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Ectopic expression of a grape aspartic protease gene, *AP13*, in *Arabidopsis thaliana* improves resistance to powdery mildew but increases susceptibility to *Botrytis cinerea*☆

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ABSTRACT

The grape aspartic protease gene, *AP13* was previously reported to be responsive, in Chinese wild *Vitis quinquangularis* cv. 'Shang-24', to infection by *Erysiphe necator*, the causal agent of powdery mildew disease, as well as to treatment with salicylic acid in *V. labrusca* × *V. vinifera* cv. 'Kyoho'. In the current study, we evaluated the expression levels of *AP13* in 'Shang-24' in response to salicylic acid (SA), methyl jasmonate (MeJA) and ethylene (ET) treatments, as well as to infection by the necrotrophic fungus, *Botrytis cinerea,* and the transcript levels of *VqAP13* decreased after *B. cinerea* infection and MeJA treatment, but increased following ET and SA treatments. Transgenic *Arabidopsis thaliana* lines over-expressing *VqAP13* under the control of a constitutive promoter showed enhanced resistance to powdery mildew and to the bacterium *Pseudomonas syringae* pv. tomato DC3000, and accumulated more callose than wild type plants, while the resistance of transgenic *A. thaliana* lines to *B. cinerea* inoculation was reduced. In addition, the expression profiles of various disease resistance- related genes in the transgenic *A. thaliana* lines following infection by different pathogens were compared to the equivalent profiles in the wild type plants. The results suggest that *VqAP13* action promotes the SA dependent signal transduction pathway, but suppresses the JA signal transduction pathway.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most economically important deciduous fruit crops, and is cultivated worldwide to produce wine, juice, raisins and table grapes [1]. However, a large variety of pathogens, such as *Botrytis cinerea* and diseases such as powdery mildew and downy mildew can affect the growth, fruit quality and yield of cultivated grapevine varieties [2]. In order to prevent or limit pathogen infection, chemical treatments are extensively used in vineyards. However, even though such control methods can effectively increase production, they can incur substantial production cost and cause environmental pollution, especially by increasing the pesticide residues, which are known to be harmful to human health [1]. Some alternative strategies to increase disease resistance, such as classical crossbreeding, are very time consuming, and the phenotypic trait in the finial generation is usually not stable. Consequently, other approaches, such as the application of elicitors of natural resistance may provide opportunities to improve resistance in cultivated crop varieties. In this context, a more extensive knowledge of the network of disease resistance related genes involved in the defense responses would be advantageous.

Abbreviations: AP, aspartic protease; SA, Salicylic acid; JA, jasmonic acid; ET, ethylene; SAR, systemic acquired resistance; PR, pathogenesis-related; MeJA, methyl jasmonate; WT, wild type; *Pst*DC3000, *Pseudomonas syringae* pv. tomato DC3000; *pad4*, *Phytoalexin deficient 4*; hpt, hours post treatment; hpi, hours post inoculation; dpi, days post inoculation; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; ORF, open reading frame; CaMV, cauliflower mosaic virus; LPS, lipopolysaccharides; ROS, reactive oxygen species; *d*, diameter; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase.

☆ The nucleotide sequence reported in this paper has been submitted to GeneBank with accession numbers KP998099.

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The induction of plant defense responses occurs via a highly complex signaling network [3,4]. In previous studies, the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have all been shown to be key mediators of resistance to different types of pathogens. SA is associated with local resistance to biotrophs and with the onset of systemic acquired resistance (SAR), while JA and ET control the resistance to necrotrophs and induce systemic resistance, which can also be triggered by root-colonizing nonpathogenic rhizobacteria [5–7].

Although the genes involved in resistance to various pathogens are far less characterized in grapevine than in the model plant *Arabidopsis thaliana*, several have been identified that may play roles in defense responses. For example, grapevine plants over-expressing *VvNPR1.1* were shown to exhibit an enhanced resistance to powdery mildew infection [8], and over-expression of *VpGLOX* from the Chinese wild vine *V. pseudoreticulata* accession 'Baihe-35-1' in *A. thaliana* conferred resistance to powdery mildew [9]. Moreover, over-expression of the transcription factor *VvWRKY1* in grapevine was reported to induce the expression of genes in the JA signaling pathway and to confer increased tolerance of downy mildew. Similarly, over-expression of *VvWRKY2* in tobacco (*Nicotiana tabacum* cv. Xanthi) enhanced broad resistance to necrotrophic fungal pathogens [2,10]. There is also evidence that two homologous WRKY genes from Chinese wild *Vitis pseudoreticulata* W. T. Wang 'Baihe-35-1', *VpWRKY1* and *VpWRKY2*, may participate in resistance to powdery mildew [11]. Finally, the *V. pseudoreticulata* EIRP1 E3 ligase has been shown to promote plant disease resistance by mediating proteolysis of the protein VpWRKY11 by the 26S proteasome [12].

As one of the four superfamilies of proteolytic enzymes, aspartic proteases (AP; EC 3.4.23) have been implicated in regulating a variety of biological processes, such as the recognition of pathogens and pests and the induction of defense responses, through limited proteolytic processing of peptide hormones, receptors, and other regulatory proteins [13–15]. Genes encoding extracellular AP enzymes that might play a role in the degradation of pathogenesis-related (PR) proteins in leaves have been identified in tobacco (*N. tabacum* L.) and tomato (*Solanum lycopersicum*) [16,17], and an AP that is expressed in potato leaves has been proposed to play a role in defense responses against pathogens and insects [18]. The expression of AP genes has also been detected in tomato leaves post-wounding or following treatments with the peptide hormone systemin or methyl jasmonate (MeJA) [19]. This AP has been suggested to be involved in intracellular protein turnover to increase amino acid pools for the synthesis of defense related proteins and/or in the defense against pathogens by hydrolyzing proteins secreted by invasive pathogens [19]. Moreover, over-expression of an AP encoded by the *A. thaliana CDR1* gene was reported to increase resistance to the virulent bacterium *Pseudomonas syringae*, suggesting a role in the activation of inducible resistance mechanisms [15]. And ectopic expression of the rice ortholog *OsCDR1* in *A. thaliana* and rice conferred enhanced resistance to bacterial and fungal pathogens, and infiltration of a *OsCDR1* fusion protein that fused at the C-terminus of glutathione-*S*-transferase (GST) of pGEX-3X expression vector into the leaves of *A. thaliana* induced the expression of *p*athogen-*related protein 2* (*PR2*), further demonstrating its induction of systemic defense responses [20,21]. In a recent study, it has been reported that the expression of a rice AP gene (*OsAP77*)was induced by pathogen infection and defense related signaling molecules, and this gene has a positive role in defensing response against fungal, bacterial and viral infections [22].

We previously identified 50 AP genes in the grape genome and characterized their transcript abundance in response to various stress conditions and hormone treatments [23]. Of these genes, it was found that the expression of *AP13* was up-regulated in Chinese wild *Vitis quinquangularis* cv. 'Shang-24' following infection with *Erysiphe necator*, in *V. labrusca* × *V. vinifera* cv. 'Kyoho' after exogenous application of SA, suggesting that this gene may confer enhanced resistance to biotrophs. In the current study, we characterized the expression of *AP13* in *V. quinquangularis* cv. 'Shang-24' after either inoculation with the fungus *B. cinerea* or treatments with SA, MeJA and ET. This was followed by gene functional analysis by characterizing the responses of transgenic *A. thaliana* lines constitutively over-expressing grape *AP13* to inoculation with *E. necator*, *P. syringae* pv. tomato DC3000 (*Pst*DC3000), and *B. cinerea*. The results elucidate the role of the *AP13* gene in biotic stress responses and, we propose, will be useful in the development of biotroph resistant crops.

2. Materials and methods

2.1. Plant materials and pathogenic bacteria

Two year old Chinese wild *V. quinquangularis* cv. 'Shang-24′ seedlings were grown in a greenhouse at the Northwest A&F University, Yangling, Shaanxi, China, and used for *B. cinerea* inoculation, SA, MeJA and ET treatments, and for the cloning and expression analysis. Wild type (WT) and transgenic *A. thaliana* lines were grown under 22 °C, 70% relative humidity and a long-day photoperiod (8 h dark/16 h light).

B. cinerea was isolated from 'Red Globe' (*V. vinifera*) and maintained on Potato Glucose Agar medium as described by Wan et al. [24]. *P. syringae* pv. tomato DC3000 (*Pst*DC3000) was grown at 28 °C on King's B medium [25] containing 50 μg/mL rifampicin. *A. thaliana* powdery mildew causing *Golovinomyces cichoracearum* isolate UCSC1 was cultured on *A. thaliana pad4* (*phytoalexin deficient 4*) mutant plants, which were grown in a plant incubator (22 °C, photoperiod 8 h dark/16 h light, light intensity 100 μmol m^{-2} s^{-1}).

2.2. Hormone treatments and B. cinerea inoculation in grape

Hormone treatments were conducted by spraying young grape leaves with 100 μM SA [26], 50 μM MeJA [27] or 0.5 g/L ET [28], followed by sampling at 1, 12, 24 and 48 h post-treatment (hpt). Leaves sprayed with sterile distilled water at the same time points were used as a control.

B. cinerea inoculation was carried out as previously described, with some modifications [29]. Conidia from 3 week old cultured *B. cinerea* were washed with sterile distilled water, suspended in 10 mL sterile distilled water, and the suspension was filtered through Miracloth (Calbiochem, Germany) to remove mycelia. The spore concentration in the inoculation suspension was adjusted to 10^5 spores/mL with sterile distilled water. Conidia were then pre-germinated for 2 h at 22 °C before inoculation [29]. Detached grapevine leaves were carefully washed and placed adaxial side down on a bed of 0.8% agar, and then sprayed with a conidia suspension supplemented with Silwett L77 (GE, USA)[0.02% (v/v)]. Trays of inoculated leaves were covered with a plastic film to ensure a relative humidity of 90–100%. Leaves sprayed with sterile distilled water at the same time served as a control. All leaves were incubated in the dark for the first 24 h and then in a 12 h dark/12 h light regime at 22 °C. Samples were frozen in liquid nitrogen 1, 12, 24 and 48 h post-inoculation (hpi) [30–32].

2.3. Gene expression analysis by quantitative real-time PCR

Total RNA was extracted from grapevine and *A. thaliana* leaves using the E.Z.N.A.® Plant RNA Kit (Omega Bio-tek, USA, R6827-01) and the RNAprep plant kit (Tiangen Biotech, China), respectively, following the manufacturers' protocols. First-strand cDNA was synthesized using PrimeScript™ RTase (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. Quantitative

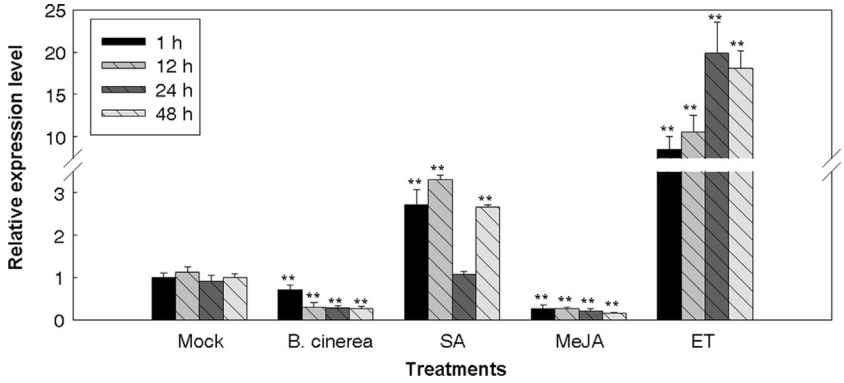

Fig. 1. Expression levels of *VqAP13* in Chinese wild *Vitis quinquangularis* cv. 'Shang-24' following various hormone treatments and *B. cinerea* infection. Data represent mean values ± SD from three independent experiments. Asterisks indicate statistical significance (**$P < 0.01$, Student's *t* test) between treatment and mock.

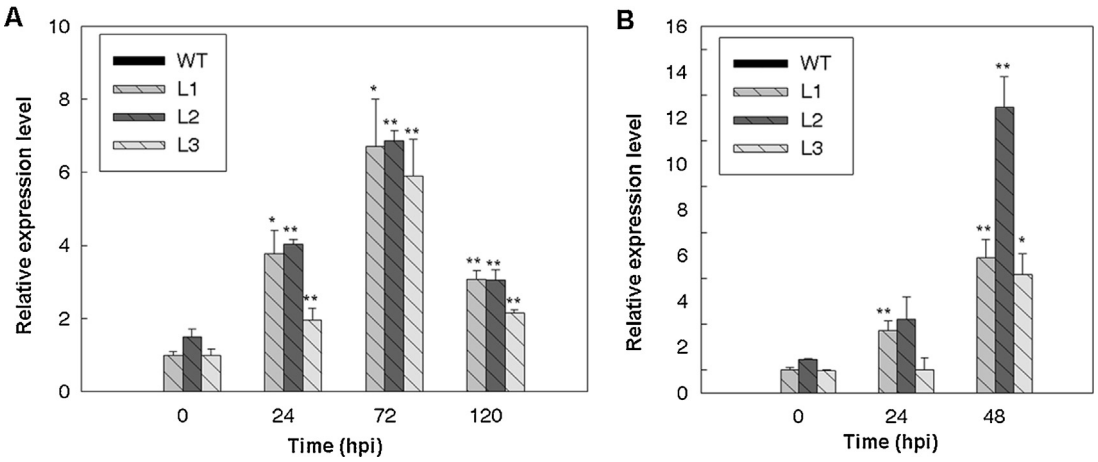

Fig. 2. The expression level of *VqAP13* in transgenic *A. thaliana* following *E. necator* and *Pst*DC3000 infection. (A) The expression level of *VqAP13* in transgenic *A. thaliana* following *E. necator* infection. (B) The expression level of *VqAP13* in transgenic *A. thaliana* following *Pst*DC3000 infection. Data represent mean values ± SD from three independent experiments. Asterisks indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the expression level of 24, 48, 72,120 and 0 h after infection.

real-time PCR (qRT-PCR) analysis was conducted using SYBR green (TaKaRa Biotechnology) and an IQ5 real-time PCR instrument (Bio-Rad, Hercules, CA, USA) with the following thermal profile: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. Each reaction was performed in triplicate for each of the three biologically replicated sets of cDNA samples. To perform the melt-curve analysis, the following program was added after 40 PCR cycles: 95 °C for 15 s, followed by a constant increase from 60 to 95 °C. Grape *Actin1* (GenBank Acc. No. AY680701) or *A. thaliana Actin1* (TAIR: AT2G37620) was used as reference genes, as appropriate. Primers used for qRT-PCR were designed and are listed in Supplementary Data 1. Relative expression levels were analyzed with the IQ5 software using the Normalized Expression Method.

2.4. Isolation and analysis of the ***VqAP13*** open reading frame

Total RNA extractions from leaves of *V. quinquangularis* cv. 'Shang-24', as well as first-strand cDNA synthesis, were carried out as described above. The *VqAP13* open reading frame (ORF) was amplified by PCR using the gene-specific primers F1 (5′-ATG CCG GCG TTG GCG G-3′) and R1 (5′-TTATAAAAA CAG GGAAGT GTA CAA C-3′). The PCR product was cloned into the pGEM®-T Easy vector (Promega, Madion, WI, USA), and the recombinant plasmid was sequenced by BGI (Shenzhen, China) and named pGEM®-T Easy-*VqAP13*.

2.5. Generation of transgenic A. thaliana plants over-expressing the ***VqAP13*** gene

The coding sequence of *VqAP13* (with *Xba*I and *Kpn*I at the 5′ and 3′ ends, respectively) was amplified from the pGEM®-T Easy-*VqAP13* vector using the gene-specific primers F2 (5′ – GCT CTA GAA TGC CGG CGT TGG CGG-3′; *Xba*I site underlined) and R2 (5′- GGG GTA CCT TATAAAAAC AGG GAA GTG TAC AAC-3′; *Kpn*I site underlined), and inserted immediately downstream of the CaMV 35S promoter in the plant over-expression vector, pCambia 2300 (Cambia, Brisbane, QLD, Australia). *Agrobacterium tumefaciens* strain GV3101 harboring the over-expression construct, pCambia2300-*VqAP13*, was used for *A. thaliana* transformation via the floral dip method [33]. T0 seeds were harvested and sown on MS medium [34] supplemented with 75 mg/L kanamycin. Three lines (L1, L2 and L3) with the strongest resistance to powdery mildew and *Pst*DC3000 were selected from 70 independent lines, and T3 homozygous lines were generated and used for all further experiments.

2.6. Inoculation of A. thaliana with pathogenic bacteria

Leaves of 4 week old T3 transgenic and WT plants were inoculated with *G. cichoracearum* as previously described [35]. Visual scoring of disease reaction phenotypes and spore count of leaves were performed 5 days post inoculation (dpi) as previously described [11,36]. Samples collected 0, 24, 72, and 120 hpi were

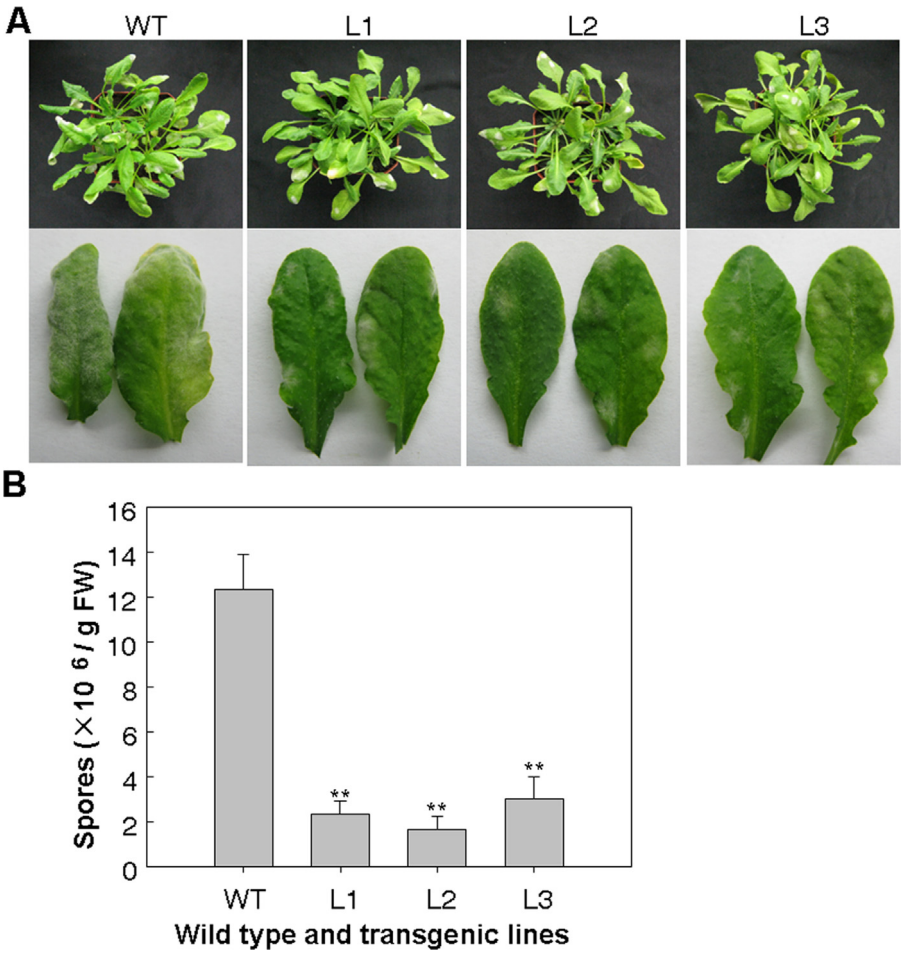

Fig. 3. The response of *VqAP13* transgenic *A. thaliana* to *E. necator* infection. (A) The incidence of transgenic and WT plants post infection for 5 days. (B) Bacterial numbers per gram of fresh leaves. Data represent mean values ± SD from three independent experiments. Asterisks indicate statistical significance (**$P < 0.01$, Student's t test) between transgenic lines and WT.

used for the expression profile analysis of disease resistance related genes.

Flg22 (Anaspec, USA) was dissolved in 10 mM $MgSO_4$ to a final concentration of 1 mM, lipopolysaccharides (LPS) (SIGMA, USA) were dissolved in sterile distilled water containing 2.5 mM $MgCl_2$ and 1 mM $CaCl_2$ to a final concentration of 0.1 mg/mL. *Pst*DC3000 was grown overnight at 28 °C in King's B [25] liquid medium containing 50 μg/mL rifampicin, and bacteria were re-suspended to an O.D. 600 of 0.0001 (~10^5 colony-forming units/mL) in 10 mM $MgCl_2$. The flg22, LPS and *Pst*DC3000 samples were infiltrated into leaves of 4-week old *A. thaliana* plants with a needleless syringe and the leaves were examined collected 18 h post injection and callose accumulation was visualized by aniline blue staining as previously described [37]. Leaves injected with 10 mM $MgCl_2$ were used as a control.

*Pst*DC3000 that had been re-suspended to 10^8 cfu/mL in 10 mM $MgCl_2$ was used to inoculate 4 week old *A. thaliana* plants. Leaves were dipped in bacterial suspension for 15 mins and the plants were then placed in a plant incubator under 90% relative humidity conditions for 24 h followed by transfer to normal growth conditions. The expression levels of disease resistance related genes were measured in leaves harvested 24 and 48 hpi. Bacterial growth within the leaves was monitored 48 h post infiltration as previously described [38].

The *B. cinerea* conidial suspension used for *A. thaliana* inoculation was as described for grape above. For each *A. thaliana* genotype, 60 leaves were detached and placed adaxial side down on humid Whatmann 3 MM paper (Whatman, USA) and then droplet inoculated with 10 μL of the conidial suspension. Samples were collected at 0, 24, 48, and 72 hpi, and were used for the assessment of antioxidant enzyme activities, levels of reactive oxygen species (ROS) and the presence of dead cells, as well as the expression levels of disease resistance related genes. Lesion diameters (d) were measured 72 h after inoculation and the size populations were defined as follows: small ($d \leq 5$ mm); medium ($5 < d < 9$ mm); and large ($d \geq 9$ mm) [31]. The detection of ROS and dead cells and antioxidant enzyme activities were carried out as previously described [39].

2.7. Statistical analysis

All experiments were repeated independently three times for each of the three biological replicates. Results are presented as means and standard errors, and were calculated using Microsoft Excel (Microsoft Corporation, USA). Paired t-tests were performed using the SPSS Statistics 17.0 software (IBM China Company Ltd. Beijing, China) to assess significant differences.

3. Results

3.1. The expression profiles of *VqAP13* after treatment with different hormones and B. cinerea inoculation

In a previous study, we found that the expression levels of *AP13* increased in *E. necator* infected Chinese wild *V. quinquangularis*

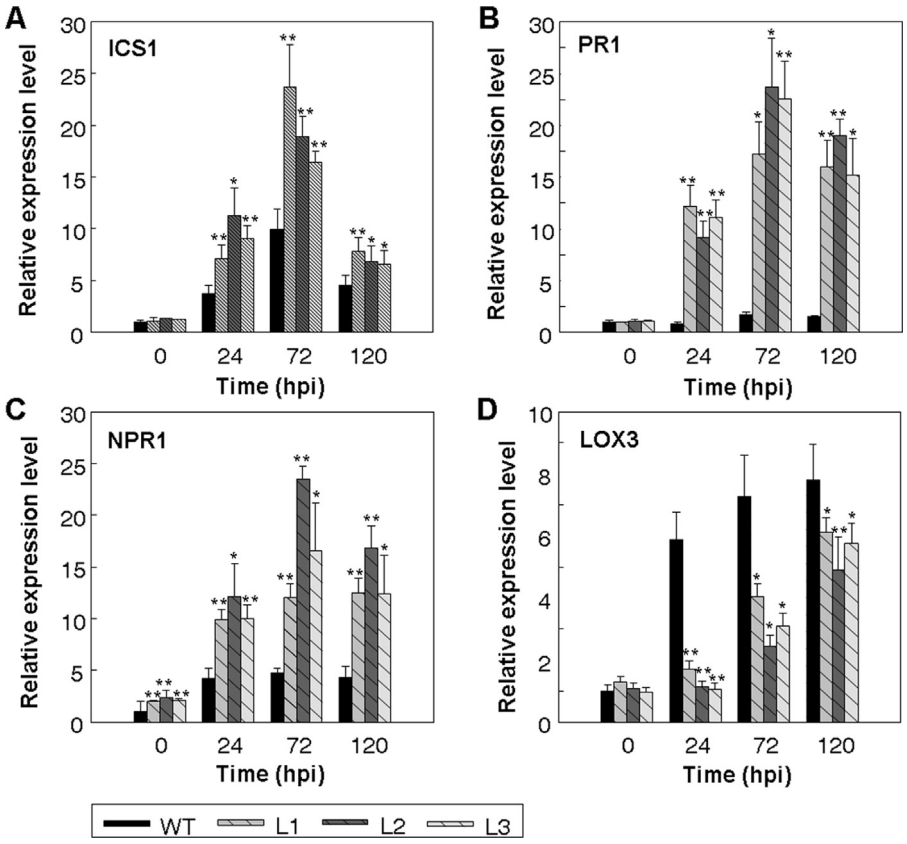

Fig. 4. Expression levels of disease resistance genes in WT and *VqAP13* transgenic *A. thaliana* plants following *E. necator* infection. (A) The relative expression levels of the SA biosynthesis gene *ICS1*. B, C. The relative expression levels of *PR1* (B) and *NPR1* (C), two genes in the SA signal transduction pathway. D. The relative expression levels of the JA biosynthesis gene, *LOX3*. Relative gene expression levels were analyzed using quantitative RT-PCR. Bars represent the mean ± SD of three independent experiments. Asterisks indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between transgenic lines and WT.

cv. 'Shang-24' and exogenous SA treated *V. labrusca* × *V.vinifera* cv. 'Kyoho' [23]. To determine the expression of *VqAP13* in response to pathogen infection, we measured the expression levels of *AP13* in *V. quinquangularis* cv. 'Shang-24' following SA, MeJA and ET treatments as well as inoculation with *B. cinerea*. The transcript levels of *VqAP13* decreased after *B. cinerea* infection and MeJA treatment, but increased following ET treatment. The transcript abundance of *VqAP13* had no significant difference with that of the mock at 24 h post SA treatment, but were higher than that of the mock at other time points (Fig. 1).

3.2. Cloning and sequence analysis of ***VqAP13***

Gene specific primers were designed according to the cDNA sequence of *AP13* available in the Grape Genome Database (12×; http://www.genoscope.cns.fr) and were used to amplify the *VqAP13* ORF. The *VqAP13* transcript sequence (1500 bp; GenBank Acc. No. KP998099) and the corresponding deduced protein sequence (499 amino acids) are shown in Supplementary data 2, highlighting the two conserved DT/SG active sites. The coding sequence of *VqAP13* from *V. quinquangularis* cv. 'Shang-24' shared 99.4% nucleotide identity with *AP13* from *V. vinifera*, and their respective deduced amino acid sequences were 99% identical.

3.3. Analysis of transgenic A. thaliana lines over-expressing ***VqAP13***

The potential role of *VqAP13* in responses to infection with different pathogens was investigated via its constitutive overexpression in *A. thaliana*. A total of 70 independent transgenic lines were generated and the three with the strongest resistance to powdery mildew and *Pst*DC3000 infection (L1, L2 and L3) were selected for generation of homozygous T3 lines. The expression patterns of *VqAP13* following *G. cichoracearum* and *Pst*DC3000 inoculation in these three transgenic *A. thaliana* lines was evaluated using quantitative RT-PCR. The transcript levels at 24, 72 and 120 hpi after the onset of with powdery mildew disease increased 2–7 times when compared with levels at 0 hpi (Fig. 2A), and 2–8 times at 24 h and 48 h post *Pst*DC3000 infection (Fig. 2B), indicating that these lines were appropriate for use in the further studies.

3.4. The response of ***VqAP13*** over-expressing A. thaliana to powdery mildew disease

To elucidate the role of *VqAP13* in disease resistance, the three transgenic *A. thaliana* lines and WT plants were inoculated with *G. cichoracearum*. As shown in Fig. 3A, the transgenic *A. thaliana* lines exhibited increased resistance to *G. cichoracearum* at 5 dpi compared to WT plants. To quantify this resistance, the number of spores on the susceptible leaves was determined and found to be significantly lower in the 3 transgenic *A. thaliana* lines at 5 dpi than in the WT plants (Fig. 3B).

Disease resistance is typically accompanied by a marked increase in the expression of defense related genes [40]. We therefore assessed the expression of several key genes known to be involved in SA and JA synthesis and their associated signal transduction pathways. The expression levels of *ICS1*, which is involved in SA biosynthesis, and *PR1* and *NPR1*, which are components of the SA signal transduction pathway, were higher post powdery mildew inoculation, and peaked at 72 hpi (Fig. 4). Furthermore, the

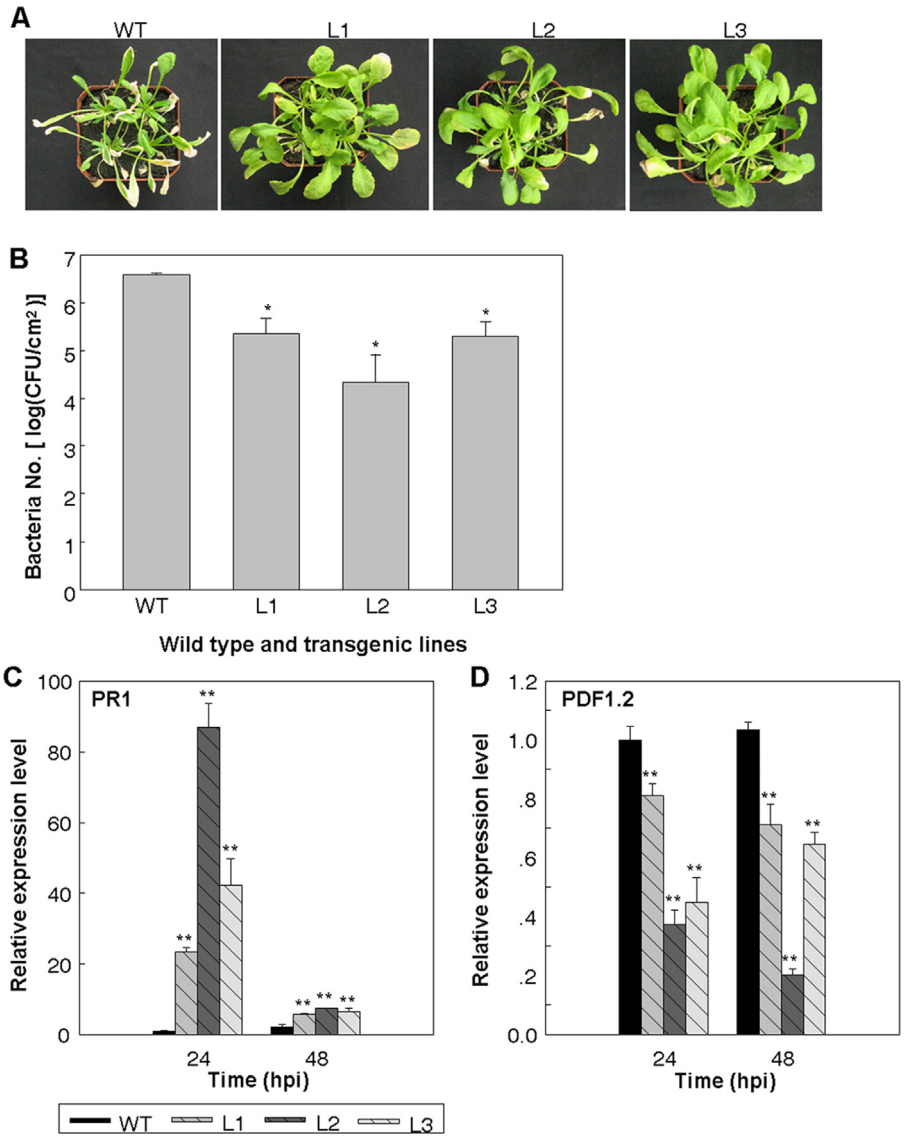

Fig. 5. The response of *VqAP13* transgenic *A. thaliana* to infection by *Pst*DC3000. (A) Changes in phenotype 5 days after infection. (B) Bacterial numbers per square centimeter 2 days after infection. (C) The relative expression levels of *PR1*. (D) The relative expression levels of *PDF1.2*. Relative gene expression levels were analyzed using quantitative RT-PCR. Bars represent the mean ± SD of three independent experiments. Asterisks indicate statistical significance (*0.01 < *P* < 0.05, Student's *t* test) between transgenic lines and WT.

expression levels of the three genes were higher in the transgenic *A. thaliana* lines than in the WT plants at 24, 72, and 120 hpi. The expression of *LOX3*, which is involved in JA biosynthesis was also greater after powdery mildew infection, but its expression levels in the transgenic *A. thaliana* lines were lower than in WT (Fig. 4D).

3.5. Over-expression of *VqAP13* improves the *Pst*DC3000 resistance of transgenic A. thaliana lines

To further examine the role of *VqAP13* in disease resistance, the 3 transgenic *A. thaliana* lines and WT plants were inoculated with *Pst*DC3000. The bacterial infection in the WT plants was severe and most of the leaves showed desiccation, while the 3 transgenic *A. thaliana* lines exhibited much less severe symptoms at 5 dpi (Fig. 5A). The bacterial numbers per unit leaf area were tested 2 dpi, and were found to be lower in the transgenic *A. thaliana* lines than in WT (Fig. 5B). We measured the expression profiles of disease resistance related genes in the *Pst*DC3000 infected transgenic *A. thaliana* and WT leaves, transcript levels of *PR1*, which is involved in the SA signaling pathways, peaked at 24 hpi and were at least 23-fold higher than in WT (Fig. 5C). Its expression subsequently decreased, but was still higher than in WT (Fig. 5C). In contrast, the transcript abundance of *PDF1.2*, which is involved in the JA signaling pathways, was lower in the transgenic *A. thaliana* lines (Fig. 5D).

3.6. Accumulation of callose in *VqAP13* over-expressing A. thaliana in response to different treatments

Previous studies have shown that the cell wall polymer, callose, may act as a physical barrier to slow pathogen invasion, and thus can contribute to plant innate immunity at early time points of infection [41–44]. Using the aniline blue staining technique, we observed that the leaves of the transgenic *A. thaliana* lines produced more callose than those of WT in response to *Pst*DC3000 infection, or application of flg22 or LPS, although the difference in callose deposition between WT and L1 in case of flg22 was not significantly, which maybe caused by the lower expression level of *VqAP13* in L1 than in other lines post flg22 injection (Fig. 6).

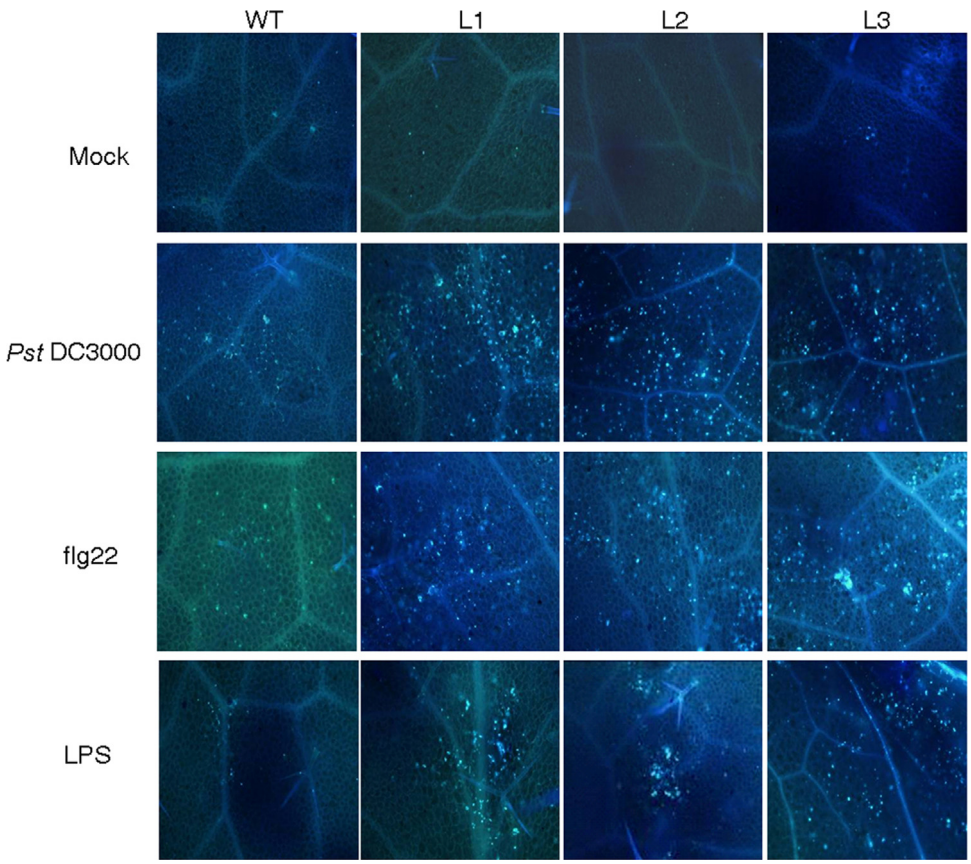

Fig. 6. Callose deposition in WT and *VqAP13* transgenic *A. thaliana* plants following *Pst*DC3000 infection, or flg22 and LPS injection. Leaves of *A. thaliana* were stained with aniline blue to locate callose depositions by epifluorescent illumination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. *Influence of* B. cinerea *infection on* ***VqAP13*** *expression levels in transgenic* A. thaliana *lines*

We previously observed that the expression of *VqAP13* was down-regulated in *V. quinquangularis* cv. 'Shang-24' in response to *B. cinerea* infection (Fig. 1). We further examined this association by measuring *VqAP13* expression in the transgenic *A. thaliana* lines and observed lower levels at 24, 48, and 72 hpi than at 0 hpi (Fig. 7).

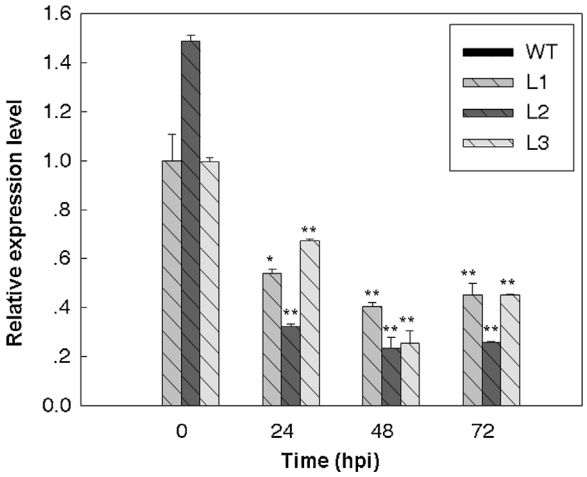

Fig. 7. The expression level of *VqAP13* in transgenic *A. thaliana* following *B. cinerea* infection. Data represent mean values ± SD from three independent experiments. Asterisks indicate statistical significance (*$0.01 < P < 0.05$,**$P < 0.01$, Student's *t* test) between the expression level of 24, 48, 72 and 0 hpi.

3.8. *Over-expression of* ***VqAP13*** *reduces the resistance of transgenic* A. thaliana *lines to* B. cinerea

The phenotypes of the leaves of *VqAP13* over-expressing *A. thaliana* lines and WT plants were observed and lesion diameters were measured 3 d post *B. cinerea* infection. Brown specks appeared on the leaves of the transgenic *A. thaliana* lines and WT 1 dpi (data not shown); however, the lesions on the transgenic *A. thaliana* lines were larger (Fig. 8A,B), and the proportions of medium size and large lesion was higher (Fig. 8C). Trypan blue staining also indicated that the area of dead cells in the transgenic *A. thaliana* lines was larger than in the WT leaves (Fig. 8D). In addition, levels of reactive oxygen species (ROS) were higher in the transgenic *A. thaliana* lines and the activities of the antioxidant enzymes, SOD, CAT and POD lower, (Fig. 9). We observed that SOD activity peaked at 24 hpi (Fig. 9C), while of CAT and POD activities peaked at 48 and 72 hpi, respectively (Fig. 9D,E).

3.9. *Altered expression of disease resistance related genes in* ***VqAP13*** *over-expressing plants post* B. cinerea *infection*

We next evaluated the expression of several disease resistance related genes involved in SA and JA synthesis and signal transduction pathways in the transgenic *A. thaliana* lines following *B. cinerea* infection. The expression of *LOX3*, which is involved in the

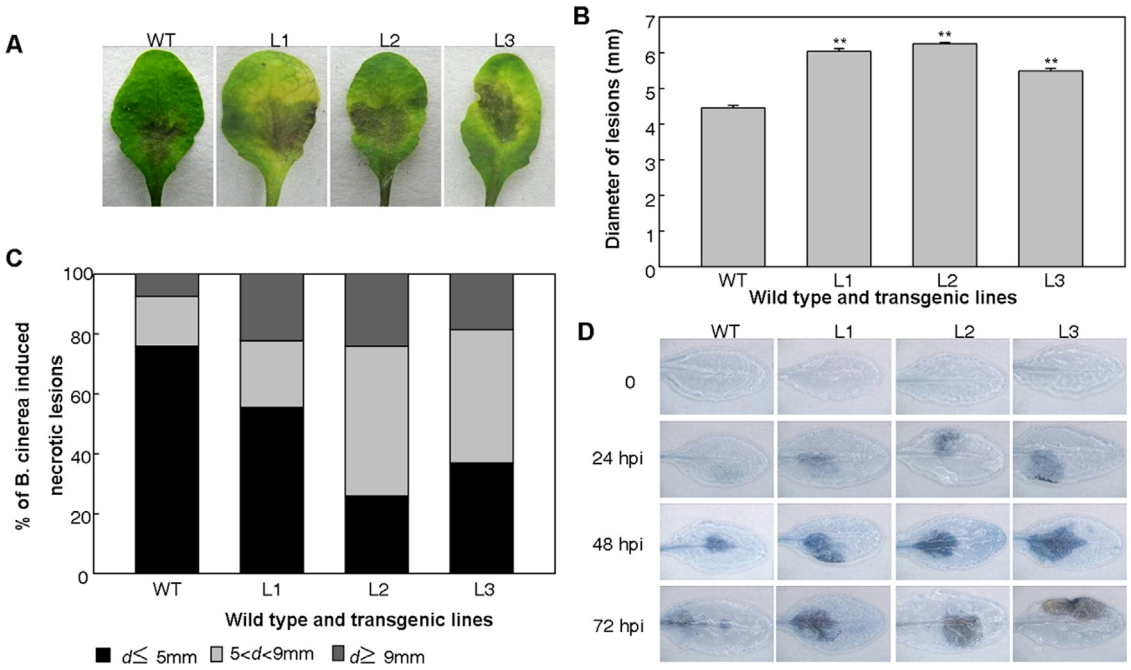

Fig. 8. The response of *VqAP13* transgenic *A. thaliana* to the infection of *B. cinerea*. (A) The phenotypes of transgenic and WT leaves three days after inoculation. (B) Average diameters of lesions three days after inoculation. Data represent mean values ± SD from three independent experiments with at least 50 leaves per sample. Asterisks indicate statistical significance (**$P < 0.01$, Student's t test) between transgenic lines and WT. (C) Symptoms three days after inoculation were scored by defining three lesion diameter (d) classes: $d \leq 5$ mm; $5 < d < 9$ mm; $d \geq 9$ mm. Data represent mean values from three independent experiments with at least 50 leaves per sample. (D) Staining with trypan blue for cell death in detached leaves from WT and transgenic lines subjected to *B. cinerea* infection for 0 h, 24 h, 48 h, 72 h. The experiment was repeated 3 times with 5–10 leaves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

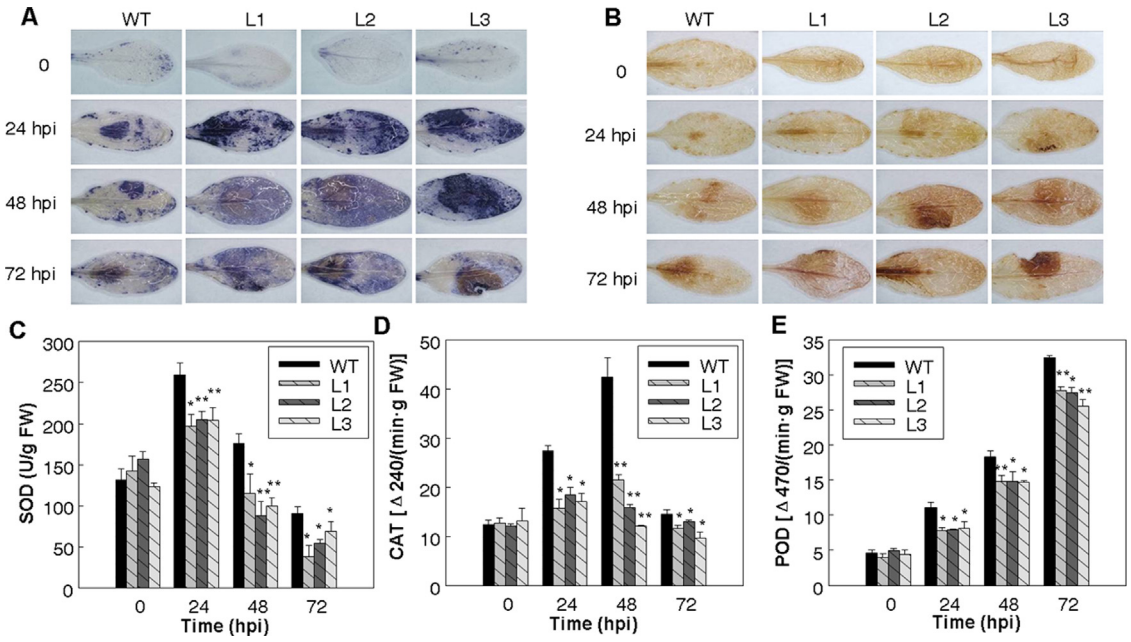

Fig. 9. ROS levels and activity assays measuring antioxidant enzymes in WT and *VqAP13* transgenic *A. thaliana* plants post *B. cinerea* infection. A, B. Histochemical staining assay detecting H_2O_2 and O_2^- accumulation with nitro blue tetrazolium (DAB) (A) and diaminobenzidine (NBT) (B) in WT and transgenic leaves. The experiment was repeated 3 times with 5–10 leaves. C-E Activity of superoxide dismutase (SOD) (C), catalase (CAT) (D) and peroxidase (POD) (E) in the leaves of WT and transgenic plants. Data represent mean values ±SD from three independent experiments. Asterisks indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's t test) between transgenic lines and WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

JA synthesis pathway increased post infection, and was higher in WT plants than in the transgenic *A. thaliana* lines (Fig. 10A). The expression of *PDF1.2* also increased continuously after infection; however, we saw no significant difference between transgenic *A. thaliana* lines and WT at 24 hpi, while at 48 and 72 hpi its expression was lower in the transgenic *A. thaliana* lines (Fig. 10B). The transcript levels of the SA biosynthesis gene, *ICS1*, also increased continuously, and were higher in transgenic *A. thaliana* lines immediately after infection and 24 hpi, but were higher in WT plants at subsequent time points (Fig. 10C). Finally, we observed that the expression of *PR1* was 4-fold greater in the transgenic *A. thaliana* lines at 24 hpi, after which expression levels declined in the trans-

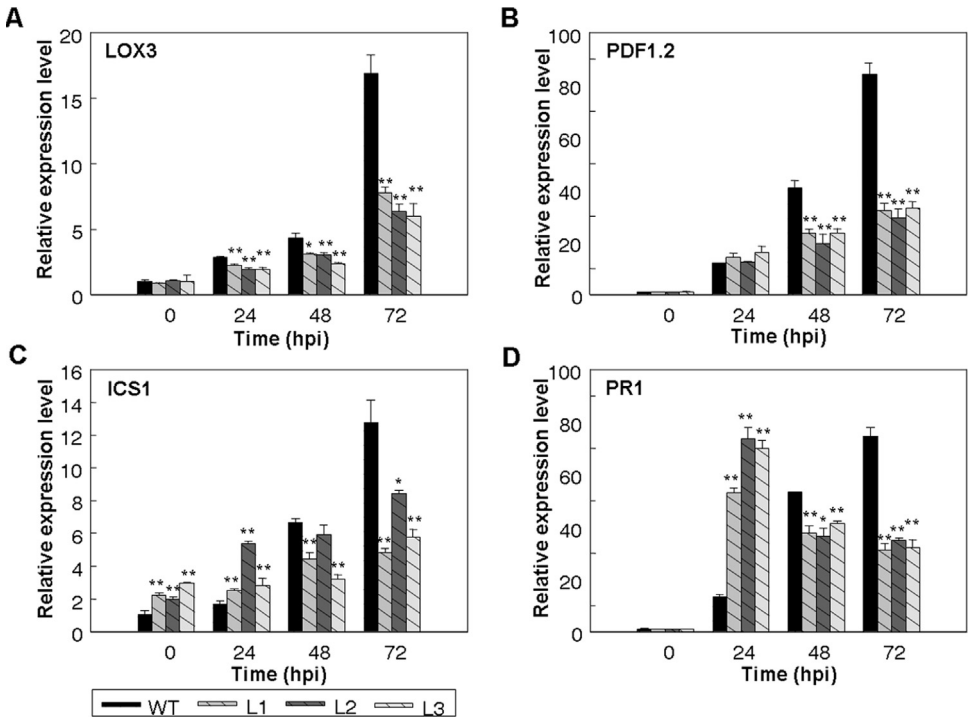

Fig. 10. Expression levels of disease resistance genes in WT and *VqAP13* transgenic *A. thaliana* plants following *B. cinerea* infection. (A) Relative expression levels of *LOX3*. (B) Relative expression levels of *PDF1.2*. (C) Relative expression levels of *ICS1*. (D) Relative expression levels of *PR1*. Relative gene expression levels were analyzed using quantitative RT-PCR. Bars represent the mean ± SD of three independent experiments. Asterisks indicate statistical significance ($^{*}0.01 < P < 0.05$, $^{**}P < 0.01$, Student's *t* test) between transgenic lines and WT.

genic *A. thaliana* lines but continued to increase in WT plants at 48 and 72 hpi (Fig. 10D).

4. Discussion

Induced disease resistance in plants relies on the ability of the host to recognize the potential pathogen and trigger an appropriate response [45]. Plants employ distinct recognition mechanisms and signal transduction pathways associated with the response to different types of invading pathogens. In these pathways, SA, JA and ET function as key signaling molecules, and form complex defense networks of synergistic and antagonistic interactions [46–48]. The SA signaling pathway is involved in resistance to biotrophic pathogens, while the JA and ET signaling pathways principally mediate resistance to necrotrophic pathogens [49]. In a previous study, we found that the expression of *AP13* in Chinese wild *V. quinquangularis* cv. 'Shang-24' was induced by infection with *E. necator* [23]. In the current study, we measured the expression of *AP13* in *V. quinquangularis* cv. 'Shang-24' following treated with SA, MeJA, ET, as well as after inoculation with *B. cinerea*, and found that it was up-regulated by SA treatment, except at 24 hpt, which was consisted with our previous study [23], and this maybe due to its interaction with other genes in SA signal pathway. Moreover, *VqAP13* was up-regulated by ET treatment, and down-regulated by MeJA treatment and *B. cinerea* infection (Fig. 1). From this we concluded that *AP13* may be promote signaling through the SA and ET signal transduction pathways, and suppress the JA signal transduction pathway. The opposite roles that *AP13* appears to play in the ET and JA signal transduction pathways may reflect their distinct regulatory networks.

VqAP13 was ectopically expressed in *A. thaliana* in order to test its function in the responses to different pathogens, specifically be evaluating infection phenotypes, as well as the expression profiles of disease resistance related genes post pathogens infection. Some studies have shown that the SA signal transduction pathway plays an important role in limiting the invasion of powdery mildew and *Pst*DC3000 [49,50]. In our study, the transcript levels of *VqAP13* in the transgenic *A. thaliana* lines increased after infection with these two pathogens (Fig. 2), and over-expression of *VqAP13* improved the resistance of the transgenic plants (Fig. 3 and Fig. 5). The increased transcript levels of *VqAP13* post the two pathogens infection might partially owe to interaction of the *cis*-acting element that involved in defense and stress responsiveness in the CaMV 35S promoter with the two pathogens. It has been suggested that *Pst*DC3000 produce a JA analog, coronatine, which suppress SA dependent defenses, thereby promoting susceptibility to this pathogen [51–53]. Thus, the increased resistance of the transgenic *A. thaliana* lines to *Pst*Dc3000 suggests a role for *VqAP13* in promoting SA dependent defense responses. In addition, after being infected by *G. cichoracearum*, the expression levels of *ICS1*, *PR1* and *NPR1*, which are involved in the SA biosynthetic and signal transduction pathways, respectively, were higher in the transgenic *A. thaliana* lines than in WT (Fig. 4A–C), while *LOX3*, which is involved in the JA biosynthetic pathway, was down-regulated in the transgenic plants (Fig. 4D). Finally, the expression of *PR1* was up-regulated in the transgenic *A. thaliana* lines by *Pst*DC3000 infection, while *PDF1.2*, which is involved in the JA signal transduction pathway, was down-regulated (Fig. 5C,D). Taken together, these results indicate that *VqAP13* promotes signaling through the SA mediated signal transduction pathway.

We also examined the effects of challenging the plants with defence response elicitors, such as the peptide flg22, which can act as an elicitor in a variety of plants [54], and LPS, which is a key component of the outer membrane of Gram-negative bacteria and serves in many experimental systems as stimulant of innate immunity [55,56]. We observed that more callose accumulated in the *VqAP13* over-expressing transgenic *A. thaliana* lines than in WT post *Pst*DC3000, flg22 and LPS injection (Fig. 6), revealing a factor that presumably contributes to the enhanced defense responses.

B.cinerea is a necrotrophic fungal pathogen that induces host cell death at very early stages of infection [48]. We observed that the expression of *VqAP13* in transgenic *A. thaliana* lines gradually decreased following *B. cinerea* infection (Fig. 7), and that the lesions on the transgenic *A. thaliana* leaves were larger than those on WT leaves (Fig. 8), implying the potential roles of *VqAP13* in suppressing the disease-resistance defense response post *B. cinerea* infection. Moreover, the expression levels of *LOX3* and *PDF1.2*, which play key roles in the JA biosynthetic and signaling transduction pathways, respectively, were lower in the transgenic *A. thaliana* lines than in WT, and gradually increased post *B. cinerea* infection (Fig. 10A,B), indicating the over-expression of *VqAP13* suppresses the biosynthesis of JA, as well as the expression of downstream genes. And meanwhile, the decreased expression level of *VqAP13* in transgenic *A. thaliana* lines post *B. cinerea* infection would be partially due to interaction of the several MeJA-response *cis*-acting regulatory elements contained in the CaMV 35S promoter with the suppressed JA biosynthetic and signal transduction pathways. It was reported that the local restriction of *B. cinerea* growth in *A. thaliana* requires SA-dependent defense responses, and that the SA signaling pathway is involved in tolerance of *B. cinerea* [57–60]. We also observed that the expression levels of *ICS1* and *PR1*, which are components of the SA biosynthesis pathway and SA signaling pathways, respectively, increased in *B. cinerea* infected WT plants. Moreover, the over-expression of *VqAP13* resulted in higher transcript levels of *ICS1* and *PR1* 24 h post infection, while levels subsequently decreased (Fig. 10C,D). These results suggested that *VqAP13* contributes to responses to *B. cinerea* infection through both the SA- and JA-dependent signal transduction pathways.

It is known that infection by *B. cinerea* is promoted by, and requires, an active cell death programme in the host [61]. Therefore, it is possible that the reduced resistance of *VqAP13* transgenic *A. thaliana* lines to *B. cinerea* is related to the potential role of *VqAP13* in plant cell death [62]. ROS have been shown to play important roles in susceptible responses of plants to infection by necrotrophic fungal pathogens, such as *B. cinerea*, and their accumulation during late stage of infection are thought to directly benefit the fungus [63]. There is considerable evidence that *B. cinerea* produces ROS and induces the generation of ROS *in planta* to promote its invasion of plant tissues [24,61,64–66]. We found that the *VqAP13* transgenic *A. thaliana* lines accumulated higher ROS levels than WT plants following *B. cinerea* infection (Fig. 9A,B), and that the activities of antioxidant enzymes in the transgenic *A. thaliana* lines were lower than those in WT (Fig. 9C–E), further suggesting that over-expression *VqAP13* resulted in a suppression of certain defence responses as a result of *B. cinerea* infection.

In conclusion, in this study we demonstrated a role for *AP13* from Chinese wild *V. quinquangularis* cv. 'Shang-24' in responses to infection by various pathogens. The characterization of transgenic *A. thaliana* lines over-expressing *VqAP13* demonstrated that this gene promotes signaling through the SA transduction pathway, but suppresses signaling through the JA pathway. Furthermore, the over-expression of *VqAP13* in *A. thaliana* enhanced the resistance of transgenic lines to powdery mildew disease and *Pst*DC3000 infection but reduce the resistance to *B. cinerea*. These results advance our understanding of the function of *VqAP13* in plants responses to biotic stresses. Future studies will investigate the underlying regulatory mechanisms of the different associated signaling transduction pathways.

Author contribution

XW, RG designed the study. RG, MT contributed to the experiment, XW performed data analysis. JZ did the quantitative real-time RT-PCR. RW and ZL assisted with the interpretation of the results. YW and XW overall provided guidance on the whole study. RG and XW wrote the manuscript. All authors approved the final manuscript.

Conflict of interest

There are no competing interests in this paper, and the authors do not have any possible conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.04.006.

References

[1] Z.G. Zhu, J.L. Shi, J.L. Cao, M.Y. He, Y.J. Wang, *VpWRKY3*, a biotic and abiotic stress-related transcription factor from the Chinese wild *Vitis pseudoreticulata*, Plant Cell Rep. 31 (2012) 2109–2120.
[2] R. Mzid, C. Marchive, D. Blancard, L. Deluc, F. Barrieu, M.F. Corio-Costet, N. Drira, S. Hamdi, V. Lauvergeat, Overexpression of *VvWRKY2* in tobacco enhances broad resistance to necrotrophic fungal pathogens, Physiol. Plantarum 131 (2007) 434–447.
[3] J. Glazebrook, W. Chen, B. Estes, H. Chang, C. Nawrath, J.-P. Metraux, T. Zhu, F. Katagiri, Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping, Plant J. 34 (2003) 217–228.
[4] B.N. Kunkel, D.M. Brooks, Cross talk between signalling pathways in pathogen defense, Curr. Opin. Plant Biol. 5 (2002) 325–331.
[5] J. Glazebrook, Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens, Annu. Rev. Phytopathol. 43 (2005) 205–227.
[6] C.M.J. Pieterse, A. Leno-Reyes, S. Van der Ent, S.C. Van Wees, Networking by small-molecule hormones in plant immunity, Nat. Chem. Biol. 5 (2009) 308–316.
[7] B.P. Thomma, I.A. Penninckx, W.F. Broekaert, B.P.A. Cammue, The complexity of disease signaling in *Arabidopsis*, Curr. Opin. Immunol. 13 (2001) 63–68.
[8] G. Le Henanff, S. Farine, F. Kieffer-Mazet, A.S. Miclot, T. Heitz, P. Mestre, C. Bertsch, J.L. Chong, *Vitis vinifera VvNPR1.1* is the functional ortholog of *AtNPR1* and its overexpression in grapevine triggers constitutive activation of PR genes and enhanced resistance to powdery mildew, Planta 234 (2011) 405–417.
[9] X. Guan, H.Q. Zhao, Y. Xu, Y.J. Wang, Transient expression of glyoxal oxidase from the Chinese wild grape *Vitis pseudoreticulata* can suppress powdery mildew in a susceptible genotype, Protoplasma 248 (2011) 415–423.
[10] C. Marchive, C. Leon, C. Kappel, P. CoutosThevenot, M.F. Corio-Costet, S. Delrot, V. Lauvergeat, Over-expression of *VvWRKY1* in grapevines induces expression of jasmonic acid pathway-related genes and confers higher tolerance to the downy mildew, PLoS One 8 (2013) e54185.
[11] H. Li, Y. Xu, Y. Xiao, Z. Zhu, X. Xie, H. Zhao, Y. Wang, Expression and functional analysis of two genes encoding transcription factors *VpWRKY1* and *VpWRKY2*, isolated from Chinese wild *Vitis pseudoreticulata*, Planta 232 (2010) 1325–1337.
[12] Y.H. Yu, W.R. Xu, J. Wang, L. Wang, W.K. Yao, Y.Z. Yang, Y. Xu, F.L. Ma, Y.J. Du, Y.J. Wang, The Chinese wild grapevine (*Vitis pseudoreticulata*) E3 ubiquitin ligase *Erysiphe necator*-induced RING finger protein 1 (EIRP1) activates plant defense responses by inducing proteolysis of the VpWRKY11 transcription factor, New Phytol. 200 (2013) 834–846.
[13] A. Domsalla, M.F. Melzing, Occurrence and properties of proteases in plant lattices, Planta Med. 74 (2008) 699–711.
[14] N.D. Rawlings, A.J. Barrett, MEROPS: the peptidase database, Nucleic Acids Res. 27 (1999) 325–331.
[15] Y.J. Xia, H. Suzuki, J. Borevitz, J. Blount, Z.J. Guo, K. Patel, R.A. Dixon, C. Lamb, An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling, EMBO J. 23 (2004) 980–988.
[16] I. Rodrigo, P. Vera, V. Conejero, Degradation of tomato pathogenesis-related proteins by an endogenous 37-kDa aspartyl endoproteinase, Eur. J. Biochem./FEBS 184 (1989) 663–669.
[17] I. Rodrigo, P. Vera, L.C. Van Loon, V. Conejero, Degradation of tobacco pathogenesis-related proteins: evidence for conserved mechanisms of

degradation of pathogenesis-related proteins in plants, Plant Physiol. 95 (1991) 616–622.
[18] M.G. Guevara, G.R. Daleo, C.R. Oliva, Purification and characterization of an aspartic protease from potato leaves, Physiol. Plantarum 112 (2001) 321–326.
[19] A. Schaller, C.A. Ryan, Molecular cloning of a tomato leaf cDNA encoding an aspartic protease, a systemic wound response protein, Plant Mol. Biol. 31 (1996) 1073–1077.
[20] B.D. Prasad, G. Creissen, C. Lamb, B.B. Chattoo, Overexpression of rice (*Oryza sativa* L.) *OsCDR1* leads to constitutive activation of defense responses in rice and *Arabidopsis*, Mol. Plant- Microbe In. 22 (2009) 1635–1644.
[21] B.D. Prasad, G. Creissen, C. Lamb, B.B. Chattoo, Heterologous expression and characterization of recombinant *OsCDR1*, a rice aspartic proteinase involved in disease resistance, Protein Expression Purif. 72 (2010) 169–174.
[22] M.M. Alam, H. Nakamura, H. Ichikawa, A. Miyao, H. Hirochika, K. Kobayashi, N. Yamaoka, M. Nishiguchi, Response of an *aspartic protease* gene *OsAP77* to fungal, bacterial and viral infections in rice, Rice 7 (2014) 9.
[23] R.R. Guo, X.Z. Xu, B. Carole, X.Q. Li, M. Gao, Y. Zheng, X.P. Wang, Genome-wide identification, evolutionary and expression analysis of the aspartic protease gene superfamily in grape, BMC Genomics 14 (2013) 554.
[24] R. Wan, X.Q. Hou, X.H. Wang, J.W. Qu, Y.J. Wang, X.P. Wang, S.D. Singer, Resistance evaluation of Chinese wild *Vitis* genotypes against *Botrytis cinerea* and different responses of resistant and susceptible hosts to the infection, Front. Plant Sci. 6 (2015) 854.
[25] E.O. King, N.K. Ward, D.E. Raney, Two simple media for the demonstration of pyrocyanin and fluorescein, J. Lab. Clin. Med. 44 (1954) 301–307.
[26] L.J. Wang, S.H. Li, Thermotolerance and related antioxidant enzyme activities induced by heat acclimation and salicylic acid in grape (*Vitis vinifera* L.) leaves, Plant Growth Regul. 48 (2006) 137–144.
[27] V. Repka, I. Fischerova, K. Silharova, Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell-suspension culture, Biol. Plantarum 48 (2004) 273–283.
[28] A. Belhadj, N. Telef, S. Cluzet, J. Bouscaut, M.F. Corio, M. Merillon, Ethephon elicits protection against *Erysiphe necator* in grapevine, J. Agr. Food Chem. 56 (2008) 5781–5787.
[29] B. Assselbergh, K. Curvers, S.C. Franca, K. Audenaert, M. Vuylateke, F. Van Breusegem, M. Hoefte, Resistance to *Botrytis cinerea* in sitiens an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis, Plant Physiol. 144 (2007) 1863–1877.
[30] K. Audenaert, G.B. De Meyer, M.M. Hofte, Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms, Plant Physiol. 128 (2002) 491–501.
[31] G. Le Henanff, C. Profizi, B. Courteaux, F. Rabenoelina, C. Gerard, C. Clement, F. Baillieul, S.C. Ordelier, S. Dhondt-Corelier, Grapevine NAC1 transcription factor as a convergent node in developmental processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance, J. Exp. Bot. 64 (2013) 4877–4893.
[32] O. Windram, P. Madhou, S. McHattie, C. Hill, R. Hickman, Arabidopsis defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis, Plant Cell 24 (2012) 3530–3557.
[33] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[34] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.
[35] S. Xiao, S. Ellwood, K. Findlay, R.P. Oliver, J.G. Turner, Characterization of three loci controlling resistance of *Arabidopsis thaliana* accession Ms-0 to two powdery mildew diseases, Plant J. 12 (1997) 757–768.
[36] S. Xiao, O. Calis, E. Patrick, G. Zhang, P. Charoenwattana, P. Muskett, The atypical resistance gene RPW8, recruits components of basal defence for powdery mildew resistance in *Arabidopsis*, Plant J. 42 (2005) 95–110.
[37] J. Ton, B. Mauch-Mani, Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose, Plant J. 38 (2004) 119–130.
[38] A. Vart, B. Hause, G. Hause, D. Scheel, J. Lee, The *Arabidopsis NHL3* gene encodes a plasma membrane protein and its overexpression correlates with increased resistance to *Pseudomonas syringae* pv. tomato DC3000, Plant Physiol. 132 (2003) 2023–2033.
[39] R.R. Guo, J. Zhao, X.H. Wang, C.L. Guo, Z. Li, Y.J. Wang, X.P. Wang, Constitutive expression of a grape aspartic protease gene in transgenic *Arabidopsis* confers osmotic stress tolerance, Plant Cell Tiss. Org. Cult. (2014) 275–287.
[40] X. Dong, M. Mindrinos, K.R. Davis, F.M. Ausubel, Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene, Plant Cell 3 (1991) 61–72.
[41] D. Ellinger, M. Naumann, C. Falter, C. Zwikowics, T. Jamrow, C. Manisseri, S.C. Somerville, C.A. Voigt, Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*, Plant Physiol. 161 (2013) 1433–1444.
[42] J.D.G. Jones, J.L. Dangl, The plant immune system, Nature 444 (2006) 323–329.
[43] B. Schwessinger, P.C. Ronald, Plant innate immunity: perception of conserved microbial signatures, Annu. Rev. Plant Biol. 63 (2012) 451–482.
[44] B.A. Stone, A.E. Clarke, Chemistry and Biology of (1,3)-β-Glucans, La Trobe University Press, Bundoora, Australia, 1992.
[45] J. Li, G. Brader, E.T. Palva, The *WRKY70* transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense, Plant Cell 16 (2004) 319–331.
[46] J. Glazebrook, Genes controlling expression of defense responses in *Arabidopsis*, Curr. Opin. Plant Biol. 4 (2001) 301–308.
[47] B.N. Kunkel, D.M. Brooks, Cross talk between signaling pathways in pathogen defense, Curr. Opin. Plant Biol. 5 (2002) 325–331.
[48] S.H. Spoel, A. Koornneef, S.M.C. Claessens, J.P. Korzelius, J.A. Van Pelt, M.J. Mueller, A.J. Buchala, J.P. Metraux, R. Brown, K. Kazan, L.C. Van Loon, X.N. Dong, C.M.J. Pieterse, NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol, Plant Cell 15 (2003) 760–770.
[49] L. Chen, L. Zhang, D. Yu, Wounding-induced *WRKY8* is involved in basal defense in *Arabidopsis*, Mol. Plant Microbe Interact. 23 (2010) 558–565.
[50] T.P. Delaney, S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessmann, E. Ward, A central role of salicylic acid in plant disease resistance, Science 266 (1994) 1247–1250.
[51] D.M. Brooks, C.L. Bender, B.N. Kunkel, The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*, Plant Pathol. 6 (2005) 629–640.
[52] J. Cui, A.K. Bahrami, E.G. Pringle, G. Hernandez-Guzman, C.L. Bender, N.E. Pierce, F.M. Ausubel, *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 1791–1796.
[53] N. Laurie-Berry, V. Joardar, I.H. Street, B.N. Kunkel, The *Arabidopsis thaliana JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*, Mol. Plant Microbe Interact. 19 (2006) 789–800.
[54] G. Felix, J.D. Duran, S. Volko, T. Boller, Plants have a sensitive perception system for the most conserved domain of bacterial flagellin, Plant J. 18 (1999) 265–276.
[55] C. Alexander, E.T. Rietschel, Bacterial lipopolysaccharides and innate immunity, J. Endotoxin Res. 7 (2001) 167–202.
[56] Y. Desaki, A. Miya, B. Venkatesh, S. Tsuyumu, H. Yamane, H. Kaku, E. Minami, N. Shibuya, Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells, Plant Cell Physiol. 47 (2006) 1530–1540.
[57] S. Ferrari, J.M. Plotnikova, G. De Lorenzo, F.M. Ausubel, Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2* but not *SID2*, *EDS5* or *PAD4*, Plant J. 35 (2003) 193–205.
[58] E.M. Govrin, A. Levine, Infection of *Arabidopsis* with a necrotrophic pathogen *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR), Plant Mol. Biol. 48 (2002) 267–276.
[59] L. Sanchez, B. Courteaux, J. Hubert, S. Kauffmann, J.H. Renault, C. Clément, F. Baillieul, S. Dorey, Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in *Arabidopsis* and highlight a central role for salicylic acid, Plant Physiol. 160 (2012) 1630–1641.
[60] L. Zimmerli, J.P. Métraux, B. Mauch-Mani, β-Aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*, Plant Physiol. 126 (2001) 517–523.
[61] J.A.L. Van Kan, Licensed to kill: the lifestyle of a necrotrophic plant pathogen, Trends Plant Sci. 11 (2006) 247–253.
[62] J.L. Chong, M.C. Piron, S. Meyer, D. Merdinoglu, C. Bertsch, P. Mestre, The SWEET family of sugar transporters in grapevine: *VvSWEET4* is involved in the interaction with *Botrytis cinerea*, J. Exp. Bot. 65 (2014) 6589–6601.
[63] T. Mengiste, Plant immunity to necrotrophs, Annu. Rev. Phytopathol. 50 (2012) 267–294.
[64] E.M. Govrin, A. Levine, The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*, Curr. Biol. 10 (2000) 751–757.
[65] E.M. Govrin, S. Rachmilevitch, B.S. Tiwari, M. Solomon, A. Levine, An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease, Phytopathology 96 (2006) 299–307.
[66] N. Temme, P. Tudzynski, Does *Botrytis cinerea* ignore H_2O_2-induced oxidative stress during infection? Characterization of *Botrytis* activator protein 1, Mol. Plant-Microbe In. 22 (2009) 987–998.