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Heat Shock Protein HSP24 Is Involved in the BABA-Induced Resistance to Fungal Pathogen in Postharvest Grapes Underlying an NPR1-Dependent Manner

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Although heat shock proteins (HSPs), a family of ubiquitous molecular chaperones, are well characterized in heat stress-related responses, their function in plant defense remains largely unclear. Here, we report the role of *VvHSP24*, a class B HSP from *Vitis vinifera*, in β-aminobutyric acid (BABA)-induced priming defense against the necrotrophic fungus *Botrytis cinerea* in grapes. Grapes treated with 10 mmol L^{-1} BABA exhibited transiently increased transcript levels of *VvNPR1* and several SA-inducible genes, including *PR1*, *PR2*, and *PR5*. Additionally, phytoalexins accumulated upon inoculation with the gray mold fungus *B. cinerea*, which coincided with the action of a priming mode implicated in pathogen-driven resistance. Intriguingly, electrophoretic mobility shift (EMSA), yeast two-hybrid (Y2H) and His pull-down assays demonstrated that the nuclear chaperone *VvHSP24* cannot modulate the transcript of *PR* genes but does directly interact with *VvNPR1 in vivo* or *in vitro*. Furthermore, we found that *VvHSP24* overexpression enhanced the transcript levels of NPR1 and SA-responsive genes (*PR1*, *PR2*, and *PR5*) and increased the resistance of transgenic *Arabidopsis thaliana* to *B. cinerea* compared with wildtype Col-0. An opposite trend between CRISPR mutants of *AtHSFB1* (the orthologous gene of *VvHSP24* in *Arabidopsis*) and wildtype plants was observed. Hence, our results suggest that *VvHSP24* has a potential role in NPR1-dependent plant resistance to fungal pathogen. BABA-induced priming defense in grapes may require posttranslational modification of the chaperone *VvHSP24* to activate *VvNPR1* transcript, leading to *PR* gene expressions and resistance phenotypes.

Keywords: β-aminobutyric acid, heat shock protein, priming resistance, NPR1, *Botrytis cinerea*, grape berries

INTRODUCTION

Grapes are cultivated worldwide with great functional importance in terms of their attractive flavor and constitution of natural phytonutrients (Dai et al., 2011). Nevertheless, the shelf life of postharvest grape berries is very limited, which is mainly ascribed to the invasion of bacterial and fungal pathogens. Indeed, gray mold disease by the fungal pathogen *Botrytis cinerea* has been recognized as one of the most severe epidemics threatening economic effectiveness in fruit crop farming (Feliziani et al., 2013). A variety of *B. cinerea* strains can secrete mycotoxins and extracellular cell wall-degrading enzymes to disintegrate and digest the host cell walls of vine crops (Gabler et al., 2003). Ongoing application of synthetic fungicides for controlling gray mold rot of grapevines is highly efficiently, but the occurrences of resistance as well as the negative impacts posed by the frequent use of fungicides have gradually threatened the environment. Hence, eco-compatible alternatives for harmful fungicides to suppress the postharvest spoilage of grapes have been urgently demanded (Mari et al., 2014).

Among a wide spectrum of emerging approaches, several phytohormones and a few organic elicitors have been described as high effectiveness against disease pressure in different kinds of agronomic fruits (Mengiste et al., 2009; Burketova et al., 2015; Romanazzi et al., 2016). To optimize defense against invading pathogens, plants gradually potentiate the systemic immunity driven by stress-induced signaling. This response has been defined as a "priming defense," which suggests that systemic reactions against pathogen invasions can be triggered (van Hulten et al., 2006). β-aminobutyric acid (BABA), a non-proteinogenic amino acid, has been well documented as a predominant priming elicitor for effective resistance induction and is very desirable for disease management in agricultural fields (Cohen et al., 2016; Baccelli et al., 2017). The biobased resistance inducer of BABA employs its defense functions via abscisic acid (ABA)-responsive and/or phosphoinositide-responsive signaling transductions (Ton et al., 2005). A series of studies utilizing various *Arabidopsis* defense pathway mutants revealed that BABA not only primes the expressions of *AtPR1*, *AtPR2*, and *AtPR5* through a salicylic acid (SA) signaling pathway but also intensifies the transcripts of a set of jasmonate acid (JA)- or ethylene (ET)-inducible genes, including *AtPDFs*, *AtVSPs,* and *AtHELs*

Abbreviations: BABA, β-aminobutyric acid; BABA-IR, BABA-induced resistance; NPR1, non-expressor of pathogenesis-related genes 1; TF, transcription factor; CDS, coding sequence; ABA, abscisic acid; ET, ethylene; PI, phosphoinositide; JA, jasmonate; SA, salicylic acid; SAR, systemic acquired resistance; AbA, aureobasidin A; CaMV, cauliflower mosaic virus; CRISPR, clustered regularly interspaced short palindromic repeats; dpi, day post infection; GFP, green fluorescent protein; GST, glutathione; His, histidine; HPLC, high performance liquid chromatography; NCBI, National Center for Biotechnology Information; MEGA, molecular evolutionary genetics analysis; NJ, neighbor-joining; iTOL, interactive tree of life; SMART, simple modular architecture research tool; TBtools, toolkit for biologists; LD, long-day; MS, Murashige and Skoog; PDA, potato dextrose agar; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR; SD/-Leu-Trp, synthetic dropout medium lacking leucine and tryptophan; SD/-Leu-Trp-His, synthetic dropout medium lacking leucine, tryptophan and histidine; SD/-Leu-Trp-His-Ade, synthetic dropout medium lacking leucine, tryptophan, histidine and adenine; WT, wildtype; X-α-gal, X-α-D-galactosidase; Y2H, yeast two-hybrid; EMSA, electrophoretic mobility shift assay.

(Zimmerli et al., 2001; Ton et al., 2002). Hence, BABA-induced resistance (BABA-IR) has been considered as an integrated disease alternative in plants and can be regulated by a signaling crosstalk involving SA, ABA, PI, JA, ET, and other hormone signaling pathways, which is dependent on the stress category (Cohen et al., 2016; AbuQamar et al., 2017; Sham et al., 2019). Similarly, the recent studies concluded that BABA-IR in peach, strawberry and grape occurs in a dose/concentration-dependent manner as a disease-dependent priming defense responding to BABA concentrations ranging from 10 to 50 mmol L^{-1}, in contrast to a direct defense triggered by relatively high concentrations ($\geq$100 mmol L^{-1} BABA) (Wang et al., 2016, 2018, 2019; Li et al., 2020b). Of note, there are metabolic costs involved in the activation of a certain defense, regardless of what type of BABA-IR is elicited (Perazzolli et al., 2011; Luna et al., 2014). Although the priming defense has some obvious ecological advantages by reducing the excessive cost of cellular resources unless plants suffer from severe hostile pressures (van Hulten et al., 2006), the critical node and its function underlying a priming defense have not been characterized in the specific plant-fungus interactions.

Biotic and abiotic stresses are environmental constraints that differ from natural or optimal conditions, impeding normal cellular functions and constituting a major hindrance to plant growth and development (Uyttewaal et al., 2010). Heat shock proteins (HSPs) are ubiquitous molecular chaperones that are recruited under diverse stressful conditions, including heat stress and other non-heat environmental stresses, such as osmotic, drought, salt, ultraviolet (UV) light, oxidative stresses, and pathogen infections (Swindell et al., 2007). HSPs/chaperones may serve as hubs in the proteostasis network by assisting in protein folding, protein assembly, compartment targeting, degradation, and signaling (Baniwal et al., 2004; Swindell et al., 2007). It has been evident that the expression of HSPs is constitutively regulated at the transcriptional level by heat shock transcription factors (HSFs), which are the terminal components of a signal transduction pathway modulating the transcript of genes responsive to distinct stimuli (von Koskull-Döring et al., 2007; Xin et al., 2010). *Arabidopsis* contains 21 HSF representatives that have been classified into three classes (A, B, and C) based on their characteristic cluster of DNA-binding domains (DBDs) and adjacent oligomerization domains (ODs, HR-A/B regions) (Nover et al., 2001; Scharf et al., 2012). Class B and Class C HSFs are characterized by the absence of the typical activator motif aromatic hydrophobic acidic (AHA), which is essential for the transcriptional activity of Class A HSFs (Guo et al., 2016). Along this line, HSF1a, HSF1b, HSF1d, and HSF1e are all responsible for basal thermotolerance, and the heat-acclimation phenotype fundamentally involves HSFA2, HSFA3, and HSFA7a (Bharti et al., 2000; Nishizawa et al., 2006; Larkindale and Vierling, 2008; Zheng et al., 2016). Expression of *AtHSFA4a* is transcriptionally elicited by numerous stresses, such as cold, drought and UV-B, and transgenic *Arabidopsis* overexpressing HSFA4a showed enhanced tolerance to those stresses (Pérez-Salamó et al., 2014). Enhancements of *AtHSFA2* and *AtHSFA7a* transcripts have been monitored during virus infection, revealing the involvement of these two HSFs in the disease resistance of *Arabidopsis*

(Sugio et al., 2009). Moreover, overexpression of HSP27 strongly augmented plant resistance to various apoptotic stimulis (Samali et al., 2001; Tanabe et al., 2010), and the multichaperone complex HSP70-HSP90 is implicated in resistance protein (R protein)-mediated immunity and is essential for proper defense signal transduction (Noël et al., 2007; Shirasu, 2009; Li et al., 2012).

For decades, HSFs/HSPs have been recognized as hallmarks of the environmental stress response, and research on most HSFs/HSPs has typically focused on their central roles in response to several abiotic stresses, in particular heat stress management, in model plants such as *Arabidopsis thaliana*, peruvian tomato (*Solanum peruvianum*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*). Nonetheless, much less is known about the functional modulation of HSFs/HSPs against biotic stresses, especially against fungal pathogens, in horticultural crops. In this research, our focus extends the exploration of the chaperone function and potential regulation of HSFs/HSPs involved in the BABA-elicited priming response to fungal infection in grape fruit.

MATERIALS AND METHODS

Plant and Fungi

Grapes (*Vitis vinifera* Cv 'Kyoho') from 6-year-old vines were purchased and hand picked from a local supplier with a standard orchard in Chuanshan District, Suining City, Sichuan Province, China. The grapes coincided with some of reliable quality parameters, including the berry diameter of 1.8–2.2 cm, total soluble solids (SCC) of 13.4–15.7%, titratable acidity (TA) of 0.64–0.72%, pH value of 3.14–3.27 and firmness of 4.7–6.3 N cm^{-2} were late-harvested at a commercial maturity stage that was identified according to the Balic et al. (2014) and Rosales et al. (2016). The harvested grapes were transported to our laboratory within 1 h by an air-condition vehicle (20°C).

Arabidopsis thaliana wildtype (WT) Columbia-0 (Col-0) plants were applied for genetic transformation, and WT and transgenic *Arabidopsis* lines were grown in a SPT-P500B illumination incubator (Darth carter Co., Hefei, China) equipped with an automatic stable controller for maintaining the R.H. at 60–70%, illumination at 2500–3000 lx and temperature at 22°C with a long-day photoperiod (light/dark cycle of 14 h/10 h) over 4 weeks.

Botrytis cinerea (Pers.: Fr.) B05.10 strain (phylogenetic analysis shown in **Supplementary Figure 1**) was isolated from decayed 'Kyoho' grape berries (Lovato et al., 2019) and cultured on PDA medium (Haibo Co., Qingdao, China) for 14 days at 26°C. The cultured medium was then rinsed with sterile water containing 0.5% (v/v) Tween-80 to produce *B. cinerea* suspensions and diluted to a final concentration of 1.0×10^5 spores mL^{-1}, as counted with a Neubauer chamber.

Grape Treatments

Intact grape berries were picked and partitioned into four lots of 360 grapes each, which were superficially sterilized by 75% (v/v) alcohol and dried on sterile filter paper at 20°C for 2 h. Two symmetrical holes were then wounded (1.5-mm-deep × 1.5-mm-diameter) at the equator of each grape with a dissecting needle.

BABA (purity ≥ 99%, Sigma Co., St. Louis, MO, United States), at 10 mmol L^{-1}, was selected as the most effective concentration in basis of our current findings (Wang et al., 2019, 2020). Grapes in each lot were exposed to the four treatments following the description of Porat et al. (2003): (1) control, berries solely mock-inoculated with sterile water; (2) BABA, berries solely elicited with BABA solution; (3) inoculation, berries solely challenged with the *B. cinerea* suspensions; and (4) BABA + inoculation, berries elicited with BABA and subsequently challenged with *B. cinerea* suspensions. All treated grapes were then ventilated on stainless steel mesh for 6 h, transferred to PE boxes (60-μm-thick walls) and arranged in incubators for 5 days under 20 ± 1°C and 85 ± 5% R.H. A total of 30 g tissues from the healthy sarcocarp (3 mm distant from the diseased holes) of 60 grapes in each lot were sliced by a sterile scalpel before the incubation (0 h) and at 1-day intervals during the incubation. Tissues taken from each individual were rapidly frozen in liquid N$_2$ and kept at −80°C until analysis. Each treatment was performed according to a completely randomized design and repeated three times, and the experiment was performed twice.

Assessment of Disease Development

If the width of the dot-inoculated area was beyond 1.5 mm, the grape could be defined to be diseased. Percentage of decaying grapes was calculated as disease incidence. The disease development of twenty berries from each triplicate was monitored continuously for 5 days at the intervals of 2 days.

Measurement of Phytoalexin Content

For phytoalexin extraction, approximately 5 g of frozen pulp was ground in liquid N$_2$ and homogenized with 25 mL of 85% (v/v) methanol in ice bath. The homogenates were transferred into a dark incubator at 4°C overnight and centrifuged at 10,000 × g for 15 min (4°C). The supernatants were retained and evaporated under nitrogen, and the residue was further dissolved in absolute methanol before filtration. The individual phytoalexin content in the prepared extractions was measured by a HPLC analysis as described by Vitrac et al. (2005).

Classification and Sequence Analysis of HSFs and HSPs

Annotated complete lists of HSFs and HSPs in *Vitis vinifera* and *Arabidopsis thaliana* were obtained from the NCBI database. Furthermore, the phylogenetic relationship among the 37 identified *Vitis vinifera* HSFs/HSPs (*VvHSFs*/*VvHSPs*) and 20 *Arabidopsis thaliana* HSFs (*AtHSFs*) was conducted using MEGA 5.0 software with the neighbor-joining (NJ) method with 1000 bootstrap replicates and further visualized via the iTOL webtool. In addition, the online web resources SMART (Letunic and Bork, 2018) and TBtools (Chen et al., 2020) were used to visualize the conserved domains and sequence conservation, respectively.

RNA Isolation and Real-Time qRT-PCR

Aliquots (5 g) of frozen tissues were ground in liquid N$_2$, and total RNA was extracted using RNAprep Pure Kit for plants (Tiangen, China). Aliquots (1 μg) of RNA were prepared to

synthesize first-strand cDNA using a PrimeScript[TM] RT reagent kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) was conducted in triplicate using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). The 10-μL qPCR mixture contained 0.2 μL of each individual 10 μmol L^{-1} primer for *VvHSP24*, SA-dependent genes including *VvNPR1*, *VvPR1*, *VvPR2,* and *VvPR5* as well as JA/ET-dependent genes including *VvPDF1.2*, *VvVSP1*, *VvHEL*, *VvFAD3*, *VvTHI2*, *VvERS1,* and *VvERF1* (listed in **Supplementary Table 1**), 1 μL cDNA template, 5 μL 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) and 3.6 μL ddH$_2$O. The thermal cycle for qRT-PCR was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s with the ABI-specified hold and melt curve stages. Amplicon lengths were optimized to 90–150 bp to ensure optimal polymerization efficiency. qRT-PCR results were normalized by the cycle threshold value (CT) using the reference gene *VvActin7* or *AtActin2* according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Relative expression levels of *HSP/HSF* and *PR* genes were calibrated, with the values for the control fruit/plant being set as 1.

Subcellular Localization

PCR-amplified *VvHSP24* was cloned into the pEAQ-GFP vector to construct the pEAQ-*VvHSP24*-GFP fusion protein and the pEAQ-GFP empty vector was used as the positive control. Then, onion bulb epidermis cells were transformed by inoculation with *A. tumefaciens* strain EHA105 harboring pEAQ-GFP and the recombinant construction of pEAQ-*VvHSP24*-GFP via an *Agrobacterium*-mediated system, respectively. Briefly, the adaxial epidermises obtained from onion bulb were cultured on Murashige and Skoog (MS) medium for approximately 1 week and then immersed overnight in the suspension of EHA105 cells containing pEAQ-*VvHSP24*-GFP or pEAQ-GFP, respectively. Following 3 days of incubation on MS medium at 26°C in darkness, these epidermises were stained with the nuclear dye 4,6-diamidino-phenylindole (DAPI, 20 μg mL^{-1}, Sigma, United States). Then, the fluorescence signal of the stained onion peels was captured by a Zeiss LSM 510 confocal laser scanning microscope with excitation wavelength set at 405 nm for DAPI fluorescence and 488 nm for GFP fluorescence and emission wavelength in the range of 450–550 nm at 3 h after DAPI staining.

Y2H Assay

Y2H assay was performed by employing the Matchmaker[TM] Gold Y2H System (Clontech). Full-length cDNA clones of *VvHSP24* and *VvNPR1* in BABA-treated, *B. cinerea*-infected berries were inserted into the pGBKT7 and pGADT7 plasmids, respectively. The recombinant plasmids, including BD-*VvHSP24* and AD-*VvNPR1*, were cotransformed into yeast strain AH109 cells, which were further smeared on double dropout medium (DDO, SD/-Leu-Trp) and cultured for about 3 days at 29°C (Li et al., 2020b). Transformed colonies were cultured on triple dropout medium (TDO, SD/-Leu-Trp-His) and quadruple dropout medium (QDO, SD/-Leu-Trp-His-Ade) with or without 40 mg L^{-1} X-α-gal to determine possible interaction.

His Pull-Down Assay

The recombinant GST-*VvNPR1* and His-*VvHSP24* proteins were obtained through fusing with pGEX-4T-1 or pET-28a vector, respectively. The recombinant proteins were transformed into *Escherichia coli* BL21 (DE3) cells and expressed at 20°C for 3 h after the addition of 0.1 mmol L^{-1} IPTG (isopropyl β-D-galactopyranoside). Bacteria expressing the GST-*VvNPR1* fusion protein were lysed by sonication and purified using glutathione agarose (Thermo Fisher Scientific, United States) following the instructions. *E. coli* harboring the His-*VvHSP24* fusion protein were isolated and purified using a B-PER (R) 6 × His Spin Purification Kit (Pierce, Rockford, IL, United States). For His pull-down assay, the purified His-*VvHSP24* fusion protein was immobilized with Ni sepharose 6 Fast Flow (GE Healthcare, code number: 17-5318-01) and mixed for 2 h at 4°C. Then, 5 g of GST-*VvNPR1* fusion protein was incubated with 15 μL of prewashed resin overnight (4°C) before being washed three times with cold phosphate-buffered saline (PBS) based on standard protocols. Pulled down proteins (6 μL) were boiled in 2× Laemmli buffer and detected by Western blotting with an anti-GST antibody (TransGen Biotech Co., Ltd., Beijing, China, HT601); the His + GST-*VvNPR1* fusion protein was used as a negative control.

EMSA Assay

The CDS fragment of *VvHSP24* was synthesized and cloned into the pCzn1 vector to produce the His-*VvHSP24* recombinant protein and introduced into ArcticExpress (DE3) chemically competent cells. Then, 0.5 mmol L^{-1} IPTG was applied to induce the expression of the target recombinant protein (His-*VvHSP24*) at 15°C for approximately 9 h and then purified using a Ni-NTA His-Bind resin column (Novagen, Gibbstown, NJ, United States). The heat stress elements (HSE: 5′-GAAnnTTC-3′), which could be recognized by HSFs/HSPs, were detected in the promoters of *PR1*, *PR2* and *PR5* (**Supplementary Data 1**), and their nucleotides adjacent to HSF motifs (approximately 50 bp) were adopted as probes, respectively. These probes were labeled at the 5′ end with biotin using Light Shift chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, United States) and incubated with the purified His-*VvHSP24*. The protein-DNA complexes formed during EMSA experiments were transferred electrophoretically to a nitrocellulose membrane after separated by SDS-PAGE. Then, the shift bands were visualized via a chemiluminescence reaction on X-ray films. The addition of 200-fold mutant probes with the mutant HSF motif served as the competitors for the 5′-labeled WT DNA probes to test for the specific of the binding assays; the biotin-EBNA control DNA + EBNA extract was used as a positive control.

Overexpression and Analysis of *Vitis vinifera* HSP24 in Transgenic *Arabidopsis* Plant

The coding region of *VvHSP24* was amplified by PCR technology from a grape berry cDNA library with gene-specific primer listed in **Supplementary Table 1**. The construction of a plasmid for overexpression of *VvHSP24* by introducing the *VvHSP24*

fragment into the binary vector pCambia 1305.1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. *Arabidopsis* plant was transformed with *A. tumefaciens* GV3101 harboring the resulting *35S:VvHSP24* construct using the floral dip method (Clough and Bent, 1998). The harvested T1 seeds were surface sterilized in 2% (v/v) sodium hypochlorite for 10 min and stratification at 4°C for 3 days and then germinated on MS medium containing 50 mg L^{-1} kanamycin (kan) for more than 7 days. The kan-resistant seedlings were sown in pots containing a 1: 2 perlite-vermiculite mixture and cultured in illumination incubator with long-day photoperiod. Ultimately, homozygous plants of the T3 generation were used for subsequent experiments.

Generation of HSFB1 Mutants by CRISPR-Cas9

Mutations were introduced into *Arabidopsis* HSFB1 through the non-homologous end joining (NHEJ) method of RNA-guided endonuclease-mediated targeted mutagenesis with the clustered regularly interspersed short palindromic repeats (CRISPR)-Cas9 system (Lowder et al., 2015; Li et al., 2019). In brief, the gene-targeting binary vector M2CRISPR, with specific guide RNA (sgRNA) cassettes under the control of the U26:U29 promoter as well as an EC promoter driving Cas9 expression, was provided by Shanghai Weidi Biotechnology Co., Ltd. sgRNA cassettes were designed using sequences corresponding to the protospacer adjacent motif (PAM) sites (set as 'NGG') on the sense or antisense strand of the targeted *AtHSFB1* gene, and the web server CRISPRdirect[1] was used to check their off-target effects. Then, two sgRNA cassettes were annealed, and PCR products were further ligated into the *Bsa*I-digested vector M2CRISPR to generate a U26 promoter-driven T1 sgRNA and U29 promoter-driven T2 sgRNA (**Supplementary Figure 4**). The primers used are provided in **Supplementary Table 1**. The resulting binary vector was used for *A. tumefaciens* GV3101-mediated genetic transformation of *Arabidopsis*. T1 generation transformants were selected on MS medium containing 50 mg L^{-1} hygromycin phosphotransferase (HYG), and the effectiveness of genome editing was evaluated using the HYG-resistant seedlings. Genomic DNA from true leaves was isolated, and the targeted genomic region was amplified by PCR using KOD FX (Toyobo Co. Ltd., Osaka, Japan) with the primers listed in **Supplementary Table 1**. The resulting PCR products were sequenced, and mutants were identified by comparison with WT sequences. INDELs (insertions and deletions) at the targeted sites were considered as mutations. The T1 mutant seedlings were transplanted into soil, and T2 generation seeds were collected. After the identification of homozygous mutants, the T2 seeds were used to produce T3 seedlings.

Inoculation of *Arabidopsis* Plant With Fungal Pathogen *B. cinerea*

Four- to five-week-old T3 transgenic and wildtype (Col-0) *Arabidopsis* were dot-inoculated with *B. cinerea* conidial

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[1] https://crispr.dbcls.jp/

suspensions (1.0 × 10^5 conidia mL^{-1}) as previously described (Xiao and Chye, 2011). Detached leaf samples were used for morphological observations at 6 dpi and leaves picked at 0, 3, and 6 dpi were used to test for the transcript levels of disease resistance-related genes.

Trypan Blue Staining

Trypan blue staining was employed to assess the rate of cell death and fungal development of *B. cinerea*-infiltrated leaves from *Arabidopsis* WT (Col-0), *VvHSP24*-overexpressing plants and CRISPR mutants at 6 dpi, as described previously, with minor modifications (Cai et al., 2020). *B. cinerea*-infected *Arabidopsis* leaves were immersed and boiled in trypan blue staining solution [0.025% (m/v) trypan blue, 25% (m/v) phenol, 25% (v/v) lactic acid, and 25% (v/v) glycerol] for 5 min and then allowed to cool naturally and soak overnight. The stained leaves were immersed in 1.25 g mL^{-1} chloral hydrate solution for more than 24 h to eliminate chlorophyll; the samples were observed by binocular stereomicroscopy (Leica EZ4D) and photographed.

Electrolyte Leakage Measurement

Estimation of electrolyte leakage was carried out as previously described (Mackey et al., 2002). Leaf disks (8 mm diameter) of *B. cinerea*-infiltrated leaves from WT and *VvHSP24*-overexpressing *Arabidopsis* were washed in 50 mL ddH$_2$O at least twice and then transferred to 10 mL ddH$_2$O and floated for 8 h at 120 rpm and 25°C. Water conductivity was detected over time using a conductivity meter (MIK-TDS210-B).

Statistical Analysis

All data were subjected to *t*-test and one-way analysis of variance according to a Tukey's test using SAS version 8.2 (SAS Inst., NC, United States); data were reported as the mean ± standard error of three replicates from one independent experiment. Different letters and asterisks indicated that the differences were statistically significant at the 0.05 or 0.01 level of the *p*-value.

RESULTS

Inhibitory Effectiveness of BABA Treatment Against *B. cinerea* Infection in Grapes

As presented in **Figure 1**, grape berries are obviously susceptible to the necrotrophic pathogen *B. cinerea* and became deteriorated after infection at 20°C. 10 mmol L^{-1} BABA treatment caused a significant inhibition in disease development in postharvest grapes, and their lesion diameter and disease incidence were 32.16 and 32.32%, respectively, lower compared with the only *B. cinerea*-inoculated fruit at 5 dpi (**Figure 1** and **Supplementary Figure 2**).

Molecular Identification and Characterization of *VvHSP24*

Given that *AtHSFB1*—a class B-HSF from *Arabidopsis*, also referred to as *AtHSF4* or *AtTBF1*—plays a positive

FIGURE 1 | Impact of BABA on the disease development of grapes altered in their responses to the fungal pathogen *B. cinerea*. Lesion diameter **(A)** and disease incidence **(B)** measured at 1, 3, and 5 days post inoculation with *B. cinerea*. Grapes were pretreated with water (gray bars) or BABA (black bars) before inoculation. Disease development is expressed as the mean ± SE of nine assessments. Different letters above the bar indicate a significant difference ($p < 0.05$) between grapes with BABA-treated or *B. cinerea*-inoculated and untreated cases.

role in the development of defense priming and systemic acquired resistance (SAR, Pajerowska-Mukhtar et al., 2012; Pick et al., 2012), we compared the amino acid sequences of HSFs/HSPs from *Vitis vinifera* specie and those of *Arabidopsis thaliana* HSFs by constructing a phylogenetic tree. According to the phylogenetic tree of *Vitis vinifera* HSFs/HSPs with *AtHSFs*, *VvHSP24* and *AtHSFB1* grouped into class B and a single conserved HSF_DNA-binding domain consisting of 94 amino acids is present at residue ranges of 11–104 and 6–99 in *AtHSFB1* and *VvHSP24*, respectively (**Supplementary Figure 3**). Hence, the expression of *VvHSP24*, an *AtHSFB1* orthologous gene in *Vitis vinifera*, might be related to grape defense. In addition, GFP-tagged *VvHSP24* was exclusively detected in the nucleus of onion epidermal cells, contrarily the positive control, i.e., GFP alone, was found in both the cytoplasm and nucleus (**Figure 2**). As a result, *VvHSP24* might be a nucleus-localized regulatory protein associated with the development of defense priming.

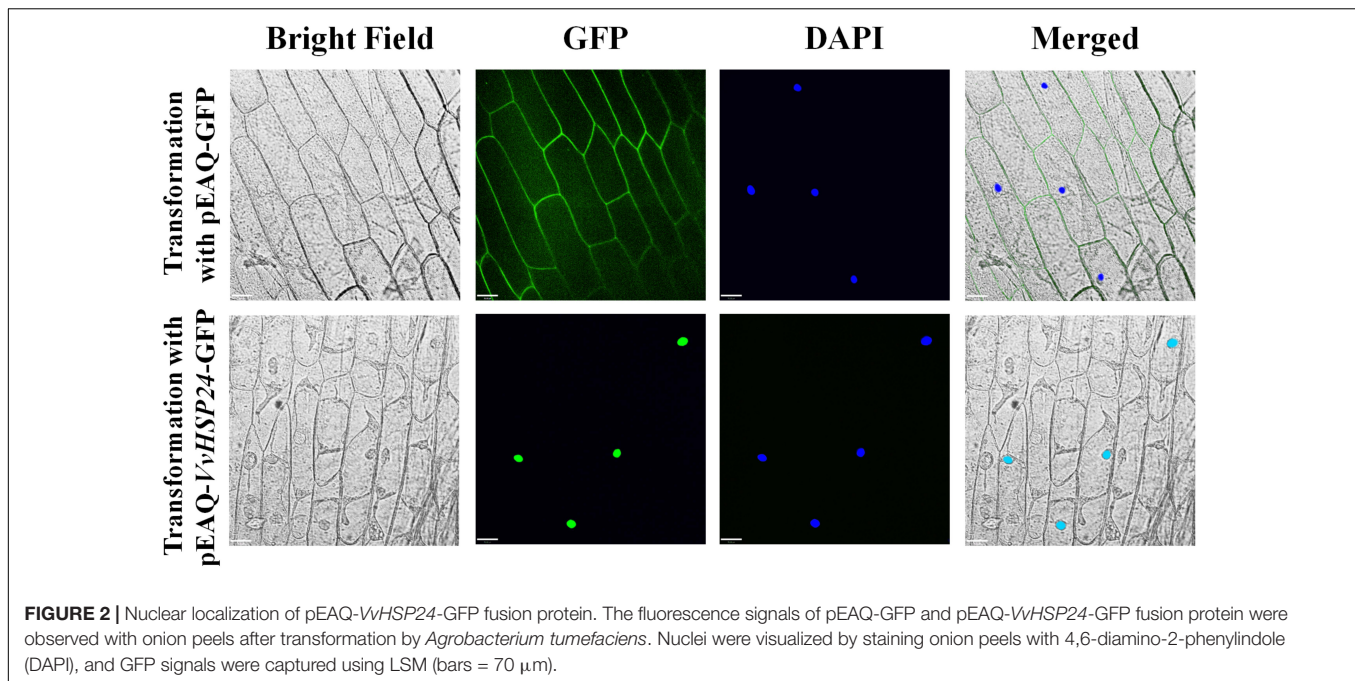### Effect of BABA Treatment on Transcript of *VvHSP24* and SA-Dependent Genes in Grapes

For analyzing the intensity of BABA-triggered SAR defense, the qRT-PCR method was used to quantify the transcript levels of the *VvHSP24* and SAR marker genes in grapes throughout 5 days of the inoculation. As shown in **Figure 3A**, treatment of grapes with 10 mmol L^{-1} BABA enhanced expression of the *VvHSP24* gene within the incubation period of 5 days, similar to the trend in our previous RNA-seq database[2] (**Supplementary Figure 5**). Thus, *VvHSP24* may indeed participate in BABA-IR in grapes upon the perception of disease stress. In addition, although grapes reacted to BABA pretreatment with enhanced transcripts of *VvNPR1* and SA-responsive genes such as *VvPR1*,

[2]https://www.ncbi.nlm.nih.gov/sra/PRJNA663112

VvPR2, and *VvPR5* following mock inoculation with ddH$_2$O, the reaction was much more pronounced in *B. cinerea*-inoculated grapes than in non-fungal-infected grapes. In BABA-treated and then *B. cinerea*-inoculated grapes, accumulation of *VvNPR1* and SA-inducible genes transiently peaked at 1 day post inoculation (dpi) followed by a gradual decrease (**Figures 3B–E**). Thus, SA-dependent priming of pathogen responses was boosted in BABA-pretreated grapes because the transient accumulation of mRNA transcript levels detected in SA-dependent marker genes is a hallmark of the priming mode for BABA-IR (Ton et al., 2005).

Effect of BABA Treatment on Transcript of JA/ET-Dependent Genes in Grapes

It is well-documented that a subset of genes, including *VvPDF1.2* (encoding a plant defensin), *VvHEL* (encoding a hevein-like protein), *VvVSP1* (encoding an acid phosphatase), *VvTHI2* (Encoding a antimicrobial thionin), *VvFAD3* (encoding a fatty acid desaturase), *VvERS1* (encoding an ethylene response sensor), and *VvERF1* (encoding an ethylene response factor), are regulated by JA or ET signaling pathway and associated with a resistance against hostile threats (Zarate et al., 2007). Thus, the transcript levels of the above genes were assessed for determining the inductive effectiveness of BABA elicitation on JA/ET-dependent defense. Throughout the whole incubation period, the treatment of *B. cinerea*-inoculated grapes with 10 mmol L^{-1} BABA did not induce the significant change in the transcript levels of *VvPDF1.2*, *VvHEL,* and *VvTHI2* compared with the only *B. cinerea*-inoculated samples (**Figures 3F,H,J**). On the contrary, an inductive effect of BABA on gene expressions of *VvVSP1*, *VvFAD3*, *VvERS1,* and *VvERF1* could be observed in the BABA-stimulated and subsequent *B. cinerea*-challenged grapes (**Figures 3G,I,K,L**), indicating that the JA/ET-responsive genes were in part implicated in the BABA-induced priming defense.

FIGURE 2 | Nuclear localization of pEAQ-*VvHSP24*-GFP fusion protein. The fluorescence signals of pEAQ-GFP and pEAQ-*VvHSP24*-GFP fusion protein were observed with onion peels after transformation by *Agrobacterium tumefaciens*. Nuclei were visualized by staining onion peels with 4,6-diamino-2-phenylindole (DAPI), and GFP signals were captured using LSM (bars = 70 μm).

Effect of BABA Treatment on Individual Phytoalexin Content in Grapes

To assess whether BABA-fueled defense was related to phytoalexin biosynthesis in grape berries, the contents of the dominant stilbenes in *Vitis vinifera* species, including *trans*-resveratrol and its oligomer ε-viniferin, were measured (Sarig et al., 1997). As depicted in **Figure 4**, the *trans*-resveratrol and ε-viniferin contents in the controls fluctuated within a very small range (from –23.72 to 17.06%) around their average values over 5 days at 20°C. BABA treatment alone was sufficient to promote stilbene synthesis in grapes, and subsequent *B. cinerea* inoculation resulted in an obvious enhancement of *trans*-resveratrol and ε-viniferin compared with the controls. It is worth noting that the amount of phytoalexin was higher in fruit treated with BABA elicitation in combination with *B. cinerea* suspension than that in non-*B. cinerea*-inoculated or non-BABA-treated grapes within 5 dpi, indicating the BABA elicitation exhibited a positive regulation on stilbene phytoalexin biosynthesis in grapes.

Direct Interaction Between *VvHSP24* and *VvNPR1*

The Y2H system was used to identify whether physical interaction occurs between *VvHSP24* and *VvNPR1* *in vivo*; meanwhile, the interaction between *VvHSP24* and *VvNPR1* *in vitro* was conducted by His pull-down. As shown in **Figure 5A**, colonies of competent yeast AH109 cotransfected with the BD-*VvHSP24* and AD-*VvNPR1* vectors grew within 4–6 days on synthetic dropout plates (SD/-Trp-Leu-His and SD/-Trp-Leu-His-Ade), and these cotransformants were blue on X-α-gal plates. Few or no colonies carrying BD-*VvHSP24* plus the empty pGADT7 vector or AD-*VvNPR1* plus the empty pGBKT7 vector were obtained. Therefore, the interaction between *VvHSP24* and *VvNPR1* was confirmed by the successful activated GAL4-responsive reporter genes.

Figure 5B illustrated that two intense bands observed at the position corresponding to the estimated sizes of His-*VvHSP24* (37 kD) or GST-*VvNPR1* (98 kD) when incubated with anti-His or anti-GST antibodies, respectively. For detection of a pulled down protein, a His-tagged *VvHSP24* fragment protein immobilized on Ni sepharose 6 Fast Flow was used as bait against the GST-*VvNPR1* fusion protein. As shown in lanes 7 and 8 of **Figure 5B**, GST-*VvNPR1* was pulled down by the His-*VvHSP24* fusion protein but not by the unfused His; hence, the *VvHSP24* protein physically interacted with *VvNPR1* *in vitro*.

VvHSP24 Cannot Directly Regulate the Transcript of *PR* Genes

qRT-PCR results showed that *PR* genes such as *PR1*, *PR2,* and *PR5* display similar long-term potentiation (LTP) trends as *VvHSP24* after BABA elicitation (**Figure 3**). In particular, previous studies have shown that HSFs/HSPs regulate the transcript of the target genes primarily by interacting with HSF sequence-binding elements (HSEs) (Nover et al., 2001; Guertin and Lis, 2010). HSEs (5′-GAAnnTTC-3′) were detected in the promoters of all *PR* genes (**Supplementary Data 1**). Hence, we tested the possibility whether *VvHSP24* could directly control *PR* gene transcripts. Unexpectedly, the His-*VvHSP24* fusion protein was unable to bind the biotin-labeled probes targeting the HSE motifs from the *PR1*, *PR2,* and *PR5* promoters; indeed, mobility shifts were not observed, and the addition of mutated HSEs had no
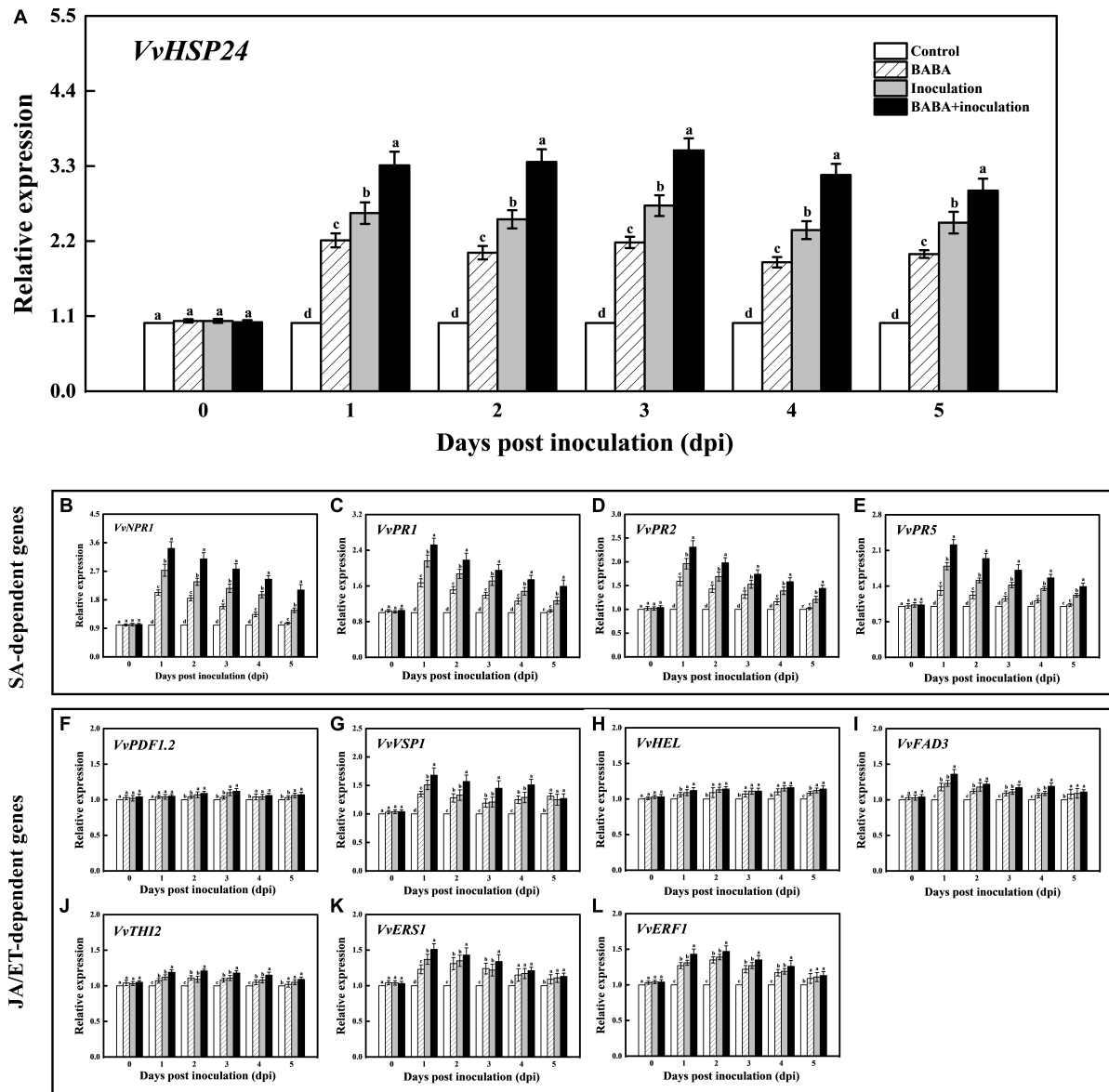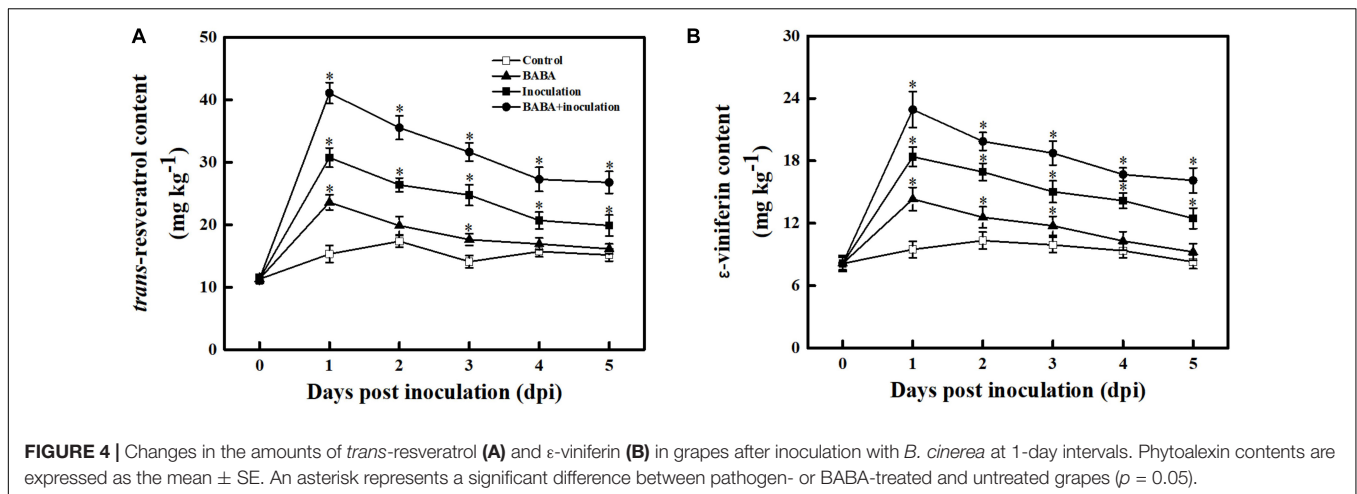
FIGURE 3 | Transcript levels of *VvHSP24* **(A)**, SA-dependent *VvNPR1* **(B)**, *VvPR1* **(C)**, *VvPR2* **(D)** and *VvPR5* **(E)** genes and JA/ET-dependent *VvPDF1.2* **(F)**, *VvVSP1* **(G)**, *VvHEL* **(H)**, *VvFAD3* **(I)**, *VvTHI2* **(J)**, *VvERS1* **(K),** and *VvERF1* **(L)** genes in grapes after inoculation with *B. cinerea* for 1-day intervals. Relative mRNA expression levels are expressed as the mean ± SE. Different letters above the bar represent a significant difference between treatments (*p* = 0.05).

effect (**Figure 6**). However, a mobility shift was produced when biotin-EBNA control DNA was incubated with EBNA extract. Thus, the *VvHSP24* could not directly regulate the transcript of *PR* genes.

Heterologous Expression of *Vitis vinifera* HSP24 Enhances the Resistance of *Arabidopsis* to *B. cinerea* and Mitigates Cell Death in Transgenic Leaves

As previously reported, *AtHSFB1* is a critical player in the development of defense priming (Pajerowska-Mukhtar et al.,

2012; Pick et al., 2012). To examine the effectiveness of *VvHSP24* (an orthologous gene of *AtHSFB1* from *Vitis vinifera*) in necrotrophic pathogen-induced defense responses, transcript levels of *VvHSP24* and pathogenesis-related genes (NPR1, PR1, PR2, and PR5) in *B. cinerea*-inoculated WT plants and *VvHSP24*-overexpressing lines were monitored. qRT-PCR analysis showed that *VvHSP24* gene expression was significantly enhanced in the *VvHSP24*-overexpressing lines, especially in OE-5, OE-7, and OE-10, in comparison with Col-0 WT (**Figures 7A,B**). As shown in **Figure 7E**, mRNA levels of *AtNPR1*, *AtPR1*, *AtPR2,* and *AtPR5* were upregulated in the three *VvHSP24*-overexpressing lines (OE-5, OE-7,

FIGURE 4 | Changes in the amounts of *trans*-resveratrol **(A)** and ε-viniferin **(B)** in grapes after inoculation with *B. cinerea* at 1-day intervals. Phytoalexin contents are expressed as the mean ± SE. An asterisk represents a significant difference between pathogen- or BABA-treated and untreated grapes ($p = 0.05$).

and OE-10) at 3 and 6 dpi compared with WT plants. To determine pathogen growth- and cell death-inducing activity, we further observed *B. cinerea*-inoculated leaves from WT and *VvHSP24*-transgenic plants by imaging after staining with lactophenol trypan blue. At 6 days after *B. cinerea* inoculation, almost all of the cells of WT leaves were basically dead and stained with trypan blue; in contrast, lesions and necrotic areas were visibly reduced in all transgenic lines with respect to dot-inoculated WT plants (**Figures 7F,G**). Coincidently, cell electrolyte leakage from transgenic leaves infiltrated with the *B. cinerea* conidial suspension was also significantly lower than that from WT plants (**Figure 7H**). Of note, heterologous expression of HSP24 from *Vitis vinifera* apparently retarded the growth of transgenic *Arabidopsis* plants, as displayed with dwarf leaves, smaller leaf area and less biomass in the transgenic lines than in the WT plants (**Figures 7C,D**). These data reflected that overexpression of *VvHSP24* can obviously alleviate cell damage and disease progression.
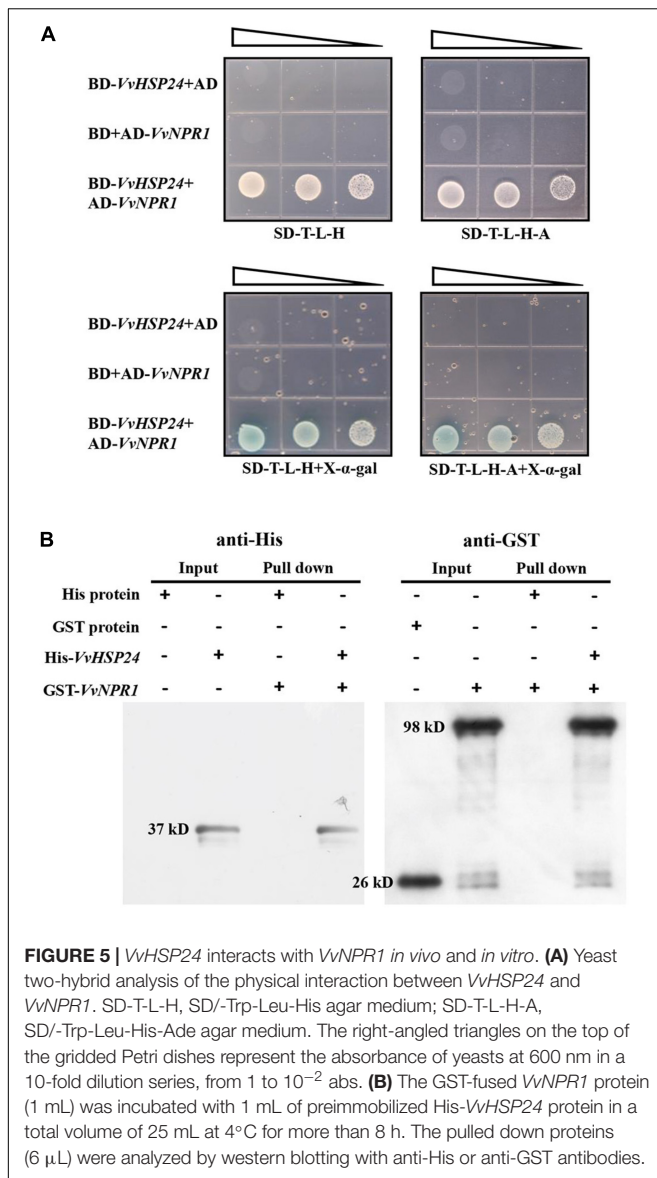
AtHSFB1 Mutants With Decreased Resistance in Response to Fungal Pathogen

Given that *AtHSFB1* is a *VvHSP24* orthologous gene in *Arabidopsis*, we harvested *Arabidopsis AtHSFB1* mutant seedlings constructed via the CRISPR-Cas9 system to evaluate the disease resistance of HSP through mutant-based verification. As depicted in **Figures 8A,B**, *AtHSFB1* transcript in all CRISPR mutants, especially in CRP-1, CRP-2, and CRP-6, were largely not expressed or drastically decreased compared with that in WT plants. Interestingly, the dominant CRISPR mutants CRP-1, CRP-2, and CRP-6 showed no apparent alteration in growth phenotypes compared with those in the WT (**Figures 8C,D**). In addition, CRISPR/Cas9-mediated *AtHSFB1* knockout lines displayed similar kinetics and intensity of pathogenesis-related gene (NPR1, *PR1*, *PR2,* and *PR5*) transcript accumulations, and all were slightly or significantly decreased when compared with the WT at 3 and 6 dpi (**Figure 8E**). Consistent

with this observation, the symptoms of fungal infection and staining level of the dot-inoculated mutants were stronger than those of WT leaves (**Figures 8F,G**). Thus, the *AtHSFB1* mutation impaired the defense expression and led to a pathogenic sensitiveness.

DISCUSSION

As sessile organisms, plants are frequently subjected to a fluctuating environment and exhibit a high degree of elasticity in survival under various stresses, suggesting broad crosstalk between plant defense responses and other biochemical and physiological processes (Khare et al., 2020). Of note, hyperactivation of resistance not only cannot terminate the wide variety of disease threats but also may entail an irreversible cost that might suppress the growth, fruit set, yield and quality of horticultural crops (Vos et al., 2013). Priming has been found to be an ecological approach of resistance owing to its capacity to respond promptly to a certain degree of biotic stress; thus, priming can balance the specific resistance and the resulting fitness costs or quality impairment in crops (Conrath et al., 2015; Ton et al., 2009). The induction of BABA on biotic resistance has been researching over half a century (Cohen et al., 2016). In this study, the resistance against *B. cinerea* fungal infection of postharvest grapes elicited by BABA at 10 mmol L^{-1} likely paralleled a priming mode. In fact, its definite role in the transient activation antimicrobial protein expression, particularly pathogenesis-related proteins (including *VvNPR1*, *VvPR1*, *VvPR2*, and *VvPR5*), and the synergistic stimulation of host-synthesized antifungal compounds such as *trans*-resveratrol and ε-viniferin occurred exclusively after combined therapy (BABA pretreatment with fungal infection; **Figures 1**, **3**, **4**; Thevenet et al., 2017). Although the infection process of the gray mold fungus *B. cinerea* could not be completely abolished by 10 mmol L^{-1} BABA, disease development was appreciably attenuated, leading to the smaller lesions and lower disease incidences during 5 days of the incubation at 20°C (**Figure 1** and **Supplementary Figure 1**). Similarly, such priming resistance

FIGURE 5 | *VvHSP24* interacts with *VvNPR1* in vivo and in vitro. **(A)** Yeast two-hybrid analysis of the physical interaction between *VvHSP24* and *VvNPR1*. SD-T-L-H, SD/-Trp-Leu-His agar medium; SD-T-L-H-A, SD/-Trp-Leu-His-Ade agar medium. The right-angled triangles on the top of the gridded Petri dishes represent the absorbance of yeasts at 600 nm in a 10-fold dilution series, from 1 to 10^{-2} abs. **(B)** The GST-fused *VvNPR1* protein (1 mL) was incubated with 1 mL of preimmobilized His-*VvHSP24* protein in a total volume of 25 mL at 4°C for more than 8 h. The pulled down proteins (6 μL) were analyzed by western blotting with anti-His or anti-GST antibodies.

against pathogen stresses in *Arabidopsis*, peach and bayberry has been observed for other priming elicitors, such as methyl jasmonate (MeJA), 2,4-epibrassinolide (EBR), benzothiadiazole (BTH), and chitosan (Beckers et al., 2009; Wang et al., 2014; De Vega et al., 2020; Li et al., 2020a,b). In addition, Wang et al. (2016) reported an *in vitro* lethal activity of BABA on the cell membrane of *B. cinerea*. Thus, the combination of the enhanced host disease resistance elicited by BABA and its direct toxic effect on pathogens supports the inherent role of BABA as a defense inducer. Although the BABA conferred a positive resistance on disease development caused by *B. cinerea*, the combination strategy of BABA and other chemical or physical inducers should be tested for gaining a more satisfactory protection from fungal attack.

Specifically, primed grapes presented much stronger transcript levels of SA-responsive defense-related genes, such

as *VvPR1*, *VvPR2*, and *VvPR5*, and stimulated disease resistance upon the artificial *B. cinerea*-inoculation over the observation period. We speculate that the mode of manner of BABA-elicited defense in grapes may be attributed to a SAR-like defense response. Although Nie et al. (2017) have recently pointed out that JA/ET-dependent signaling pathways have been shown to be involved in systemic resistance against *B. cinerea* in *Arabidopsis*, a set of JA/ET-dependent defensive genes (*VvPDF1.2*, *VvHEL*, and *VvTHI2*) were not promoted in *B. cinerea*-inoculated grapes after the BABA elicitation in our present study. In contrast, the transcriptional data exhibited a determinable induction of BABA on gene expressions of *VvVSP1*, *VvFAD3*, *VvERS1*, and *VvERF1* in grapes. Noticeably, either of *VvPDF1.2*, *VvTHI2*, or *VvHEL* can directly participate in defense expression against biotic stress (Oh et al., 1999; Bertini et al., 2012), but the other JA/ET-dependent genes including *VvVSP1*, *VvFAD3*, *VvERS1*, and *VvERF1* have been reported to be relevant to the plant development, ethylene perception, metabolic process or mechanical wounding healing, rather than the direct pathogenic response (Nath and Watson, 1980; Puttick et al., 2009; Liu and Wen, 2012). Hence, this BABA-IR in grapes is mainly associated with the expression of SA-dependent SAR. These results were in part consistent with our most current findings, showing that the BABA-activated resistance in grapes and peaches is dependent on SA signaling but not the JA/ET signaling pathways (Li et al., 2020c; Wang et al., 2021). Takahashi et al. (2004), Koornneef et al. (2008), and van der Does et al. (2013) also elucidated that the SA-dependent SAR reaction suppressed the transduction of JA signaling and the corresponding expression levels of JA-dependent defensive genes in model plant *Arabidopsis thaliana*. However, the obvious increases in the transcript levels of *VvVSP1*, *VvFAD3*, *VvERS1*, and *VvERF1* in BABA-treated grapes provided a valuable clue that BABA may affect the fruit physiology and quality due to the genetic regulation of metabolic flow between the fruit defense and development, which will lead us to research the relationship between the induced resistance and its allocated metabolic influence. Coincidentally, the protection of *Arabidopsis* and tobacco plants by BABA against infection by the necrotrophic fungus *B. cinerea*, virulent pathogen tobacco mosaic virus (TMV) or avirulent pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) through the burst of the SA-dependent SAR pathway has been reported (Siegrist et al., 2000; Zimmerli et al., 2001; Slaughter et al., 2012). These results suggest that BABA-IR might be dependent on the SA-dependent defense pathway and several potential factors may propagate the SA signal.

Heat shock proteins are members of multigene families encoding molecular chaperones, and have long been recognized as heat stress-inducible proteins. However, a large number of evidence have pointed out that HSPs become activated when plants undergo other stresses, including drought, salt, cold, heavy metals, oxidative stresses and pathogen invasion, demonstrating extensive transcriptional overlap among heat and non-heat stress response pathways, and the essential role of HSPs/chaperones in the crosstalk of multistress resistance (Swindell et al., 2007; Jacob et al., 2017). A report by Pick et al. (2012) indicated that HSFB1, a class B heat shock factor, promotes primed expression of a subset of defense-related
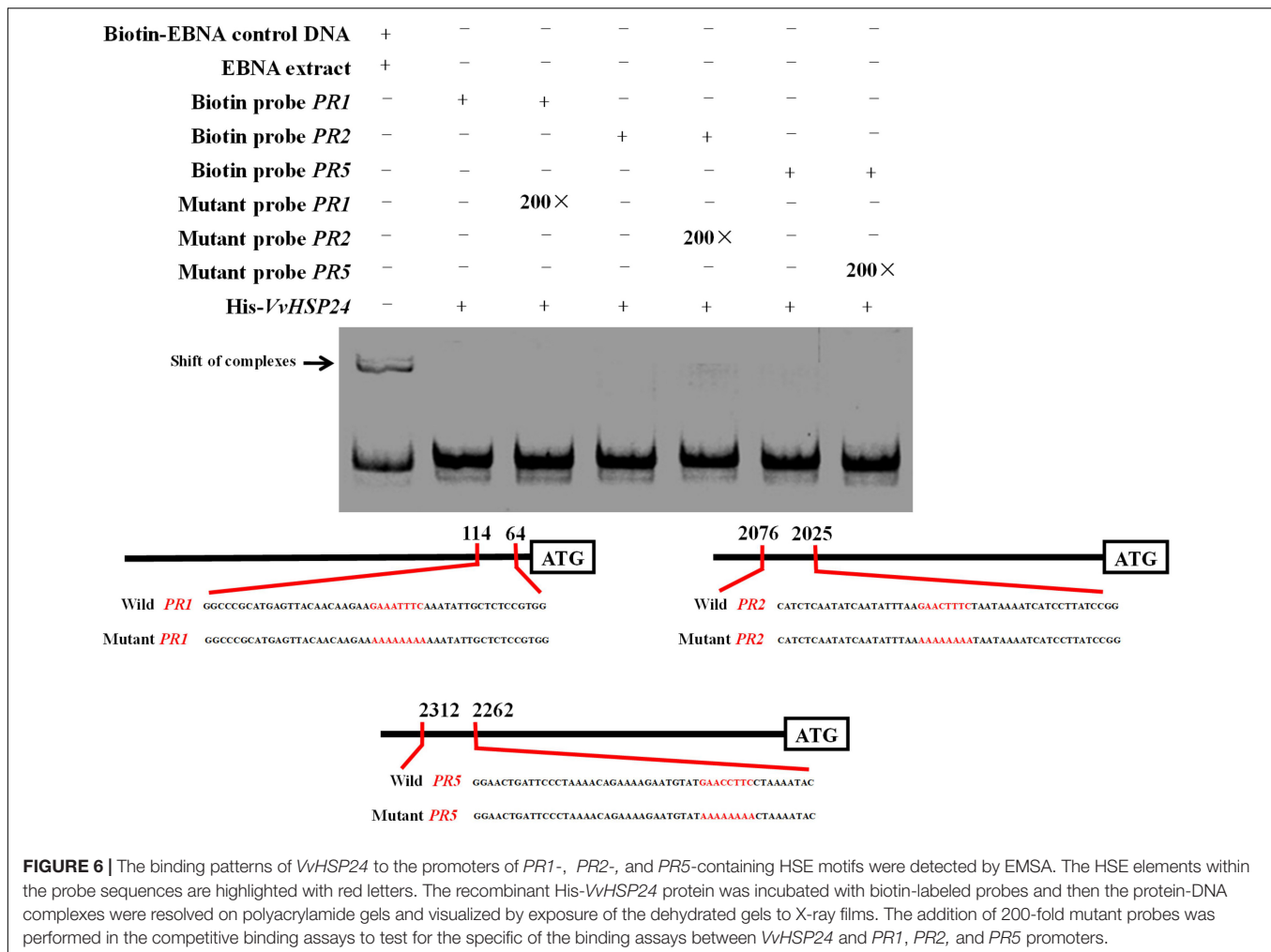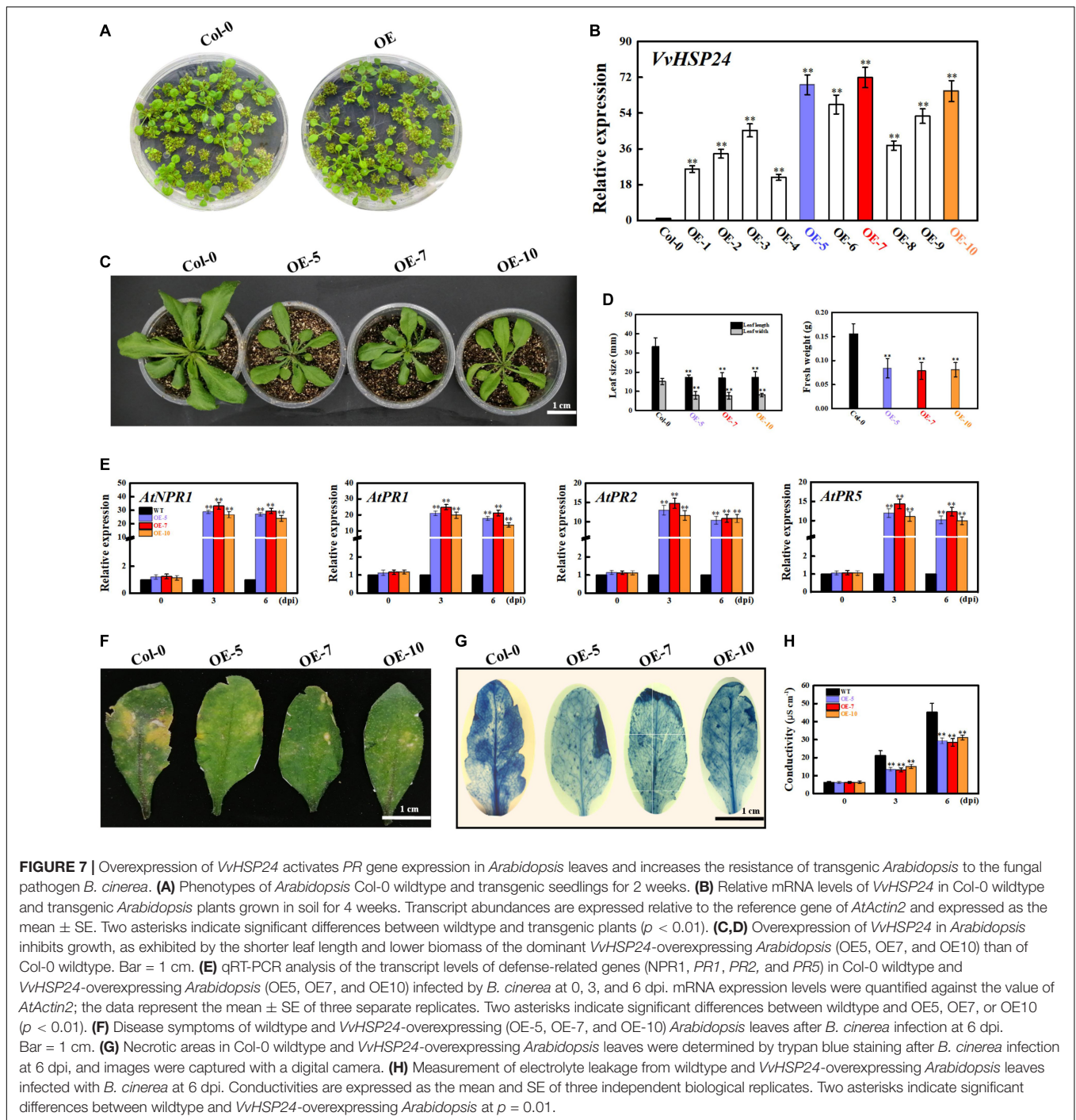
Biotin-EBNA control DNA	+	−	−	−	−	−
EBNA extract	+	−	−	−	−	−
Biotin probe *PR1*	−	+	+	−	−	−
Biotin probe *PR2*	−	−	−	+	+	−
Biotin probe *PR5*	−	−	−	−	−	+
Mutant probe *PR1*	−	−	200×	−	−	−
Mutant probe *PR2*	−	−	−	−	200×	−
Mutant probe *PR5*	−	−	−	−	−	200×
His-*VvHSP24*	−	+	+	+	+	+

FIGURE 6 | The binding patterns of *VvHSP24* to the promoters of *PR1-*, *PR2-*, and *PR5*-containing HSE motifs were detected by EMSA. The HSE elements within the probe sequences are highlighted with red letters. The recombinant His-*VvHSP24* protein was incubated with biotin-labeled probes and then the protein-DNA complexes were resolved on polyacrylamide gels and visualized by exposure of the dehydrated gels to X-ray films. The addition of 200-fold mutant probes was performed in the competitive binding assays to test for the specific of the binding assays between *VvHSP24* and *PR1*, *PR2,* and *PR5* promoters.
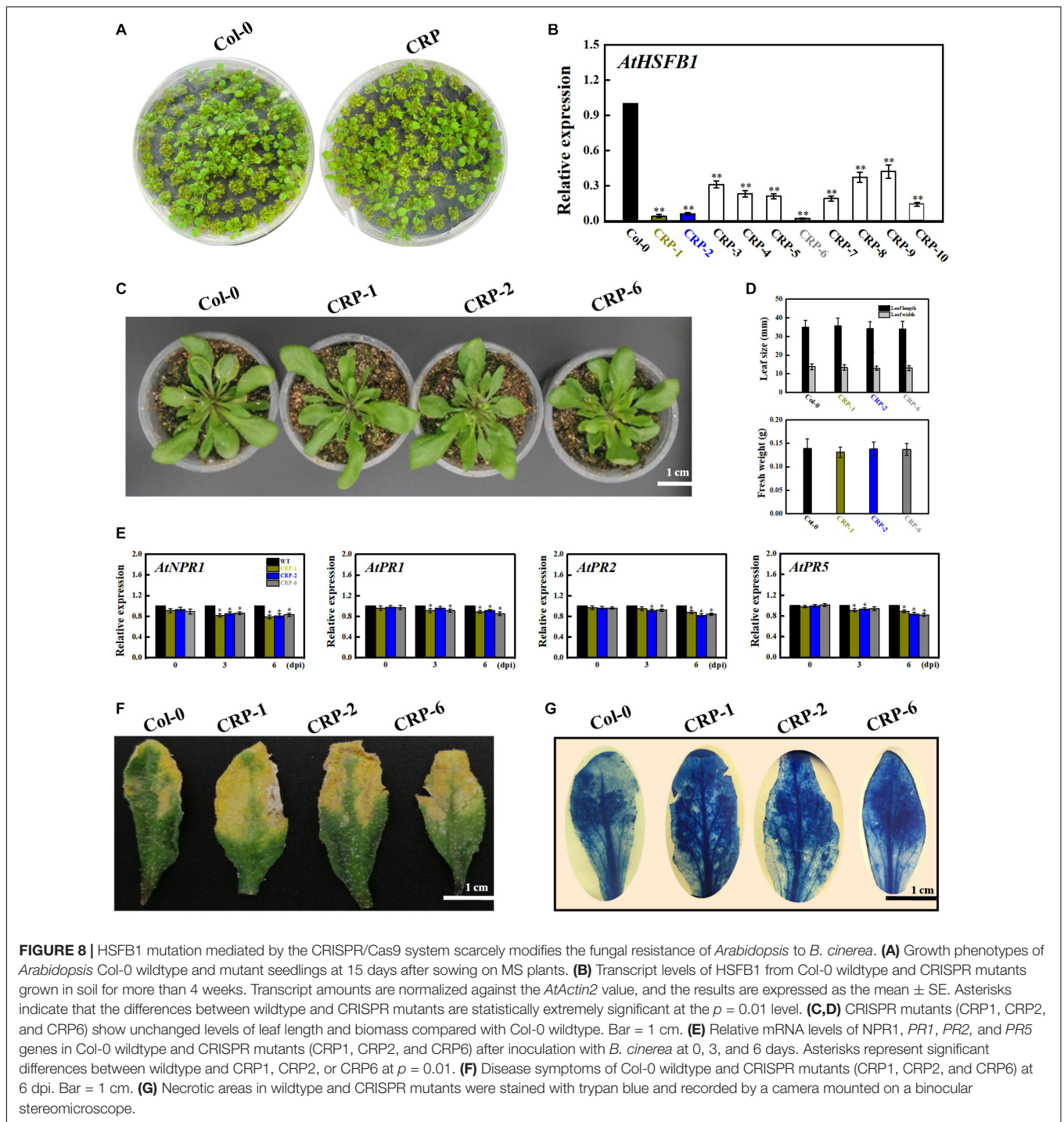
genes and is a potential candidate for SAR in *Arabidopsis* against *Pseudomonas syringae*. Windram et al. (2012) also implied a crosstalk mediating heat stress and *B. cinerea* infection. Similarly, a series of notable transcriptomic analysis by Sham et al. (2014, 2015, 2017) have emphasized that HSPs shared signaling pathways responding to biotic and abiotic stresses in *Arabidopsis*. In those cases, the specific HSP17.4 or HSP26.5-P was up- or down-regulated by the *B. cinerea* infection respectively, indicating that HSPs may be implicated in defense function. Hence, to explore whether a credible heat shock factor that also participates in the development of defense priming and SAR in grape berries might exist, we first comparatively analyzed the phylogenetic relationship between *Vitis vinifera* HSFs/HSPs and *Arabidopsis thaliana* HSFs (*AtHSFs*). A HSP (*VvHSP24*) with close sequence similarity to *AtHSFB1* from *Arabidopsis* was identified and isolated (**Supplementary Figure 3**). Multiple alignment and conserved domain analysis of *VvHSP24* and *AtHSFB1* further revealed that both factors contain a highly conserved HSF_DNA-binding domain consisting of a total of 94 amino acids. Furthermore, *VvHSP24* and *AtHSFB1* group into class B, indicating that *VvHSP24*, as an ortholog of *AtHSFB1*, might exert a certain effect

on SAR in grapes against the necrotrophic fungus *B. cinerea* (**Supplementary Figure 3**). Indeed, we found that treatment of grapes with a formulation of BABA increased the expression of *VvHSP24* mRNA by several-fold, and the transcript level of *VvHSP24* was significantly activated by *B. cinerea* fungal infection (**Figure 3A**). Together, these observations support our assumption that *VvHSP24* is a candidate for BABA-induced systemic resistance in grape berries. Intriguingly, most *npr1* (for non-expresser *PR* genes) alleles and NPR1 paralog null mutants exhibit enhanced disease susceptibility phenotype and are incapable of expressing *PR* genes (including *PR1*, *PR2,* and *PR5*) or developing SAR in their response to SA or its analog INA (2,6-dichloroisonicotinic acid) and benzothiadiazole (BTH); this reveals the positive function of NPR1 in SA-mediated activation of *PR* genes and SAR development (Canet et al., 2010). Because the dominant transgenic *Arabidopsis* plants overexpressing *VvHSP24* exhibited potentiated resistance to necrotrophic fungal pathogens, including dozen-fold increased expression of *VvNPR1* and the resulting constitutive expression of SA-responsive *PR1*, *PR2,* and *PR5* genes and decreased levels in necrotic areas and cell death (**Figures 7E–H**), it can be deduced that *VvHSP24* functions in the defense signaling

FIGURE 7 | Overexpression of *VvHSP24* activates *PR* gene expression in *Arabidopsis* leaves and increases the resistance of transgenic *Arabidopsis* to the fungal pathogen *B. cinerea*. **(A)** Phenotypes of *Arabidopsis* Col-0 wildtype and transgenic seedlings for 2 weeks. **(B)** Relative mRNA levels of *VvHSP24* in Col-0 wildtype and transgenic *Arabidopsis* plants grown in soil for 4 weeks. Transcript abundances are expressed relative to the reference gene of *AtActin2* and expressed as the mean ± SE. Two asterisks indicate significant differences between wildtype and transgenic plants ($p < 0.01$). **(C,D)** Overexpression of *VvHSP24* in *Arabidopsis* inhibits growth, as exhibited by the shorter leaf length and lower biomass of the dominant *VvHSP24*-overexpressing *Arabidopsis* (OE5, OE7, and OE10) than of Col-0 wildtype. Bar = 1 cm. **(E)** qRT-PCR analysis of the transcript levels of defense-related genes (NPR1, PR1, PR2, and PR5) in Col-0 wildtype and *VvHSP24*-overexpressing *Arabidopsis* (OE5, OE7, and OE10) infected by *B. cinerea* at 0, 3, and 6 dpi. mRNA expression levels were quantified against the value of *AtActin2*; the data represent the mean ± SE of three separate replicates. Two asterisks indicate significant differences between wildtype and OE5, OE7, or OE10 ($p < 0.01$). **(F)** Disease symptoms of wildtype and *VvHSP24*-overexpressing (OE-5, OE-7, and OE-10) *Arabidopsis* leaves after *B. cinerea* infection at 6 dpi. Bar = 1 cm. **(G)** Necrotic areas in Col-0 wildtype and *VvHSP24*-overexpressing *Arabidopsis* leaves were determined by trypan blue staining after *B. cinerea* infection at 6 dpi, and images were captured with a digital camera. **(H)** Measurement of electrolyte leakage from wildtype and *VvHSP24*-overexpressing *Arabidopsis* leaves infected with *B. cinerea* at 6 dpi. Conductivities are expressed as the mean and SE of three independent biological replicates. Two asterisks indicate significant differences between wildtype and *VvHSP24*-overexpressing *Arabidopsis* at $p = 0.01$.

pathway upstream of *VvNPR1*. This scenario would require the generation of the corresponding *VvHSP24* mutation for confirmation. However, constitutive expression of NPR1 and SA-responsive genes in the CRISPR mutants of *AtHSFB1* (an orthologous gene of *VvHSP24* in *Arabidopsis*) was only slightly suppressed compared with that in the WT (**Figure 8E**). This finding elucidated that the NPR1-conferred resistance phenotype does not depend on HSP/chaperone; thus, HSP/chaperone is not a direct upstream regulator of NPR1.

If HSP/chaperone is not an upstream regulator of NPR1, where does it function in the defense signaling pathway? The presence of ankyrin repeats but the lack of a DNA-binding domain indicate that NPR1 may execute its regulatory role in SAR through interaction with other proteins (Dong et al., 2001). Therefore, it is possible that HSP24 physically interacts with NPR1 to stimulate the development of SAR against *B. cinerea* fungal infection in grape berries. As depicted in **Figures 2, 5**, *VvHSP24* was identified as a nuclear chaperone that interacted

FIGURE 8 | HSFB1 mutation mediated by the CRISPR/Cas9 system scarcely modifies the fungal resistance of *Arabidopsis* to *B. cinerea*. **(A)** Growth phenotypes of *Arabidopsis* Col-0 wildtype and mutant seedlings at 15 days after sowing on MS plants. **(B)** Transcript levels of HSFB1 from Col-0 wildtype and CRISPR mutants grown in soil for more than 4 weeks. Transcript amounts are normalized against the *AtActin2* value, and the results are expressed as the mean ± SE. Asterisks indicate that the differences between wildtype and CRISPR mutants are statistically extremely significant at the $p = 0.01$ level. **(C,D)** CRISPR mutants (CRP1, CRP2, and CRP6) show unchanged levels of leaf length and biomass compared with Col-0 wildtype. Bar = 1 cm. **(E)** Relative mRNA levels of NPR1, *PR1*, *PR2,* and *PR5* genes in Col-0 wildtype and CRISPR mutants (CRP1, CRP2, and CRP6) after inoculation with *B. cinerea* at 0, 3, and 6 days. Asterisks represent significant differences between wildtype and CRP1, CRP2, or CRP6 at $p = 0.01$. **(F)** Disease symptoms of Col-0 wildtype and CRISPR mutants (CRP1, CRP2, and CRP6) at 6 dpi. Bar = 1 cm. **(G)** Necrotic areas in wildtype and CRISPR mutants were stained with trypan blue and recorded by a camera mounted on a binocular stereomicroscope.

directly with the *VvNPR1* in both *in vivo* and *in vitro* systems, as expected. Additionally, the results of EMSA (**Figure 6**) show that *VvHSP24* did not have the capacity of activating the transcript of the *PR* genes directly, implying that the protein binding activity of *VvHSP24* to *VvNPR1* is a prerequisite for *PR* expressions and consequent defense functions. In contrast to the resistance of transgenic *Arabidopsis* plants overexpressing *VvHSP24* against fungal pathogens over 6 dpi, SA-responsive *PR* gene expressions

and resistance to the necrotrophic fungus *B. cinerea* in the CRISPR mutants CRP1, CRP2 and CRP6 were not constitutively repressed. Moreover, an interesting observation of our study is the finding that unlike the limited influence of CRISPR mutation on transgenic plants, the high production of *VvHSP24* in transgenic *Arabidopsis* overexpressing this gene can be beneficial for enhancing plant fungal resistance but can cause deleterious effects on plant growth (**Figures 7C,D**, **8C,D**). Such negative

influence of *VvHSP24* overexpression on biomass and leaf length may occur because *VvHSP24* initiates specific transcriptional reprogramming that prioritizes defense over growth-related costs. In fact, Pajerowska-Mukhtar et al. (2012) found that *AtHSFB1*, the *VvHSP24* orthologous gene in *Arabidopsis*, plays a major role in the growth-toward-defense transition. Although overexpressing the stress protein HSP24 distinctly elevated the expression levels of *NPR1* and SA-inducible genes, the CRISPR mutants only presented a slight decrease in NPR1, *PR1*, *PR2,* and *PR5* transcript levels. Conversely, a more persuasive hypothesis is that HSPs/chaperones might not separately affect the defense signaling pathway downstream of *VvNPR1*, leading to *PR* gene expressions and resistance phenotypes. One probable explanation is that a second protein or factor, in addition to HSP/chaperone, is required for NPR1 activation in the defense signaling pathway. The CRISPR mutants might not totally bypass the positive function of this second protein or factor. In support of this possibility, Yu et al. (2001), Desveaux et al. (2002), Després et al. (2003), and Dong (2004) demonstrated that in addition to TGAs, the single-stranded DNA-binding protein Whirly1 and WRKY transcription factors are involved in the NPR1-mediated SAR network. Similarly, our previous studies showed that the involvement of TGA1 and WRKY transcription factors in the activation of fungal resistance progresses in peaches and grapes in an NPR1-dependent manner (Li et al., 2020c,d; Wang et al., 2020). Indeed, the NPR1 protein tends to form a large-scale oligomer under natural conditions, which seriously impedes its cellular functions (Tada et al., 2008), and the main chaperone function of HSPs is to modulate the depolymerization of bound substrates (Wang et al., 2004; Haslbeck et al., 2015). Thus, the stress protein HSP24 might control an important switch that, depending on fungal stress, may differentially disaggregate NPR1 into an active monomer to regulate NPR1-dependent SA signaling pathway. Similarly, Ramakrishna et al. (2003) found that the HSP viscosity 1 (*visl*) acts a positive function in pectin depolymerization. Moreover, BABA-IR, as exhibited in our and other studies, is a crosstalk mechanism through a convergence of several signaling pathways, such as the SA, ABA, PI, JA, and ET pathways, further suggesting the existence of shared components other than NPR1, TGAs, WRKYs and Whirly among these defense pathways. In terms of the SA signaling pathway, our current results suggest that BABA-IR in grapes can be fueled by the *VvNPR1-VvHSP24* complex when *VvNPR1* oligomers become disaggregated, resulting in producing active monomers that propagate the SA signal and induce *PR* gene expressions.

CONCLUSION

We characterized a HSP, *VvHSP24*, as a potential candidate in NPR1-dependent plant resistance to the necrotrophic fungus *B. cinerea*. Furthermore, NPR1 is an essential regulator in the development of SAR and a key regulatory protein in activating transcripts of SA-inducible genes, but this capacity of *VvNPR1* might rely on posttranslational modification of the *VvHSP24* chaperone and their subsequent interaction after the perception of invading disease stress by plant cells. Our study

reports the previously unclarified function of HSPs/chaperones and offers insight for uncovering the regulatory network of disease resistance in agronomic fruits. Given that the HSPs usually generate upon the plant suffers from various stresses, further researches should concentrate on the elaboration of the explicit molecular or metabolic modes of the cross-tolerance in postharvest fruit. Meanwhile, identification of stress-related HSPs and their functional co-activator under biotic stress may be definitely conducive to the studies on induced resistance.

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

KW, SC, YZ, and FX conceived the experiments. CLi, SC, KW, CLe, NJ, YJ, and LQ conducted the experiments. CLi and KW analyzed the data and wrote the manuscript. All the authors read, commented, revised, and approved the final revision 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. 646147/full#supplementary-material

Supplementary Figure 1 | Tree analysis of the isolated *Botrytis cinerea* strain with the *Botrytis cinerea* family. Accession numbers of sequences: *Botrytis cinerea* B05.10, MH427722.1; *Botryotinia fuckeliana* T4, AJ422103.1; *Botrytis cinerea* Bo.CC166, MH427722.1; *Botrytis cinerea* strain B23, AJ422103.1; *Botrytis cinerea* BOT 40, KU145363.1; *Botrytis cinerea* BOT 5, KX772771.1; *Botrytis cinerea* BOT 16, KX781162.1; *Botrytis cinerea* BOT 61, KX781165.1; *Botryotinia fuckeliana* strain Minhang, AY694146.1; *Botrytis cinerea* BOT 74, KX781167.1; *Botrytis cinerea* strain SCB7-5, KR080285.1; *Botrytis cinerea* Bot 68, KU145391.1; *Botrytis cinerea* strain SCB2-2, KR080283.1; *Botrytis cinerea* strain SCB7-4, KP165498.1; *Botrytis cinerea* strain SCB5-2, KP165495.1; *Botrytis cinerea* strain BC-8, KP141792.1; *Botryotinia fuckeliana* species; AY674786.1; *Botrytis cinerea* strain KBC-13, KP141795.1; *Botrytis cinerea* strain SL,

KU936083.1; *Botrytis cinerea* strain BC-10, KP141793.1; *Botrytis cinerea* strain KBC-14, KP141794.1; *Botrytis cinerea* strain BC-1, KP141790.1; *Botrytis cinerea* strain AC1, KU936079.1; *Botrytis cinerea* strain AR, KU936082.1; *Botrytis cinerea* strain SV, KU936085.1.

Supplementary Figure 2 | Grape fruit displays significant differences in visual appearances under four treatments (control, BABA, inoculation, and BABA + inoculation). BABA treatment and pathogen inoculation were conducted independently twice, with similar results. Representative grapes were photographed and compared after the 5 dpi at 20°C.

Supplementary Figure 3 | Phylogenetic and domain analyses of *VvHSPs/AtHSFs*. **(A)** Three classes of HSFs/HSPs are shown in the phylogenetic tree. *VvHSP24* and *AtHSFB1* are in bold in purple and group into class B. The sequences of 57 HSFs/HSPs from *Vitis vinifera* and *Arabidopsis thaliana* were aligned with Clustal W, and the phylogenetic analysis was made with MEGA (NJ method; bootstrap of 1000). **(B)** Identical amino acids between *VvHSP24* and its closest related protein *AtHSFB1* are shaded in black. In addition, a single conserved HSF_DNA-binding domain consisting of a total of 94 amino acids was visualized by TBtools software; it occurs at residue ranges of amino acids 6–99 in *VvHSP24* and 11–104 in *AtHSFB1*.

Supplementary Figure 4 | Schematic diagram of the M2CRISPR/Cas9-*AtHSFB1* vector.

Supplementary Figure 5 | Heat map of *VvHSPs* in pairwise comparisons of BABA-vs.-control, inoculation-vs.-control, BABA + inoculation-vs.-control, and BABA + inoculation-vs.-inoculation.

Supplementary Figure 6 | His-tagged protein was detected by a gel image analysis (un-cropped gels).

Supplementary Figure 7 | In the His pull-down assay, GST-*VvNPR1* fusion protein was efficiently pull-downed by His-*VvHSP24* fusion protein (un-cropped gels).

Supplementary Figure 8 | EMSA of *VvHSP24* binding to the HSE elements of *PR1*, *PR2,* and *PR5* (un-cropped blots).

Supplementary Table 1 | Primer sequence of the used genes.

Supplementary Data 1 | Promoter sequences of *VvPR1*, *VvPR2*, and *VvPR5*. HSEs (GAAnnTTC) and the translation start site (ATG) are marked in red and yellow boxed, respectively.

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