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Research article

The jasmonate-ZIM domain gene *VqJAZ4* from the Chinese wild grape *Vitis quinquangularis* improves resistance to powdery mildew in *Arabidopsis thaliana*

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ABSTRACT

Grape (*Vitis vinifera* L.) is one of the most widely cultivated and economically important fruits. Most cultivated varieties of grape are highly susceptible to fungal diseases, and one of the most pervasive is powdery mildew, caused by *Uncinula necator*. The jasmonate-ZIM domain (JAZ) family proteins are critical for plant responses to environmental stresses. Here, we report the characterization of *VqJAZ4*, a jasmonate-ZIM domain gene isolated from *Vitis quinquangularis*, a Chinese wild *Vitis* species that exhibits high tolerance to several kinds of fungi. Subcellular localization assay indicated that the VqJAZ4 protein is targeted to the nucleus. The *VqJAZ4* gene was strongly induced by *U. necator* inoculation, as well as by the defense-related hormones methyl jasmonate (MeJA) and salicylic acid (SA). The upregulation of *VqJAZ4* after inoculation was dependent on its promoter sequences. Expression of *VqJAZ4* in *Arabidopsis thaliana* improved resistance to powdery mildew. Histochemical staining assays indicated that plants expressing *VqJAZ4* displayed a larger number of dead cells and stronger reactive oxygen species (ROS) burst than non-transgenic control (NTC) plants. Expression analysis of several disease-related genes suggested that *VqJAZ4* expression enhanced defense responses though SA and/or JA signaling pathways. We also found that *VqJAZ4*-expressing Arabidopsis showed increased susceptibility to *Botrytis cinerea*. Taken together, these results provide evidence that *VqJAZ4* may play an important role in response to fungal pathogens in grape, and may represent a candidate for future grape molecular breeding for disease resistance.

1. Introduction

Plants have evolved sophisticated strategies to overcome invasion by pathogens. The underlying molecular mechanisms associated with plant immune responses are intricate and complex. Two general and complementary defense systems have been characterized in plants. The first, termed pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), is activated by interaction between plant transmembrane proteins called pattern recognition receptors (PRRs) and PAMPs. The second defense system, effector-triggered immunity (ETI), is activated by recognition of pathogen effector proteins by host intracellular receptors. This is typically accompanied by a reactive oxygen species (ROS) burst and hypersensitive response (HR) including cell death. Both mechanisms lead to activation of downstream defense-related genes and crosstalk among different phytohormonal pathways (Jones and Dangl, 2006).

The phytohormone jasmonate (JA) has various functions during vegetative growth, development and senescence. JA can also act as a signal in plant response to wounding, pathogen infection and herbivore attack (Wasternack and Feussner, 2018). Jasmonoyl-L-isoleucine (JA-Ile) serving as the endogenous bioactive JA is perceived by a receptor complex consisting of the F-box protein CORONATINE INSENSITIVE1 (COI1) and several JASMONATE-ZIM DOMAIN (JAZ) transcriptional repressors (Chini et al., 2016; Fonseca et al., 2009; Sheard et al., 2010). An important advance in understanding the JA signaling pathway was the discovery of JASMONATE-ZIM DOMAIN (JAZ) transcriptional repressors (Chini et al., 2007; Thines et al., 2007). In the absence of JA-Ile, JAZ proteins inhibit downstream JA-responsive transcription

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factors, such as MYC2, by recruiting the corepressors TOPLESS (TPL) and TPL-related proteins (TPRs) through the adaptor protein Novel Interactor of JAZ (NINJA), as well as by hampering the interaction between MEDIATOR25 (MED25) and the transcription initiation complex (An et al., 2017; Zhang et al., 2015). In the presence of JA-Ile, COI1 interacts with JAZ proteins, leading to their proteolysis via the ubiquitin-26S proteosome pathway. This results in evacuation of NINJA-TPL from transcription sites, and subsequent derepression of JA-dependent genes (Pauwels et al., 2010).

JAZ proteins share two highly conserved domains. The ZIM domain, characterized by a TIF[F/Y]XG motif, is typically located in the middle of the protein.This domain functions to mediate interactions with NINJA and regulate the dimerization among JAZ proteins (Chini et al., 2009; Chung and Howe, 2009). The Jas domain characterized by a $SLX_2FX_2KRX_2RX_5PY$ signature is near the carboxyl (C) terminus, and is critical for interaction with COI1 and most transcription factors (TFs). Two positively charged amino acids near the amino (N) terminus of the Jas domain were found to be necessary for JA-dependent COI1-JAZ interactions (Melotto et al., 2008). In addition, this domain contains the bipartite JAZ degron consisting of a conserved α-helix and a loop, which is essential for the perception of the hormone (Sheard et al., 2010).

To date, the molecular function of some JAZ proteins is well understood by genetic, biochemical and structural analyses. Extensive studies have revealed that JAZ proteins participate in a wide range of biological processes in plants, including development, secondary metabolites biosynthesis, and response to abiotic and biotic stresses. For example, three JAZ proteins from Arabidopsis (AtJAZ1, AtJAZ8, and AtJAZ11) were found to interact with AtMYB21 and AtMYB24, two R2R3-MYB transcription factors that participate in JA-induced stamen maturation (Song et al., 2011). A novel JAZ gene in tobacco, *NaJAZh*, positively regulated nicotine levels, and silencing of this gene via RNA interference caused leaf necrosis (Oh et al., 2012). A recent study indicated that in Catharanthus, JAZ proteins are also involved in the regulation of monoterpene indole alkaloids (MIAs) biosynthesis (Patra et al., 2018). Rice OsJAZ1 protein acts as a transcriptional regulator of *OsbHLH148*-mediated drought response (Seo et al., 2011). Coronatine (COR), a mimic of JA-Ile, is a virulence factor secreted by *Pseudomonas syringae* that can promote entry of bacteria into plant tissues (Katsir et al., 2008). During infection of Arabidopsis, AtJAZ5 collaborates with AtJAZ10 to restrain COR phytotoxicity and pathogen reproduction, but the associated molecular mechanisms remain unknown (De et al., 2016). An Arabidopsis decuple *jaz* mutant, *jazD*, defective in 10 JAZ genes, exhibited robust resistance to *Botrytis cinerea*, but showed slow growth (Guo et al., 2018).

Grapevine (*Vitis vinifera* L.) is a widely cultivated fruit crop that has a long history and wide range of uses, including fresh or possessed into raisins juice and wine. However, *V. vinifera* is well known for its high susceptibility to various fungal diseases which decrease yield and fruit quality. The causal agent of powdery mildew, *Uncinula necator* (Schw.) Burr., is a biotrophic pathogen that negatively impacts grapevine growth and fruit ripening (Wang et al., 1995). In recent years, extensive use of chemical fungicides to control powdery mildew has raised environmental concerns and has contributed to the evolution of fungicide-resistant strains. The development of genomic resources for grapevine, including a high-quality draft genome sequence, has facilitated novel approaches to control powdery mildew. China is one of the major grape-producing countries, and a Chinese wild grapevine species, *Vitis. quinquangularis*, has been reported to be tolerant to a number of fungal pathogens (Wang et al., 1995). This species therefore has been targeted as a resource for examining the defence-related genes. In the current study, we characterized the putative roles of JAZ4 from *V. quinquangularis* clone Shang-24 in resistance to powdery mildew. Expression pattern analysis showed that *VqJAZ4* can be strongly induced by defence-related phytohormone treatments and *U. necator* inoculation, suggesting that *VqJAZ4* may have an important role in plant biotic responses. In order to elucidate the biological functions, we over-expressed this gene in Arabidopsis and analyzed the responses of the transgenic lines to inoculation with *Golovinomyces cichoracearum* as well as *B. cinerea.*

2. Materials and methods

2.1. Plant material, growth conditions and pathogen

Vitis quinquangularis clone Shang-24 and *V. vinifera* cv. Red Globe plants were grown in the grapevine germplasm resource vineyard at Northwest A&F University, Yangling, Shaanxi, China (34°20′N, 108°24′E). *Arabidopsis thaliana* ecotype Columbia (Col-0) and *Nicotiana benthamiana* were grown in a growth chamber under the following conditions: 22 °C, 50% relative humidity, and a long-day photoperiod (16 h-light/8 h-dark). *Botrytis cinerea* was cultured on Potato Dextrose Agar (PDA) medium in the dark at 23 °C. *Golovinomyces cichoracearum* was grown on Arabidopsis *pad4* (*phytoalexin deficient 4*) mutant plants at 21 °C, 50% relative humidity and a long-day photoperiod (16 h-light/8 h-dark). *Uncinula necator* was maintained on leaves of *V. vinifera* cv. Cabernet Sauvignon.

2.2. Bioinformatic analysis

The conserved domains of VqJAZ4 were identified using the SMART program (http://smart.embl-heidelberg.de/). Open reading frame translations of JAZ genes from other plants were obtained from the Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/index.jsp), the *Oryza sativa* genome database (TIGR; http://rice.plantbiology.msu.edu) and the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment was performed by DNAMAN software (Version 5.2.2.0, Lynnon Biosoft, USA). A phylogenetic analysis was carried out using MEGA (version 5.05) software and the neighbor-joining (NJ) method, with bootstrap test replicated 1000 times.

2.3. Subcellular localization assays in Nicotiana benthamiana

The *VqJAZ4* open reading frame (ORF) lacking a termination codon was cloned into the pEarleyGate101 vector. The construction and non-modified vector control were transformed into *Agrobacterium tumefaciens* strain GV3101, and introduced into 4-week-old *Nicotiana benthamiana* leaves by infiltration as previously described (Withers et al., 2012). The infiltrated tobacco plants were grown for an additional 48 h in a growth chamber under 23 °C, 50% relative humidity and a long-day photoperiod (16 h-light/8 h-dark). DAPI (4,6-diamidino-2-phenylindole dihydrochloride) was used to identify the nucleus. Yellow fluorescent protein (YFP) signals were observed with a confocal microscope (Olympus FV1000, Japan).

2.4. Transcriptional activity assay

The Matchmaker™ Gold yeast two-hybrid system (Clontech, Mountain View, CA, USA) was used for transcriptional activity assays. The full-length coding sequence of *VqJAZ4* was cloned into the pGBKT7 vector. Interactions between pGBKT7-P53 and pGADT7-T7, as well as pGBKT7 vector, were used as positive and negative controls. All constructions were transformed into the Y2HGold yeast strain and selected on SD/-Trp and SD/-Trp/X-α-gal/Aba medium, respectively.

2.5. Phytohormone treatments and pathogen inoculation

To investigate the expression level of *VqJAZ4* under different hormone treatments and in response to pathogen, young leaves from *V. quinquangularis* clone Shang-24 plants were sprayed with a solution of 100 μM salicylic acid (SA), 50 μM methyl jasmonate (MeJA), or 0.5 g/L ethylene (ET). Leaves sprayed with sterile distilled water served as the control. Samples were collected at 0, 1, 6, 12 and 24 h after treatment. The procedure for inoculation with *U. necator* was as described previously (Wang et al., 1995). Briefly, naive leaves were lightly contacted with leaves harboring colonies of *U. necator* and kept in a controlled-climate room. Leaves contacted with pathogen-free leaves were used as the control. Samples were collected at 0, 24, 48, 72, 96 and 120 h after treatment. All experiments were performed with three biological and technological replicates.

2.6. RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from Arabidopsis and various tissues of grapevine using the EZNA Plant RNA Kit (Omega Bio-tek, USA, R6827-01), and first-strand cDNA was synthesized using Prime Script TMR Tase (TaKaRa Biotechnology, Dalian, China). qRT-PCR was performed on a Bio-Rad CFX96 TM instrument with SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Biotechnology), and cycling parameters were: 95 °C for 30s, 42 cycles of 95 °C for 10s, 60 °C for 30s. *VvACTIN1* and *AtACTIN2* were used as endogenous controls. At each time point of each treatment, five leaves from five plants were combined to form one sample. Three biological and technical replicates were analyzed for each experiment. Relative expression levels were analyzed with CFX software using the Normalized Expression Method. Gene-specific primers used for expression analysis are shown in Table S1.

2.7. Transient expression and GUS assays

The *VqJAZ4* promoter (from −1465 to −1) was cloned and inserted into the pC0380GUS vector to construct PVqJAZ4-GUS. Sequences were analyzed for potential *cis* elements using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plancare/html/). PCR primers used in the vector construction are listed in Table S1. Agrobacterium-mediated transient transformation assays were performed using the vacuum infiltration method (Xu et al., 2010). Detached leaves of Red Globe (*V. vinifera*) were kept at 23 °C for 48 h before inoculation with *U. necator*. At 24 h after inoculation, the grape leaves were collected and then immersed in β-glucuronidase (GUS) staining solution [0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 100 mM NaH_2PO_4, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Biosynth AG), 10 mM Na_2EDTA and 0.1% Triton X-100, pH 7.0] for detection of GUS activity. Chlorophyll was removed with 70% ethanol at 37 °C. Quantitative GUS assays were conducted with a Hitachi 850 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan) as previously described (Xu et al., 2010).

2.8. Heterologous expression of VqJAZ4 in arabidopsis

The coding sequence of *VqJAZ4* was amplified using oligonucleotide primers designed using the Ensembl Plants (http://plants.ensembl.org/index.html) resource, and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). After the correct sequence was confirmed by direct sequencing, the PCR products were introduced into the pCAMBIA-2300 vector (Cambia, Brisbane, QLD, Australia) under the control of the CaMV35S promoter. The fusion constructs were transformed into *A. tumefaciens* GV3101, and transformation of Arabidopsis (accession Col-0) was carried out using the floral dip method (Clough and Bent, 1998). Seeds from the T0 plants were screened on MS medium supplemented with 75 mg/L kanamycin. T3 homozygous lines from three independent transgenic lines were analyzed used for further experiments.

2.9. Pathogen inoculation assays in arabidopsis

Four-week-old, T3 transgenic lines and non-transgenic control (NTC) plants were inoculated with *G. cichoracearum* as previously described (Li et al., 2010). Leaf samples collected 0, 24, 72, and 120 h post-inoculation (hpi) were used for histochemical staining and for analyses of expression of defense-related genes. The number of spores generated was determined at 7 days post-inoculation (dpi), as previously described (Li et al., 2010). Specifically, infected leaves were weighed and placed in 50 mL tubes containing 40 mL of sterilized water and 0.02% of Tween 20, then tubes were vortexed thoroughly. The resulting spore suspension was diluted 1:10 with 0.02% Tween 20 solution and spores were counted with a hemocytometer under a dissecting microscope.

Inoculation with *B. cinerea* was carried out according to a protocol previously described with some modifications (Wan et al., 2015). The spore suspensions used for inoculation were obtained by harvesting the spores in a solution containing 4% maltose and 1% peptone, and then adjusted to a concentration of 1.5×10^6 spores/mL. Leaves from four-week-old Arabidopsis plants were inoculated with a 10 μL droplet of *B. cinerea* spore suspension. Inoculated leaflets were maintained in a growth chamber under high temperature and humidity. Leaf samples collected 0, 24, 48, and 72 hpi were used for histochemical staining and analyses of expression of defense-related genes.

2.10. Histochemical staining

Hydrogen peroxide (H_2O_2) accumulation was detected by diaminobenzidine (DAB) staining (Wan et al., 2015). Infected leaves were incubated in 1 mg/mL DAB solution (Sigma, Beijing), and kept in darkness for 8 h. Chlorophyll was removed by incubation in 80% (v/v) ethanol at 80 °C for 2 h. The measurement of H_2O_2 accumulation was carried out using a hydrogen peroxide kit, according to the manufacturer's instructions (Nanjing Bio Ins., Jiangsu, China).

Dead cells were visualized by trypan blue staining as previously described (Serrano et al., 2014). Leaves were soaked in boiled trypan blue solution (5 mg trypan blue powder dissolved in 5 mL glycerol, 5 mg phenol, 5 mL 85% (v/v) lactic acid and 5 mL sterile water) for 3 min, and decolored with 2.5 g/mL chloral hydrate for 3 days. For quantification of dead cells, Arabidopsis leaves (0.5 mm in diameter) were stained with 0.25% Evans blue (Sigma) for 30 min. The dye was extracted with 1 mL of 50% methanol supplemented with 1% SDS for 1 h at 50 °C. Absorbance at 600 nm was determined after a 10-fold dilution of the extracts (Ahn et al., 2007).

Aniline blue staining was performed to visualize callose deposition (Ellinger et al., 2013). Infected leaves were immersed into 95% (v/v) ethanol overnight and then transferred into aniline blue (dissolved in 150 mM k_2HPO_4 (pH 9.5)) for 24 h prior to microscopy (Olympus BX53, Tokyo, Japan). For quantitative examination of callose, Arabidopsis leaves were immersed in ethanol until the chlorophyll was gone. After centrifugation for 15 min at 10000 × *g*, the leaf pellet was resuspended in 320 μL DMSO and boiled for 30 min. The sample was supplemented with the solution [400 μL 0.1% (w/v) aniline blue, 600 μL 1 M glycine/NaOH (pH 9.5) and 200 μL 1 M HCl]. The control was not supplemented with aniline. The level of callose fluorescence was analyzed as described (Kohler et al., 2000).

2.11. Statistical analysis

Data was analyzed using Microsoft Excel (Microsoft Corporation, USA) and SigmaPlot (v. 12.5, Systat Inc., CA, USA). Significant differences were determined by Student's t-test and one-way ANOVA computed by SPSS 17.0 software (IBM China Company Ltd., Beijing). All experiments were conducted with three technical and biological replicates.

2.12. Accession numbers

The accession numbers of the genes used in this study are as follows: AtJAZ1 (AT1G19180), AtJAZ2 (AT1G74950), AtJAZ3 (AT3G17860), AtJAZ4 (AT1G48500), AtJAZ5 (AT1G17380), AtJAZ6 (AT1G72450), AtJAZ7 (AT2G34600), AtJAZ8 (AT1G30135), AtJAZ9 (AT1G70700), AtJAZ10 (AT5G13220), AtJAZ11 (AT3G43440), AtJAZ12 (AT5G20900), AtACTIN2 (AT3G18780), AtCOI1 (AT2G39940), AtMYC2 (AT1G32640), AtPDF1.2 (AT5G44420), AtVSP2 (AT5G24770), AtICS1 (AT1G74710), AtPR1 (AT2G14610), AtNPR1 (AT1G64280), AtLOX3 (AT2G35980); OsJAZ1 (AK061602), OsJAZ2 (AK073589), OsJAZ3 (AK070649), OsJAZ4 (AK120087), OsJAZ5 (AK061842), OsJAZ6 (AK065604), OsJAZ7 (AK108738), OsJAZ8 (AK065170), OsJAZ9 (AK103459) OsJAZ10 (AK059441) OsJAZ11 (AK107750) OsJAZ12(AK107003); SlJAZ1 (Solyc07g042170), SlJAZ2 (Solyc12g009220), SlJAZ3 (Solyc03g122190), SlJAZ4 (Solyc12g049400), SlJAZ5 (Solyc03g118540), SlJAZ6 (Solyc01g005440), SlJAZ7 (Solyc11g011030), SlJAZ8 (Solyc06g068930), SlJAZ9 (Solyc08g036640), SlJAZ10 (Solyc08g036620), SlJAZ11 (Solyc08g036660), SlJAZ12 (Solyc01g009740), VvJAZ1 (XM_002284819), VvJAZ2 (XM_002262714), VvJAZ3 (XM_003634778), VvJAZ4 (XM_002272327), VvJAZ5 (XM_002277733), VvJAZ6 (XM_002277769), VvJAZ7 (XM_002277916), VvJAZ8 (CBI30922), VvJAZ9 (XM_002277121), VvJAZ10 (XM_002263220), VvJAZ11 (XM_002282652), VvACTIN1 (AY680701), VqJAZ4 (MN172367).

3. Results

3.1. Cloning and characterization of VqJAZ4

The cloned VqJAZ4 cDNA was 861 bp long and would encode a protein of 286 amino acids. This protein contains a TIFY domain in the middle (residues 123–158) and a Jas domain near its C terminus (residues 227–256), both regions of which show strong amino acid sequence conservation among JAZ proteins from various plants. (Fig. 1A). VqJAZ4 shows only five amino acid differences from the homologous *Vitis vinifera* VvJAZ4 (Fig. S1). Protein sequences of VqJAZ4, together with JAZ proteins from Arabidopsis (*Arabidopsis thaliana;* AtJAZ1-12; Vanholme et al., 2007), rice (*Oryza sativa*; OsJAZ1-12; Ye et al., 2009), tomato (*Solanum lycopersicum*; SlJAZ1-12; Chini et al., 2017) and grape (*Vitis vinifera*; VvJAZ1-11; Zhang et al., 2012) were used to perform multiple sequence alignment and phylogenetic analysis. As shown in Fig. 1, AtJAZ1, AtJAZ2, and SlJAZ1 shared high sequence identity to VqJAZ4 (40%, 35.9% and 34.5%, respectively). Apart from VvJAZ4, VvJAZ9 had the closest relationship with VqJAZ4 in grape, with 41.5% sequence identity. Of the *Oryza sativa* proteins, VqJAZ4 was most homologous to OsJAZ7 (30.1%), and had similar homology with OsJAZ6 and OsJAZ8 (28.8% and 25.9% identity, respectively).

3.2. The VqJAZ4 protein is targeted to the nucleus

Most JAZ proteins in Arabidopsis studied to date have been shown to be localized in the nucleus, as have their interactions with downstream transcription factors such as MYC2 (Thines et al., 2007; Withers et al., 2012). In order to visually determine the subcellular localization of VqJAZ4, the coding region of *VqJAZ4* was tagged with a C-terminal YFP sequence and transiently expressed in *Nicotiana benthamiana* leaf epidermal cells. At 48 h after transformation, fluorescence in control cells transformed with YFP alone was observed in both the cytoplasm and nucleus, whereas epidermal cells transformed with VqJAZ4-YFP showed YFP fluorescence localized in the nucleus (Fig. 2A). We also evaluated its expression in vegetative and floral structures of *V. quinquangularis* clone Shang-24. As shown in Fig. 2B, *VqJAZ4* was relatively strongly expressed in the flower and root, moderately in the leaf and fruit, and was expressed relatively weakly in the stem. In addition, as a potential repressor in JA signaling pathway, VqJAZ4 did not exhibit any transcriptional activity in yeast (Fig. S2).

3.3. Expression analysis of VqJAZ4

In order to gain insight into potential function of *VqJAZ4*, we examined the expression profiles of *VqJAZ4* in response to phytohormone treatments and powdery mildew infection. The results showed that *VqJAZ4* was induced by MeJA and SA treatments (Fig. 3A and B), suggesting that this gene may be involved in JA and SA signaling pathways. *VqJAZ4* expression was not significantly altered following ET treatment, in spite of some minor decline in the process (Fig. 3C). After leaves were inoculated with *U. necator*, the transcript level of *VqJAZ4* increased between 48 hpi and 72 hpi, then decreased gradually until 120 hpi (Fig. 3D). These transcriptional responses indicated that *VqJAZ4* may be involved in the regulation of the responses to multiple phytohormones and powdery mildew.

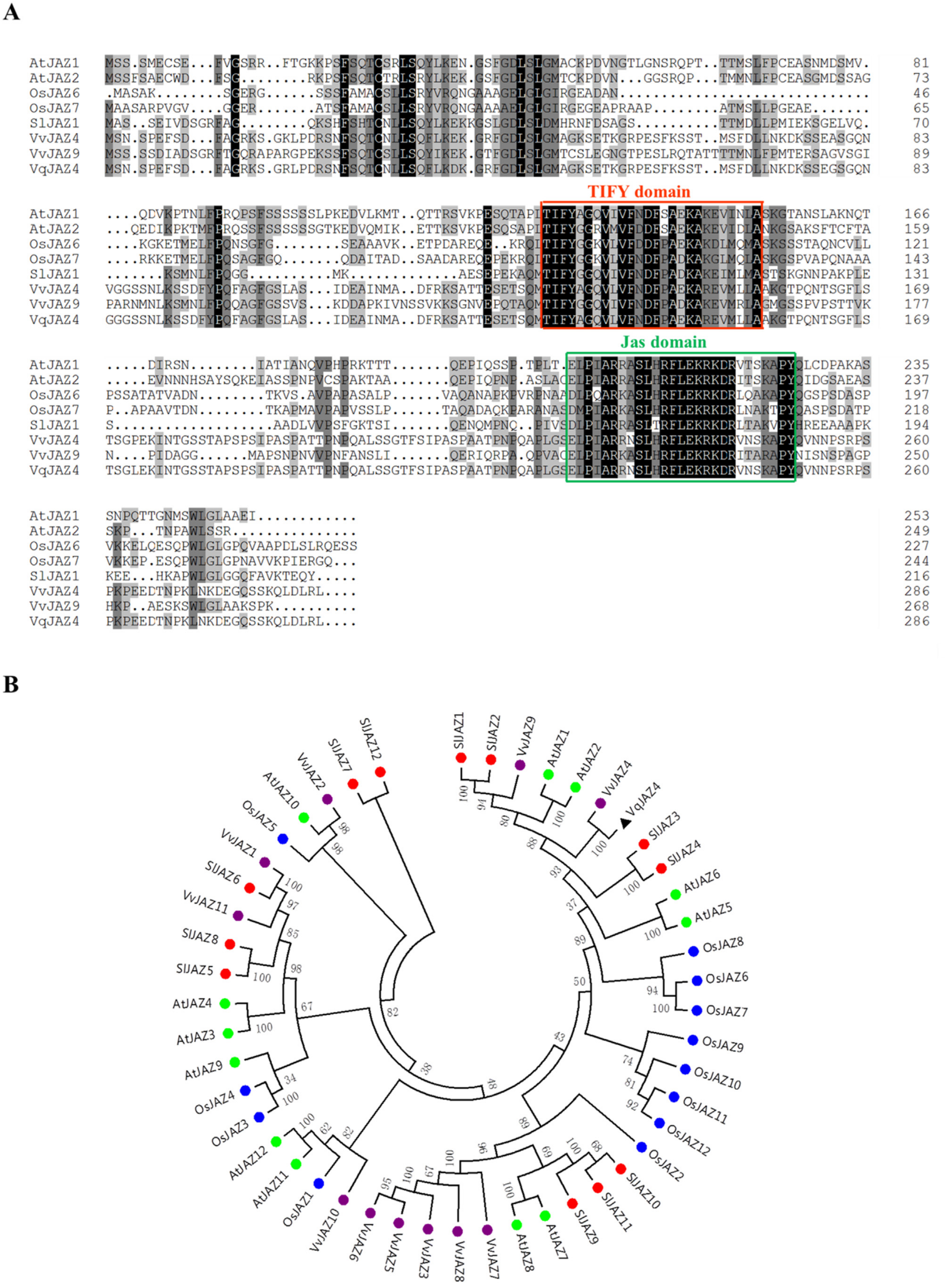
3.4. VqJAZ4 promoter activity is induced upon U. necator inoculation

To gain insight into regulation of the *VqJAZ4* gene, a 1465-bp segment of the *VqJAZ4* promoter was cloned from *V. quinquangularis* clone Shang-24. This region contained several potential *cis*-acting elements, including a CAAT-box and two TATA-boxes typical for promoters, a CGTCA motif involved in JA responsiveness, a TCA element involved in SA responsiveness, an MBS element involved in drought responsiveness, and two TC-rich repeats involved in defense and stress response (Figs. 4A and S3).

To evaluate the activity of PVqJAZ4 in response to powdery mildew disease progression, the full-length promoter (from −1465 to −1) was fused to the GUS reporter gene. The resulting constructs were transformed into leaves of 'Red Globe' (*V. vinifera*), followed by inoculation with *U. necator* or sterile water (mock treatment). Non-modified vector (pC3080GUS) was used as the control. As shown in Fig. 4B, the wild type (WT) and pC0380GUS showed much lower levels of GUS activity in this assay. However, when PVqJAZ4 transformed leaves were inoculated with *U. necator*, the infected leaves exhibited stronger GUS activity than mock treatment at 24 hpi. Further fluorometric assays indicated that GUS activity of *VqJAZ4* promoter was increased 2.01-fold in pathogen-inoculated leaves over mock treatment (Fig. 4C).

3.5. Heterologous expression of VqJAZ4 in arabidopsis confers tolerance to powdery mildew

To further assess the potential role of *VqJAZ4* in resistance to powdery mildew, we fused the *VqJAZ4* coding sequence to the strong, constitutive viral 35S promoter, and introduced this into wild-type Arabidopsis plants. A total of 44 independent transgenic lines were obtained; three homozygous T3 lines were selected and designated L1, L2 and L3 for further experiments (Fig. 5B). No obvious differences were seen between the NTC and transgenic plants in the absence of *G. cichoracearum* infection during the vegetative growth stages. When plants were challenged with *G. cichoracearum*, only small amounts of mycelia developed on transgenic leaves, whereas the leaves of control plants were entirely covered (Fig. 5A). Consistent with this, relatively few spores were recovered from leaves of transgenic plants, compared with controls (Fig. 5C). Moreover, histochemical staining assays showed that transgenic lines had higher levels of cell death and H_2O_2 accumulation than NTC plants, indicating that ectopic expression of *VqJAZ4* activated a HR response in the transgenic Arabidopsis. Aniline blue staining showed that transgenic lines had stronger callose deposition, which is known as a common response to fungal attack (Ellinger et al., 2013) (Fig. 5D). In addition, spectrophotometric estimation indicated the amount of dead cells in transgenic lines was 2–3 times higher than that in NTC plants (Fig. 5E). Similarly, transgenic lines exhibited a 1.5–2.5-fold enhancement in the level of H_2O_2 compared to controls (Fig. 5F). Quantitative measurement of callose was also conducted, and this suggested that the transgenic plants accumulated 1.25-2-fold more callose than NTC plants (Fig. 5G).



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AtJAZ1   MSS.SMECSE...FVGSRR..FTGKKPSFSQTCSRLSQYLKEN.GSFGDLSLGMACKPDVNGTLGNSRQPT..TTMSLFPCEASNMDSMV.   81
AtJAZ2   MSSFSAECWD...FSG........RKPSFSQTCTRLSRYLKEK.GSFGDLSLGMTCKPDVN...GGSRQP...TMMNLFPCEASGMDSSAG   73
OsJAZ6   .MASAK........SGERG......SSSFAMACSLLSRYVRQNGAAAGELGLGIRGEADAN..............................   46
OsJAZ7   MAASARPVGV....GGER.......ATSFAMACSLLSRYVRQNGAAAAELGLGIRGEGEAPRAAP........ATMSLLPGEAE.......   65
SlJAZ1   MAS..SEIVDSGRFAG........QKSHFSHTCNLLSQYLKEKKGSLGDLSLDMHRNFDSAGS..........TTMDLLPMIEKSGELVQ.   70
VvJAZ4   MSN.SPEFSD...FAGRKS.GKLPDRSNFSQTCNLLSQFLKEK.GRFGDLSLGMAGKSETKGRPESFKSST..MSFDLLNKDKSSEASGQN   83
VvJAZ9   MSS.SSDIADSGRFTGQRAPARGPEKSSFSQTCSLLSQYIKEK.GTFGDLSLGMTCSLEGNGTPESLRQTATTTTMNLFPMTERSAGVSGI   89
VqJAZ4   MSN.SPEFSD...FAGRKS.GRLPDRSNFSQTCNLLSQFLKDK.GRFGDLSLGMAGKSETKGRPESFKSST..MSFDLLNKDKSSEGSGQN   83

AtJAZ1   ....QDVKPTNLFPRQPSFSSSSSSLPKEDVLKMT..QTTRSVKPESQTAPITIFYAGQVIVFNDFSAEKAKEVINLASKGTANSLAKNQT  166
AtJAZ2   ...QEDIKPKTMFPRQSSFSSSSSSGTKEDVQMIK..ETTKSVKPESQSAPITIFYGGRVMVFDDFSAEKAKEVIDLANKGSAKSFTCFTA  159
OsJAZ6   ....KGKETMELFPQNSGFG........SEAAAVK..ETPDAREQE..KRQITIFYGGKVLVFDDFPAEKAKDLMQMASKSSSTAQNCVLL  121
OsJAZ7   ....RKKETMELFPQSAGFGQ.......QDAITAD..SAADAREQEPEKRQITIFYGGKVLVFNDFPADKAKGLMQLASKGSPVAPQNAAA  143
SlJAZ1   .......KSMNLFPQGG..........MK.............AESEPEKAQMTIFYGGQVIVFNDFPADKAKEIMLMASTSKGNNPAKPLE  131
VvJAZ4   VGGSSNLKSSDFYPQFAGFGSLAS...IDEAINMA..DFRKSATTESETSQMTIFYAGQVLVFNDFPAEKAREVMLIAAKGTPQNTSGFLS  169
VvJAZ9   PARNMNLKSMNLFPQQAGFGSSVS...KDDAPKIVNSSVKKSGNVEPQTAQMTIFYGGQVIVFNDFPADKAKEVMRLAGMGSSPVPSTTVK  177
VqJAZ4   GGGSSNLKSSDFYPQFAGFGSLAS...IDEAINMA..DFRKSATTESETSQMTIFYAGQVLVFNDFPAEKAREVMLIAAKGTPQNTSGFLS  169

AtJAZ1   .....DIRSN........IATIANQVPHPRKTTT.......QEPIQSSP.TPLT.ELPIARRASLHRFLEKRKDRVTSKAPYQLCDPAKAS  235
AtJAZ2   .....EVNNNHSAYSQKEIASSPNPVCSPAKTAA.......QEPIQPNP.ASLACELPIARRASLHRFLEKRKDRITSKAPYQIDGSAEAS  237
OsJAZ6   PSSATATVADN........TKVS.AVPAPASALP......VAQANAPKPVRPNAADLPQARKASLHRFLEKRKDRLQAKAPYQGSPSDASP  197
OsJAZ7   P..APAAVTDN........TKAPMAVPAPVSSLP......TAQADACKPARANASDMPIARKASLHRFLEKRKDRLNAKTPYQASPSDATP  218
SlJAZ1   S..................AADLVVPSFGKTSI.......QENQMPNQ..PIVSDLPIARRASLTRFLEKRKDRLTAKVPYHREEAAAPK  194
VvJAZ4   TSGPEKINTGSSTAPSPSIPASPATTPNPQALSSGTFSIPASPAATPNPQAPLGSELPIARRNSLHRFLEKRKDRVNSKAPYQVNNPSRPS  260
VvJAZ9   N....PIDAGG......MAPSNPNVVPNFANSLI.......QERIQRPA.QPVACELPIARKASLHRFLEKRKDRITARAPYNISNSPAGP  250
VqJAZ4   TSGLEKINTGSSTAPSPSIPASPATTPNPQALSSGTFSIPASPAATPNPQAPLGSELPIARRNSLHRFLEKRKDRVNSKAPYQVNNPSRPS  260

AtJAZ1   SNPQTTGNMSWLGLAAEI............                                                             253
AtJAZ2   SKP...TNPAWLSSR...............                                                             249
OsJAZ6   VKKELQESQPWLGLGPQVAAPDLSLRQESS                                                             227
OsJAZ7   VKKEP.ESQPWLGLGPNAVVKPIERGQ...                                                             244
SlJAZ1   KEE...HKAPWLGLGGQFAVKTEQY.....                                                             216
VvJAZ4   PKPEEDTNPKLNKDEGQSSKQLDLRL....                                                             286
VvJAZ9   HKP..AESKSWLGLAAKSPK..........                                                             268
VqJAZ4   PKPEEDTNPKLNKDEGQSSKQLDLRL....                                                             286
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Fig. 1. Multiple sequence alignment and phylogenetic relationship of the jasmonate-ZIM domain gene *VqJAZ4*. **A.** Multiple alignment of VqJAZ4 with related proteins from various plants. The TIFY domain and Jas domain are marked with red and green frames, respectively. **B.** Phylogenetic analysis of VqJAZ4 with other known JAZ proteins from different plants including *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* and *Vitis vinifera*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

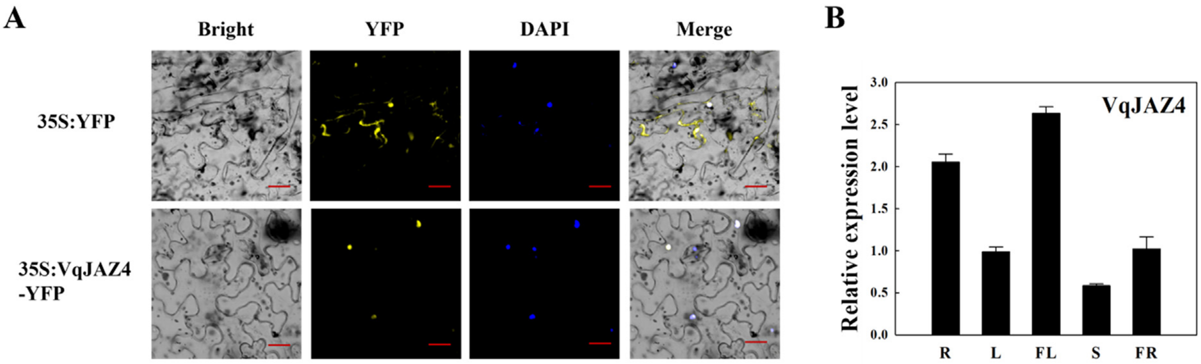

Fig. 2. Subcellular localization and tissue-specific expression pattern of VqJAZ4. **A.** VqJAZ4 was localized in the nucleus of tobacco leaf epidermal cells. The nucleus was stained with DAPI and the YFP signal was observed by confocal microscopy. From left to right: bright-field, YFP, DAPI and merged. YFP, yellow fluorescent protein. Scale bars = 50 µm. **B.** Tissue-specific expression pattern of *VqJAZ4* in *V. quinquangularis* clone Shang-24. R, root; L, leaf; FL, flower; S, stem; FR, fruit. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

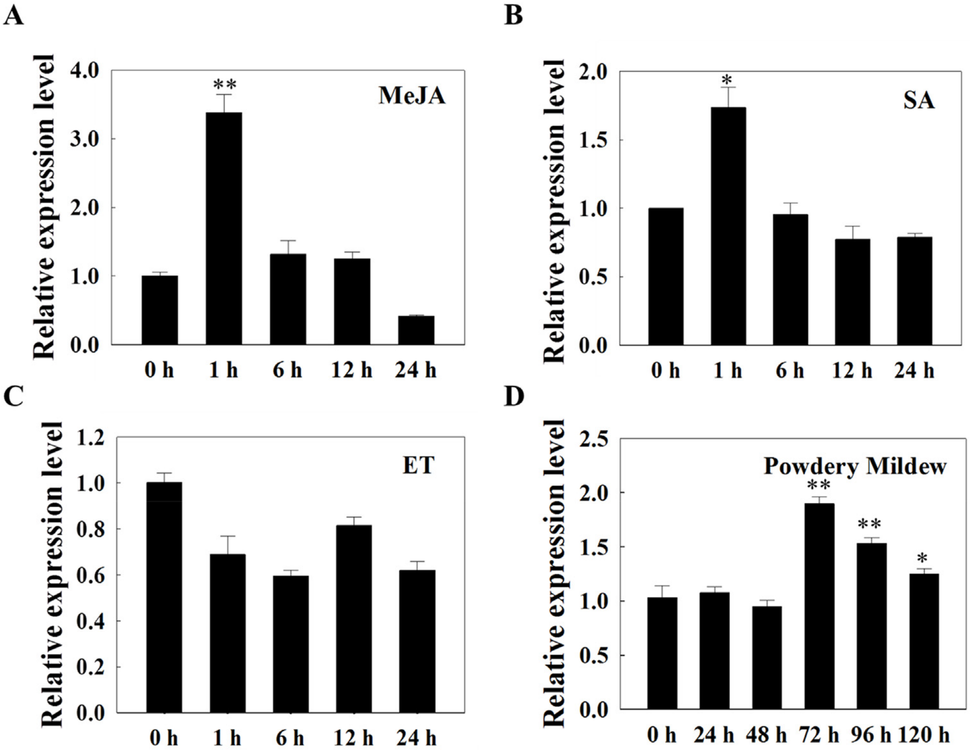

Fig. 3. Expression analysis of *VqJAZ4*. **A-D.** Expression of *VqJAZ4* in response to phytohormone treatments (MeJA, SA and ET) and *U. necator* inoculation. Double-distilled water was used as a control. Error bars represent the mean ± SE of three replicates. Asterisks indicate statistical significance (**P < 0.01, *P < 0.05, Student's *t*-test).

3.6. VqJAZ4 alters the expression of defense-related genes in arabidopsis

To investigate the mechanism of the enhanced resistance to powdery mildew conferred by *VqJAZ4*, we evaluated the expression of several marker genes involved in JA and SA signaling after inoculation with *G. cichoracearum*. *AtCOI1* and *AtMYC2* are critical components that interact directly with JAZ proteins (Chini et al., 2007; Thines et al., 2007). The expression of *AtCOI1* in transgenic lines increased 24 h after inoculation, then decreased at 72 and 120 hpi, whereas expression of *AtMYC2* decreased 24h after inoculation, then increased marginally at 72 and 120 hpi. Neither of these genes showed an obvious expression response in NTC plants, at least through 72 hpi (Fig. 6A and B). The expression levels of the JA-related genes *AtPDF1.2* and *AtVSP2* were lower in the transgenic Arabidopsis compared with control plants at 72 hpi and 120 hpi (Fig. 6C and D). *AtICS1*, *AtPR1* and *AtNPR1* positively regulate SA signaling (Loake and Grant, 2007). *AtICS1* expression showed an initial increase at 24 hpi, reaching a peak at 72 hpi and declining again at 120 hpi. *AtPR1* expression was induced strongly and increased continuously after inoculation. *AtNPR1* expression increased between 24 hpi and 72 hpi and had decreased by 120 hpi. Interestingly, expression of these three genes in all three transgenic lines were higher from that seen in the NTC plants at most time points, indicating that expression of *VqJAZ4* can enhance the endogenous molecular response to *G. cichoracearum* (Fig. 6E–G).

3.7. Heterologous expression of VqJAZ4 in arabidopsis enhances susceptibility to B. cinerea

Previous studies have shown that JAZ proteins are involved in *B. cinerea* resistance (Guo et al., 2018; Jiang and Yu, 2016). Based on this, we evaluated the response of *VqJAZ4*-expressing plants to *B. cinerea*. Interestingly, we found that *VqJAZ4*-expressing lines exhibited more intense symptoms and larger necrotic lesions compared with NTC plants (Fig. 7A and B). We also measured cell death and ROS levels by trypan blue and DAB staining. As shown in Fig. 7C, a larger number of dead cells and higher levels of H_2O_2 were observed in transgenic lines

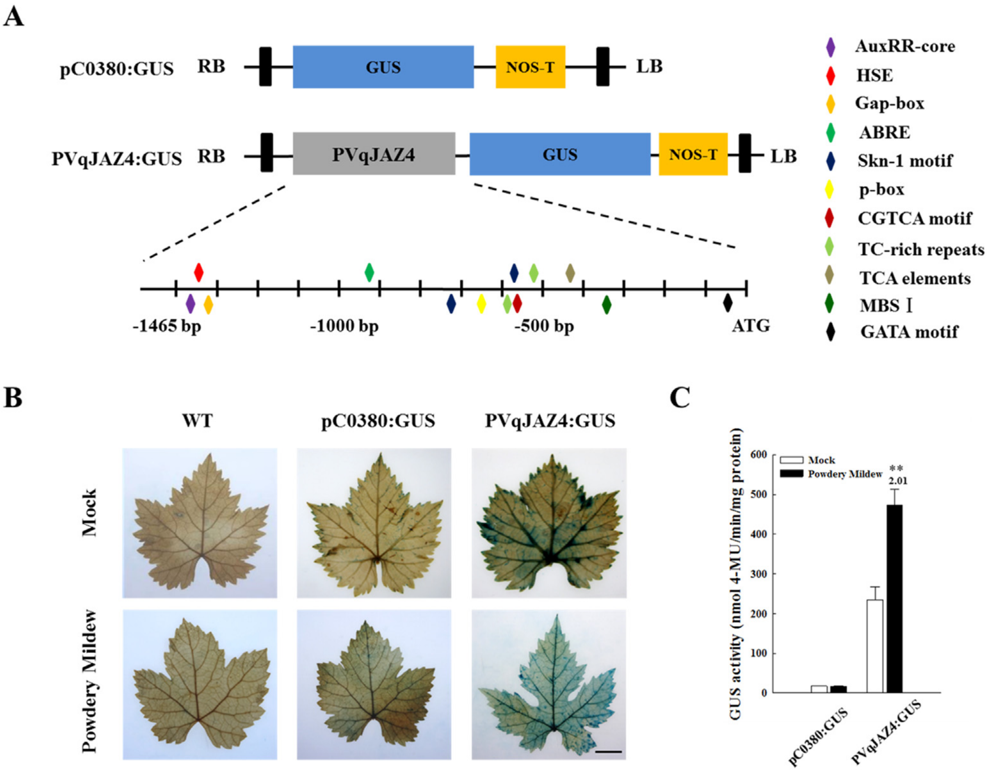

Fig. 4. Structure and activity analysis of the *VqJAZ4* promoter. **A.** Schematic diagram of expression vector constructions. The locations of *cis*-element sequences are marked on the promoter. LB, left border; GUS, β-glucuronidase; NOS-T, Nos terminator; RB, right border. **B.** Histochemical assay for GUS expression driven by *VqJAZ4* promoter in transiently transformed grapevine leaves following mock treatment (sterile water) and inoculation with *U. necator*. WT, wild type. Scale bar = 1 cm. **C.** Quantitative measurement of GUS activity in transiently transformed grapevine leaves expressing each construct. Error bars represent the mean ± SD of three replicates. Numbers above the bars indicate the relative fold induction of GUS activity. Asterisks indicate statistical significance (**P < 0.01, *P < 0.05, Student's *t*-test) between the inoculated leaves and mock-treated leaves.

compared with control plants. Quantitative analyses further confirmed these results (Fig. 7D and E).

To help elucidate the function of *VqJAZ4* in disease susceptibility in transgenic Arabidopsis, we examined the expression of three important components of JA signaling, *AtLOX3*, *AtMYC2* and *AtPDF1.2* at different time points after inoculation. Transcript levels of *AtLOX3* in all plants declined gradually following *B. cinerea* inoculation, with the three transgenic lines having lower levels than the NTC plants (Fig. 7F). Conversely, the expression of *AtMYC2* and *AtPDF1.2* increased strongly at 24, 48, and 72 hpi in control plants, but showed little induction in transgenic lines (Fig. 7G and H). Taken together, these data suggest that heterologous expression of *VqJAZ4* in Arabidopsis increases susceptibility to *B. cinerea*, and that this was associated with decreased expression of *AtLOX3*, *AtMYC2* and *AtPDF1.2*.

4. Discussion

JAZ proteins act as a critical component of signaling networks that promote the adaptation of plants to various biotic stresses (Kazan and Manners, 2012). In recent years, more and more studies have attempted to elucidate the function of JAZ proteins in response to pathogen attack. Here, we identified the jasmonate-ZIM domain protein-encoding gene, *VqJAZ4*, from the Chinese wild grapevine *V. quinquangularis*. Subcellular localization assay indicated that VqJAZ4 is a nuclear protein. We also evaluated the response to *G.cichoracearum* as well as *B. cinerea* in Arabidopsis *VqJAZ4*-expressing lines.

The VqJAZ4 protein contains a typical TIFY (also called ZIM) domain in its central region and Jas domain near its C terminus (Fig. 1A), as previously described (Vanholme et al., 2007). Our phylogenetic analysis showed that VqJAZ4 shares a close relationship with AtJAZ1, AtJAZ2 and SlJAZ3 in *A. thaliana* and *S. lycopersicum* (Fig. 1B). Chromatin immunoprecipitation (ChIP) assays demonstrated that AtWRKY57 directly bound to the *AtJAZ1* promoter, and promoted expression of *AtJAZ1* to suppress resistance to *B. cinerea* infection (Jiang and Yu, 2016). The AtJAZ2 protein physically interacted with MYC and NAC transcription factors to control stomatal reopening during bacterial invasion (Gimenez-Ibanez et al., 2017). In tomato, the C-terminal 157 amino acids of SlJAZ3 were found to be essential and sufficient for JA–Ile/COR-dependent binding of SlCOI1 (Katsir et al., 2008). Moreover, VqJAZ4 was found to be localized in the nucleus and lacked transcriptional activity (Figs. 2A and S2), suggesting that it is likely to be functioning via interaction with other transcription mediators, as was previously described for TIFY proteins belonging to Group II in Arabidopsis (Vanholme et al., 2007).

Previous studies revealed that JAZ1/TIFY10a transcripts were induced by both salt treatment and the elicitor Harpin in *V. rupestris*, indicating that these abiotic and biotic stresses share downstream factors (Ismail et al., 2012). However, there is scarce direct evidence that JAZ proteins can affect pathogen resistance in grape. Expression analysis indicated *VqJAZ4* was significantly increased upon exposure to *U. necator* inoculation (Fig. 3D), suggesting that *VqJAZ4* may participate in the response to powdery mildew. In order to further understand the expression pattern of *VqJAZ4*, we cloned the promoter of this gene. GUS assays in transiently transformed grapevine leaves showed that the endogenous promoter was induced upon *U. necator* inoculation (Fig. 4B and C). The analysis of promoter sequence identified a TCA element, which was proposed to be involved in SA-mediated defense, and TC-rich repeats, which were shown to function in defense and stress response (Fig. 4A). It is therefore accepted that these motifs are excellent candidates for regulatory elements important for the transcriptional response of *VqJAZ4* to powdery mildew. Furthermore, we introduced *VqJAZ4* into Arabidopsis to evaluate its role in response to powdery mildew. After inocultion with *G. cichoracearum*, a larger number dead cells and more massive production of H_2O_2 were observed in the transgenic plants inoculated relative to the control (Fig. 5D–F). Accumulation of ROS and programmed cell death (PCD) are associated with activation of the SA signaling pathway (Loake and Grant, 2007), so we hypothesize that expression of *VqJAZ4* enhanced SA-dependent basal defence in transgenic Arabidopsis.

Phytohormones are widely known to display either synergistic or antagonistic interactions in response to external stimuli. As transcriptional repressors in the JA pathway, JAZ proteins often work as a

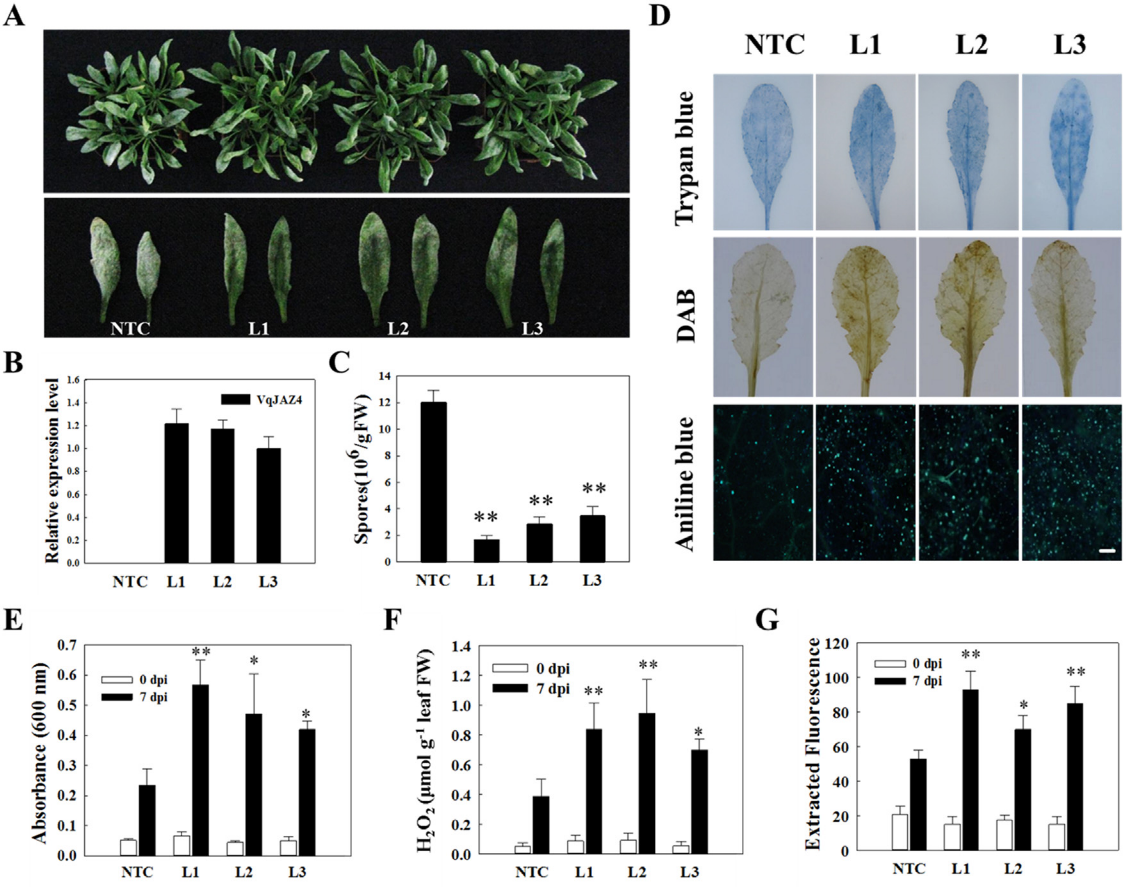

Fig. 5. Response of *VqJAZ4*-expressing Arabidopsis lines to *G. cichoracearum* inoculation. **A.** Phenotype of transgenic lines (L1, L2 and L3) and non-transgenic control (NTC) plants 7 days post-inoculation (dpi) with *G. cichoracearum*. **B.** qRT-PCR analysis of *VqJAZ4* in transgenic seedings. **C.** Quantitative analysis of spore numbers recovered from infected leaves. **D.** Detection of dead cells (upper panels), H_2O_2 (middle panels) and callose deposition (lower panels) in the NTC plants and transgenic lines 7 dpi. Scale bar = 100 μm. **E-G.** Quantification of dead cells, H_2O_2 content and callose in the leaves of NTC plants and transgenic lines at 0 and 7 dpi. Error bars represent the mean ± SD of three replicates. One-way ANOVA followed by Tukey test was performed for data in C, E, F and G. Asterisks indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$).

convergence node in the crosstalk among hormones. For instance, GA attenuates JA-mediated defense and conversely, JA could inhibit GA-mediated growth. These effects are accomplished through interaction between JAZ and DELLA proteins. Two Arabidopsis ET-stabilized transcription factors, EIL1 and EIN3, integrate JA and ET signaling by interacting with JAZs to modulate downstream gene expression (Kazan and Manners, 2012). Earlier studies showed that inhibition of JA signaling conferred by SA was mediated partly through the suppression of downstream components of JA, such as the genes *PDF1.2* and *VSP2*, and that this process might be related to the degradation of JAZ proteins. Another finding was that EIN3 and EIL1, which interact with JAZ repressors, could also suppress *SID2*, a gene required for SA biosynthesis. When JAZ proteins are degraded via the 26S-proteasome pathway, *EIN3* and *EIL1* are derepressed, allowing them to repress the biosynthesis of SA (Thaler et al., 2012). Given this, we evaluated the transcript levels of some JA and SA marker genes. *AtMYC2*, a representative and key TF gene repressed by JAZ proteins, functions as a master regulator of JA-mediated internal and external stimuli (Boter et al., 2004; Kazan and Manners, 2013; Lorenzo et al., 2004). *AtPDF1.2* and *AtVSP2* are two critical JA-responsive defense genes acting downstream of JA signaling (Kazan and Manners, 2013). The down-regulated expression of these genes observed at most time points after inoculation in transgenic Arabidopsis suggests that heterologous expression of *VqJAZ4* suppresses the JA signaling pathway (Fig. 6A–D). *AtICS1* is essential for pathogen-induced SA biosynthesis. *AtNPR1* plays a key role in SA-mediated systemic acquired resistance (SAR) and is involved in the crosstalk with JA. *AtPR1* is a presumed defense effector genes activated by elevated SA levels (Loake and Grant, 2007). Up-regulated expression of these SA-related genes in transgenic lines indicates that VqJAZ4 may activate more intense SA-mediated defense response to the pathogen (Fig. 6E–G). Our results suggest that JA and SA may act antagonistically in the regulation of transgenic Arabidopsis disease resistance against powdery mildew. A previous study showed that AtMYC2 can bind to the promoters of ANAC019, ANAC055, and ANAC072, and activates their expression. These NAC TFs are apt to exert the inhibitory effect on SA accumulation (Kazan and Manners, 2013). It has also been reported that ENHANCE DISEASE RESISITANT 1 (EDR1) assumes a central role in the crosstalk between JA and SA signaling pathway in defense against powdery mildew. Specifically, EDR1, an important regulator of SA signaling in Arabidopsis, can prevent MYC2-mediated repression of plant defensins through phosphorylation (Hiruma et al., 2011). However, the potential regulatory mechanism of VqJAZ4 mediated JA/SA interplay remains to be explored.

It was also found that *VqJAZ4*-overexpressing transgenic Arabidopsis became more sensitive to *B. cinerea* (Fig. 7A). Given the negative role of JAZ proteins in JA signaling, one of the possible reasons may be that ectopic expression of *VqJAZ4* suppresses the activation of JA-response genes in Arabidopsis, thus hampering resistance to pathogen mediated by the JA signaling pathway. Based on this, we checked the expression of three important hormone-related genes, including the JA biosynthesis gene *AtLOX3*, *AtMYC2* and *AtPDF1.2*. We found that the transcript levels of these genes were relatively lower in

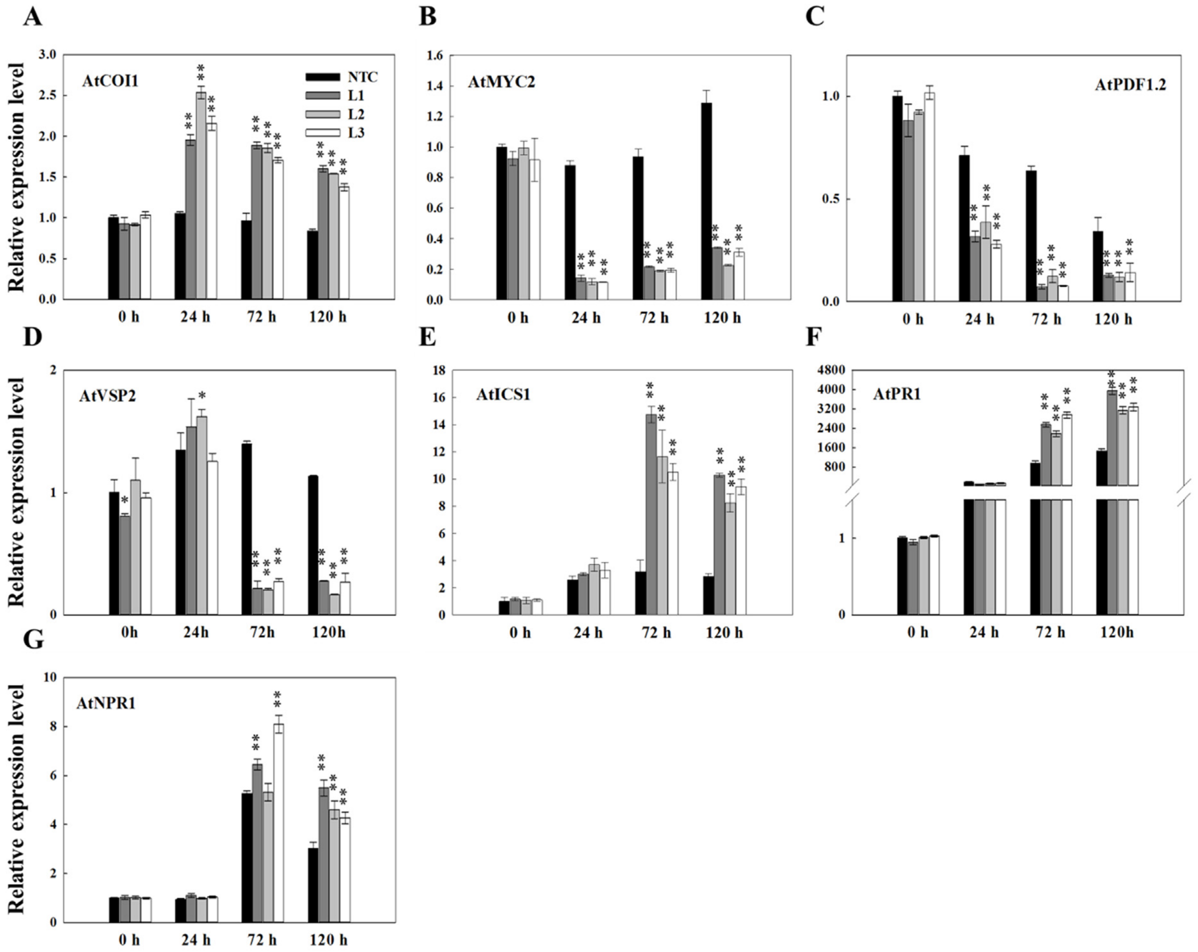

Fig. 6. Expression of *VqJAZ4* and defense-related genes in transgenic Arabidopsis and NTC plants following *G. cichoracearum* inoculation. Error bars represent the mean ± SE of three replicates. Asterisks indicate statistical significance (*0.01 < P < 0.05, **P < 0.01, one-way ANOVA followed by Turkey test).

transgenic plants compared with the control at different time points, though they displayed similar expression pattern after inoculation (Fig. 7F–H). Meanwhile, JA has been reported to promote phytoalexin synthesis in grapevine, which has a positive role in response to fungal infections (Tassoni et al., 2005), so we suspect that the presence of *VqJAZ4* in Arabidopsis might also reduce the production of phytoalexin resulting in increased susceptibility to *B. cinerea*. Given the negative role of *VqJAZ4* in response to *B. cinerea* and redundancy of JAZ family proteins in Arabidopsis (Chini et al., 2007; Guo et al., 2018), new genome editing technologies such as CRISPR/Cas system can be applied to achieve functional loss of *VqJAZ4* or multiple redundant JAZ genes simultaneously.

In conclusion, in the present study we characterized a novel grapevine JAZ gene, *VqJAZ4*, which was verified to encode a nuclear-localized protein. We found that *VqJAZ4* and its promoter could be highly induced by *U. necator* inoculation and that heterologous expression of this gene in *Arabidopsis* led to enhanced tolerance to powdery mildew disease. Further studies are needed to investigate the endogenous role of *VqJAZ4* in grapevine, which is complicated by the recalcitrance of grapevine to genetic transformation.

Contributions

XW and GZ designed the study. GZ and XY performed the experiments. SZ, YZ, ZL and XZ contributed to data analysis. GZ, XW and SVN wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

All authors declare that there are no conflicts of interest.

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Abbreviations

PRRs	pattern-recognition receptors
PTI	pattern-triggered immunity
ETI	effector-triggered immunity
HR	hypersensitive response
ROS	reactive oxygen species
NJ	neighbor joining
ORF	open reading frame
SA	salicylic acid
MeJA	methyl jasmonate
ET	ethylene
JA	jasmonic acid
YFP	yellow fluorescent protein
hpi	hours post inoculation
MS	Murashige and Skoog
NTC	non-transgenic control

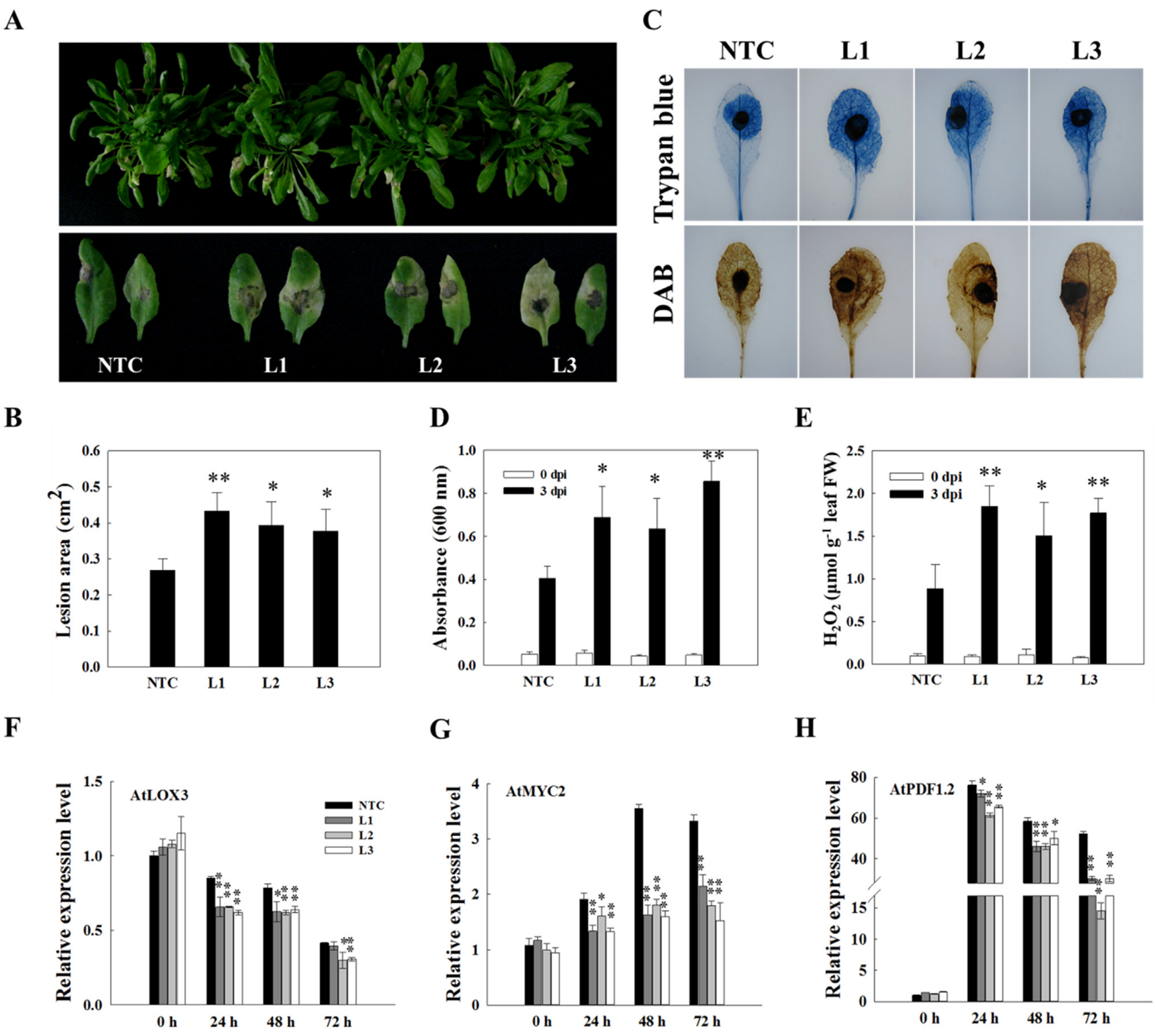

Fig. 7. Effect of heterologous *VqJAZ4* expression on response to *B.cinerea* in Arabidopsis. **A.** Phenotype of transgenic lines (L1, L2 and L3) and NTC plants 3 dpi with *B.cinerea*. **B.** Analysis of the lesion area at 3 dpi. Error bars represent the mean ± SD from three independent experiments. **C.** Visualization of cell death (upper panels) and H_2O_2 (lower panels) at 3 dpi. **D and E.** Quantification of dead cells and H_2O_2 content in the leaves of NTC plants and transgenic lines at 0 and 3 dpi. Error bars represent the mean ± SD from three independent experiments. **F–H.** Expression analysis of defense-related genes in NTC plants and transgenic Arabidopsis following *B.cinerea* inoculation. Error bars represent the mean ± SE of three replicates. One-way ANOVA followed by Tukey test was performed for data in B, D and E. Asterisks indicate statistical significance (*0.01 < P < 0.05, **P < 0.01).

CaMV	cauliflower mosaic virus
dpi	days post-inoculation
DAB	diaminobenzidine.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.018.

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