	Stomatal conductance (cm s^{-1})
HD 2643	TS_C	25.33 ± 0.67^{a}	0.76 ± 0.001^{c}	0.24 ± 0.003^{b}	3.49 ± 0.019^{b}
	TS_GA	26.67 ± 0.33^{a}	0.79 ± 0.003^{b}	0.25 ± 0.003^{ab}	3.51 ± 0.035^{b}
	TS_PBZ	27 ± 0.58^{a}	0.8 ± 0^{a}	0.26 ± 0.007^{a}	3.97 ± 0.072^{a}
	LS_C	14.67 ± 0.33^{b}	0.67 ± 0.004^{a}	0.32 ± 0.006^{b}	4.15 ± 0.026^{b}
	LS_GA	15 ± 0.58^{b}	0.68 ± 0.007^{a}	0.32 ± 0.003^{b}	4.47 ± 0.083^{a}
	LS_PBZ	17.33 ± 0.33^{a}	0.68 ± 0.003^{a}	0.34 ± 0.009^{a}	4.59 ± 0.013^{a}
DBW 14	TS_C	26 ± 0.58^{b}	0.78 ± 0.004^{b}	0.33 ± 0.006^{b}	3.53 ± 0.047^{b}
	TS_GA	27.33 ± 0.33^{ab}	0.78 ± 0.004^{ab}	0.35 ± 0.003^{ab}	3.55 ± 0.034^{b}
	TS_PBZ	27.67 ± 0.33^{a}	0.79 ± 0.002^{a}	0.35 ± 0.006^{a}	3.74 ± 0.046^{a}
	LS_C	15 ± 0.58^{b}	0.66 ± 0.004^{b}	0.44 ± 0.003^{a}	4.29 ± 0.053^{a}
	LS_GA	16.67 ± 0.88^{ab}	0.67 ± 0.001^{b}	0.44 ± 0.006^{a}	4.39 ± 0.043^{a}
	LS_PBZ	19 ± 0.58^{a}	0.69 ± 0.005^{a}	0.47 ± 0.012^{a}	4.47 ± 0.06^{a}
HD 2189	TS_C	23 ± 0.58^{b}	0.78 ± 0.004^{c}	0.24 ± 0.007^{b}	3.4 ± 0.037^{b}
	TS_GA	22.67 ± 0.33^{b}	0.81 ± 0.005^{b}	0.24 ± 0.006^{b}	3.47 ± 0.026^{b}
	TS_PBZ	25.67 ± 0.33^{a}	0.85 ± 0.003^{a}	0.27 ± 0.003^{a}	3.64 ± 0.067^{a}
	LS_C	12.67 ± 0.33^{a}	0.57 ± 0.006^{c}	0.33 ± 0.003^{a}	4.46 ± 0.052^{a}
	LS_GA	14.33 ± 0.88^{a}	0.61 ± 0^{b}	0.34 ± 0.003^{a}	4.62 ± 0.041^{a}
	LS_PBZ	13.67 ± 0.88^{a}	0.62 ± 0.006^{a}	0.34 ± 0.006^{a}	4.52 ± 0.058^{a}
HD 2833	TS_C	25 ± 0.58^{b}	0.8 ± 0.006^{a}	0.3 ± 0.003^{b}	2.71 ± 0.042^{b}
	TS_GA	26.67 ± 0.33^{a}	0.8 ± 0.001^{a}	0.31 ± 0.006^{ab}	2.88 ± 0.038^{ab}
	TS_PBZ	26.67 ± 0.33^{a}	0.81 ± 0.004^{a}	0.32 ± 0.006^{a}	3 ± 0.094^{a}
	LS_C	14 ± 0.58^{b}	0.59 ± 0.005^{b}	0.33 ± 0.003^{b}	3.33 ± 0.034^{b}
	LS_GA	16 ± 0.58^{a}	0.6 ± 0.006^{ab}	0.33 ± 0.003^{b}	3.35 ± 0.026^{b}
	LS_PBZ	16.67 ± 0.33^{a}	0.61 ± 0.005^{a}	0.35 ± 0.003^{a}	3.65 ± 0.025^{a}

The different letters (as superscript) in a column denotes significant difference among the treatments within the given variety and stress level according to LSD test at $P < 0.05$. The value follows ± represent the standard error ($n = 5$).

TS, Timely sown; LS, Late Sown; C, Control; GA, GA3 spray; PBZ, Paclobutrazol spray.

22.6, and 10.4% in tolerant cultivars, and 24, 22.6, 24.5, and 20.4% in susceptible cultivars, respectively at $P < 0.05$. The mean value of H_2O_2 content in plants under non-stressed condition was 0.9 μmol H_2O_2 g dry wt^{-1}, while it was 1.54 μmol H_2O_2 g dry wt^{-1} under stressed conditions. H_2O_2 content was about 55–87% higher under heat stress as compared to non-stressed plants. PBZ reduced the H_2O_2 content significantly ($P < 0.05$) in all the cultivars under stressed conditions. The magnitude of reduction was 9 and 12% under non-stressed and stressed conditions, respectively. The effect of PBZ spray was most evident in cultivar DBW 14 and HD 2189 under stressed environmental conditions.

Relative Gene Expression

In the RT-PCR study, the genes of GA biosynthesis and degradation pathways showed a significant change in gene expression level under different stress environments on the application of GA_3 and PBZ. The genes that showed a significant change in their RT-PCR expression study were selected for the RT-qPCR study (**Supplementary Figure 1**). Application of GA_3 slightly decreased KO expression, GA20ox, and GA3ox in non-stressed plants, whereas in stressed plants, upregulation in gene expression level was observed (**Figure 3**). The expression level of GA degrading enzyme GA2ox was upregulated on GA_3 application in both cultivars, but the increase in expression was very high in TS plants compared to LS plants. Expression of GA degrading enzyme GA2ox was upregulated in both cultivars understudied environmental conditions on GA_3 application, but the increase was very high in TS plants compared to LS. PBZ application drastically decreased the mRNA level of KO by 10–20 times. The PBZ application also significantly decreased gene expression of other genes like GA20ox, GA3ox, and GA 2ox.

The relative gene expression level of Ta EXp A2 and Ta Exp A6(a) was higher in tolerant cultivar HD 2643, and Ta Exp A4 was higher in the susceptible cultivar. Under stress conditions, the expression of all three genes was downregulated. The Ta Exp A2 gene expression had a maximum decrease under stress. Expression of all three studied cell expansins gene, i.e., Ta Exp 2, Ta Exp A4, and Ta Exp A6(a) was upregulated on GA_3 application under both conditions. Expression of Ta ExpA2 showed a maximum increase, and an increase in expression was higher under stressed condition on application of GA_3 (**Figure 4**).

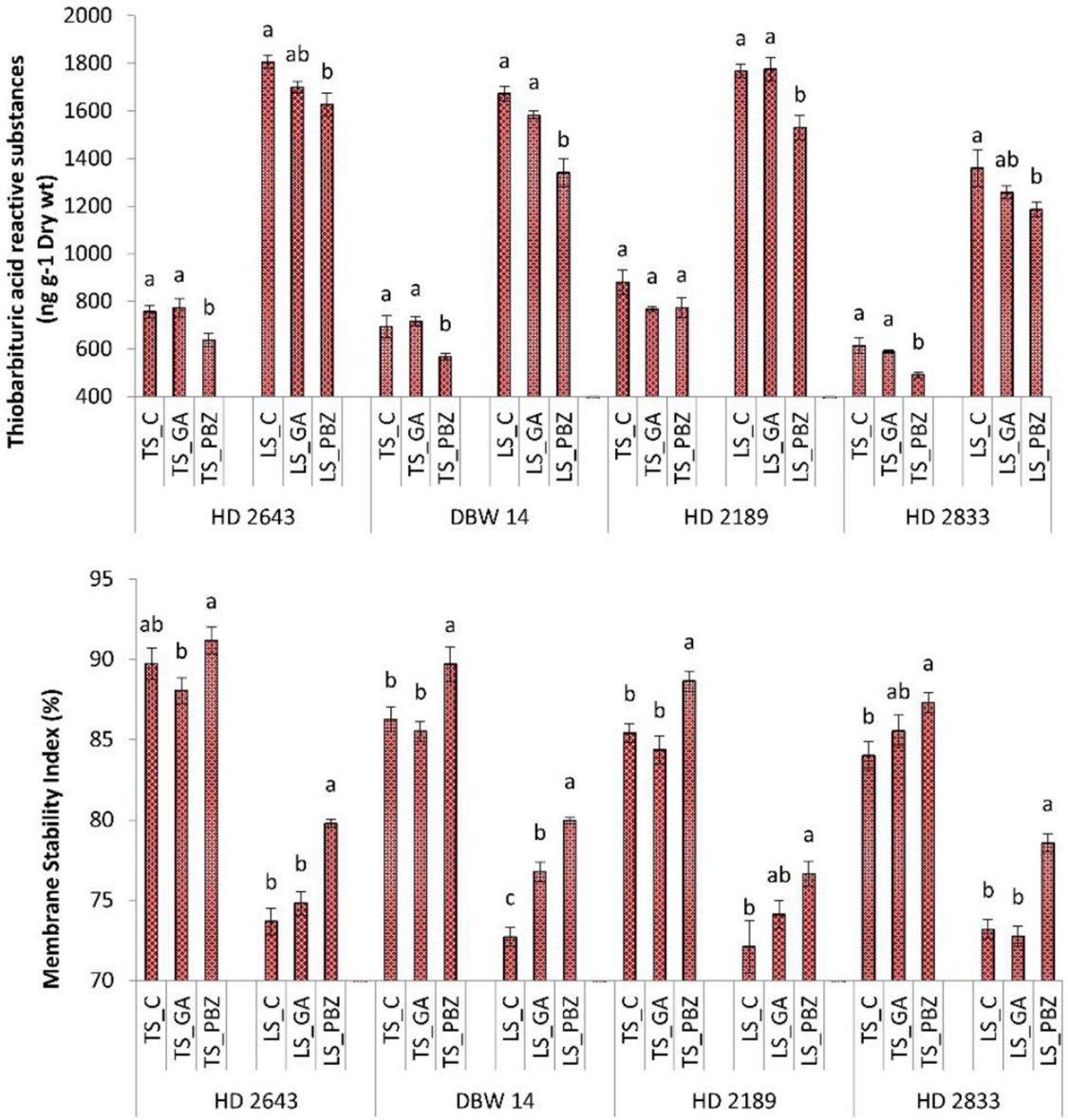

FIGURE 2 | Effect of post-anthesis application of gibberellic acid (100 ppm) and paclobutrazol (50 ppm) on lipid peroxidation (TBARS content ng^{-1} dry wt); membrane stability index (%) in wheat exposed to timely and late sown conditions. The different letters on bar denotes significant difference among the treatments within the given variety and stress level according to LSD test at $P < 0.05$. The error bar represents the standard error. Value on the ordinate is the mean of five replicates. On the abscissa, HD 2643, DBW 14, HD2189, and HD2833 are varieties; TS and LS denote timely sown and late sown of stress treatment, respectively; C, GA, and PBZ denote control, GA3, and paclobutrazol of spray treatment, respectively.

DISCUSSION

Abiotic stress leads to alteration in phytohormones levels and decreased plant growth (Abdulaziz et al., 2020). Stress conditions alter hormonal homeostasis, stability, content, biosynthesis, and compartmentalization in the plant. Abiotic stresses like salinity, cold, and drought also cause a decrease in endogenous GA level (Yang et al., 2001; Achard, 2006; Magome et al., 2008; Tuna et al., 2008; Alonso-Ramírez et al., 2009; Ahmad, 2010). Akin to other stresses, our study also observed a reduction in endogenous GA_3 content under terminal heat stress in studied wheat cultivars. We report a decrease in transcript level of GA biosynthesis pathway genes, i.e., CPS, EKS, KO, KAO, GA20ox, GA3ox, and GA2ox under stress.

The expression study results supported our finding that reduced GA content was due to decreased biosynthesis of gibberellin, even though the transcript level of GA degrading enzyme GA2ox decreased under stress. The decrease in GA2ox activity could have prevented the

TABLE 3 | Effect of post-anthesis application of gibberellic acid (100 ppm) and paclobutrazol (50 ppm) on superoxide dismutase content (unit min^{-1} mg^{-1} protein); glutathione reductase (Δ A_{412} min^{-1} mg^{-1} protein); ascorbate peroxidase (μmol ascorbate oxidized min^{-1} mg^{-1} protein); catalase activity (nmol H_2O_2 min^{-1} mg^{-1} protein); H_2O_2 content (μmol H_2O_2 g dry wt^{-1}) in wheat exposed to timely and late sown conditions.

Variety	Stress _Spray	Suseroxide dismutase (unit min^{-1} mg^{-1} protein)	Ascorbate peroxidase activity (μmol asc. oxidized $min^{-1}mg^{-1}$protein)	Catalase activity (nmol H_2O_2 min^{-1} mg^{-1}protein)	Glutathione reductase (nmol H_2O_2 min^{-1} mg^{-1}protein)	H_2O_2 content (μmol H_2O_2 g $drywt^{-1}$)
HD 2643	TS_C	3.98 ± 0.21^{a}	5.14 ± 0.14^{b}	1.93 ± 0.03^{b}	1.08 ± 0.03^{b}	0.88 ± 0.03^{a}
	TS_GA	4.23 ± 0.26^{a}	6.9 ± 0.22^{a}	1.91 ± 0.05^{b}	1.09 ± 0.03^{ab}	0.91 ± 0.03^{a}
	TS_PBZ	4.65 ± 0.13^{a}	6.99 ± 0.22^{a}	2.16 ± 0.08^{a}	1.21 ± 0.04^{a}	0.94 ± 0.04^{a}
	LS_C	9.13 ± 0.15^{c}	13.01 ± 0.33^{b}	6.4 ± 0.2^{b}	1.67 ± 0.08^{b}	1.49 ± 0.02^{a}
	LS_GA	9.9 ± 0.15^{b}	12.71 ± 0.69^{b}	6.37 ± 0.12^{b}	1.76 ± 0.06^{b}	1.44 ± 0.05^{a}
	LS_PBZ	9.61 ± 0.41^{a}	15.13 ± 0.66^{a}	7.5 ± 0.08^{a}	1.89 ± 0.02^{a}	1.32 ± 0.04^{b}
DBW 14	TS_C	5.2 ± 0.07^{c}	3.41 ± 0.4^{b}	2.05 ± 0.04^{a}	1.02 ± 0.06^{a}	0.88 ± 0.05^{a}
	TS_GA	5.75 ± 0.13^{b}	3.16 ± 0.31^{b}	2.1 ± 0.06^{a}	0.99 ± 0.06^{a}	0.8 ± 0.05^{ab}
	TS_PBZ	6.65 ± 0.23^{a}	5.59 ± 0.31^{a}	2.08 ± 0.1^{a}	1.08 ± 0.05^{a}	0.71 ± 0.03^{b}
	LS_C	11.15 ± 0.25^{b}	13.07 ± 0.41^{b}	5.54 ± 0.07^{b}	1.72 ± 0.01^{a}	1.36 ± 0.01^{a}
	LS_GA	10.43 ± 0.41^{b}	13.88 ± 0.64^{ab}	4.72 ± 0.33^{b}	1.67 ± 0.03^{a}	1.25 ± 0.05^{b}
	LS_PBZ	14.57 ± 0.63^{a}	15.35 ± 0.42^{a}	7.11 ± 0.5^{a}	1.85 ± 0.1^{a}	1.18 ± 0.03^{b}
HD 2189	TS_C	4.33 ± 0.32^{b}	6.4 ± 0.27^{b}	2.09 ± 0.03^{a}	1.03 ± 0.05^{a}	0.85 ± 0.02^{a}
	TS_GA	4.6 ± 0.4^{b}	6.99 ± 0.32^{ab}	2.06 ± 0.05^{a}	1.08 ± 0.01^{a}	0.79 ± 0.02^{ab}
	TS_PBZ	5.87 ± 0.15^{a}	7.73 ± 0.27^{a}	2.11 ± 0.14^{a}	1.08 ± 0.05^{a}	0.75 ± 0.04^{b}
	LS_C	5.76 ± 0.32^{b}	11.19 ± 0.64^{b}	4.66 ± 0.21^{b}	1.41 ± 0.03^{b}	1.6 ± 0.08^{b}
	LS_GA	6.12 ± 0.15^{b}	11.13 ± 0.23^{b}	4.97 ± 0.14^{b}	1.47 ± 0.02^{b}	1.81 ± 0.06^{a}
	LS_PBZ	7.49 ± 0.18^{a}	14.33 ± 0.32^{a}	6.12 ± 0.21^{a}	1.62 ± 0.04^{a}	1.39 ± 0.01^{c}
HD 2833	TS_C	4.2 ± 0.21^{b}	2.84 ± 0.19^{b}	2.07 ± 0.05^{a}	1.03 ± 0.06^{a}	1 ± 0.1^{a}
	TS_GA	4.87 ± 0.15^{b}	2.68 ± 0.27^{b}	2.26 ± 0.04^{a}	1.03 ± 0.06^{a}	0.93 ± 0.03^{a}
	TS_PBZ	6.69 ± 0.31^{a}	3.64 ± 0.13^{a}	2.32 ± 0.14^{a}	1.05 ± 0.04^{a}	0.81 ± 0.04^{a}
	LS_C	7.12 ± 0.16^{b}	7.65 ± 0.35^{b}	5.15 ± 0.04^{b}	1.35 ± 0.03^{b}	1.69 ± 0.03^{a}
	LS_GA	7.6 ± 0.36^{ab}	7.91 ± 0.42^{ab}	5 ± 0.16^{b}	1.37 ± 0.03^{b}	1.78 ± 0.06^{a}
	LS_PBZ	8.4 ± 0.15^{a}	8.98 ± 0.18^{a}	6.07 ± 0.29^{a}	1.7 ± 0.08^{a}	1.5 ± 0.07^{b}

The different letters (as superscript) in a column denotes significant difference among the treatments within the given variety and stress level according to LSD test at $P < 0.05$. The value follows ± represent the standard error ($n = 5$).

TS, Timely sown; LS, Late Sown; C, Control; GA, GA3 spray; PBZ, Paclobutrazol spray.

further degradation of bioactive gibberellins so that plants could maintain a minimum level of endogenous gibberellins under stress.

Exogenous gibberellins/PBZ may regulate the level of active gibberellins in the plant (Tonkinson et al., 1997). We observed that exogenous spray of GA_3 and PBZ significantly ($P < 0.05$) induced and inhibited endogenous GA_3 level, respectively, in tolerant and susceptible cultivars. Susceptible and tolerant varieties did not respond differently toward the application of both growth regulators. PBZ inhibited KO, a cytochrome P450 monooxygenase class enzyme of the GA biosynthesis pathway (Tuna et al., 2008); thus, a highly significant decrease in the KO enzyme transcript level was seen in all cultivars on PBZ application. Inhibition of this enzyme significantly also reduced the level of endogenous GA in plants. Baninasab and Ghobadi (2011) reported downregulation of KO, GA20ox, and GA3ox under ambient and high-temperature conditions on PBZ application. A relatively more significant decrease in GA biosynthesis was observed in TS plants due to PBZ application. The higher decrease in expression level of GA biosynthesis genes is may be attributed to the more active GA biosynthesis mechanism of TS plants than in LS plants. GA_3 application upregulated GA2ox (GA degrading enzyme) expression in both types of cultivars, and it can be inferred that over-accumulation of bioactive gibberellin induced negative feedback regulation and increased its degradation to maintain the optimum level of hormone. In contrast, PBZ application downregulated the expression of GA2ox further to reduce the degradation of 656 bioactive gibberellins in plants. Tuna et al. (2008) also observed a decrease in expression of GA2ox activity on PBZ application under salinity stress.

Generally, under stress conditions, simultaneous ROS generation and consequent enhanced antioxidant system activity have been observed in plants (Almeselmani et al., 2009; Wang et al., 2014). The trade-off between ROS generation and the antioxidant enzyme system decides the tolerance ability of the plant under stress. In conformity to previous studies, we also observed an increase in ROS, i.e., under the heat-stressed environment in all cultivars, H_2O_2 had a relatively higher increase in susceptible cultivars than tolerant cultivars. In

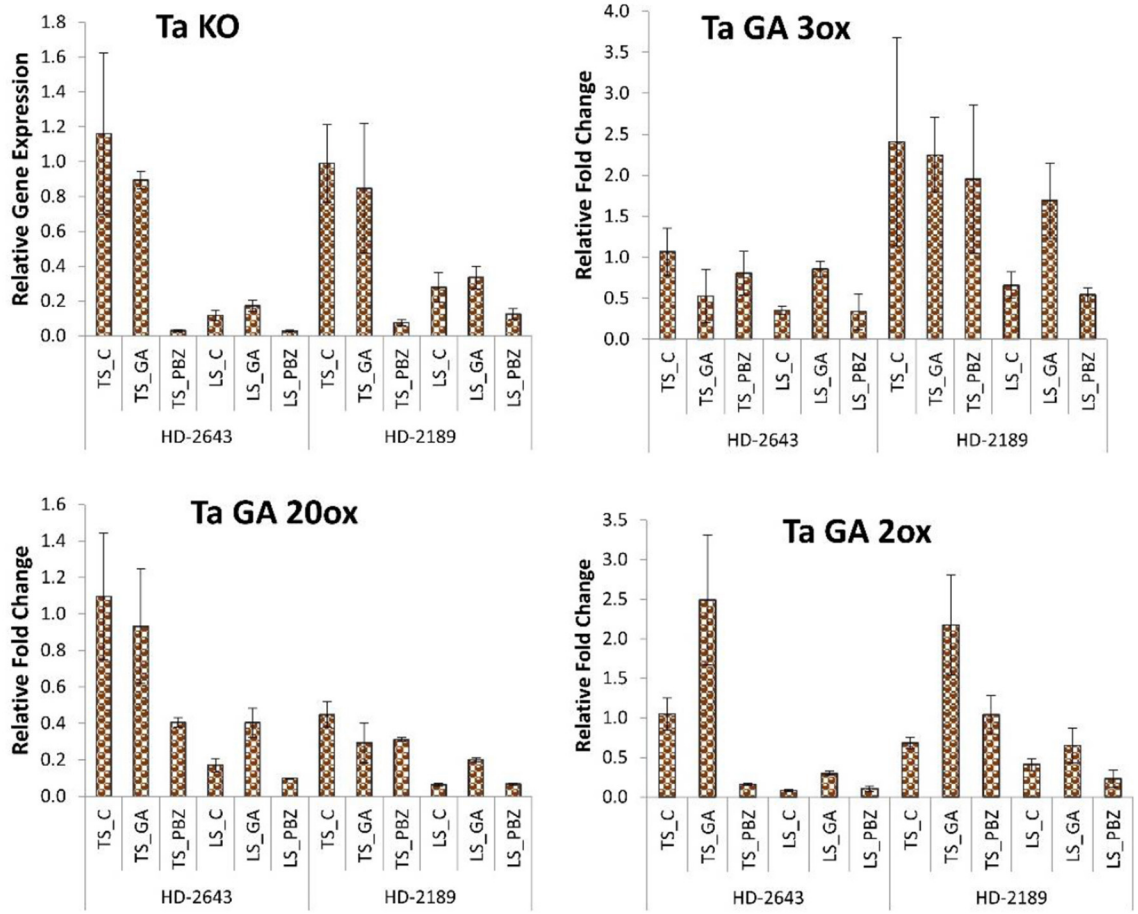

FIGURE 3 | Relative fold change in expression of GA biosynthesis and degradation genes, i.e., Kaurene oxidase (KO), GA 3oxidase, GA 20 oxidase, and GA 2 oxidase in developing grain. The error bar represents the standard error. Value on the ordinate is the mean of three replicates. On the abscissa, HD 2643 and HD 2189 are varieties; TS and LS denote timely sown and late sown of stress treatment, respectively; C, GA, and PBZ denote control, GA3, and paclobutrazol of spray treatment, respectively.

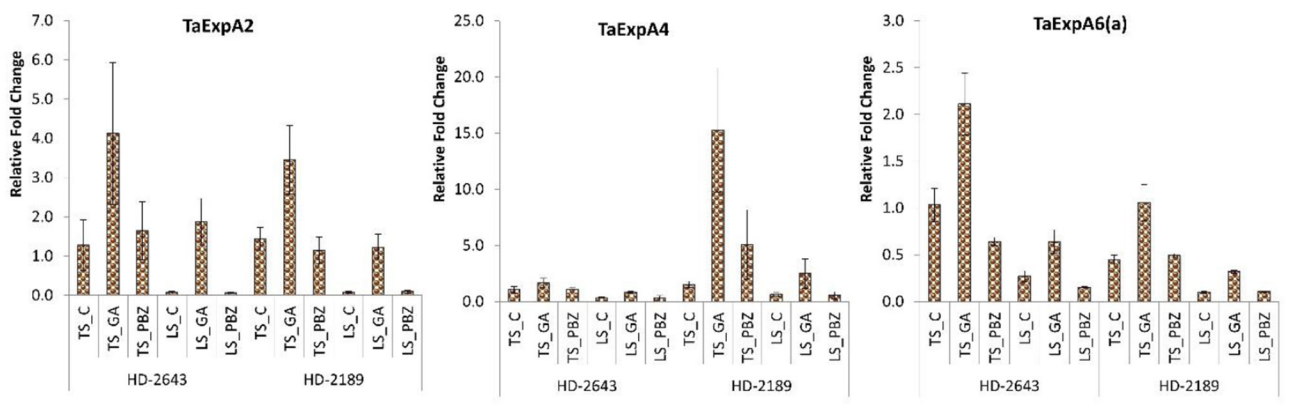

FIGURE 4 | Relative fold change in expression of wheat cell expansin genes, i.e., Ta Exp 2, Ta Exp A4, and Ta Exp A6(a) in developing grain. The error bar represents the standard error. Value on the ordinate is the mean of three replicates. On the abscissa, HD 2643, and HD 2189 are varieties; TS and LS denote timely sown and late sown of stress treatment, respectively; C, GA, and PBZ denote control, GA3, and paclobutrazol of spray treatment, respectively.

tolerant cultivars, activity of antioxidant enzymes like superoxide dismutase, ascorbate peroxidase, peroxidase, catalase, and glutathione reductase was recorded higher in comparison to susceptible cultivars. The increase in ROS such as superoxide, hydroxyl ion, and hydrogen peroxide may be attributed to an increase in free-electrons for O_2 due to excessive excitation of photosystems. The increased ROS level has various effects on peroxidation of membrane lipid and accumulation of malondialdehyde compound, such as increases in electrolyte leakage from the cell, leading to damage to the membrane (Senaratna et al., 1988; Hasanuzzaman et al., 2020) and PBZ-induced stress protection through an efficient antioxidant mechanism (Zhou and Leul, 1998; Soumya et al., 2017). To date, comparative analysis of exogenous PBZ/GA_3-mediated stress tolerance through GA_3 biosynthesis inhibition/induction has been less explored. We observed that PBZ conferred stress protection by reducing oxidative damage caused by ROS through an increased level of antioxidant enzymes, but not through GA_3 biosynthesis inhibition. The observation further supported that exogenous GA_3-mediated GA_3 biosynthesis induction did not significantly affect the activity of the antioxidant enzymes.

Erstwhile studies have shown a significant decrease in electrolyte leakage of susceptible wheat varieties as compared to tolerant varieties (Almeselmani et al., 2009). The electrical conductivity of the leachate from heat-shocked seedlings of susceptible cultivars was higher than seedlings of tolerant cultivars grown in a controlled environment (ElBasyoni et al., 2017). Similarly, we also observed higher Malonaldehyde (MDA) accumulation and an increase in electrolyte leakage of LS plants. The PBZ application increased membrane thermostability by reducing lipid peroxidation-mediated damage to the unsaturated lipid of the membrane. Such plants could scavenge more ROS generated under stress due to high availability of the antioxidant enzymes (Chakraborty and Tongden, 2005). In general, no significant effect of GA_3 application was seen on lipid peroxidation and electrolyte leakage. It could be due to the non-significant effect of GA_3 on antioxidant enzyme activity. On the contrary, electrolyte leakage was enhanced by GA_3 foliar application under salinity by Tuna et al. (2008). Our results indicate that a higher level of endogenous GA_3 is not associated with an antioxidant defense system and membrane stability.

High-temperature damages to the photosystem electron transport chain reduced the activity of rubisco and rubisco activase. The solubility of CO2 decreases under high temperature. It increases photorespiration, thus reducing photosynthesis rate under stress (Wahid et al., 2007; Parry et al., 2011). Photochemical efficiency is the ratio of variable to maximum fluorescence (Fv/Fm), an indicator of photosystem II efficiency, which positively correlates with photosynthesis rate. Thus, a higher Fv/Fm ratio is associated with enhanced stress tolerance (Parry et al., 2011; Sharma et al., 2015; Faseela et al., 2020). We found that photosynthesis rate and Fv/Fm ratio decreased in plants under LS condition. The decrease in photosynthesis rate and Fv/Fm ratio was found to be cultivar-dependent. Tolerant cultivars maintained a higher photosynthesis rate and Fv/Fm ratio in a stressed environment than susceptible ones. In tolerant cultivars, the presence of more efficient photosystems and heat stress-tolerant enzymes of photosynthesis, i.e., rubisco and rubisco activase, have been reported previously (Feng et al., 2014; Brestic et al., 2018). In PBZ-treated plants, photosynthesis rate and Fv/Fm ratio were higher than those of non-treated plants under LS conditions. These results were in agreement with the findings of previous researchers (Mahoney et al., 1998; Sharma et al., 2015). Results indicated that PBZ could decrease the heat-induced photo-inhibition by protecting photosystem II. The PBZ application increased photosynthesis rate by reducing ROS-mediated damage to photosynthesis machinery and photosynthetic pigments in plants. Transpiration rate and stomatal conductance were higher in stressed plants as compared to their respective control. Our results were also in agreement with the findings of Feng et al. (2014) and Sharma et al. (2015). The GA_3/PBZ application did not affect stomatal conductance and transpiration rate under both temperature conditions in studied cultivars. Thus, we concluded that the decline in photosynthesis rate under heat stress was not the consequence of stomatal limitation.

LS plants were exposed to different temperatures and photoperiods during their life cycle compared to TS plants, which was a deviation from standard requirement (Nagar et al., 2015). This reduced plant growth and development, resulting in diminished height, tiller number, and dry matter accumulation. High temperature increased the rate of development, but at the same time, reduced the duration of crop growth and grain filling. The compensatory effect of grain filling rate was not adequate to overcome the decrease in duration, leading to a decrease in seed setting and 1,000 grain weight, ultimately causing a significant reduction in yield (Reynolds et al., 2012; Tan et al., 2015). High temperature diverted the photo-assimilates from developing stress tolerance mechanism to survive under stress, which led to a decrease in the availability of photo-assimilates for the development of reproductive organs (Wahid et al., 2007; Janda et al., 2019; Hasanuzzaman et al., 2020). The rate of starch deposition in wheat grains was decreased by >30% at temperatures between 30 and 40°C, with the early grain filling being the most critical stage (Stone and Nicolas, 1994). Reduction in growth parameters and yield traits was higher in susceptible cultivars than tolerant cultivars because of stress condition. Decreases in active photosynthesis area and the photosynthesis rate were less in tolerant cultivars than sensitive cultivars. Application of PBZ and GA_3 had no significant effect on growth parameters. At anthesis, growth regulators applied, but by that time, the plant had already achieved its maximum growth. In contradiction, Khan and Samiullah (2003) also observed an increase in total dry matter in *Brassica juncea* when 10^{-5}M GA3 was applied at 40 days after sowing.

The smaller grain size under stress reduces grain weight potential, leading to a significant decrease in final grain yield. Grain weight depends on the number and size of cells in the pericarp and endosperm; it determines the sink capacity of the grain (Kaur et al., 2011). Additionally, cell size plays a more significant role than cell numbers in developing endosperm and pericarp, as they determine the grain expansion ability during grain development. Cell expansion enzymes also play a determining role in the process of cell wall extension and

cell size (McQueen-Mason et al., 1992; Lizana et al., 2010). We reported a decrease in mRNA level of expansin proteins, i.e., TaExp A2, TaExp A4, and TaExpA6a under stress. Thus, this decrease reduced the cell expansin enzyme activity; which, in turn, decreased cell size in pericarp and endosperms, limited the accumulation of starch in endosperm, and reduced the grain size. Smaller grains and a decrease in test weight under stress might also be due to decreased cell expansins enzyme activity. The increase in endogenous gibberellic acid level in developing grains might have contributed to a gain in 100 seed weight, as the spray of GA_3 coincided with the most sensitive period of grain weight determination. Gibberellins regulate grain filling duration in crop plants, contributing to individual grain weight (Wang et al., 2006), and GA_3 is also associated with the grain filling process, like an increase in mobilization of storage reserve, which leads to an increase in mean grain weight. Furthermore, Yang et al. (2001) revealed a positive correlation between content of the gibberellins and the rate of embryo development during the grain filling stage in rice. We reported the increase in transcript level of three cell expansin genes, i.e., TaExp A2, TaExp A4, and TaExp A6(a), in developing seed on exogenous application of GA_3. Choi et al. (2006) reported an increase in transcript level of cell wall expansins gene OsEXP4 after 30 min of exogenous application of gibberellin in rice. Our results also demonstrated that GAs contents in grains were positively related to the increased grain weight, which caused an increase in grain storage space due to an increase in pericarp cell expansion rate and endosperm cell division. This was caused by an increase in cell expansins gene expression.

In general, PBZ application improved the grain yield and 1,000-seed weight with a relatively more significant increase in susceptible cultivars. The efficient antioxidant defense system of PBZ-treated plants contributed to a higher photosynthesis rate under stress and, consequently, increased photo-assimilate availability for grain development. PBZ did not affect the cell expansin genes activity, which implied that the cell expansins-mediated mechanism does not play a role in PBZ-treated plants. Harvest index showed the non-significant effect on GA_3/PBZ application, which suggests that GA_3 and PBZ were unable to change the ratio of grain yield to biomass, agreeing with the results of Baninasab and Ghobadi (2011). The study concludes that the inhibitory/inducing effect of PBZ/GA_3 application on endogenous GA_3 level is not solely responsible for the thermo-protection under terminal heat stress in wheat. We report that an increased level of endogenous GA_3 did not significantly influence the antioxidant enzyme activity thus indicates that PBZ would have provided thermotolerance through another mechanism instead of the GA_3 inhibitory mechanism. Other mechanisms of conferring stress tolerance by PBZ could be through the enhanced synthesis of proline, abscisic acid, and salicylic acid biosynthesis (Nivedithadevi et al., 2012; Khan et al., 2015; Soumya et al., 2017; Kousar et al., 2018). We reported that an increase in levels of endogenous GA_3 brought no change in thermotolerance traits, but had a positive effect on grain yield and test weight. An increase in cell expansin genes activity might have, in part, contributed to an increase in individual grain weight.

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

VS, AA, and GS proposed the hypothesis and designed the experiment. SN and RSR conducted the experiment. SN, SK, and RD analyzed the data and wrote the manuscript. SN and NS standardized the GA extraction protocol. 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.2021.692252/full#supplementary-material

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Exogenous 6-Benzyladenine Improves Waterlogging Tolerance in Maize Seedlings by Mitigating Oxidative Stress and Upregulating the Ascorbate-Glutathione Cycle

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The synthetic cytokinin 6-benzyladenine (6-BA) regulates plant growth and prevents the negative consequences of various forms of abiotic stress, including waterlogging in crop plants. The present study aimed to investigate the effects of exogenous 6-BA on the growth, oxidative stress, and ascorbate-glutathione (AsA-GSH) cycle system in the inbred SY-MY13 (waterlogging-resistant) and SY-XT1 (waterlogging-sensitive) seedlings of waxy corn in conditions of waterlogging stress. The results demonstrated that waterlogging stress causes chlorosis and necrosis in waxy corn leaves, inhibiting growth and leading to the accumulation of reactive oxygen species (ROS), which induces oxidative stress and, in turn, reduces membrane lipid peroxidation and the disruption of membrane homeostasis. This is specifically manifested in the increased concentrations of superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), in addition to increased relative electrical conductivity (REC%) values. The SY-MY13 strain exhibited growth superior to that of SY-XT1 when waterlogged due to its excellent waterlogging resistance. Thus, exogenous 6-BA was found to be effective in enhancing the growth of plants stressed by waterlogging in terms of the weight of the shoots and roots, shoot height, and leaf area. In addition to this, exogenous 6-BA also reduced the accumulation of O_2^-, H_2O_2, and MDA, increased ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR) activity, and enhanced ascorbic acid (AsA), and reduced glutathione (GSH) concentration through the regulation of the efficiency of the AsA-GSH cycle system in maize plants. Hence, the application of exogenous 6-BA can alleviate waterlogging-induced damage and improve waterlogging tolerance in waxy corn via the activation of the AsA-GSH cycle system and the elimination of ROS.

Keywords: AsA-GSH cycle, oxidative stress, waterlogging stress, waxy corn, 6-benzyladenine

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INTRODUCTION

Climate change has compounded global environmental risks, affecting crop growth and jeopardizing food production and economic growth worldwide (Li et al., 2017; Tiwari et al., 2017; Maraci et al., 2018). Of the heightened environmental risks, waterlogging has already been identified as among the most critical abiotic stresses affecting crop productivity

(Wright et al., 2017; Fukao et al., 2019). Waterlogging stress is defined as the saturation of the soil around the roots of crops with water, which creates a low-oxygen (hypoxic) environment because of the limited diffusion of gas in water (Xu et al., 2016; Ren et al., 2017).

Waterlogging stress modifies the balance between the endogenous production and neutralization of reactive oxygen species (ROS), such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), etc., causing the accumulation of ROS that leads to oxidative stress in plants (Posso et al., 2018; Anee et al., 2019; Begum et al., 2019; Malhi et al., 2019; Park and Ju, 2019). Xiao et al. (2020) concluded that the exposure of peach seedlings to waterlogging induced the accumulation of intracellular reactive ROS, in turn, causing apoptosis and impeding their growth and development. Lou et al. (2017) observed that excess ROS increased lipid peroxidation, leading to the dysregulation of several physiological mechanisms in pakchoi. In addition, excess ROS also destroys physiological membrane activity, causing cell swelling and rupture (Zhang et al., 2007; Geetika et al., 2016; Sarwar et al., 2019).

Plants minimize the accumulation of ROS *via* complex and highly efficient enzymatic and non-enzymatic antioxidants (Ahmad et al., 2010, 2019; Kohli et al., 2019). Specifically, the ascorbate-glutathione (AsA-GSH) cycle has been reported to reduce the negative impact of ROS (Jung et al., 2020; Tan et al., 2020; Wang M. et al., 2020). The AsA-GSH cycle consists of the enzymes ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), and the non-enzymatic molecules ascorbic acid (AsA), dehydroascorbic acid (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) (Ahmad et al., 2010, 2019; Kohli et al., 2019). After preferentially donating an electron to APX, AsA becomes oxidized to monodehydroascorbate (MDHA) or DHA, while APX reduces H_2O_2 to H_2O. MDHA is subsequently reduced to AsA by MDHAR, and DHA is reduced to AsA by GSH-dependent DHAR (Ahmad et al., 2010, 2019; Kohli et al., 2019). In a parallel reaction, GSH becomes oxidized by DHAR to generate GSSG, which is reduced back to GSH by GR (Foyer and Noctor, 2011; Kuo et al., 2020; Yildiztugay et al., 2020). Furthermore, the AsA-GSH cycle can regulate oxidative damage caused by environmental stresses, such as chromium stress in kenaf cultivars (Niu et al., 2018), waterlogging stress in wheat (Wang X. et al., 2016), drought stress in maize (Guo et al., 2020), and saline stress in rice (Islam et al., 2017). Thus, plants reduce the negative consequences of waterlogging stress through the up- or down-regulation of enzymatic and non-enzymatic antioxidants. Such studies have contributed to our understanding that the AsA-GSH cycle regulates the physiological response of plants to stress (Alam et al., 2019; Ahanger et al., 2020; Ahmad et al., 2020; Jan et al., 2020; Kaya et al., 2020).

Nevertheless, waterlogging stress results in several pernicious consequences that require sustainable and effective measures. One such intervention for alleviating waterlogging stress is supplementation with a plant growth regulator (PGR). PGR are often used to increase plant productivity, tolerance to stress, and self-repair qualitatively similar to the physiological and biological effects of phytohormones (Ali et al., 2015; Neill et al., 2019). Studies have demonstrated that PGRs can significantly increase the efficiency of the AsA-GSH cycle by removing the oxidative damage caused by the accumulation of ROS induced by environmental stress. Examples include heavy metal stress (Khan et al., 2019), salt stress (Alam et al., 2019), drought stress (Wang H. H. et al., 2016), and heat stress (Li Z., et al., 2016). Cell division is the most fundamental process of any system of biotic growth and development and core-to-tissue growth in all organisms (Alim et al., 2012; Meng et al., 2020). Cytokinins are central regulators of plant growth and development, playing important regulatory functions in physiological processes, including cell division, apical dominance, plant growth, photomorphogenesis, nutrient translocation, and leaf senescence (Paul et al., 2016; Wang Y. et al., 2016; Jan et al., 2019). In addition, cytokinins are also implicated in the regulation of plant immune signaling networks, suggesting that the growth and defense of plants are intimately connected (Van der Does et al., 2013; Sorensen et al., 2018). 6-benzyladenine (6-BA) is in a class of synthetic cytokinin PGRs that can significantly increase levels of endogenous cytokinins in crop plants, which otherwise decrease significantly when experiencing environmental stress (Hu et al., 2020; Prerostova et al., 2020). Thus, supplemental 6-BA allows plants to overcome the negative effects of various types of abiotic stress. Treatment with 6-BA has been shown to enhance photochemical efficiency, increase growth, and reduce Na^+ accumulation in ryegrass in a saline environment; thus effectively preventing the inhibition of growth in plants experiencing salt stress (Ji et al., 2019). The exogenous application of 6-BA could significantly reduce toxicity caused by cadmium (Cd) and uranium (U) on rapeseed, subsequently reducing malondialdehyde (MDA) and H_2O_2 levels and increasing antioxidant levels (Chen et al., 2020). The addition of 6-BA also suppressed heat-induced leaf senescence and oxidative damage in ryegrass (Zhang et al., 2019) and enhanced drought stress tolerance in sweet potato plants (Li et al., 2020). Spraying wheat plants with 6-BA prior to becoming waterlogged has also been found to decrease MDA levels in leaves, reduce plant senescence, and facilitate the production of dry matter (Wang X. Y. et al., 2020). It has also been reported that exogenous 6-BA significantly increased the activity of the leaf defense system in maize and is effective in reducing the adverse effects of the accumulation of ROS caused by waterlogging on plant physiological traits (Hu et al., 2020).

Waxy corn, a variety of maize expressing the only amylopectin, has been extensively planted in China and many other countries (Ketthaisong et al., 2014). However, maize (*Zea mays* L.) is considered sensitive to variations in its environment, especially during the seedling stage (Xie et al., 2017; Wang et al., 2018; Babu et al., 2020). In particular, maize

Abbreviations: AsA-GSH, ascorbate-glutathione; ROS, reactive oxygen species; MDA, malondialdehyde; APX, ascorbate peroxidase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; AsA, ascorbic acid; DHA, dehydroascorbic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; PGRs, plant growth regulators; FW, fresh weight; DW, dry weight; REC%, relative electric conductivity.

is highly sensitive to waterlogging stress, which in the early growth stage, significantly inhibits its growth and development. Waterlogging during the seedling stage causes the greatest delay to growth (Ren et al., 2014), causing the leaves to be withered and yellow, decreasing the maximum green leaf area, reducing photosynthetic efficiency, and exacerbating oxidative damage (Tang et al., 2010; Wu et al., 2018; Yao, 2021). In particular, grain yield decreases most significantly when plants are waterlogged during the seedling stage compared with other stages (Ren et al., 2018b). In addition to heavy rainfall caused by extreme weather, waterlogging due to the lack of drainage in low-lying areas is also a leading factor (Visser et al., 2003; Barik et al., 2019). Therefore, improving tolerance to waterlogging is critical for the production of waxy corn that experiences waterlogging stress. However, to the best of our knowledge, no study has investigated the role of 6-BA-induced tolerance to waterlogging stress in waxy corn. Based on previous studies, which have explored the role of 6-BA in other plants undergoing different forms of stress (Ji et al., 2019; Chen et al., 2020; Hu et al., 2020; Li et al., 2020; Wang X. Y. et al., 2020), we hypothesized that 6-BA scavenges ROS by the promotion of the AsA-GSH cycle system and alleviates the adverse effects of oxidative damage from waterlogging stress in waxy corn seedlings, thereby enhancing tolerance to waterlogging in waxy corn seedlings. Hence, the effect of leaf-sprayed 6-BA in two genotypes of waxy corn seedlings when waterlogged was investigated by comparing morphology, ROS metabolism, cell stability, and the AsA-GSH cycle to verify whether 6-BA prevents oxidative damage from waterlogging stress in waxy corn seedlings. Such an approach may represent a cost-effective and environmentally friendly method to solve the problems of waterlogging stress in waxy corn production.

MATERIALS AND METHODS

Plant Materials and Experimental Design

Pot experiments were conducted at the Research and Education Center of Agronomy, Shenyang Agricultural University (Shenyang, China) in 2020 using two waxy corn inbred lines, namely, SY-MY13 (waterlogging-resistant) and SY-XT1 (waterlogging-sensitive), provided by the Specialty Corn Institute, Shenyang Agricultural University. Pots that were 12 cm in height and 10 cm in diameter were filled with 1 kg of conventional tilled brown soil (from a depth of 0–to 20 cm) with a composition of 32.45 g.kg^{-1} of organic matter, 121.86 of mg.kg^{-1} of alkali-hydrolyzable nitrogen, 9.47 mg.kg^{-1} of available phosphorus, and 114.31 mg.kg^{-1} of available potassium. The experiment was conducted under a rain shelter covered with polyethylene film to exclude any natural precipitation in the experiment. Seeds were sown into each pot on May 12. Three healthy seedlings were retained after germination, with normal watering until the fourth leaf stage. The experiments were conducted using an entirely randomized design. The 6-BA (analytically pure) used in this study was obtained from Ryon Biological Technology Co., Ltd. (Shanghai, China) and dissolved in distilled water containing 0.01% of Tween-20 to a final concentration of 0.5 mM. The concentrations of 6-BA were selected from the results of a previously reported study (Chen et al., 2013). The 6-BA was evenly sprayed on the leaves of waxy corn seedlings. There were four treatment groups for each inbred line, namely, (1) CK: normal watering conditions; (2) CK + 6-BA: 0.5 mM of 6-BA plus normal watering conditions; (3) W: waterlogged; (4) W + 6-BA: 0.5 mM of 6-BA plus waterlogging. Treatments started on June 6. Pots from the same group were placed into plastic boxes (61 × 42 × 12 cm). Prior to starting the waterlogging stress treatments, 0.5 mM of 6-BA was applied as a foliar spray on all the leaves of each treatment group until they were completely moistened. After 24 h, water was added to the plastic boxes to a depth of 3 cm above the soil surface in the waterlogged groups. There were 40 pots in each treatment group. After 7 days, the whole plants and three fully expanded leaves from the bottom of the plants were collected for analysis and measurement. All measurements were performed in triplicates and the mean values were recorded.

Determination of Growth Parameters

Growth parameters were measured in accordance with an earlier study (Wang et al., 2021). Shoot height and leaf dimensions were measured using a ruler. Leaf area was calculated in accordance with the following formula:

$$\text{Leaf area (cm}^2) = \text{L} \times \text{W} \times 0.75$$

where L represented leaf length, W was leaf width, and 0.75 was a factor used for maize seedlings that consider leaf shape (Hussain et al., 2019).

Each whole plant was subdivided into shoots and roots. Roots were cleaned with tap water to remove adherent soil and then dried with absorbent paper. The fresh weight (FW) of the shoots and roots were individually measured using an analytical balance (*UQINTIX65-1CN*, Sartorius, Göttingen, Germany) and were then loaded into sample bags after 2 h of drying in an oven at 105°C. After that, the shoots and roots were dried to a constant weight at 80°C. The dry weight (DW) of each shoot and root was then measured using the analytical balance.

Roots were placed in an acrylic tray (400 × 300 mm) in a 1 cm depth of water. The length, volume, diameter, and surface area of the roots were measured through scanning using a flatbed scanner (*1680*, Epson, Nagano, Japan) and analysis with the WinRHIZO root analysis software (*Pro 2007*, Regent Instruments, Québec, Canada).

Quantification of Lipid Peroxidation and Relative Electrical Conductivity

Malondialdehyde content was measured in accordance with a method described by Ohkawa et al. (1979). A total of 0.5 g of fresh leaves were ground and homogenized in 5 ml of 5% trichloroacetic acid (TCA) then centrifuged at 12,000 × *g* for 10 min. The supernatant (2 ml) was retained and mixed with TCA (5%) containing thiobarbituric acid (TBA, 0.5%), placed in a water bath for 20 min at 100°C, then rapidly cooled and centrifuged at 12,000 *g* for 10 min. The absorbances of the supernatant at 450, 532, and 600 nm were recorded using a microplate reader (*1510*, Thermo Fisher, USA). Relative electrical

conductivity (REC%) was determined using a conductivity meter (*DDSJ-380F*, Rex, Shanghai, China) and measured in accordance with a method described by Li A. X. et al. (2015). Briefly, 0.1 g of fresh leaves were immersed in deionized water, and the electrical conductivity of the solution was measured after 3 h, termed EC1. The solution was placed in a water bath for 30 min at 100°C, then cooled to room temperature, shaken, and its electrical conductivity measured, termed EC2. REC% was calculated using the following formula:

$$REC\% = EC1/EC2 \times 100\%$$

Histochemical Staining and Determination of O_2^- and H_2O_2

Histochemical staining for O_2^- and H_2O_2 was conducted in accordance with a method described by Xia et al. (2009), with modifications. The three lowest fully expanded leaves were washed with distilled water, blotted dry, then placed into a test tube containing 50 ml of 0.5 mg/ml nitrotetrazolium blue chloride (NBT) and a reaction solution (potassium phosphate buffer, pH 7.8), respectively, prior to incubation at 25°C in the dark for 2 h. Leaves were also placed in 50 ml of 1 mg/ml diaminobenzidine (DAB) reaction solution (Tris-HCl buffer, pH 3.8), which was incubated at 25°C in the dark for 24 h. The solutions in the individual tubes were replaced with a bleaching solution (90% ethanol) and placed in a water bath at 90°C for 15 min until the leaves were fully bleached; after which, they were photographed.

To measure O_2^- and H_2O_2, a method described by Xu et al. (2012) was followed. A total of 0.5 g of fresh leaves were ground in liquid nitrogen, suspended in 50 mM of phosphate-buffered saline (PBS, pH 7.8), and then centrifuged at 12,000 × *g* for 15 min. The concentration of O_2^- in the supernatant was measured using a microplate reader (*1510*, Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 580 nm. For measurements of H_2O_2, 0.5 g of fresh leaves were ground in liquid nitrogen, suspended in 100 mM of PBS (pH 7.8) containing 1% (w/v) polyvinyl pyrrolidone (PVP), and then centrifuged at 12,000 × *g* for 20 min. The concentration of H_2O_2 in the supernatant was measured using a microplate reader at a wavelength of 350 nm.

Measurements of ASA-GSH Cycle Enzyme Activity

A 0.5-g quantity of fresh leaves was ground in liquid nitrogen, then homogenized in 10 ml of an extraction buffer (pH 7.8) containing 25 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2% PVP, 2 mM of ascorbic acid, and 0.2 mM of ethylenediaminetetraacetic acid (EDTA). The homogenized solution was centrifuged at 12,000 × *g* for 20 min at 4°C. The activity of the AsA-GSH cycle enzymes in the supernatant was measured. The activities of APX and DHAR were calculated in accordance with a method published by Nakano and Asada (1980), GR activity was calculated in accordance with GarcíaLimones et al. (2002), and MDHAR activity was calculated in accordance with (Aravind and Prasad, 2005).

Measurements of Concentration of AsA-GSH Cycle Metabolites

A 0.5-g quantity of fresh leaves was ground in liquid nitrogen and then homogenized in 10 ml 10% (w/v) trichloroacetic acid. The suspension was centrifuged at 15,000 × *g* for 15 min at 4°C. The concentrations of AsA, DHA, GSH, and GSSG were measured in the supernatant. In particular, AsA and DHA concentrations were quantified using a dithiothreitol assay (Hodges et al., 1996) against calibration curves prepared from AsA and DHA standards. Concentrations of GSH and GSSG were measured using a 2-vinylpyridine assay (Griffith, 1980) against calibration curves constructed using GSH and GSSG standards.

Statistical Analysis

Data were analyzed using a one-way ANOVA, after which, a least significant difference (LSD) test was conducted. Differences were considered significant for $P < 0.05$. Analysis was performed with the *SPSS v19.0 software* (*SPSS Inc.*, Chicago, IL, USA). Data were plotted using the Origin 2017 software (*OriginLab,* Massachusetts, USA).

RESULTS

Plant Morphology

When grown in normal conditions, plants in the CK and 6-BA groups displayed no apparent differences, as illustrated in **Figure 1**. Waterlogging stress injuries in the waxy corn seedlings were characterized by chlorosis, wilting, and necrosis. However, the extent of the symptoms was considerably reduced in the SY-MY13 plants due to their excellent waterlogging resistance properties. Plants treated with 6-BA when stressed with waterlogging, on the other hand, did not exhibit visible damage in either inbred lines.

Plant Growth and Development

The growth parameters directly reflected the adaptability of the plants to the growing environment (Chen D. Q. et al., 2016; Kosar et al., 2021), as presented in **Tables 1**, **2**. Treatment with 6-BA significantly increased the shoot height of SY-MY13 plants, although there were no significant differences for other growth parameters between the two inbred lines in well-drained conditions. Waterlogging significantly reduced the fresh and dry shoot weight of the SY-XT1 plants but had no significant effect on the SY-MY13 strain. Leaf area and fresh and dry root weight were reduced by 17.9, 31.8, and 48.1% in the SY-MY13 strain and 31.5, 49.9, and 59.4% in the SY-XT1 plants compared with those of the CK group, respectively. Similarly, root length and surface area were reduced by 32 and 10.4% in SY-MY13 plants and 22.8 and 12% in SY-XT1 plants, respectively. In addition, the root diameter of the two inbred lines increased by 21.7 and 13.4%, respectively. Treatment with 6-BA also significantly enhanced the growth and development of both waxy corn inbred lines exposed to waterlogging stress. The dry weight of shoots, plant height, leaf area, and root diameter increased by 15.7, 9.8, 15, and 10.4% in SY-MY13 and 47.4, 9.9, 26.3, and 14.9% in SY-XT1 plants treated with 6-BA when waterlogged, respectively. The same treatment also increased the fresh and dry weight of

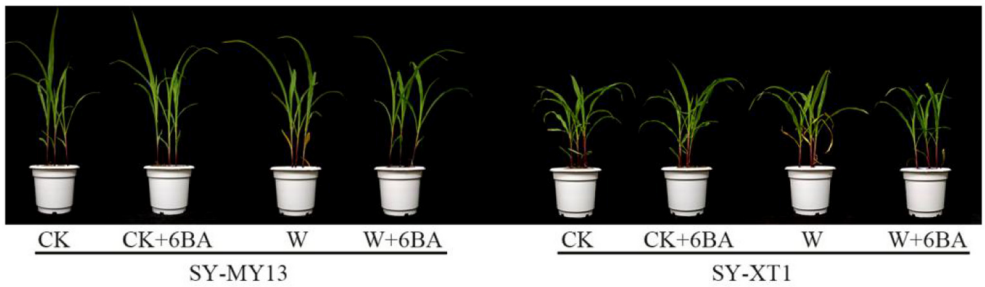

FIGURE 1 | Effects of 6-BA on the phenotype of two inbred lines with different treatments. CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions.

TABLE 1 | Effects of 6-benzyladenine (6-BA) on the biomass of two waxy corn inbred lines experiencing waterlogging stress.

Genotype	Treatment	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
SY-MY13	CK	3.78 ± 0.208cd	0.522 ± 0.019c	2.64 ± 0.257a	0.239 ± 0.012a
	CK+6BA	3.93 ± 0.100bc	0.535 ± 0.008c	2.33 ± 0.290ab	0.242 ± 0.021a
	W	3.61 ± 0.253d	0.538 ± 0.006c	1.80 ± 0.164c	0.124 ± 0.023b
	W+6BA	3.80 ± 0.227cd	0.638 ± 0.021a	1.16 ± 0.148d	0.139 ± 0.018b
SY-XT1	CK	4.17 ± 0.088ab	0.547 ± 0.013bc	2.24 ± 0.240b	0.229 ± 0.024a
	CK+6BA	4.27 ± 0.228a	0.544 ± 0.036bc	2.43 ± 0.050ab	0.237 ± 0.014a
	W	2.73 ± 0.100e	0.304 ± 0.01d	1.13 ± 0.062d	0.093 ± 0.007c
	W+6BA	3.75 ± 0.114cd	0.578 ± 0.029b	1.49 ± 0.107c	0.148 ± 0.014b

CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters represent significant differences ($P < 0.05$).

roots by 31.9 and 59.1%, respectively, in SY-XT1 compared with waterlogging stress.

Oxidative Stress and Histochemical Staining

Histochemical staining techniques have previously been used to detect H_2O_2 and O_2^- in plants (Xu et al., 2012). Compared with CK, the CK + 6-BA group displayed a significantly lower H_2O_2 content in SY-XT1, but displayed no significant difference in SY-MY13 plants (**Figure 2C**). In addition, there was no significant difference in the changes in O_2^- content in the CK and CK + 6-BA groups between the two inbred lines (**Figure 2D**). The histochemical staining of the leaves also revealed that there was less H_2O_2 and O_2^- accumulation when plants were waterlogged following pretreatment with 6-BA compared with waterlogged plants that were untreated (**Figures 2A,B**). As shown in **Figures 2C,D**, the leaves of the two inbred lines displayed higher levels of H_2O_2 and O_2^- accumulation when waterlogged compared with those that were well-drained, with H_2O_2 and O_2^- levels in SY-MY13 significantly increasing by 13.6 and 208.1% and in SY-XT1 by 40.9 and 405.1%, respectively, compared with the CK groups. Pretreatment with 6-BA resulted in a significant decrease in both H_2O_2 and O_2^- levels in the SY-MY13 plants, reducing by 10.2 and 34% and in SY-XT1 by 35.6 and 17.6%, respectively, compared with those that were waterlogged (**Figures 2A–D**).

Oxidative Damage to Cellular Membranes

Both MDA concentration and REC% reflect the extent of damage to cell membranes (Farooq et al., 2020). As displayed in **Figure 3**, pretreatment with 6-BA in plants that were watered normally exhibited a significantly reduced MDA concentration in SY-MY13 plants, but showed no effect on SY-XT1. It also had no significant effect on REC% in either of the two inbred lines. The MDA content and REC% increased in both inbred lines when the plants were waterlogged compared with the CK groups, although the increase was greater in SY-XT1 plants (140 and 307.2%) compared with SY-MY13 (42.1 and 254.1%). Furthermore, pretreatment with 6-BA resulted in a significant decrease in MDA levels and REC% compared with those that were waterlogged. The MDA concentration and REC% of SY-MY13 only increased by 9.5 and 22.6%, with the same observed in SY-XT1 by 27.8 and 17.9%, respectively.

Enzymatic and Non-enzymatic Metabolites of the AsA-GSH Cycle

By using the enzymatic defense system in the AsA-GSH cycle, plants can scavenge ROS generated by stress conditions (Niu et al., 2018; Abd-Allah et al., 2019). As identified in the two inbred lines, spraying 6-BA increased APX activity compared with well-drained plants, although the difference was only significant for SY-MY13. In addition, spraying 6-BA slightly elevated GR and

TABLE 2 | Effects of 6-BA on the growth of two waxy corn inbred lines experiencing waterlogging stress.

Genotype	Treatment	Shoot height (cm)	Leaf area (cm^2)	Root length (cm)	Root surface area (cm^2)
SY-MY13	CK	45.0 ± 2.646b	153.2 ± 18.292bc	2,114.7 ± 259.427a	494.7 ± 50.615a
	CK+6BA	49.2 ± 0.289a	160.3 ± 11.884bc	2,093.2 ± 128.438a	496.5 ± 7.904a
	W	43.3 ± 1.155b	125.6 ± 4.346d	1,438.0 ± 13.169bc	443.1 ± 7.057bcd
	W+6BA	48.0 ± 1.732a	147.8 ± 9.464c	1,217.1 ± 97.011cd	417.8 ± 14.544cd
SY-XT1	CK	37.8 ± 0.289cd	181.6 ± 6.255a	1,695.6 ± 193.975b	467.9 ± 21.746ab
	CK+6BA	38.8 ± 0.764cd	184.0 ± 4.611a	1,613.5 ± 222.672b	457.2 ± 34.470abc
	W	36.3 ± 2.081d	124.5 ± 3.243d	1,309.5 ± 38.747cd	411.8 ± 3.076d
	W+6BA	40.3 ± 1.528c	168.9 ± 3.175ab	1,096.2 ± 164.101d	401.0 ± 24.899d

CK, normal watering condition only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters represent significant differences ($P < 0.05$).

MDHAR activity in the two inbred lines compared with well-drained plants, although the differences were not significant (**Figure 4**). Thus, waterlogging clearly affects enzymes in the AsA-GSH cycle, negatively. Furthermore, the activity of APX and GR decreased significantly compared with the CK group when waterlogged in SY-MY13 by 37.3 and 24.4% and SY-XT1 plants by 43.4 and 68.6%, respectively (**Figures 4A,B**). The activity of DHAR and MDHAR in SY-XT1 plants declined significantly, by 50 and 43%, while there were no significant differences in DHAR and MDHAR activity in SY-MY13 plants (**Figures 4C,D**). Pretreatment with 6-BA for the two waxy corn inbred lines exposed to waterlogging stress conditions also resulted in an increasing trend in the activity of AsA-GSH cycle enzymes relative to that of non-sprayed plants. The activity of APX, GR, and MDHAR increased significantly in SY-MY13 and SY-XT1 plants by 137.5, 57.8, and 60% and 136.4, 373.8, and 116.9%, respectively (**Figures 4A,B,D**). The activity of DHAR in SY-XT1 plants also increased significantly by 94.4% (**Figure 4C**).

The metabolites in the AsA-GSH cycle can both directly scavenge ROS and act as enzymatic substrates that reduce ROS (Raja et al., 2020). The concentrations of AsA, GSH, and their oxidized forms for the different treatments are displayed in **Figures 5A–D**. Spraying 6-BA in well-drained plants increased the GSH content of the two inbred lines and decreased the GSSG content of SY-XT1. Higher ratios of GSH/GSSG were accompanied by changes in AsA and GSH pools in SY-MY13 and SY-XT1 plants. Waterlogging increased GSH content in SY-MY13 plants by 79.6% but affected SY-XT1 to a greater extent, with AsA and GSH levels decreasing by 25.7 and 69.2% and those of DHA and GSSG increasing by 79.2 and 23.8%. Thus, there was a significant decline in the AsA/DHA ratio, by 14.7 and 58.7%. In contrast, the GSH/GSSH ratio in SY-MY13 plants increased by 74.4%, while it decreased by 75.5% in SY-XT1 plants. Furthermore, 6-BA pretreatment enhanced AsA and GSH content and reduced DHA and GSSG levels when plants were waterlogged. The levels of AsA and GSH in SY-MY13 plants increased by 44.7 and 18.9% and in SY-XT1 by 61.5 and 751.4%, respectively. The levels of DHA and GSSG in the SY-MY13 strain decreased by 16.1 and 5% and in SY-XT1 by 37.9 and 42.8%, respectively. Treatment with 6-BA significantly increased AsA/DHA and GSH/GSSH ratios compared with waterlogging stress treatment, with these ratios in the 6-BA-sprayed groups increasing significantly when compared with the non-sprayed groups when waterlogged in SY-MY13 plants, by 72.6 and 25.6% and in SY-XT1 by 162.6 and 111.4%, respectively.

DISCUSSION

Waterlogging stress adversely affects the growth of many terrestrial crops, causing chlorosis and necrosis in their leaves, inhibiting shoot and root growth, and decreasing dry matter accumulation (Komatsu et al., 2013; Wang et al., 2017; Kaya et al., 2019; Men et al., 2020). The inhibitory effects of waterlogging stress on plant growth and development are likely due to hypoxic conditions in the root zone that hamper root respiration, resulting in limited nutrient uptake and transport (Tan et al., 2010; Qi et al., 2016; Wang et al., 2019). In the present study, waterlogging stress injuries in waxy corn seedlings were characterized by chlorosis, wilting, and necrosis (**Figure 1**), which cause the repression of seedling growth (**Tables 1**, **2**) as confirmed by Zhu et al. (2016). In addition, waterlogging also significantly reduced the fresh and dry weight of shoots in SY-XT1 plants, while there was no significant effect on SY-MY13, which may be due to the waterlogging-sensitive line more readily losing biomass when exposed to waterlogged conditions (Striker, 2012). Furthermore, cytokinin content decreased when plants were placed in stressful conditions, which represents a limiting factor for the growth of plants (Shams and Yildirim, 2021). Cytokinins allow the optimal growth and development of plants by promoting cell division and tissue growth and delaying leaf senescence (Davies, 2010; Kim et al., 2018). Furthermore, 6-BA is in a class of synthetic cytokinin PGRs that can significantly increase levels of endogenous cytokinins in crop plants (Hu et al., 2020; Prerostova et al., 2020). In the present study, after spraying with 6-BA, the two waxy corn inbred lines exhibited reduced injuries in terms of growth and had greater leaf area, shoot height, and dry weight compared with the non-sprayed plants when waterlogged. This proves the hypothesis that 6-BA

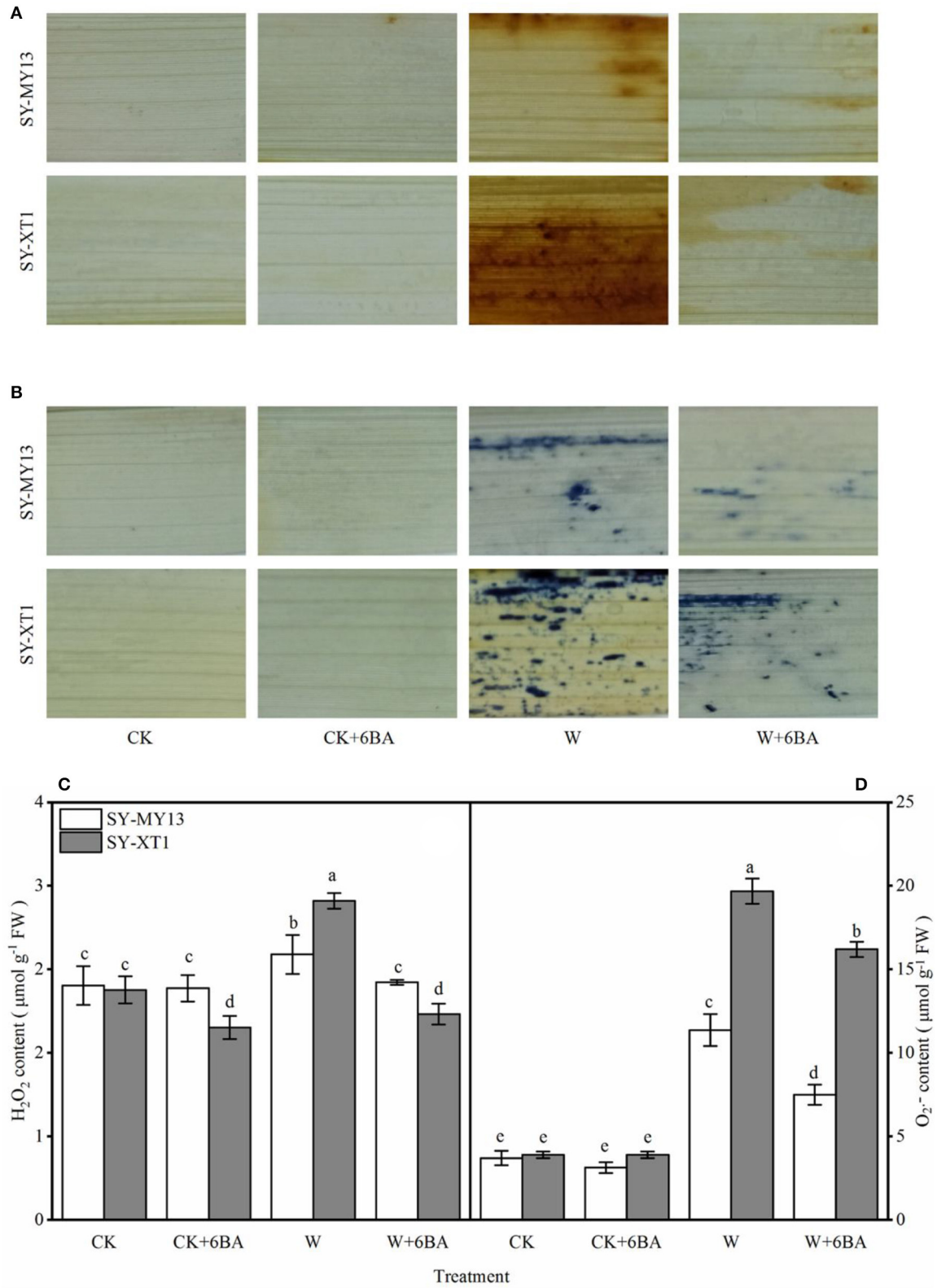

FIGURE 2 | Effects of 6-BA on histochemical staining of H_2O_2 **(A)** and O_2^- **(B)**, Concentration of H_2O_2 **(C)** and O_2^- **(D)** in two waxy corn inbred lines in waterlogged conditions. CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, Addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters were significantly different ($P < 0.05$).

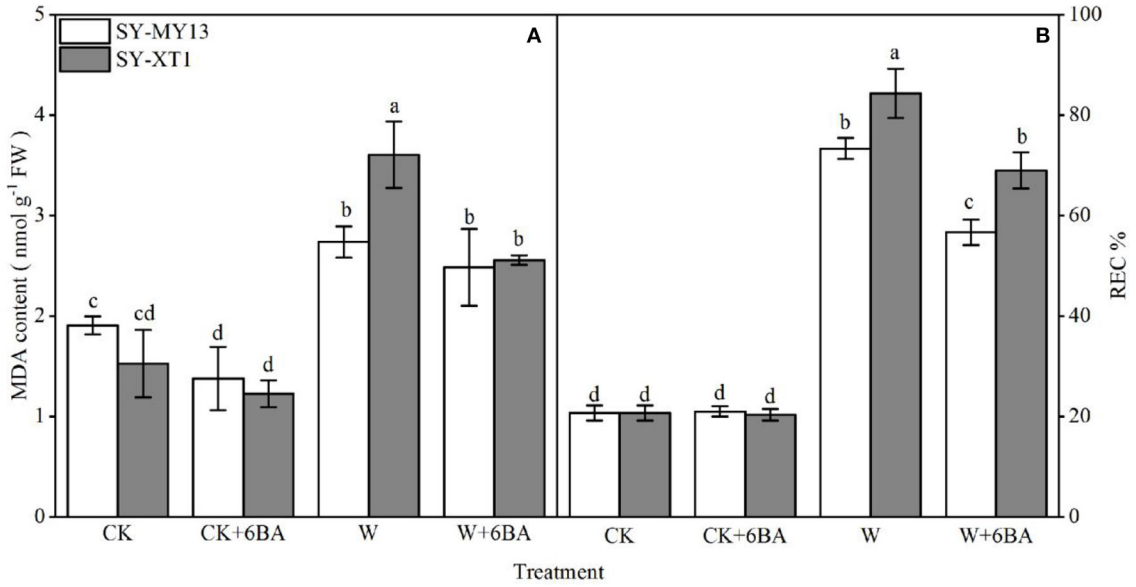

FIGURE 3 | Effects of 6-BA on malondialdehyde (MDA) concentration **(A)** and relative electric conductivity (REC%) **(B)** in two waxy corn inbred lines experiencing waterlogging. CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters were significantly different ($P < 0.05$).

acts positively to regulate the response to waterlogging stress in waxy corn seedlings. Plant biomass has also been shown to increase, for example, by the application of PGRs capable of producing growth hormones (Cohen et al., 2009). Therefore, the exogenous application of 6-BA can mitigate the adverse effects of waterlogging stress on plant growth.

Waterlogging results in low O_2 levels in plant tissues that can, in turn, lead to excess ROS production *via* the disruption of the balance of ROS generation and detoxification (Posso et al., 2018; Anee et al., 2019; Park and Ju, 2019). The first ROS to be generated is usually O_2^-, with the radicals forming H_2O_2 spontaneously by dismutation (Mori and Schroeder, 2004). Excess H_2O_2 production when plants are waterlogged affects multiple physiological processes because H_2O_2 is a strong uncharged oxidant molecule (Castro-Duque et al., 2020; Cen et al., 2020). Waterlogging stress inflicts significant damage on waxy corn seedlings, as suggested by the greater levels of O_2^- and H_2O_2 in the leaves of both the SY-MY13 and SY-XT1 inbred lines used in the current study (**Figures 2C,D**), which also demonstrated the histochemical staining of O_2^- and H_2O_2 at the tissue level in the leaves of waxy corn (**Figures 2A,B**). The ROS injury of the bio membranes also causes lipid peroxidation and disrupts membrane homeostasis (Shu et al., 2012; Song et al., 2021). In the study, the waterlogging stress induction of ROS causing membrane damage was also reflected by the increased MDA content and REC% (**Figure 3**). The application of exogenous 6-BA reduced damage in the seedlings when waterlogged by significantly reducing the waterlogging-induced production of H_2O_2 (**Figure 2C**), O_2^- (**Figure 2D**), MDA (**Figure 3A**), and REC% (**Figure 3B**) compared with those of waterlogging alone. Moreover, histochemical staining indicated that the levels of O_2^- and H_2O_2 in tissues were lower in the leaves (**Figures 2A,B**). Furthermore, endogenous cytokinin levels when plants experience environmental stress can be enhanced by the application of synthetic cytokinins, which can offset stress-induced premature senescence of plants, in addition to reducing damage due to ROS and lipid peroxidation; thus, effectively improving the adaptability of plants experiencing stress (Gujjar and Supaibulwatana, 2019). Our studies revealed that pretreatment with 6-BA not only decreased MDA content but also suppressed ROS accumulation and electrolyte leakage in waxy corn seedlings when exposed to waterlogging stress. These findings demonstrated that 6-BA has excellent potential for use in applications that maintain the integrity of cellular membranes through the prevention of lipid peroxidation against waterlogging-induced oxidative damage, which is a possible principal mechanism by which waterlogging stress is alleviated in maize plants. Interactions between cytokinin signaling and ROS production and scavenging systems have also been demonstrated in arabidopsis (Nishiyama et al., 2012). Feng et al. (2004) also demonstrated that 6-BA can significantly reduce oxidative damage in poplar, the rate of O_2^- generation, and H_2O_2 and MDA levels becoming effectively reduced, indicating that reduced oxidative damage due to 6-BA was associated with the promotion of the scavenging system for ROS. Ren et al. (2018a) reported that exogenous 6-BA increases the tolerance of maize seedlings to waterlogging stress by the protection of physiological processes from oxidative damage and enhancement of the metabolism of ROS. These findings suggest that exogenous 6-BA enhances the tolerance of waxy corn

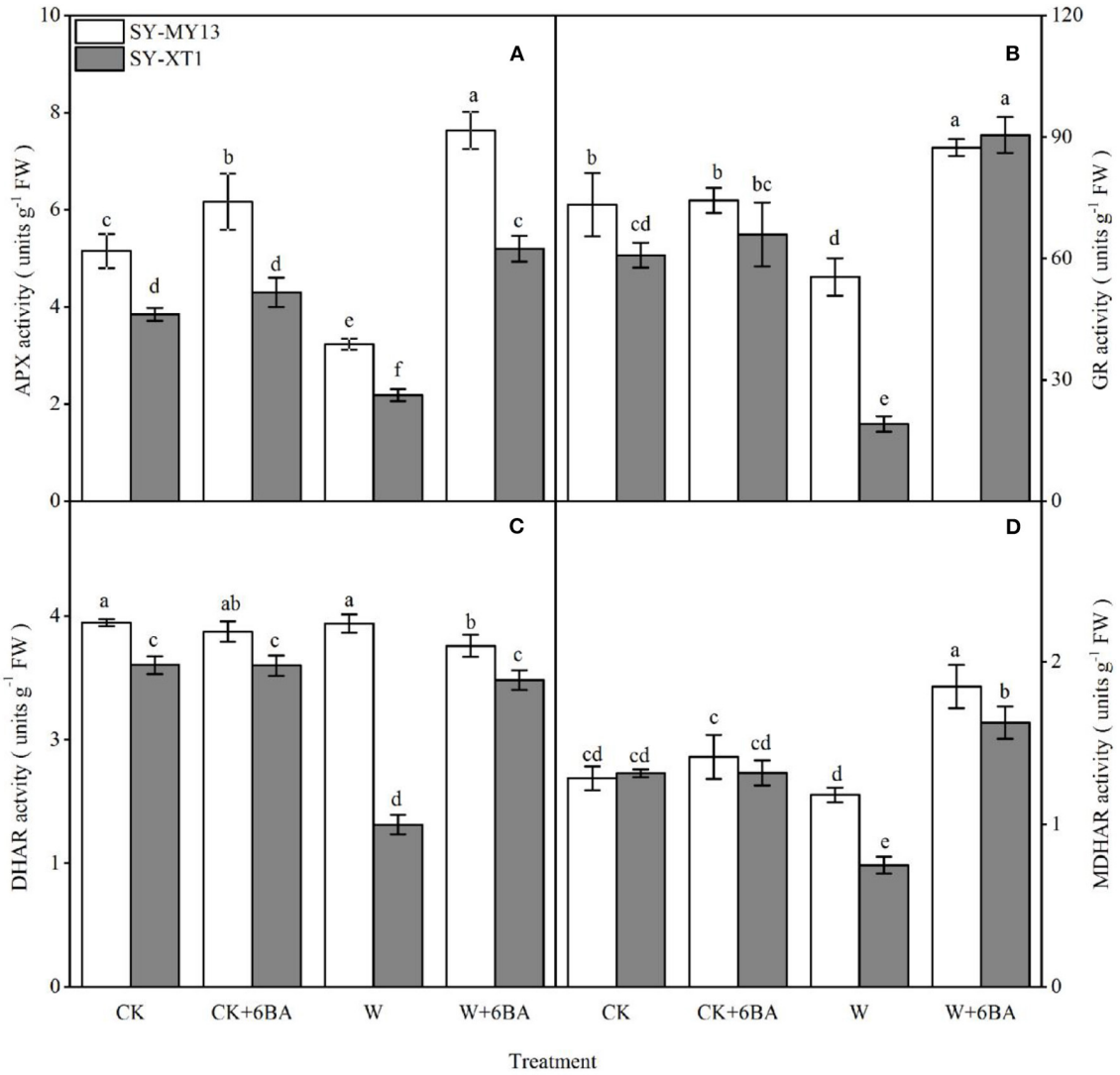

FIGURE 4 | Effects of 6-BA on ascorbate peroxidase (APX) **(A)**, glutathione reductase (GR) **(B)**, dehydroascorbate reductase (DHAR) **(C)**, and monodehydroascorbate reductase (MDHAR) **(D)** activity in two waxy corn inbred lines experiencing waterlogging. CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters were significantly different ($P < 0.05$).

seedlings to waterlogging by the attenuation of ROS-induced oxidative damage.

Waterlogging stress-induced hypoxic injury generally results in oxidative stress by inducing the production of ROS. The antioxidant system in plants plays a critical role in ROS scavenging and is positively correlated with waterlogging tolerance (Da-Silva and Do-Amarante, 2020; Mira et al., 2021). Ascorbate peroxidase is a pivotal enzyme in the AsA-GSH cycle; since, it utilizes AsA as a substrate to reduce H_2O_2 to H_2O (Gordon et al., 2013; Ghosh and Biswas, 2017; Mir et al., 2018). Glutathione reductase is also involved in the defense against oxidative stress, in addition to the regeneration of GSH (Kaur et al., 2018; Guo et al., 2019). Furthermore, both DHAR and MDHAR are key to maintaining AsA concentration (Zhang et al., 2015; Juszczak et al., 2016; Raja et al., 2020). In the present study, the AsA-GSH cycle was activated when SY-MY13 plants were exposed to waterlogging stress, enabling normal metabolic activity through exposure to excess ROS. In contrast, the AsA-GSH cycle of SY-XT1 plants operated at a relatively low level, and so were more vulnerable to ROS damage. Thus, the current data indicate that plants with higher efficiencies in the AsA-GSH cycle can resist waterlogging stress that can be further increased by 6-BA supplementation in waxy corn plants. In the present study, increased APX, GR, DHAR, and MDHAR activity were observed in waxy corn seedlings supplemented with 6-BA exposed to waterlogging stress (**Figure 4**). Improvements in the tolerance of waxy corn to waterlogging are associated with a reduction in H_2O_2 content and attributable to the

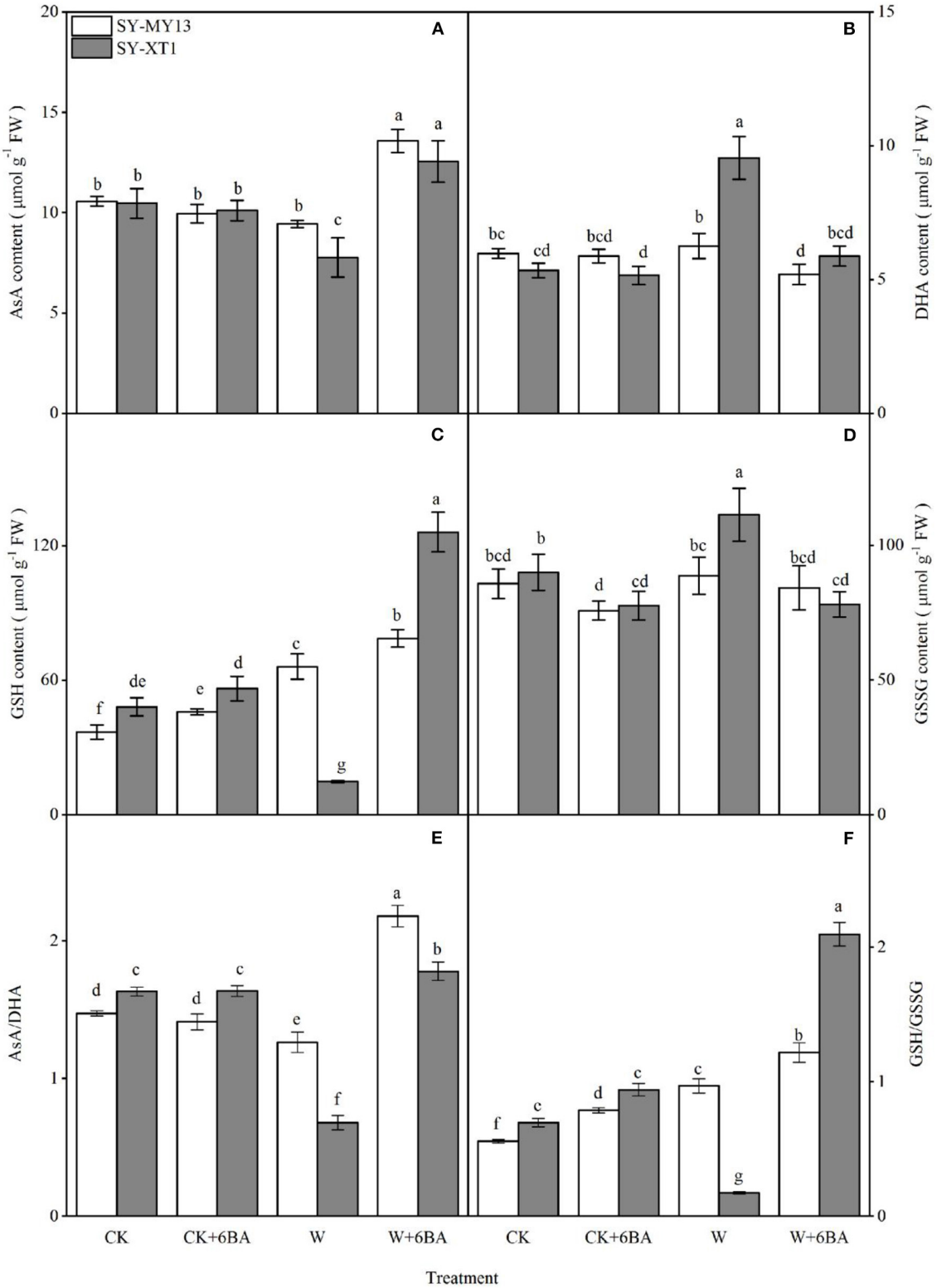

FIGURE 5 | Effects of 6-BA on ascorbic acid (ASA) **(A)**, dehydroascorbic acid (DHA) **(B)**, reduced glutathione (GSH) **(C)**, and oxidized glutathione (GSSG) concentrations **(D)** and the ratios AsA/DHA **(E)** and GSH/GSSG **(F)** in two waxy corn inbred lines in waterlogged conditions. CK, normal watering conditions only; CK + 6-BA, 0.5 mM of 6-BA plus normal watering conditions; W, waterlogged; W + 6-BA, addition of 0.5 mM of 6-BA plus waterlogging stress conditions. Data represent means ± SD of three replicates. For each variable, means with different lowercase letters were significantly different ($P < 0.05$).

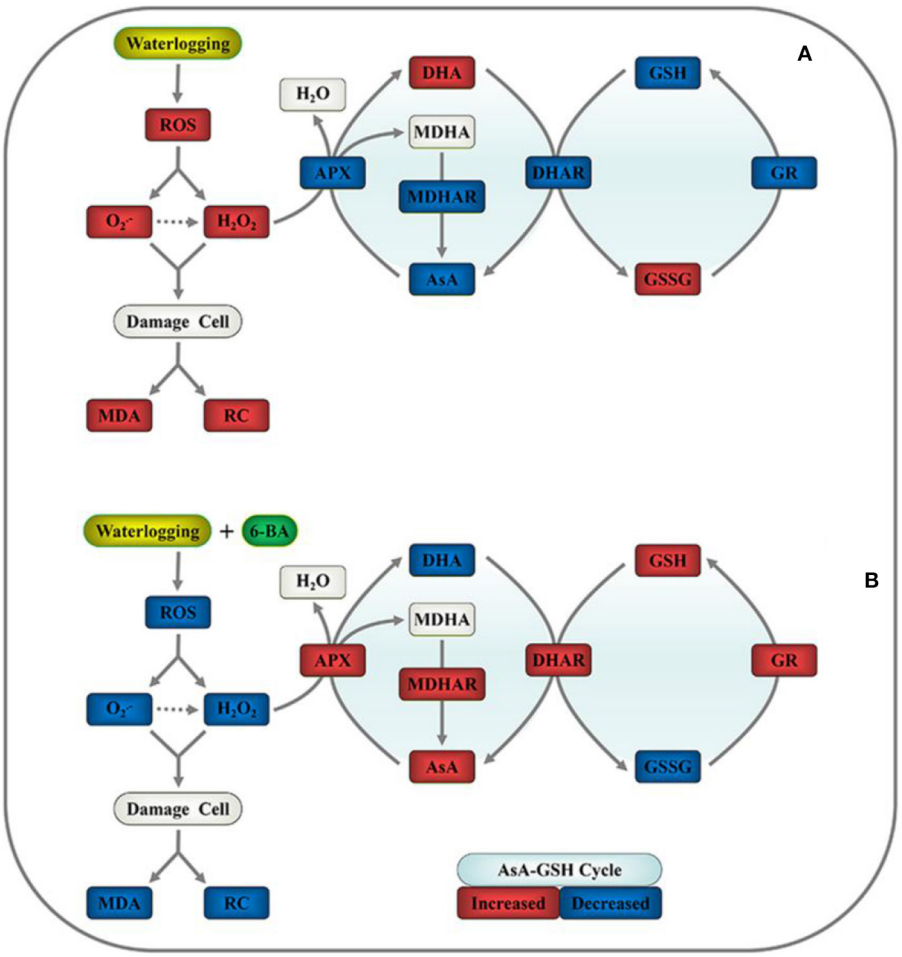

FIGURE 6 | Proposed mechanism for 6-BA-induced waterlogging tolerance in waxy corn seedlings through the mitigation of oxidative stress and sustaining homeostasis of the ascorbate-glutathione (AsA-GSH) cycle. **(A)** Plant growth in waterlogged conditions and **(B)** plant growth after the application of 0.5 mM of 6-BA in waterlogged conditions. Red indicates upregulation, and blue indicates downregulation.

enhanced activity of APX. In addition, increased MDHAR activity suggests that it can act synergistically with APX to increase the efficiency of the AsA-GSH cycle and maintain the redox status and antioxidant activity of the AsA pool. These results are further supported by the observations of Jiang et al. (2019) and Prerostova et al. (2020), who observed that high levels of cytokinins positively affect environmental stress tolerance in arabidopsis due to the cytokinins preventing oxidative stress by the activation of APX, which removes excess ROS. In a study by Porcher et al. (2021), cytokinins were also shown to act, in part, through the induction of the AsA-GSH scavenging pathways, which decrease internal H_2O_2 concentrations in buds that were observed during bud bursting events, thereby regulating ROS homeostasis in the buds of roses. A study by Chen and Yang (2013) also found that APX and GR activity in cucumber fruits treated with 6-BA increased after suffering chill injuries by reducing increased membrane permeability and lipid peroxidation, delaying increases in O_2^- and H_2O_2 levels, and maintaining higher levels of total antioxidant capacity. The application of 6-BA led to the increased activity of the AsA-GSH cycle enzymes when experiencing environmental stress-induced oxidative damage, as has been previously observed in tomatoes and eggplants (Chen J. L. et al., 2016; Singh et al., 2018). The results of the present study reveal that exogenous 6-BA is able to coordinate relative internal balance and the high activity of APX, GR, DHAR, and MDHAR, thus allowing adaptation to stress and enabling the balance between the production and scavenging of ROS through the modulation of the levels of the AsA-GSH cycle enzymes. This provides a strong guarantee that AsA and GSH highly efficient AsA-GSH cycle enzymes that are effective in removing ROS caused by waterlogging stress, can be regenerated, thus reducing ROS-induced oxidative damage, subsequently relieving the deleterious effects caused by waterlogging stress, and enhancing the waterlogging tolerance of waxy corn seedlings.

Ascorbic acid and GSH are the most abundant hydrophilic non-enzymatic antioxidants in cells and play a critical role in reducing oxidative stress induced by waterlogging (Pulido et al., 2010; Bai et al., 2013). As a substrate, AsA scavenges H_2O_2 and

prevents lipid peroxidation (Noctor et al., 2018). In contrast, GSH is a thiol-based antioxidant that prevents lipid peroxidation and contributes to AsA regeneration (Foyer and Halliwell, 1976; Racz et al., 2020). Furthermore, both DHA and GSSG are oxidized forms of AsA and GSH, respectively. The results of the present study indicate that waterlogging decreases AsA and GSH levels but elevates DHA and GSSG in waxy corn seedlings, relative to untreated plants, showing that waterlogging stress clearly leads to a modified oxidation-reduction status in cells through interaction with pools of AsA and GSH (**Figure 5**). This is due to reductive molecules (AsA and GSH) being involved in the elimination of ROS, resulting in the increased consumption of the AsA and GSH pools. Moreover, decreased DHAR and MDHAR activities under waterlogging stress also resulted in the lack of supplementation to AsA and GSH pools (**Figures 4C,D**), which leading to the accumulation of a large quantity of oxidized molecules (DHA and GSSG). Li Z. et al. (2015) considered that the GSH/GSSG ratio increased with increasing cytokinin concentration, allowing the maintenance of higher levels of antioxidants, particularly AsA, and thus enhancing the antioxidant capacity of plants. Therefore, the interplay between cytokinins and the AsA-GSH cycle may represent the mechanism by which environmental tolerance is regulated in plants. The AsA-GSH cycle is an efficient antioxidant system for the elimination of excessive ROS production through the maintenance of the AsA/DHA and GSH/GSSG ratios. The data in the present study demonstrate that the AsA/DHA and GSH/GSSH ratios of the waterlogging-sensitive line were both significantly lower than those of CK plants, suggesting that waterlogging stress breaks the balance between the reduced and oxidized forms of AsA and GSH in the AsA-GSH cycle. The concentrations of AsA and GSH and the AsA/DHA and GSH/GSSG ratios increased when 6-BA was applied exogenously to seedlings affected by waterlogging stress (**Figures 5A,C,E,F**), helping to reduce the ROS responsible for oxidative damage, which is reflected in the reduced levels of MDA and REC% (**Figure 3**). Recent investigations have demonstrated that GSH levels increase substantially in plants to which cytokinins were added compared with untreated controls (Porcher et al., 2021). Similar results have been demonstrated in studies on exogenous 6-BA applied to *Solanum melongena* after suffering oxidative damage (Wu et al., 2015). The present study illustrated that exogenous 6-BA reinstates the reductive status of AsA and GSH pools, which enhances the activity of the AsA-GSH cycle and allows a reduction in the ROS that cause oxidative damage, and enhances the tolerance of waxy corn seedling to waterlogging stress.

Figure 6 proposes a simple mechanism by which exogenous 6-BA mitigates waterlogging stress in waxy corn seedlings by reducing ROS-induced oxidative stress and sustaining the homeostasis of the AsA-GSH cycle. The application of exogenous 6-BA to waxy corn seedlings when experiencing waterlogging stress significantly decreased the production of O_2^-, H_2O_2, MDA, and REC% values relative to those observed in seedlings subjected to waterlogging stress alone. As presented above, the proposed mechanism indicates that the application of 6-BA to waxy corn seedlings when experiencing waterlogging stress promotes AsA-GSH cycle activity, thereby contributing to decreased ROS accumulation in plants, alleviating lipid peroxidation of cell membranes, and maintaining their stability, which also increases waterlogging tolerance in waxy corn seedlings.

CONCLUSION

The present data clearly demonstrated that waterlogging stress causes chlorosis and necrosis in waxy corn leaves, subsequently inhibiting growth and leading to ROS accumulation, which induces oxidative stress resulting in membrane lipid peroxidation and the disruption of membrane homeostasis. Exogenous 6-BA ameliorates waterlogging-induced oxidative stress in waxy corn seedlings by stimulating the components of the AsA-GSH cycle and maximally improving the adaptation of waxy corn seedlings to waterlogging stress, thus providing a cost-effective and environmentally friendly method of sustainable crop production, especially in areas vulnerable to waterlogging stress. Therefore, the results reported in this study provided invaluable insights into the critical role of 6-BA in the regulation of the AsA-GSH cycle system and a better understanding of waterlogging tolerance in waxy corn seedlings.

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

FL and MZ conceived and designed the study, obtained financial support, provided the study material, and helped revise the manuscript. JW performed the experiments, collected data, analyzed data, interpreted data, and maintenance drafted the manuscript. DW helped perform the experiments and participated in the discussion. All authors contributed to the article and approved the submitted version.

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Comparative Physiological and Proteomic Analyses Reveal the Mechanisms of Brassinolide-Mediated Tolerance to Calcium Nitrate Stress in Tomato

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Secondary salinization caused by the overaccumulation of calcium nitrate [$Ca(NO_3)_2$] in soils due to excessive fertilization has become one of the major handicaps of protected vegetable production. Brassinolide, a bioactive plant steroid hormone, plays an important role in improving abiotic stress tolerance in plants. However, whether and how brassinolide (BR) can alleviate $Ca(NO_3)_2$ stress remains elusive. Here, we investigated the effects of exogenous BR on hydroponically grown tomato (*Solanum lycopersicum* L.) plants under $Ca(NO_3)_2$ stress through proteomics combined with physiological studies. Proteomics analysis revealed that $Ca(NO_3)_2$ stress affected the accumulation of proteins involved in photosynthesis, stress responses, and antioxidant defense, however, exogenous BR increased the accumulation of proteins involved in chlorophyll metabolism and altered the osmotic stress responses in tomatoes under $Ca(NO_3)_2$ stress. Further physiological studies supported the results of proteomics and showed that the exogenous BR-induced alleviation of $Ca(NO_3)_2$ stress was associated with the improvement of photosynthetic efficiency, levels of soluble sugars and proteins, chlorophyll contents, and antioxidant enzyme activities, leading to the reduction in the levels of reactive oxygen species and membrane lipid peroxidation, and promotion of the recovery of photosynthetic performance, energy metabolism, and plant growth under $Ca(NO_3)_2$ stress. These results show the importance of applying BR in protected agriculture as a means for the effective management of secondary salinization.

Keywords: calcium nitrate, brassinolide, stress responses, antioxidative defence, energy metabolism

INTRODUCTION

The development of protected agriculture has made crop production possible beyond the seasonal barriers (Henry, 2019). However, with the fast-growing development of protected agriculture, secondary salinization in the continuous cropping soil has become increasingly common, which is attributed to excessive fertilization and intensive farming. Secondary salinization has a drastic

adverse effect on crop production in protected agriculture (Zhu et al., 2021). As it is different from open-field cultivation, the amount of nitrogen fertilizer used in greenhouse vegetable farming is large, with the main anion being nitrate (NO_3^-) and the cation being calcium cation (Ca^{2+}) in the soils (Shu et al., 2016; Zhu et al., 2021). Calcium nitrate accumulation is one of the main reasons for secondary salinization in greenhouse soils (Niu et al., 2019).

Calcium nitrate stress not only affects photosynthesis and respiration but also causes severe oxidative damage and metabolic disorder, due to the overproduction of reactive oxygen species (ROS), including oxygen ($O_2^{\cdot -}$), hydroxide ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2; Zhang et al., 2008; Li et al., 2015; Shu et al., 2016; Soares et al., 2016; Fan et al., 2017; Zhen et al., 2018). Induced lipid peroxidation, increased electrolyte leakage, and disrupted protein functions are some common consequences of excessive ROS accumulation. However, in response to salt stress such as secondary salinization, plants recruit an antioxidant defense system comprising of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase, and ascorbate peroxidase (APX) to scavenge different types of ROS. In addition, the genes encoding glutathione *S*-transferase and glutathione POD are up-regulated to protect plants from salinization (Poonam et al., 2015).

Proteomics can not only describe the complete proteome of an organism but also compare and identify specific proteins affected by different physiological conditions. Under salinity stress, obvious lignification occurs in the roots of tomato plants, which is closely related to the preferential increase of S-adenosyl methionine. Calcium nitrate stress increases the accumulation of lignin biosynthesis-related proteins in cucumber roots followed by protein modification and the accumulation of degradation-associated proteins, while it decreases the accumulation of sugar metabolism-associated proteins (An et al., 2016). Exogenous spermidine can augment the accumulation of glycolysis-related proteins and fructose-6-phosphate biosynthesis by the action of fructokinase in calcium nitrate [$Ca(NO_3)_2$-] treated cucumber plants, thereby modulating carbohydrate and energy metabolism in plants (Du et al., 2018).

Brassinolide is a multifunctional steroid phytohormone that modulates plant growth and stress responses (Bishop and Yokota, 2001; Wang et al., 2019; Chi et al., 2020; Yan et al., 2020). Brassinolide induces cell elongation by activating the plasma membrane (PM) H^+-ATPase, as described in the "acid-growth theory" (Anzu et al., 2019). Brassinolide stimulates gibberellin (GA) accumulation by modulating the expression of GA biosynthesis-related genes, thus presenting a synergy with GA in cell elongation (Li and He, 2013; Li et al., 2013; Tong et al., 2014). Genetic and biochemical studies have revealed that brassinolide (BR) binds to the extracellular domain of a receptor kinase (BRI1) to initiate the phosphorylation/dephosphorylation cascade, subsequently modulating gene expression (Yang and Komatsu, 2004; Tang et al., 2008).

Several studies showed that exogenous BR enhances photosynthetic performance, ion homeostasis, and the antioxidant system, resulting in improved plant growth, and development under salt stress (El-Mashad and Mohamed, 2012; Yuan et al., 2014; Arif et al., 2017; Ahmad et al., 2018; Ahammed et al., 2020; Su et al., 2020). An increased BR level due to the enhanced expression of the *DET-2* gene in *Arabidopsis* is positively correlated to increased CAT and SOD activities under low temperatures (Tanveer et al., 2019). Importantly, exogenous BR influences protein biosynthesis as well as the degradation of damaged proteins. For instance, BR alters the accumulation of heat shock proteins, proteases, and antioxidant enzymes in plants (Li et al., 2013, 2018; An et al., 2016). However, the effect of BR and $Ca(NO_3)_2$ on plant proteome and the potential mechanism of stress mitigation remain elusive.

In this study, using a set of physiological and proteomics analyses, we investigated the role of exogenous BR in modulating the growth, photosynthesis, and antioxidant defense of hydroponically grown tomato seedlings under $Ca(NO_3)_2$ stress. Our findings reveal crucial mechanisms involved in the BR-enhanced tolerance to $Ca(NO_3)_2$ stress, which could be useful to the development of effective strategies for the management of secondary salinization in protected agriculture.

MATERIALS AND METHODS

Plant Materials and Treatments

Tomato (*Solanum lycopersicum* L. cultivar "Money Maker") seeds were soaked in water at 55°C for 25 min and then sown in a tray containing a vermiculite matrix. The seedlings with three leaves were transferred to a 1/2-strength Yamasaki tomato nutrient solution (Li C. et al., 2016) for 7 days. Subsequently, the seedlings were transferred to fresh 1/2-strength Yamasaki tomato nutrient solution (Stanleygroup, Shandong, China) without (control) or with (i) foliar spraying with 24-epibrassinolide (BR, 0.1 μmol/L; 24-Epicastasterone, Yuanye Biotechnology Co., Shanghai, China), (ii) root exposure to $Ca(NO_3)_2$ solution (Ca, 100 mmol/L), (iii) foliar spraying with 0.1 μmol/L BR plus root exposure to 100 mmol/L $Ca(NO_3)_2$ solution (Ca+BR) for continued cultivation for 5 days. The concentration used for spraying BR (0.1 μmol/L) was selected based on previous studies (Nie et al., 2019; Li et al., 2020). The entire foliar portion was sprayed with 0.1 μmol/L BR or distilled water once every 2 days, and salt stress treatment was carried out on the day of the second spray. The osmotic potential of 100 mmol/L $Ca(NO_3)_2$ was –0.97 MPa at 25°C, which was determined using a WESCOR Vapro 5600 osmometer (WESCOR, ELITechGroup, Inc, Stoneham, MA, United States). Leaf samples were taken from three randomly selected tomato seedlings with uniform growth for each treatment at different time points. Briefly, all leaflets from the second and third fully expanded leaves of the selected tomato seedlings were collected 1, 3, and 5 days after the salt stress treatment, and mixed abundantly for physiological analysis and protein extraction. The harvested leaf samples were immediately frozen in liquid nitrogen before storing in ultra-low temperature refrigerators to determine the H_2O_2, malondialdehyde (MDA), ascorbic acid (AsA), SOD, POD, CAT, and other indicators. The plant material taken 5 days after the salt stress treatment was divided into aboveground and underground parts. They were put in the oven at 105°C for 15 min and kept at 60°C until a constant

weight was attained. The second leaf from the top was dried for the amino acid analysis.

Measurements of Biochemical and Physiological Parameters

The fresh weight (FW) of the leaves was measured by a rapid weighing method as described by Zhang et al. (2018). The chlorophylls of the second fully expanded leaves were extracted with acetone (80%) and the chlorophyll content was determined by monitoring the absorbance at 645 and 663 nm (Ahmad et al., 2018). The protein content and proline content were determined as described by Bradford (1976) and Bates et al. (1973), respectively. Three samples were used for the determination of the chlorophyll and proline content. Photosynthetic measurements were performed on fully expanded leaves (the second leaf from the top) on 5 days of salinity treatment at 10:00–11:00 a.m. A standard leaf chamber (2×3 cm^2) fitted on a portable photosynthesis system (6400XT, Li-Cor Inc., Lincoln, NY, United States) was used at ambient relative humidity: 50–60%; carbon dioxide (CO_2); 400 $\mu mol \cdot mol^{-1}$; flow rate: 500 $\mu mol\ s^{-1}$; vapor pressure deficit < 2, and photosynthetically active radiation: 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Each leaf was allowed sufficient time for equilibration in the chamber until constant readings were obtained. The photosynthetic parameters such as the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), transpiration rate (Tr), the instantaneous water use efficiency (WUE), calculated through Pn/Tr, and the stomatal limitation (Ls) defined as 1-Ci/Ca (Ca denotes the atmospheric CO_2 concentration) were measured according to Hao et al. (2019). The microstructure of the leaf was prepared by the paraffin sectioning method as described by Li et al. (2020). The leaf sections were observed and photographed under an optical microscope.

Amino acid extraction and determination were carried out as described by Ohtsuki et al. (2016). Approximately 0.1 *g* of the oven-dried sample was soaked in 2% 5-sulfosalicylic acid (1.5 ml) for 24 h. After the centrifugation at 13,681 $\times$ *g* for 10 min, the supernatant (800 µl) was diluted to 5 mL with 0.02 mmol/L hydrochloric acid (HCl) and then the diluted supernatant was filtered through a 0.45 µm filter membrane (Linghanglab, Tianjin, China) and placed in a 1.5 ml injection bottle (Linghanglab, Tianjin, China). An amino acid analyzer (L-8800, HITACHI, Chiyoda City, Tokyo, Japan) was used to determine the content of different amino acids. Each treatment was determined with three biological replicates and three technical replications.

The H_2O_2 and MDA levels were measured as described by Ahmad et al. (2018). Briefly, the fresh leaf tissue (0.3 *g*) was homogenized with 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 $\times$ *g* for 10 min. Approximately, an equal volume of supernatant, 100 mM potassium phosphate buffer (pH 7.0), and 1M potassium iodide were mixed and the absorbance was noted at 390 nm with a spectrophotometer (Shimadzu UV-2450, Japan). The H_2O_2 content was expressed as $nmol \cdot g^{-1}$ FW. The MDA content was measured on the basis of thiobarbituric acid reaction (Bailly et al., 1996). In brief, a 0.5 *g* sample was macerated in 8 ml of 0.1% (w/v) TCA and centrifuged at 4,830 $\times$ *g* for 10 min at 4°C. The supernatants were obtained and mixed with 0.5% (w/v) of TCA made in 5% (w/v) TCA. The reaction mixture was heated at 100°C on a water bath for 20 min; afterward, the mixture was put on ice to stop the reaction. After cooling, a step of centrifugation at 7,888 $\times$ *g* for 10 min was done and the absorbance was taken at 450, 532, and 600 nm.

Enzyme extraction has been improved with reference to the Gong et al. (2005) method. One gram of leaf sample was ground into a homogenate, using 8 ml of a pre-cooled sodium phosphate buffer (0.05M, pH7.0). The homogenate was transferred into a 15 ml tube and centrifuged at 4°C 9,661 $\times$ *g* for 20 min. The supernatant obtained was used for the determination of the activity of enzymes, such as SOD (EC 1.15.1.1), POD (EC 1.11.1.7), and CAT (EC 1.11.1.6). The SOD activity was analyzed using nitroblue tetrazolium (NBT) as described by Gong et al. (2005). The SOD activity was expressed as units (U) of SOD mg^{-1} of protein, in which one SOD unit is defined as the amount of enzyme that inhibits 50% of the reduction rate of NBT. The determinations of the POD and CAT activity were according to the method of Yang with slight modifications (Yang et al., 2010). The POD and CAT activities were expressed as 1U with a change of 1 per minute in OD_{470} and OD_{240}, respectively, and expressed as U mg^{-1} protein. The non-enzymatic antioxidant AsA contents were measured using a V_C (vitamin C, ascorbic acid) Assay Kit (A009, Jiancheng, Nanjing, China) by noting the absorbance at 536 nm (Quan et al., 2015).

Protein Extraction, Digestion, Isobaric Tags for Relative and Absolute Quantification Labeling, and Strong Cation Exchange Chromatography

The total protein was extracted from the fully expanded second and third leaves of the tomato seedlings from top to bottom. Briefly, 1 *g* of the leaf sample from every biological replicate were finely crushed in liquid nitrogen and mixed with 10% (w/v) TCA/acetone solution having 65 mM dithiothreitol (DTT) for 1 h (−20°C). Afterward, the extracted sample was centrifuged for 45 min at 10,000 $\times$ *g* and the obtained pellet was vacuum-dried and solubilized in 1/10 volume of SDT buffer (4% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), and 150 mM Tris–HCl, pH 8). After being incubated for 3 min, the suspended solution was ultrasonicated (80 w, 10 s ultrasonication at a time, every 15 s, and 10 times), and re-incubated at 100°C for 3 min followed by a step of centrifugation at 13,000 $\times$ *g* at 25°C for 10 min. The protein content in each sample was calculated using a Bicinchoninic acid Protein Assay Reagent (Promega, Madison, WI, United States) and the samples were stored at −80°C until use. Protein digestion was performed according to the FASP procedure described by Wiśniewski et al. (2009) and the resulting peptide mixture was marked according to the instructions of the manufacturer (AB SCIEX, Framingham, MA, United States) with 8-plex isobaric tags for relative and absolute

quantification (iTRAQ). The detailed description related to the sample preparation, digestion, and analysis is provided in the **Supplementary Methods**.

Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (MS/MS)

The LC-MS/MS experiment was performed on a Q Exactive Mass Spectrometer coupled to an Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific Waltham, MA, United States). A volume of 10 μl of each fraction was injected for the LC-MS/MS analysis. The instrument was run with peptide recognition mode enabled and the detailed information related to the sample preparation and instrument conditioning is provided in the **Supplementary Methods**. *The LC-MS/MS analysis was carried out at HooGen Biotech, Shanghai, China.* The MS/MS spectra were searched using the Mascot search engine (Matrix Science, London, United Kingdom; version 2.2) embedded in Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA, United States) against the UniProt *S. lycopersicum* database (35,921 sequences, download at 20180118) and the decoy database. The search parameters are provided in the **Supplementary Methods**. Differentially modulated proteins were identified as proteins with fold change (FC) ratio > 1.20 or <0.83 ($P < 0.05$; Li G. et al., 2017; Zhong et al., 2017).

Bioinformatic Analysis

The Gene Ontology (GO) program Blast2GO[1] was adopted to annotate differential expression proteins (DEPs) to create histograms of the GO annotation based on their role in the biological process, molecular function, and cell components. For the DEPs pathway analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, using the KEGG automatic annotation server (KAAS) program[2] was used. The GO enrichment analysis of each module was performed using Cytoscape v3.7.2.

Parallel Reaction Monitoring Analysis

Additionally, the protein accumulation determined by the iTRAQ analysis was further quantified and analyzed through LC-Parallel reaction monitoring (PRM) MS. Complete information related to the LC_PRM/MS analysis is appended in the **Supplementary Methods**. The analysis of the raw data was carried out *via* the Skyline 3.5.0 software (MacCoss Lab, University of Washington, United States; Peterson et al., 2012), where the intensity of every signal given specific peptide sequence can be measured for each sample after the normalization of each protein with the reference standard.

Statistical Analysis

All physiological data were checked for statistical significance using ANOVA and presented as the mean ± SD of three biological replicates. Duncan's multiple range test was applied to compare the means at the $P < 0.05$ level in SPSS (version-21.0). The proteomic experiments were also repeated with three independent biological replicates. The 95% confidence (Z score = 1.96) was set to pick the proteins whose distribution was removed from the main distribution. The cut-off values for the up-regulated or down-regulated proteins were taken as 1.2- or 0.83-fold, respectively (Li M. et al., 2017; Zhong et al., 2017).

[1]https://www.blast2go.com/

[2]http://www.genome.jp/kaas-bin/kaas_main

RESULTS

Brassinolide Alleviated $Ca(NO_3)_2$ Stress-Induced Growth Inhibition in Tomato Seedlings

To elucidate the effects of $Ca(NO_3)_2$-induced salt stress, the growth of tomato seedlings in response to $Ca(NO_3)_2$ treatment was investigated. As shown in **Figure 1**, $Ca(NO_3)_2$ stress repressed shoot biomass. However, the foliar application of BR alleviated the $Ca(NO_3)_2$-induced shoot growth inhibition in tomato seedlings (**Figures 1A,B**).

Observations of the microstructure of the tomato leaves showed that $Ca(NO_3)_2$ stress disrupted the arrangement of the epidermis, palisade mesophyll, and spongy mesophyll in tomato leaves (**Figure 1C**). The intercellular space enlarged, the palisade mesophyll and spongy mesophyll deformed, and the boundaries became blurred after $Ca(NO_3)_2$ stress. Foliar spraying with BR also affected the mesophyll structure in the tomato leaves. Compared with the control, the palisade mesophyll elongated, and the spongy mesophyll arranged chaotically, while the boundaries between the spongy mesophyll, the palisade mesophyll, and the epidermal cells became clear and neatly arranged in the BR-treated tomato leaves. Compared with $Ca(NO_3)_2$ stress alone, the thickness of the upper epidermal cells increased, and the cells were tightly arranged in the Ca+BR treatment. Moreover, the palisade mesophyll was arranged regularly, and an increased number of spongy mesophyll cells with clear cell boundaries were observed in the leaves of Ca+BR-treated tomato seedlings.

Identification of Differentially Changed Proteins

To elucidate the molecular mechanisms underlying BR-alleviated $Ca(NO_3)_2$ stress, we performed proteomics analysis and revealed the differentially changed proteins (DCPs) using the iTRAQ technique (**Figure 2A**). A total number of 419,939 secondary mass spectrums were obtained in the $Ca(NO_3)_2$ stress and/or BR-treated seedlings. Among these spectra, 117,757 spectra were matched to the 25,486 identified peptides. Finally, a total number of 25,486 unique peptides and 5,670 proteins were determined [$P < 0.05$, FC > 1.2] (**Supplementary Figure 1A** and **Supplementary Tables 1, 2**). There were 63.47% of the proteins that included at least two peptides (**Supplementary Figure 1B**) and the most enriched protein masses are 20–30 and 30–40 kDa, followed by 40–50, 50–60, and 10–20 kDa proteins (**Supplementary Figure 1C**).

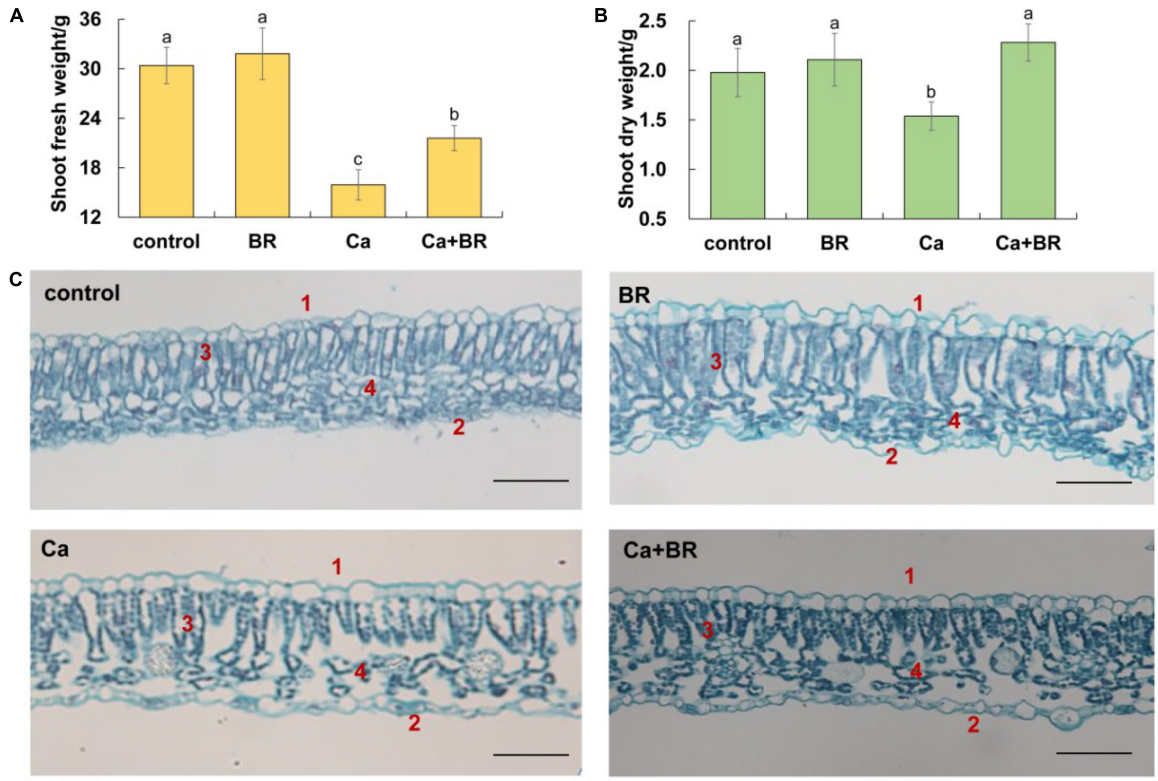


FIGURE 1 | Brassinolide alleviated calcium nitrate [$Ca(NO_3)_2$]-induced growth inhibition in tomato seedlings. **(A)** Shoot fresh weight. **(B)** Shoot dry weight. **(C)** Effects of brassinolide (BR) on the leaf microstructure in $Ca(NO_3)_2$-treated tomato seedlings. 1, Upper epidermis; 2, Lower epidermis; 3, Palisade mesophyll; and 4, Spongy mesophyll. BR, 0.1 μmol/L BR; calcium (Ca), 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying with exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$). Scale bars = 100 μm.

Compared with the control, 469 proteins showed significantly changed accumulation (244 with increased accumulation and 225 with decreased accumulation) in the leaves of $Ca(NO_3)_2$-treated seedlings (**Figure 2B**). Moreover, a total of 172 (79 with increased accumulation and 93 with decreased accumulation) and 442 (251 with increased accumulation and 191 with decreased accumulation) proteins with significantly different accumulation were identified in the BR/control and Ca+BR/Ca comparison groups, respectively (**Figure 2B**).

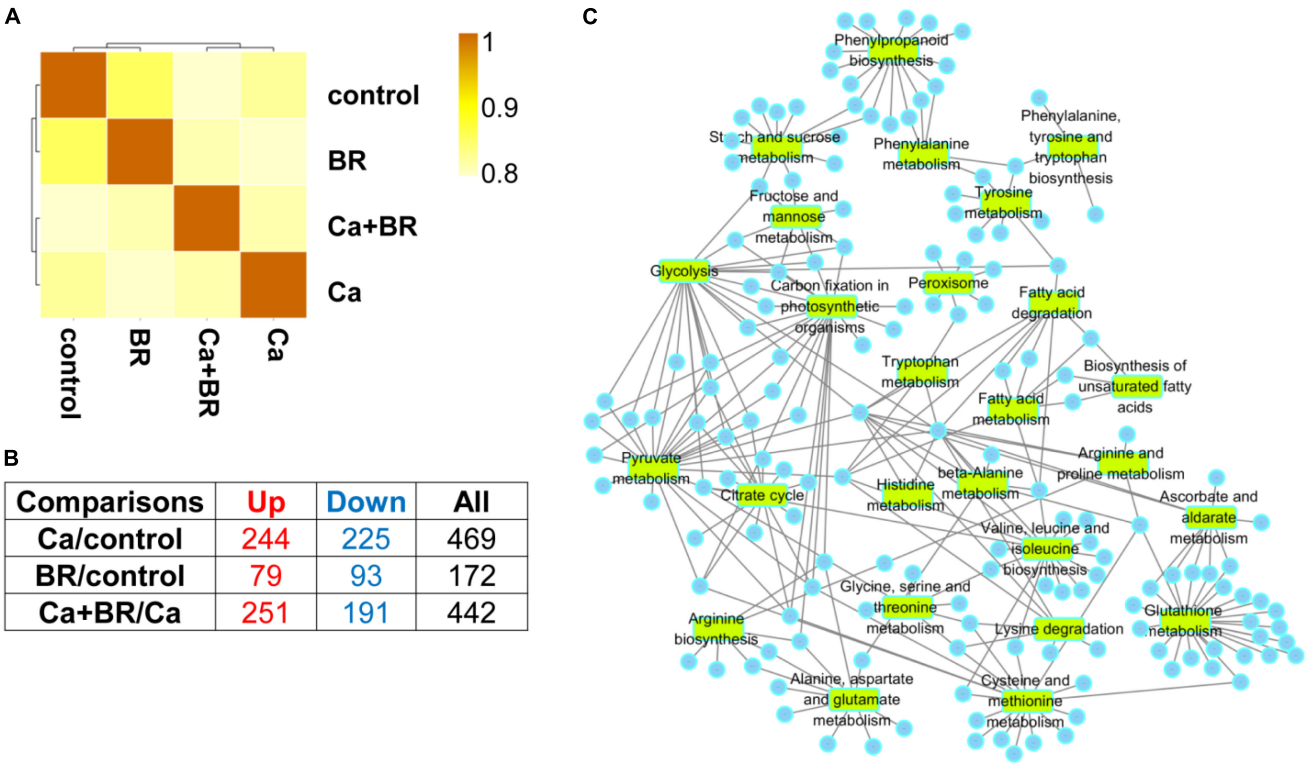
Functional Annotation of Differentially Changed Proteins

Functional annotations of the 172, 469, and 442 DCPs belong to the BR/control, Ca/control, and Ca+BR/Ca groups, respectively, (**Figure 2B**; **Supplementary Figure 2**; and **Supplementary Tables 4–7**) showed that the DCPs in the Ca/control were annotated into 47 functional terms, including 11 molecular function terms, 16 cellular component terms, and 20 biological process terms (**Supplementary Figure 2A**). Additionally, 442 DEPs in the Ca+BR/Ca were annotated to 47 functional groups, including 18, 17, and 12 terms in biological process, cellular component, and molecular function, respectively (**Supplementary Figure 2B**). The proteins were selected based on different GO terms shown in **Figure 2C**.

The DCPs were then blasted KEGG genes to retrieve their KEGG ortholog (KOs) and were subsequently mapped to the pathways in KEGG (**Supplementary Figure 3**). In the $Ca(NO_3)_2$-treated seedlings, the KEGG pathways were enriched in carbohydrate metabolism including pyruvate metabolism, glycolysis, glyoxylate, and dicarboxylate metabolism, and carbon fixation in the photosynthetic organisms involved in energy metabolism (**Supplementary Figure 3A**). In the Ca+BR-treated seedlings, the KEGG pathways were enriched in photosynthesis, carbon metabolism, propionate metabolism, pyruvate metabolism, and glycolysis (**Supplementary Figure 3C**). These results collectively indicated that compared with $Ca(NO_3)_2$ stress, foliar spraying with BR largely affected carbohydrate and energy metabolism pathways.

Validation of Isobaric Tags for Relative and Absolute Quantification Data for Selected Proteins by Parallel Reaction Monitoring

The iTRAQ data were subsequently confirmed using Skyline software. The detailed data of the 25 target peptide fragments



Comparisons	Up	Down	All
Ca/control	244	225	469
BR/control	79	93	172
Ca+BR/Ca	251	191	442

FIGURE 2 | The Spearman correlation coefficient analysis for proteomics data **(A)** and the number of the differentially changed proteins from the leaves of tomato seedlings subjected to 100 mmol/L of $Ca(NO_3)_2$ (Ca), foliar spraying with 0.1 μmol/L BR, or their combinations **(B)**. Up, up-regulated differentially changed proteins. Down, down-regulated differentially changed proteins. **(C)** Selected broad Gene Ontology (GO) terms in the leaves of tomato seedlings subjected to $Ca(NO_3)_2$ stress (Ca), foliar spraying with BR, or their combinations. Circles represent the differentially changed proteins in different pathways.

are shown in **Supplementary Table 8**. The accumulation of several antioxidant-related proteins, such as P30264, Q6X1D0, K4ASJ5, and K4ASJ6, and the light-harvesting complex chlorophyll A-B binding protein (P27524) elevated in the leaves of the $Ca(NO_3)_2$-treated seedlings. As shown in **Supplementary Tables 4, 6, 8**, the iTRAQ validation showed that $Ca(NO_3)_2$ stress increased the accumulation of Chlorophyll a-b binding protein (P27524, P27489, K4CXU8, K4C768) and ferredoxin (K4D1V7) and decreased the accumulation of cytochrome P450-type monooxygenase 97A29 (D2CV80). These results were consistent with the iTRAQ results, which indicated that $Ca(NO_3)_2$ stress affected photosynthesis and antioxidant defense in the leaves of the tomato seedlings.

Foliar spraying with BR increased the accumulation of antioxidant-related proteins such as POD (K4ASJ6, K4ASJ5) and cysteine proteinase 3 (Q40143), the proteins involved in the KEGG pathways of biosynthesis of amino acids (sly01230), photosynthesis-antenna proteins (sly00196), and protein processing in the endoplasmic reticulum (sly04141; **Supplementary Tables 4, 5, 8**).

Exogenous BR also affected the photosynthesis-related proteins under $Ca(NO_3)_2$ stress. Compared with $Ca(NO_3)_2$ stress, the accumulation of chlorophyll a-b binding protein (K4DC08) down-regulated, while that of PSBR (Q40163), PsbQ 543931 (Q672Q6), psbH (A0A0C5CEE1), and ferredoxin-1 (Q43517) up-regulated in the Ca+BR treatment (**Supplementary Tables 4, 7, 8**). Moreover, the changes in protein accumulation detected by the PRM assay were consistent with the iTRAQ results, indicating that the iTRAQ results were sufficiently valid.

Brassinolide Alleviated $Ca(NO_3)_2$-Induced Photosynthetic Inhibition

Proteomics analysis revealed that exogenous BR affected the accumulation of proteins involved in chlorophyll metabolism and photosynthesis in tomatoes under $Ca(NO_3)_2$ stress (**Figure 3A**). The key enzyme in the metabolism of porphyrin and chlorophyll is nicotinamide adenine dinucleotide phosphate (NADPH)-protochlorophyllide oxidoreductase (K4DCQ6). Calcium nitrate stress down-regulated the accumulation of K4DCQ6 in the tomato leaves. Moreover, in the pathway of porphyrin and chlorophyll metabolism, the significant increase of red chlorophyll catabolite reductase (Q1ELT8) under $Ca(NO_3)_2$ stress enhanced the electron transport in chlorophyll decomposition (**Figure 3A**). Foliar spraying with BR had no significant effect on the red chlorophyll catabolite reductase (Fragment; Q1ELT8) under salt stress, but K4DCQ6 was up-regulated in the "Ca+BR/Ca" group, and thereby modulating

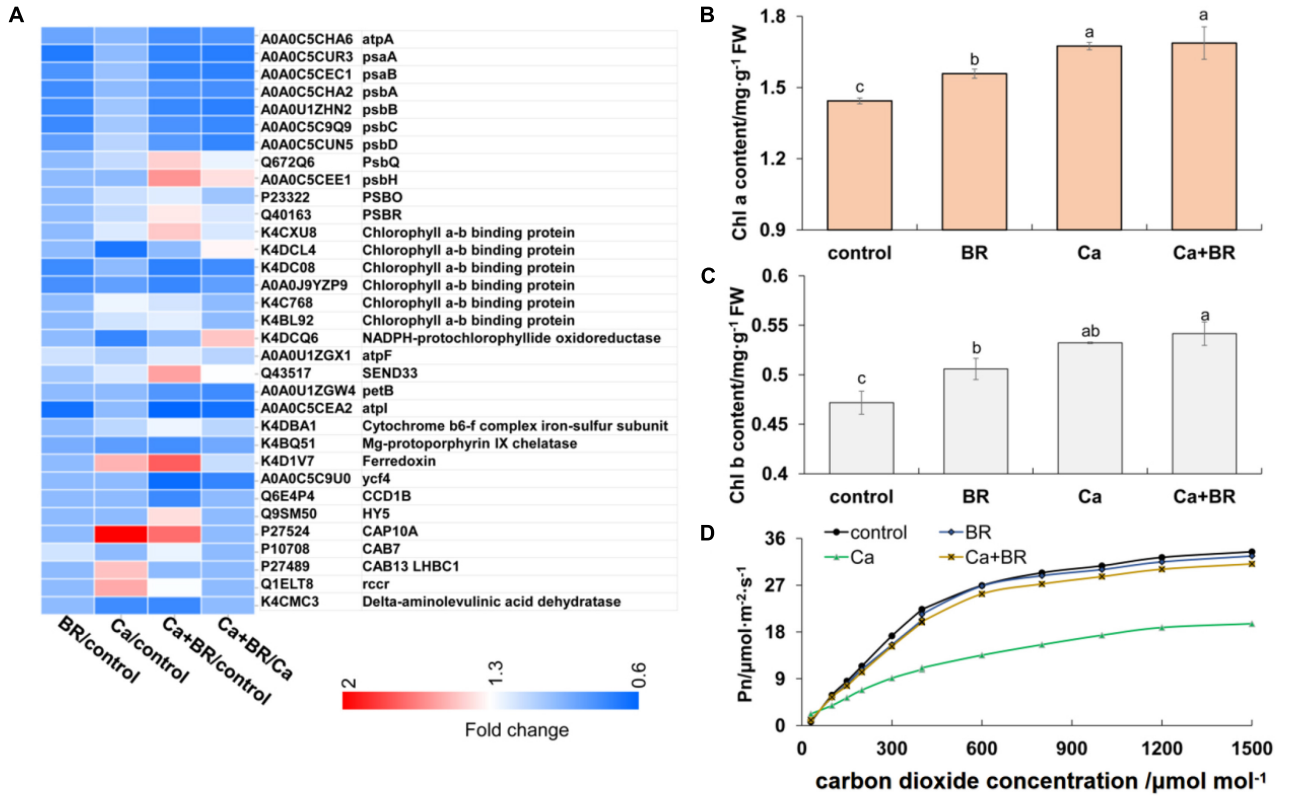


FIGURE 3 | BR alleviated $Ca(NO_3)_2$-inhibited photosynthesis in tomato seedlings. **(A)** Heatmap of the differentially changed proteins involved in photosynthesis. **(B)** Chlorophyll a content. **(C)** Chlorophyll b content. **(D)** Photosynthesis-CO_2 response curve. BR, 0.1 μmol/L BR; Ca, 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying with exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$).

chlorophyll levels in the Ca+BR treatment (**Figure 3A**). Meanwhile, $Ca(NO_3)_2$ stress increased the chlorophyll contents in the tomato leaves; however, foliar spraying with BR did not affect the chlorophyll contents in the $Ca(NO_3)_2$-treated tomato seedlings (**Figures 3B,C**). Altogether, these results indicated that BR alleviated $Ca(NO_3)_2$ stress by improving the function and stability of the photosynthetic system in tomato leaves. Next, we evaluated the effects of BR and $Ca(NO_3)_2$ on the photosynthetic parameters. Foliar spraying with BR decreased the WUE and *Ls* in the tomato leaves (**Figure 3D** and **Table 1**). Calcium nitrate stress inhibited the *Pn* and *Tr*, and decreased the *Gs* and *Ci*, but increased the *Ls* and WUE in the tomato leaves (**Figure 3D** and **Table 1**). However, foliar spraying with BR improved the *Pn*, *Tr*, *Gs*, and *Ci*, but decreased the WUE and *Ls* in the $Ca(NO_3)_2$-treated seedlings (**Figure 3D** and **Table 1**).

Brassinolide Alleviated $Ca(NO_3)_2$-Induced Oxidative Damage in Tomato Seedlings

The overproduction of ROS and subsequent oxidative damage commonly occur under salt stress (Alexander et al., 2020; Yang et al., 2020). Proteomics analysis showed that $Ca(NO_3)_2$ stress affected the accumulation of proteins involved in stress responses and antioxidant defense, and exogenous BR increased the accumulation of antioxidant enzymes and proteins involved in the responses to osmotic stress in tomatoes under $Ca(NO_3)_2$ stress (**Figure 4A**). To further confirm the proteomics results, we investigated the levels of H_2O_2 and oxidative damage in the $Ca(NO_3)_2$-treated tomato plants. Calcium nitrate stress significantly increased the content of H_2O_2 and MDA at 3 and 5 days after the treatment, respectively. However, foliar spraying with BR significantly reduced the H_2O_2 accumulation and MDA content in the leaves of $Ca(NO_3)_2$-treated tomato seedlings after 3 and 5 days of treatment, respectively (**Figures 4B,C**).

The time-course of the antioxidant enzyme activity showed the differential effects of $Ca(NO_3)_2$ and BR. For instance, $Ca(NO_3)_2$ significantly increased the activity of SOD after 1 and 5 days, but not after 3 days of treatment. Unlike this trend, $Ca(NO_3)_2$ stress significantly increased the activity of POD and CAT 1 day after treatment but it reduced the activity of POD and CAT in the tomato leaves after 3 and 5 days compared with the control (**Figures 5B,C**). However, compared with the seedlings only exposed to $Ca(NO_3)_2$ stress, the activity of these three enzymes was significantly increased by BR treatment after 1 day of $Ca(NO_3)_2$ stress. Foliar spraying with BR reduced $Ca(NO_3)_2$-induced SOD activity (**Figure 5A**) and improved the CAT activity in the leaves of $Ca(NO_3)_2$-treated tomato seedlings

TABLE 1 | Effects of exogenous BR on the photosynthetic parameters in the leaves of tomato seedlings under $Ca(NO_3)_2$ stress.

	Pn	Ci	Gs	Tr	WUE	Ls
Control	17.11 ± 1.20b	265.39 ± 10.83b	0.15 ± 0.03c	2.39 ± 0.18b	7.11 ± 0.42b	0.44 ± 0.03b
BR	20.77 ± 0.31a	296.44 ± 4.79a	0.22 ± 0.01a	3.54 ± 0.28a	5.90 ± 0.42c	0.36 ± 0.01c
Ca	10.86 ± 0.69d	191.81 ± 12.41c	0.07 ± 0.01d	1.15 ± 0.26c	9.52 ± 1.76a	0.61 ± 0.02a
Ca+BR	14.51 ± 0.94c	255.68 ± 10.49b	0.17 ± 0.02b	3.30 ± 0.09a	4.54 ± 0.10d	0.39 ± 0.03c

Error bars represent the SD (n = 3). Different letters indicate values that were significantly different at $P < 0.05$ according to Duncan's test.

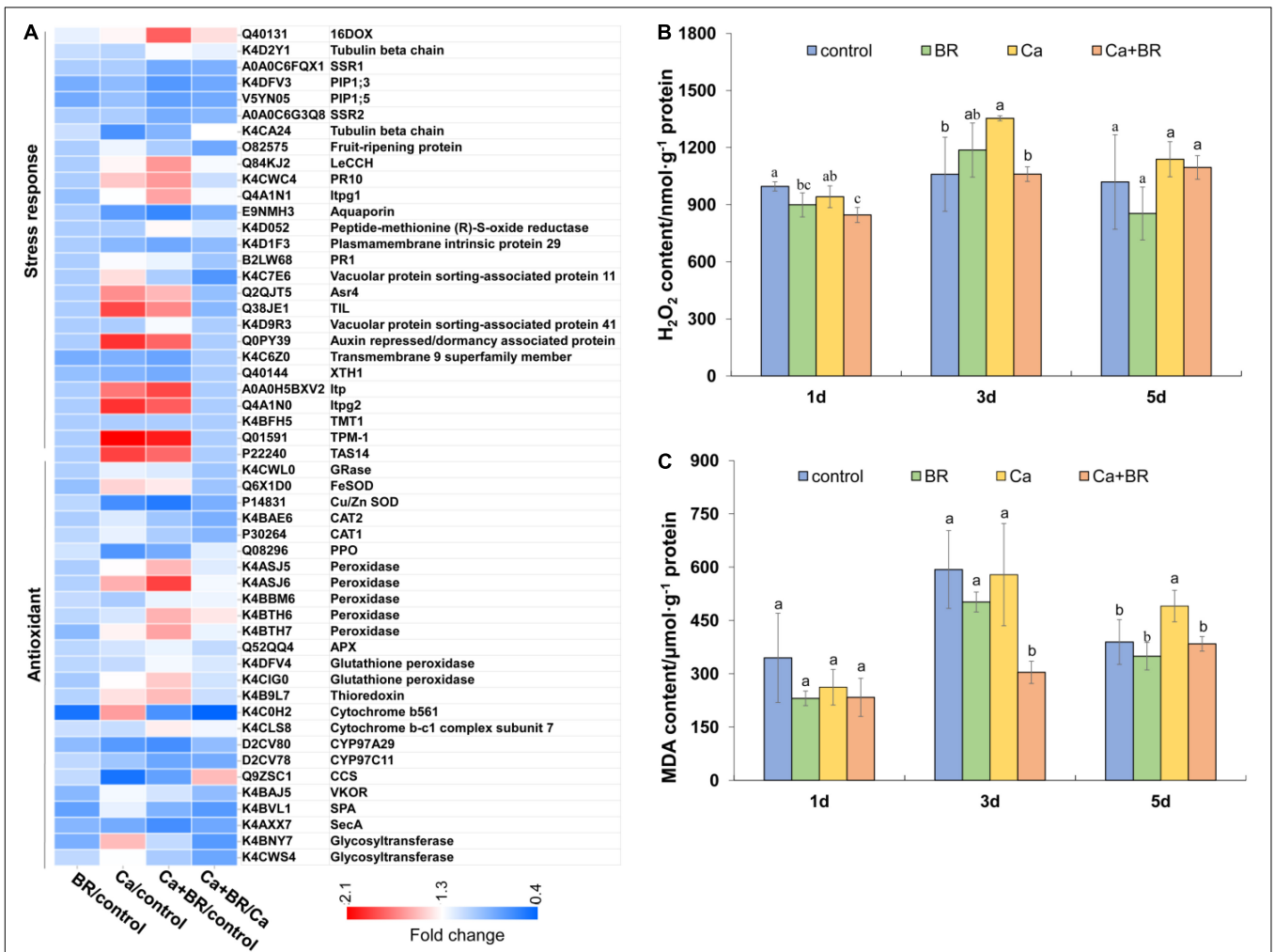


FIGURE 4 | The degree of membrane lipid peroxidation in the leaves of tomato seedling subjected to $Ca(NO_3)_2$, foliar spraying with BR, or their combinations. **(A)** Heatmap of the differentially changed proteins involved in the antioxidant system. **(B)** Hydrogen peroxide (H_2O_2) content. **(C)** Malondialdehyde (MDA) content. BR, 0.1 μmol/L brassinolide; Ca, 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying with exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$).

after 3 and 5 days of treatment (**Figure 5C**). Meanwhile, foliar spraying with BR further reduced POD activity after 3 days in the $Ca(NO_3)_2$-treated tomato seedlings (**Figure 5B**). However, the activity of POD in the Ca+BR-treated tomato seedlings was not significantly different from that in the only $Ca(NO_3)_2$-treated tomato seedlings after 5 days of treatment.

Calcium nitrate stress markedly increased the content of proline (Pro) in the tomato leaves throughout the experimental period. However, exogenous BR significantly reduced the $Ca(NO_3)_2$-induced increases in the Pro content after 3 and 5 days of $Ca(NO_3)_2$ stress, and the effect of BR was more profound with the prolongation of the treatment time (**Figure 6A**). Meanwhile, the AsA content differentially fluctuated with time in the $Ca(NO_3)_2$-treated tomato seedlings (**Figures 6A,B**). Compared with the control, the AsA content decreased after 1 day, however, it eventually increased after 5 days of $Ca(NO_3)_2$ stress

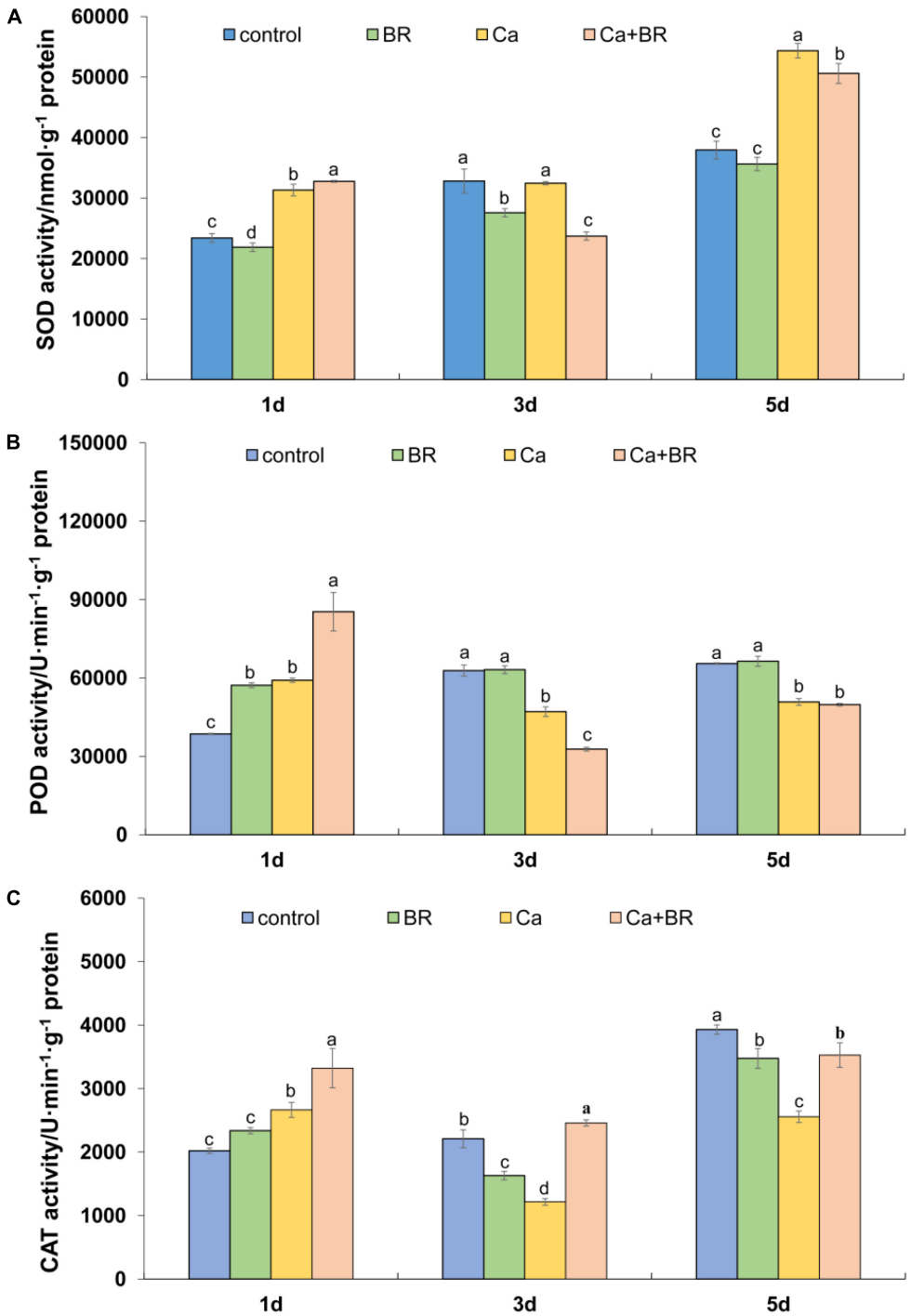


FIGURE 5 | Antioxidant enzyme activity in the leaves of the tomato seedling subjected to $Ca(NO_3)_2$, foliar spraying with BR, or their combinations. **(A)** Superoxide dismutase (SOD) activity. **(B)** Peroxidase (POD) activity. **(C)** Catalase (CAT) activity. BR, 0.1 μmol/L brassinolide; Ca, 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying with exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$).

(**Figure 6B**). Calcium nitrate alone or combined with BR did not alter the AsA accumulation at 3 days of the $Ca(NO_3)_2$ treatment. Also, the BR treatment on the $Ca(NO_3)_2$-treated tomato seedlings did not affect the AsA content compared with the only $Ca(NO_3)_2$-treated tomato seedlings after 3 and 5 days of the $Ca(NO_3)_2$ treatment. These results collectively

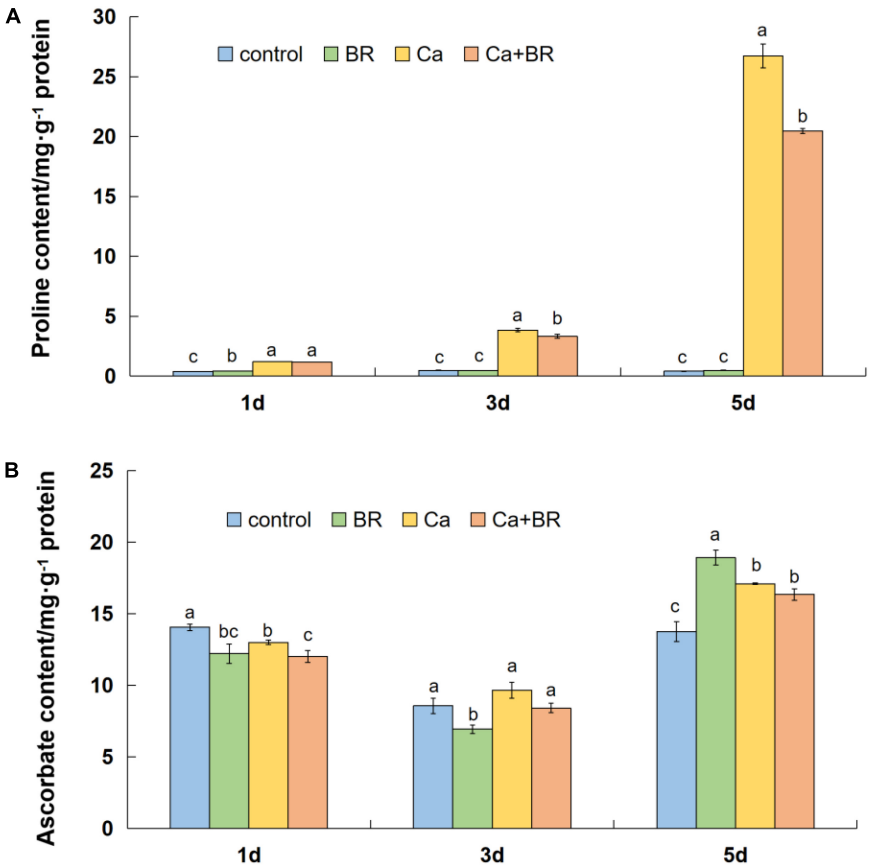


FIGURE 6 | (A) Proline and **(B)** ascorbic acid content in the leaves of the tomato seedlings subjected to $Ca(NO_3)_2$, foliar spraying with BR, or their combinations. BR, 0.1 μmol/L brassinolide; Ca, 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$).

supported the proteomics data and indicated that exogenous BR alleviated $Ca(NO_3)_2$-induced growth inhibition by increasing the antioxidant enzyme activity, thus, reducing the level of membrane lipid peroxidation, finally improving $Ca(NO_3)_2$ stress tolerance in tomatoes.

Brassinolide Affected the Contents of Amino Acids in the Tomato Leaves Under $Ca(NO_3)_2$ Stress

The proteomic analysis showed that $Ca(NO_3)_2$ stress disrupted the amino acid metabolism in the tomato leaves (**Figure 7A**). The phenylalanine (Phe) content increased under $Ca(NO_3)_2$ stress, while BR decreased the Phe content of the salt-stressed tomato seedlings. This change was negatively correlated with the decrease of PAL (K4CQI0) accumulation in the Ca/control and the increase of K4CQI0 accumulation in the Ca+BR/Ca (**Figures 7**, **8** and **Supplementary Table 7**). Moreover, the levels of amino acids such as threonine (Thr), glutamate (Glu), alanine (Ala), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), histidine (His) and proline (Pro) increased in the $Ca(NO_3)_2$-treated tomato seedlings, thereby reprogramming the primary metabolism of plants to salt stresses, while after foliar spraying with BR, all the amino acid content decreased in the $Ca(NO_3)_2$-treated tomato seedlings (**Figures 7**, **8**). All of the amino acids tested, except glycine (Gly), cysteine (Cys), and tyrosine (Tyr), showed markedly increased content in the leaves of the $Ca(NO_3)_2$-treated seedlings. Foliar spraying with BR decreased the contents of amino acids which were otherwise induced in the $Ca(NO_3)_2$ treatment (**Figure 7B** and **Supplementary Table 9**).

Brassinolide Improved the Contents of Soluble Sugars and Soluble Proteins in $Ca(NO_3)_2$-Treated Seedlings

Soluble sugars and soluble proteins, as intracellular osmoregulatory substances, play important roles in modulating plant responses to salt stress. The KEGG pathway analysis involved in carbohydrate metabolism indicated that five pathways were containing 20 identified DCPs that were enriched in the $Ca(NO_3)_2$-treated plants (viz.) glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, propanoate metabolism, pyruvate metabolism, citrate cycle (TCA cycle). The $Ca(NO_3)_2$ treatment also up-regulated the accumulation of malate dehydrogenase (K4B6N4, K4DCV3, K4CW40), thereby promoting the TCA cycle; in contrast, they down-regulated the

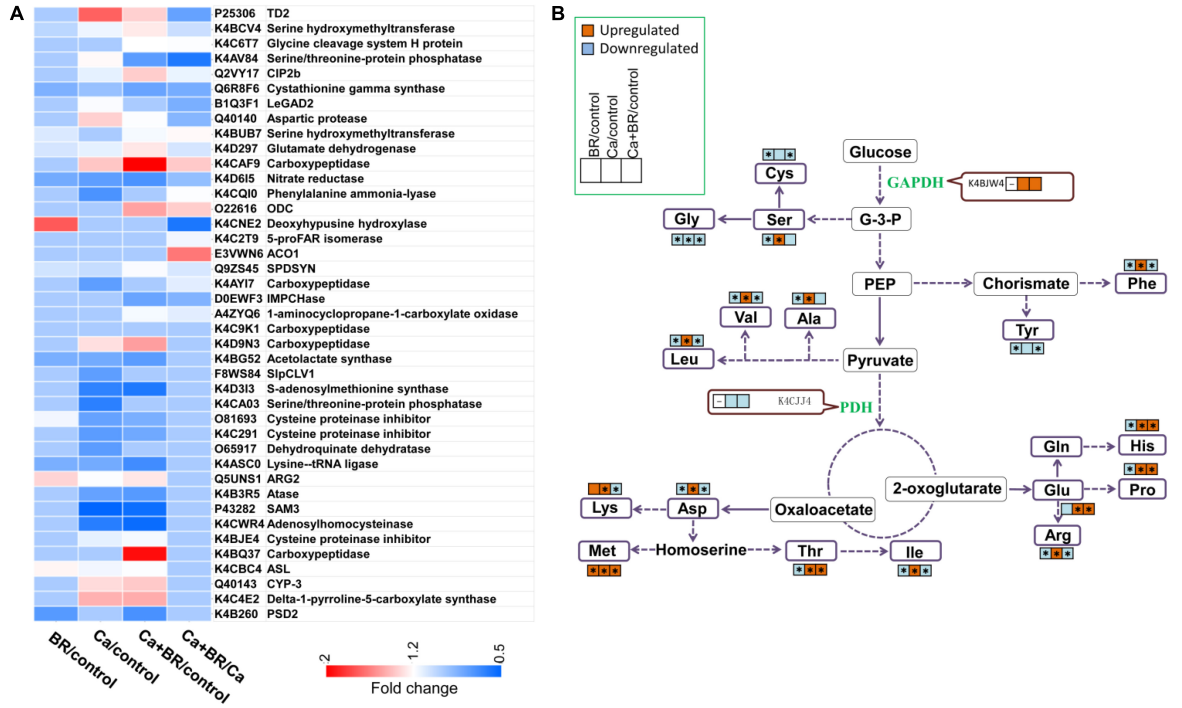


FIGURE 7 | Changes in the content of amino acids and enzymes involved in the glycolysis and TCA cycle. **(A)** Heatmap of the differentially changed proteins involved in amino acid metabolism. **(B)** Amino acids or the enzymes that showed significantly higher or lower accumulation in the leaves of tomato seedlings compared with those in the untreated control seedlings are represented by red (up-regulated) and blue (down-regulated) boxes. Asterisks indicate statistically significant differences ($P < 0.05$).

accumulation of pyruvate dehydrogenase E1 component subunit beta (K4CJJ4) in the tomato seedlings (**Supplementary Table 10**). The proteomic analysis also showed that BR modulated the sugar and protein metabolism pathways in the $Ca(NO_3)_2$-treated tomato seedlings (**Supplementary Table 10**). We thus speculated if BR alleviated salt-induced stress by increasing the contents of osmoregulatory substances. To address this question, we measured the levels of soluble sugars and soluble proteins in the tomato seedlings. Calcium nitrate stress increased the content of soluble sugar throughout the study period. Although foliar spraying with BR decreased the soluble sugar content after 1 day of salt stress, BR further increased the content of leaf soluble sugars after 3 and 5 days of salt stress compared with the tomato seedlings only treated with $Ca(NO_3)_2$. The soluble protein content more or less increased in the $Ca(NO_3)_2$-treated tomato seedlings throughout the study period, whereas foliar spraying with BR increased the content of the soluble proteins significantly at 3 days after the $Ca(NO_3)_2$ treatment (**Figures 9A,B**). Notably, the positive regulatory effect of BR was more profound on the soluble sugars than soluble proteins under $Ca(NO_3)_2$ stress after 5 days.

DISCUSSION

Secondary salinization caused by $Ca(NO_3)_2$ deposition in greenhouse soil is a major problem in tomato cultivation (Zhang et al., 2020). In this study, we found that $Ca(NO_3)_2$ stress repressed photosynthesis and induced oxidative damage in tomato seedlings. However, foliar spraying with BR showed a protective effect on alleviating $Ca(NO_3)_2$ stress by modulating the antioxidant capacity, photosynthesis, energy supply, and carbon/nitrogen metabolism processes in tomato seedlings (**Figure 8**).

Salt stress triggers excessive H_2O_2 accumulation, which induces lipid peroxidation and subsequently, oxidative damage (Yang and Guo, 2017). Thus ROS removal is a key strategy to minimize salt-induced oxidative damage, which largely relies on the efficient function of the antioxidant system (Wang and Huang, 2019; Wang et al., 2019). Previous studies showed that BR application elevated the activity of antioxidant enzymes, including ascorbate peroxidase (APX) activity in tomatoes, which conferred tolerance to oxidative stress (Claussen, 2005; Guo et al., 2018). Consistent with this, we found that exogenous BR-induced the rapid elevation of the activity of antioxidant enzymes such as SOD, POD, and CAT repressed H_2O_2 burst 1 day after $Ca(NO_3)_2$ stress (**Figure 5**), and thereby exerting a protective effect of BR on plants in response to the initial phase of $Ca(NO_3)_2$ stress. Although a few inconsistencies were found between H_2O_2 accumulation and antioxidant enzyme activity upon BR treatment during the later phases, a significant decrease in the MDA content in the Ca+BR treatment indicated the alleviation of $Ca(NO_3)_2$-induced oxidative stress after 5 days of salt treatment. It is to be noted that H_2O_2 also plays

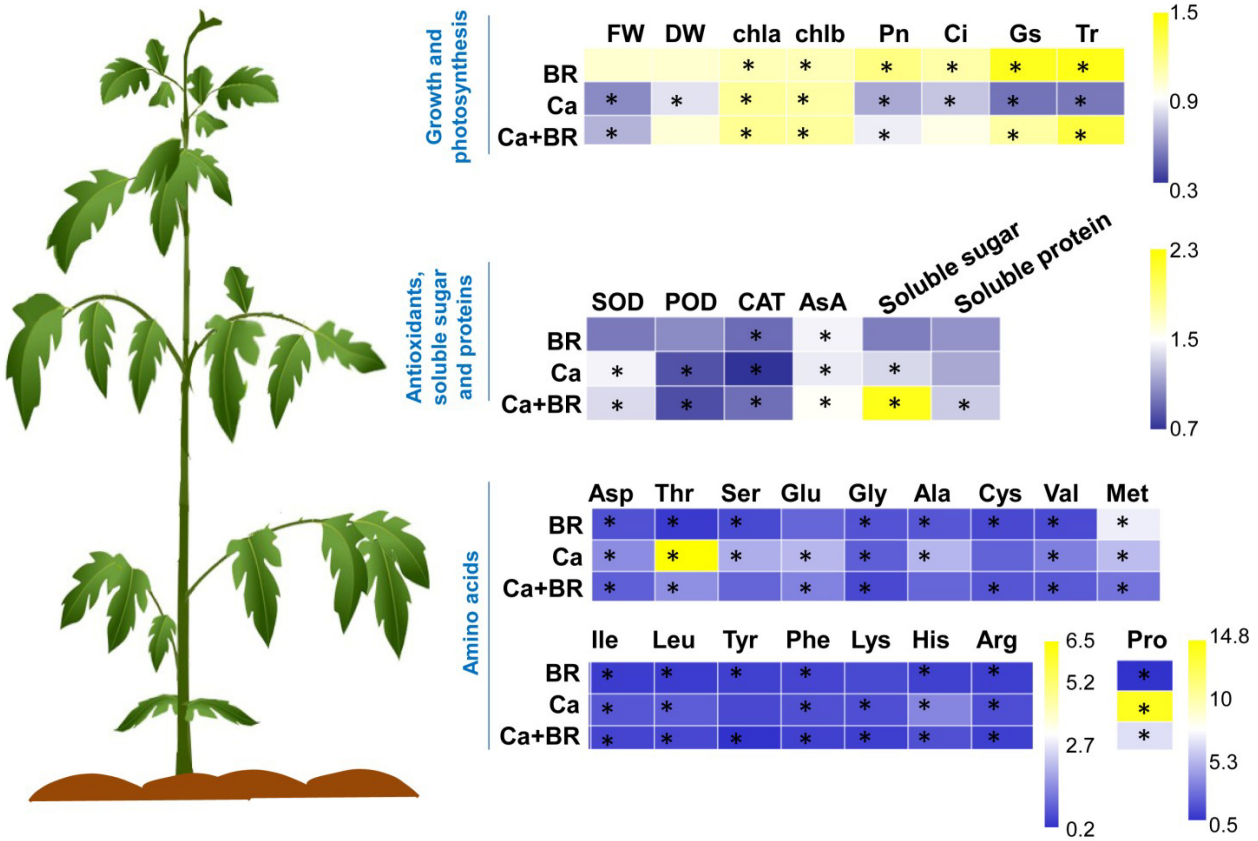


FIGURE 8 | Brassinolide alleviated $Ca(NO_3)_2$-induced growth inhibition by modulating photosynthesis, antioxidants, osmoregulatory substances, and amino acid contents in tomato seedlings. The heatmap represents the fold change of each value compared with the corresponding untreated control. Asterisks indicate that values were significantly different compared with the untreated control plants at $P < 0.05$.

a signaling role and it mediates BR-induced stress tolerance (El-Mashad and Mohamed, 2012). From that context, indifferent H_2O_2 accumulation between Ca and Ca+BR after 5 days of stress cannot be conducive to the inefficacy of BR treatment in minimizing ROS. In the study on water dropwort, it was found that stress conditions could promote the accumulation of Pro and soluble sugar in plants (Kumar et al., 2021). However, the effects of exogenous BR on Pro accumulation under stress were inconsistent in different studies. There are pieces of evidence that showed a positive correlation between the accumulation of Pro and stress tolerance in plants (Claussen, 2005), but the role of Pro in the osmotolerance of plants is also controversial (Nanjo et al., 1999). In our study, foliar spraying with BR reduced Pro accumulation in salt-stressed tomatoes, and we speculate that Pro might act as a stress indicator. Moreover, exogenous BR-induced the differential regulation of Pro, soluble sugar, and soluble protein contents from 1 to 5 days of $Ca(NO_3)_2$ stress, indicating the more complex role of BR action in the production of antioxidant and osmotic adjustment substances, which appears to be specific to the temporal context, but increased the soluble sugar content and decreased the MDA content after 5 days which signified the role of BR in protecting plants from long-term osmotic stress and oxidative damage.

Under abiotic stress, plants develop thicker leaves with a thicker palisade mesophyll and a higher ratio of palisade mesophyll to spongy mesophyll thickness accompanied by lower photosynthetic pigment content (Shi et al., 2014; Zhou et al., 2017). Moreover, adverse environmental factors often lead to disorganized leaf palisades and spongy mesophylls, thus causing reduced photosynthesis (Ivanova et al., 2009; Shi et al., 2014; Zhou et al., 2017, 2020; Wang et al., 2020). Consistent with this, $Ca(NO_3)_2$ stress disrupted the arrangement and structure of the epidermis, palisade mesophyll, and spongy mesophyll in the tomato leaves in the current study. On the contrary, BR plays a vital role in leaf morphogenesis (Zhang et al., 2019). Long-term dark treatment represses leaf primordia development in *Arabidopsis*, while BR supplementation induces leaf bud development in the seedlings under the long-term dark treatment (Nagata et al., 2000). In rice, *BR-deficient dwarf1* (*brd1*) mutants showed an obvious defect in the elongation of the stem and leaves (Hong et al., 2002). Consistent with these results, we found that exogenous BR improved the structure of the epidermis, palisade mesophyll, and spongy mesophyll in tomato leaves (**Figure 1C**). Moreover, BR has parallel links to cell-cycle progression (through S-phase Cyclin D-CDK and the anaphase-promoting complex) and cell-wall functions (through cell-wall

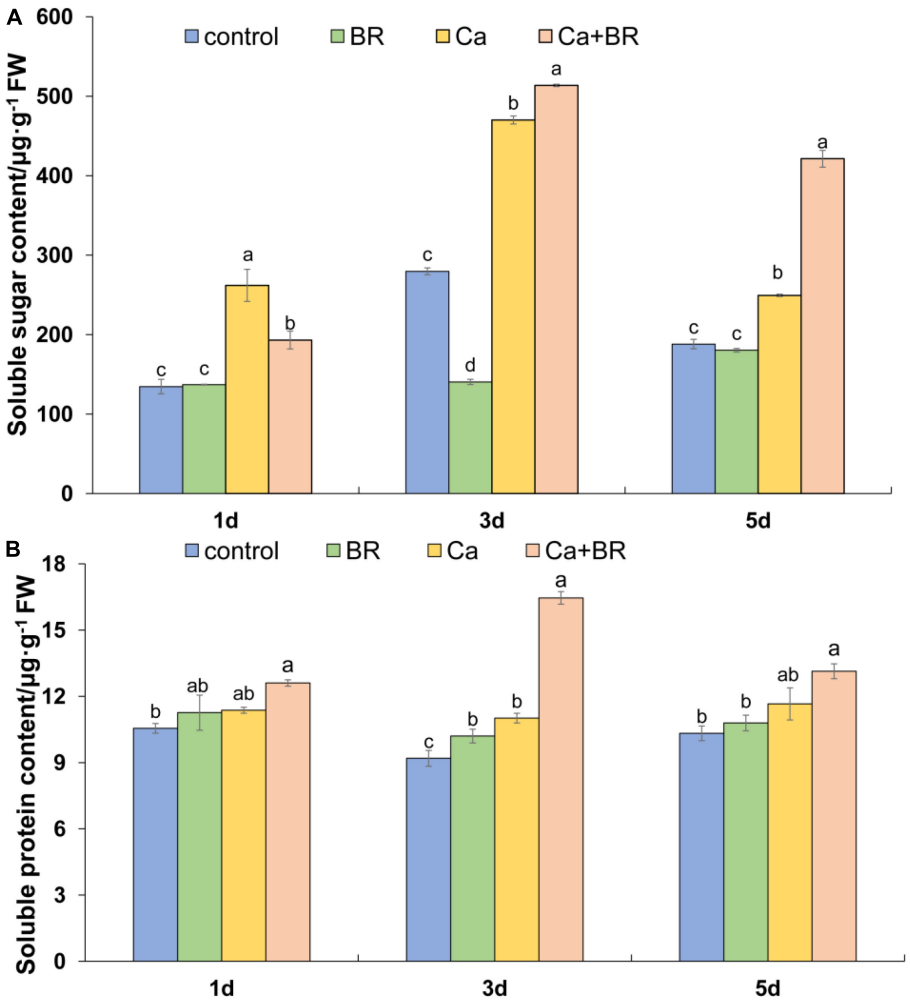


FIGURE 9 | (A) Soluble sugar and **(B)** soluble protein content in the leaves of tomato seedlings subjected to $Ca(NO_3)_2$, foliar spraying with BR, or their combinations. BR, 0.1 μmol/L brassinolide; Ca, 100 mmol/L $Ca(NO_3)_2$; Ca+BR, 100 mmol/L $Ca(NO_3)_2$ stress plus foliar spraying with exogenous BR. Each data point represents the mean of three independent biological replicates (mean ± SD). Different letters above the bars indicate statistically significant differences ($P < 0.05$).

extensibility or microtubule dynamics), which in turn affects the extension and morphology of the leaves (Kuluev et al., 2014). Mesophyll cells are the main sites of photosynthesis in plants. Neatly arranged and intact palisade mesophyll could promote light capture in plants (Jiang et al., 2011). The zigzag and continuous spongy mesophyll is conducive to optimal gas exchange, so its structural integrity ensures the normal operation of photosynthetic reaction. Exogenous BR application reduced the extent of damage of salt stress to the mesophyll structure, which might contribute to enhanced photosynthesis. However, the molecular mechanisms underlying the BR-mediated development of leaf primordium, leaf expansion, and leaf dorsiventral polarity establishment require further in-depth research.

Plants regulate the photosynthetic rate by controlling the opening and closing of the stomata, nonetheless, the stomata may behave differently in response to salt stress (Oh et al., 2019). Studies have shown that salt stress inhibits the photosynthetic process in leaves mainly *via* stomatal and non-stomatal limitations (Yang et al., 2010). Despite the increasing *Ls*, $Ca(NO_3)_2$ stress significantly decreased *Pn*, *Gs*, *Tr*, and *Ci* in the leaves of the tomato seedlings, indicating that the decrease in *Pn* under $Ca(NO_3)_2$ stress was probably caused by the stomatal restriction. On the other hand, spraying BR could reduce the *Ls* of $Ca(NO_3)_2$-stressed seedlings, suggesting that exogenous BR might decrease the stomatal restriction of tomato leaves under $Ca(NO_3)_2$ stress, and enhance their photosynthetic capacity. These results collectively indicated that the reduction in the photosynthetic efficiency of the $Ca(NO_3)_2$-treated seedlings is related to stomatal constraints.

Salt stress represses the plant photosystem (PS) I and II activity, and causes damage to the PS I components, chlorophyll A-B binding proteins, and light-harvesting complex of chlorophyll A-B binding proteins, and thereby inhibits photosynthesis (Shunichi and Norio, 2008). Several studies have indicated that BR plays an important role in the

regulation of photosynthesis (Li X. et al., 2016). Foliar spraying with BR improves the photosynthetic characteristics, actual photochemical efficiency, and quantum efficiency of leaves under normal temperature and short-term low-temperature stress (Zhang et al., 2020). In addition, BR treatment under low-temperature stress enhances the activity of PS II and antioxidant enzymes and protects the photosynthetic membrane from oxidative damage in cucumber plants (Fariduddin et al., 2011). Ferredoxin is involved in transferring PS I electrons to nicotinamide adenine dinucleotide phosphate ($NADP^+$), and generating a reducing force of NADPH, leading to the promotion of CO_2 assimilation in the Calvin cycle (Jiang et al., 2017), thus improving photosynthesis. Foliar spraying with BR up-regulated the accumulation of ferredoxin-1 (Q43517) in the "Ca+BR/Ca" group, which enhanced the PS I electron transfer in the $Ca(NO_3)_2$-treated tomato leaves. However, several proteins (PsaA, PsaB, psbC, psbB, PsbA, and PsbD) involved in the stabilization of PS I and PS II were down-regulated in the "Ca+BR/Ca" group (**Figure 3A**). This result could be explained by the fact that the application of exogenous BR might prevent excessive electron transfer under $Ca(NO_3)_2$ stress, thereby providing a protective mechanism to the photosynthetic system.

The KEGG pathway analysis indicated that the $Ca(NO_3)_2$ treatment affected the TCA cycle and carbon metabolism process in tomato seedlings. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme involved in glycolysis (Zhang et al., 2011), has a positive correlation with stress tolerance in plants (Pelah et al., 1997). A previous study found that BR treatment increased the abundance of proteins involved in sugar synthesis (such as sucrose synthase, sorbitol dehydrogenase, and IRX15-LIKE-like) in apple nursery trees. In addition, the contents of starch, sucrose, fructose, glucose, and total soluble sugar were increased in BR-treated leaves (Zheng et al., 2018), suggesting that BR affects sugar contents by modulating carbohydrate metabolism-related proteins. In this study, we found that $Ca(NO_3)_2$ stress up-regulated GAPDH (K4BJW4) in tomato leaves, while foliar spraying with BR further up-regulated the accumulation of GAPDH (K4BJW4; **Supplementary Table 10**). These results suggested that BR promoted carbon metabolism (including glycolysis and TCA cycle) in salt-stressed tomato seedlings, thereby improving salt tolerance in plants.

Plants mitigate the damage caused by stress by controlling the absorption, synthesis, and degradation of different amino acids (Li et al., 2019). We found that $Ca(NO_3)_2$ stress disrupted amino acid metabolism in tomato plants. Similarly, Martino et al. (2003) found that the contents of Glu, Gln, Asp, and Asn in spinach leaves decreased, while Ile, Leu, and Tyr increased after 43 days of sodium chloride (NaCl) treatment. Moreover, Wu et al. (2014) found that the contents of Tyr and Phe were negatively correlated, while the contents of Asp and Glu were positively correlated with the resistance of rice to saline-alkali stress. Our results further supported the hypothesis that free amino acids in plant organs are key indicators of plant tolerance to stress (Van et al., 2020). These results collectively indicated that exogenous BR application reprogrammed amino acid metabolism in the $Ca(NO_3)_2$-treated tomato seedlings. Taken together, the findings revealed that BR promoted sugar metabolism and amino acid metabolism in $Ca(NO_3)_2$-stressed tomato seedlings, thereby improving salt tolerance in tomato plants.

CONCLUSION

In summary, our results showed that $Ca(NO_3)_2$ stress inhibited plant growth by inducing oxidative stress, and repressing photosynthesis and amino acid accumulation in tomato seedlings. Proteomics analyses further revealed that $Ca(NO_3)_2$ treatment modulated the accumulation of proteins involved in photosynthesis, stress response, and antioxidant defense. Foliar spraying with BR improved photosynthesis efficiency, sugar and amino acid metabolism, energy supply, and defense responses by increasing the accumulation of proteins involved in the photosynthesis, TCA cycle, and antioxidant defense of $Ca(NO_3)_2$-treated tomato seedlings. These physiological results combined with proteomics analyses provided a deep insight into $Ca(NO_3)_2$-mediated salt stress and the roles of BR in improving the stress tolerance of tomato plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: PeptideAtlas, with DataSet Identifier PASS01682 (www.peptideatlas.org/PASS/PASS01682).

AUTHOR CONTRIBUTIONS

YZ and YS designed and supervised the research. YZ, HC, YS, YL, and SL performed most experiments. BL and LB characterized the phenotypes. YZ, MK, JX, and YS analyzed the data and 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.2021.724288/full#supplementary-material

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