



## Research article

The cytological basis of powdery mildew resistance in wild Chinese *Vitis* species

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## ABSTRACT

The wild Chinese grapevines (*Vitis* spp.) show varying levels of resistance to powdery mildew caused by *Erysiphe necator* that is an economically important disease of cultivated grapevines (*Vitis vinifera*). However, little information is available regarding the cytological mechanisms of powdery mildew resistance in these wild relatives. Here, we studied the cytological responses of three wild Chinese grapevine accessions after they were infected with *E. necator* (En) NAFU1 in comparison to the susceptible *V. vinifera* cv. ‘Thompson Seedless’ grape. The hyphal growth and sporulation of En NAFU1 were significantly restricted in wild species compared to ‘Thompson Seedless’, which appears to be associated with early cell wall deposition at the attempt sites, encasement of haustoria, and hypersensitive response-like cell death of penetrated epidermal cells. Moreover, endogenous free salicylic acid (SA) was more abundant in wild Chinese *Vitis* species than in ‘Thompson Seedless’ under pathogen-free condition. During En NAFU1 colonization, SA conjugates accumulated higher in wild grapevines than in ‘Thompson Seedless’. In addition, the species-specific expression patterns of defense-associated genes during En NAFU1 colonization indicated that mechanisms underlying powdery mildew resistance are divergent among different wild Chinese *Vitis* species. These results contribute to understanding of mechanisms underlying defense responses of wild Chinese *Vitis* species against powdery mildew.

## 1. Introduction

Grapevine, with over 7000 year's cultivation history, has irreplaceable economical, cultural, and social significance (Jaillon et al., 2007). However, the current viticulture relies on frequent and costly use of chemical fungicides worldwide, since grapevines were constantly challenged with devastating fungal diseases, especially with powdery mildew, throughout their growing seasons (He, 2012; Qiu et al., 2015). The powdery mildew caused by an obligate biotrophic fungus *Erysiphe necator* Schw. (syn. *Uncinula necator* (Schw.) Burr.) has remained a focus for disease management efforts since 1840s, for most grape cultivars are derived from Eurasian *Vitis vinifera* species that is susceptible to powdery mildew (Gadoury et al., 2012). Heavy use of fungicides could control this disease, but has negative impact on environment and a big threat to human health, and results in a rapid spread of fungicides resistance in pathogen populations (Jones et al., 2014; Mulero et al.,

2015). From a long-term goal, enhancing powdery mildew resistance of grapevine cultivars through genetic improvement could sustain grape and wine industries.

Wild relatives and varieties have been widely used for crop genetic improvement (Feechan et al., 2011; Witek et al., 2016). Increasing evidences support that *E. necator* originates on the *Vitis* species that are native to North America (Brewer and Milgroom, 2010). As a result of coevolution, American wild grapevines, including *Muscadinia rotundifolia* (syn. *Vitis rotundifolia*), *V. rupestris*, *V. riparia*, and *V. aestivalis*, develop strong resistance to most *E. necator* isolates worldwide (Gadoury et al., 2012; Blanc et al., 2012; Feechan et al., 2013; Fung et al., 2008). Through forward genetic approaches, grape breeders have identified powdery mildew resistance gene loci in *M. rotundifolia*, including *RUN1*, *RUN2*, and *REN5* (Blanc et al., 2012; Barker et al., 2005). Eastern Asia is also an origin center of wild *Vitis* species. Field observations of over forty years and resistance evaluations

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demonstrated that the wild Chinese *Vitis* species show varying levels of resistance to powdery mildew (He, 2012; Gao et al., 2016; Wan et al., 2007). Several wild Chinese grapevine accessions from *V. pseudoreticulata*, *V. romanetii*, and *V. piasezkii* exhibit high resistance to powdery mildew isolates. Moreover, three dominant powdery mildew resistance loci, designated *REN4*, *REN6*, and *REN7*, respectively, have been identified in *V. romanetii* and *V. piasezkii* (Mahani et al., 2012; Ramming et al., 2011; Pap et al., 2016). Most loci, including *RUN1*, *RUN2*, *REN5*, *REN4*, *REN6*, and *REN7*, confer post-penetration resistance against powdery mildew via inducing programmed cell death (PCD) responses (Blanc et al., 2012; Barker et al., 2005; Ramming et al., 2011; Pap et al., 2016). However, *REN4*-mediated resistance is associated with encasement of haustoria by callose-rich materials (Qiu et al., 2015; Ramming et al., 2011). In addition, *REN6* contributes to stronger ability in restriction of hyphal growth than *RUN1*, *RUN4*, and *RUN7*, in the same genetic background and in response to the same American *E. necator* isolate (Qiu et al., 2015; Pap et al., 2016). These results indicate that different cellular and molecular mechanisms might be used by wild Chinese and wild American *Vitis* species during their interactions with *E. necator*. It is, therefore, of particular interest to understand the cellular and molecular mechanisms of the interactions between wild Chinese grapevines and *E. necator*.

Defense mechanisms of Arabidopsis or cereal crops in response to powdery mildew have been well studied (Douchkov et al., 2016; Wang et al., 2016; Ellinger et al. 2013, 2014). In brief, two processes are responsible for the resistance: one is cell wall deposition, and the other one is hypersensitive response-like cell death. These processes are interacted and resulted in penetration resistance or programmed cell death (PCD) associated resistance, respectively (Jones and Dangl, 2006; Jones et al., 2016; Boutrot and Zipfel, 2017). In a previous report, we evaluated the resistance of wild Chinese grapevines to *En* NAFU1 using detached leaves and screened a total of 13 resistant wild Chinese grapevine accessions, 19 moderately resistant accessions, and 25 susceptible accessions (Gao et al., 2016). However, little information is available regarding the cytological mechanism of powdery mildew resistance in these germplasm (He, 2012; Gao et al., 2016; Wan et al., 2007). In this study, we report the cytological difference of resistant and susceptible grapevines in response to *E. necator*. We speculated that cell wall deposition and encasement of haustorium are essential to the powdery mildew resistance of wild Chinese grapevines. Moreover, the wild Chinese grapevines exhibited robust post-penetration resistance to *E. necator*, which means that nucleotide-binding domain, leucine-rich repeat proteins (NB-LRRs) mediated PCD-associated resistance function well in wild Chinese grapevines. Thus, wild Chinese *Vitis* species are important germplasm for resistance improvement of cultivated grapes.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The wild Chinese grapevines used in this study were mainly collected from the Mountains in Shaanxi Province, Hubei Province, and Jiangxi Province in 1980s, and were maintained in the grapevine germplasm resources orchard at Northwest A&F University in Yangling, Shaanxi Province, China. Here, we rooted the wild Chinese grapevines (*V. pseudoreticulata* accession ‘Baihe-35-1’, *V. piasezkii* accession ‘Baishui-40’, *V. romanetii* accession ‘Baihe-22’, and *V. hancockii* accession ‘Lingye’) and *V. vinifera* cv. Thompson Seedless by cutting in individual pots (diameter: 14 cm, height: 10 cm) containing soil mix (perlite: vermiculite: peat, 1:1:2, v:v:v), respectively. They were grown in a greenhouse with temperature ranging from 22 °C to 27 °C, a relative humidity ranging from 70% to 93% and without supplemental lighting until they developed ten leaves. The grapevines were watered daily and a fertilizing solution was supplemented once a week. For each grapevine, nine independent plants that grow well were selected for powdery mildew inoculation.

### 2.2. Artificial inoculation and microscopic observations

At the 10-leaf-stage, inoculations were conducted by touching the adaxial epidermis of the third and fourth fully expanded leaves (from the shoot apex) using heavily infected ‘Thompson Seedless’ leaves inoculated with an aggressive grapevine powdery mildew isolate *Erysiphe necator* NAFU1 (*En* NAFU1), which was obtained from a heavily infected powdery mildew *V. vinifera* cv. ‘Rizamat’ plant in a vineyard located in the Northwest of China during the summer of 2011 and maintained in laboratory as previously described (Gao et al., 2016). After inoculation, the grapevines were placed in a growth chamber at a temperature of 25 °C and 20 °C (day and night, respectively) with a photoperiod of 14 h of light and a relative humidity ranging from 72% to 80%. Inoculated leaves were collected at 0, 1, 3, 5, 7 and 20 days post-inoculation (dpi). Fungal structures and dead host cells in the inoculated leaves were visualized by trypan blue staining (Gao et al., 2016). Sections were mounted on glass slides, stained with 1% aqueous toluidine blue (dissolved in 1% sodium tetraborate) for 5–10 s, and then washed down the staining solution with water prior to examination with a microscope. The samples were then examined using an Olympus BX51 microscope (Japan). Hyphal length of *En* NAFU1 was directly measured under Olympus BX51 using the cellSens operation software. To evaluate the spore amount, 0.5 g grapevine leaves infected with *En* NAFU1 at 20 dpi were cut into small pieces and immersed in 3 mL 0.01% Tween-20. After a strong vortex to disrupt spores thoroughly, spores in suspension were calculated with a hemocytometer under a microscope. All samples at each time point were collected from three independent grapevines, and triplicate inoculation assays were carried out, respectively.

### 2.3. Transmission electron microscopy (TEM)

*En* NAFU1-infected grapevine leaves (7 dpi) were examined by TEM. The infected leaves were randomly cut into pieces with a length of 3 × 1 mm and fixed immediately in 4% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 40–50 min under vacuum, then overnight at 4 °C. The specimens were fourth washed (10 min) with 0.1 M PBS (pH 6.8) and post fixed in 1% osmium tetroxide (OsO<sub>4</sub>) for 2.5 h at room temperature in the dark, and then washed five times in 0.1 M PBS (pH 6.8). Next, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100%) for 10 min each and embedded in LR White resin with gentle rotation for 48 h. After polymerization (48 h at 60 °C), semi-thin (2 μm) sections were cut using a microtome (Leica, RM2265, Germany) with glass knives. Semi-thin (2 μm) sections were observed using a bright-field light microscope (Olympus BX51) in order to locate infection sites. Ultra-thin (80 nm) sections of these areas were cross-sectioned using a Leica Ultracut UCT ultramicrotome (Germany) with a diamond knife (Diatome, ultra 35°, Germany) and then mounted on 200-mesh copper grids (China). Finally, sections were visualized using a HITACHI HT7700 transmission electron microscope (Japan) operating at 80 kV. Samples were collected from three independent grapevines in triple inoculation assays, respectively.

### 2.4. Measurement of salicylic acid (SA)

To measure the content of SA and SA conjugates in wild Chinese grapevine accessions ‘Baihe-35-1’, ‘Baishui-40’, ‘Lingye’, and *V. vinifera* cv. ‘Thompson Seedless’ before and post *En* NAFU1 inoculation, three leaves were randomly chosen from each grapevine and pooled to form one biological replicate. Three independent biological replicates were sampled. Leaf samples were immediately frozen and ground in liquid nitrogen. Measurement of SA levels was modified from previous protocols (Yalpani et al., 1991; Fung et al., 2008). 100 mg of leaf tissues was extracted for measuring SA and the organic phase of SA and SA conjugates were dried in fume hood overnight. The dried extracts were

suspended in 1 mL of 50% methanol by vortex. 5  $\mu$ L of samples was injected. SA was analyzed using ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem spectrometry (UPLC/ESI-MS/MS) (Agilent Technologies). Flow rate is 1.2 mL/min. Quantification of SA concentration was determined in a linear range of 5–500 ng/mL calibration curve for salicylic acid (Sigma-Aldrich).

### 2.5. Other analyses

Trypan blue staining for fungal structures and dead cells were performed as previous descriptions (Gao et al., 2016; Xiao et al., 2003). Real-time quantitative PCR assays were carried out as described by Hu et al., (2016). *EF1* (elongation factor 1, EC959059) and *Actin1* (AY847627) were used as reference genes (Selim et al., 2012). The accession number of defense-associated genes and the primers used for real-time RT-qPCR are listed in Supplementary Table 1. All of the experiments were performed independently in triplicate and similar results were obtained. The data were processed with IBM SPSS 18.0 software and were presented as mean  $\pm$  standard deviation of three independent experiments and were tested for statistical significance by paired Student's *t*-test (<http://www.physics.csbsju.edu/stats/>) or Duncan's multiple range test.

## 3. Results

### 3.1. Hyphal growth and sporulation of *En NAFU1* were restricted in wild Chinese *Vitis* species

To further explore cytological mechanism of powdery mildew resistance in wild Chinese grapevines, we selected four wild Chinese grapevine accessions (*V. pseudoreticulata* accession 'Baihe-35-1', *V. piasezkii* accession 'Baishui-40', *V. rotundifolia* accession 'Baihe-22', and *V. hancockii* accession 'Lingye') and used 10-leaf-stage cuttings as materials for *En NAFU1* inoculation while used the *V. vinifera* cv. 'Thompson Seedless' cuttings as susceptible control. As shown in Fig. 1A, the infected leaves of 'Thompson Seedless' and 'Lingye' plants were covered with an abundance of white velvety mycelia, in contrast, the infected leaves of 'Baihe-35-1', 'Baishui-40', and 'Baihe-22' were covered only with little of whitish 'powder' at 20 dpi. Quantification of the hyphal length per colony at 3 dpi and 5 dpi, respectively, revealed that 'Thompson Seedless' supported fungal growth, with values more than 2 to 3-fold greater than those obtained from the infected leaves of the four wild grapevines (Fig. 1B–C). *En NAFU1* developed conidiophores on leaves of 'Thompson Seedless', 'Lingye', and 'Baihe-22' plants at 7 dpi, but did not produce visible spores on leaves of 'Baihe-35-1' and 'Baishui-40' (Fig. 1B). Moreover, over 90% penetrated epidermal cells had undergone hypersensitive response-like cell death (HR-like) in infected leaves of 'Baihe-35-1' and 'Baishui-40' during 5 dpi to 7 dpi, whereas few HR-like responses were observed in infected leaves of 'Thompson Seedless', 'Lingye', and 'Baihe-22' (Fig. 1B). As a consequence, the number of spores per mg of infected leaf tissues at 20 dpi significantly less in 'Baihe-35-1' and 'Baishui-40' than in 'Thompson Seedless' (Fig. 1D).

### 3.2. Structural difference in the epidermal cells of four grapevine genotypes

To further explore the cytological strategies used by wild Chinese grapevines to restrict the hyphal growth and sporulation of *En NAFU1*, we selected four accessions to elucidate their structural difference of leaf epidermal cells. Here, the 'Baihe-22' was not selected for further analysis, since its morphological feature is apparently different with that of other *V. rotundifolia* grapevines and seems to be an inter-species hybrid. As shown in Fig. 2, in all leaf sections, a one-cell-thick layer was coated on the outer side with a cuticle to form an adaxial epidermis, without stomata (Fig. 2A–B). Beneath the epidermis lies the palisade parenchyma which made up of tubular, tightly packed cells that contain

abundant chloroplasts and usually are arranged in one layer (Fig. 2A–B). Following the palisade parenchyma is the spongy parenchyma which consists of 3–4 cell layers of anomalous cells that contain fewer chloroplasts than palisade parenchyma cells (Fig. 2A–B). There are many intercellular spaces in the spongy parenchyma (Fig. 2A–B). The abaxial epidermis is one layer like the adaxial epidermis, but contains more tortuous cells and connected with the substomatal cavity (Fig. 2A). Notably, the palisade parenchyma and spongy parenchyma contain electron dense materials in 'Baishui-40', particularly in 'Lingye' (Fig. 2B). We observed the cuticle of the upper leaf surface and the chloroplasts. There were some differences between the four grapevines in morphology of cuticle that complex cuticle organization was observed in wild Chinese grapevines (Fig. 2C). However, the characteristics of chloroplast did not display obvious difference between the four grapevines (Fig. 2D).

### 3.3. Papilla formed at the attempt sites in powdery mildew-resistant grapevines

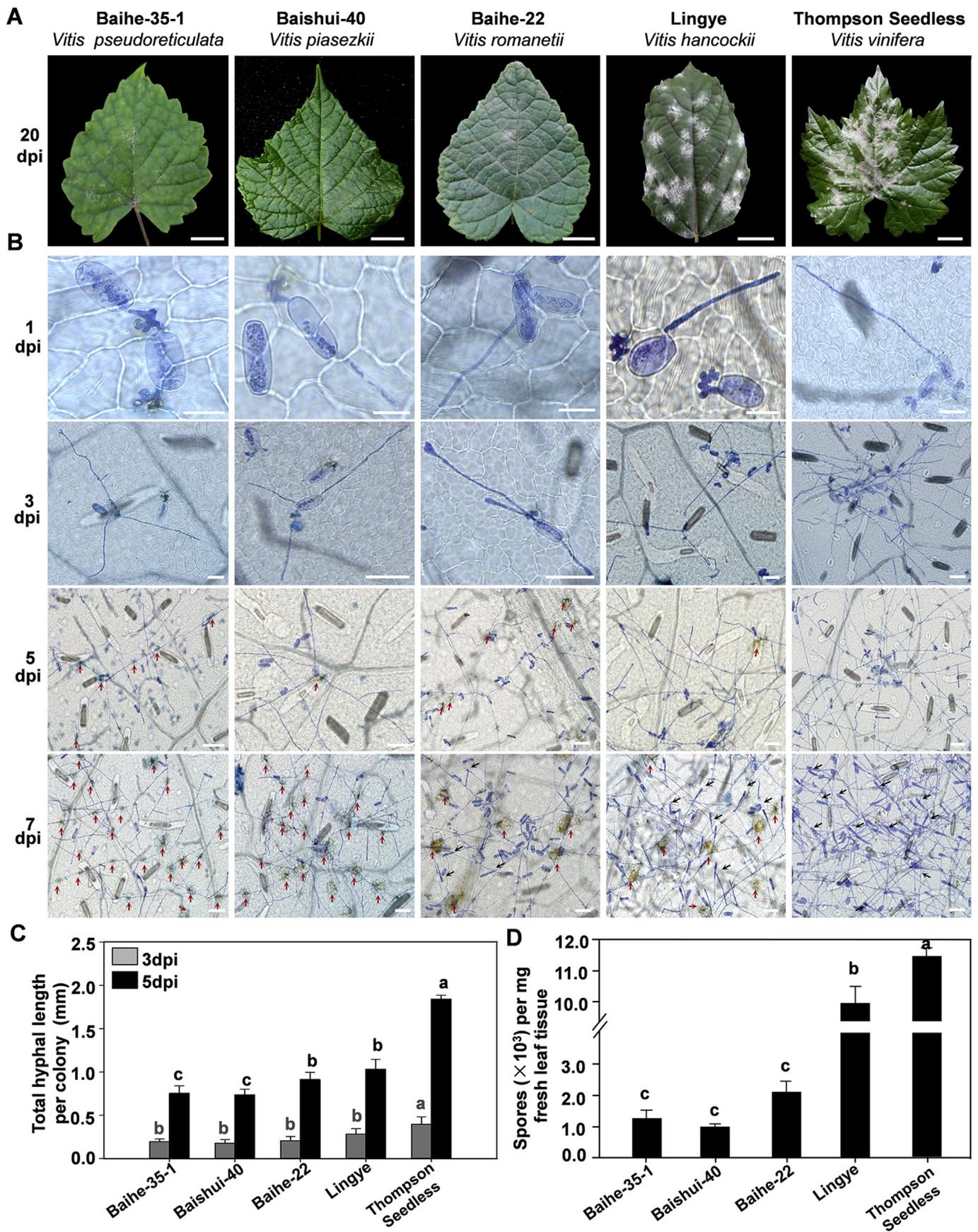
As shown in Fig. 3, preliminary observation of trypan blue stained samples revealed that haustoria of *En NAFU1* were abnormally formed and poorly developed in 'Baihe-35-1' and 'Baishui-40' leaves, whereas haustoria of *En NAFU1* were normally developed in 'Lingye' and 'Thompson Seedless' leaves (Fig. 3A–D). Observation of semithin sections using light microscopy showed striking differences between 'Baihe-35-1', 'Baishui-40', 'Lingye', and 'Thompson Seedless' leaves infected by *En NAFU1* (Fig. 3E to P). In serial sections of 'Baihe-35-1' and 'Baishui-40', massive cell wall deposition, called papillae, were observed beneath fungal attempt sites or nearby the penetrated epidermal cells, whilst abnormally formed and poorly developed haustoria of *En NAFU1* were found in the vicinity of papillae (Fig. 3E–G). Apparently, papillae were intensely stained with toluidine blue and appeared usually as hemispherical (Fig. 3E–H). By contrary, papillae were seldomly observed in 'Lingye' and 'Thompson Seedless', whilst the haustoria of *En NAFU1* were well rounded and detected with high frequency in serial sections (Fig. 3K to P).

### 3.4. Haustoria of *En NAFU1* were encased in resistant grapevines

The compositions of haustoria were manifested as disappearance of haustorial main body in 'Baihe-35-1' and distortion of haustorial main body in 'Baishui-40' and 'Lingye', respectively (Fig. 4A, C, 4E, and 4G). By contrast, the haustoria developed in susceptible 'Thompson Seedless' were characterized by an oval haustorial main body surrounded by few lobes, and the occurrence of abundant defined fungal organelles, labeled with electron-dense granular material, in haustoria bodies, indicating that the haustoria were metabolically active in 'Thompson Seedless' (Fig. 4G). Meanwhile, few haustoria in 'Thompson Seedless' were found to partially embed into electron-dense deposit within the extrahaustorial membrane, but most haustoria in 'Baihe-35-1', 'Baishui-40', and 'Lingye' were fully embedded into electron-dense deposit within the extrahaustorial membrane (Fig. 4B, D, 4F, and 4H), indicating that the haustoria of *En NAFU1* were encased in wild Chinese grapevines. Notably, in 'Baihe-35-1', massive multivesicular bodies accumulated in cytosol of the penetrated epidermal cells and the neck of each haustorium was surrounded by a multilayered collar, which are likely the lignified and thickened secondary cell wall (Fig. 4A–B).

### 3.5. SA and SA conjugates were elevated in wild Chinese *Vitis* species

Salicylic acid (SA) is an important signal molecule in host response to biotrophic pathogens, including local defense and systemic acquired resistance (Fu and Dong, 2013). Increases in the endogenous levels of SA and its conjugates (SAG) is associated with the activation of pathogenesis-related (PR) gene expression and disease resistance (Yang et al., 2015; Fan and Dong, 2002). To assess the variation of SA content

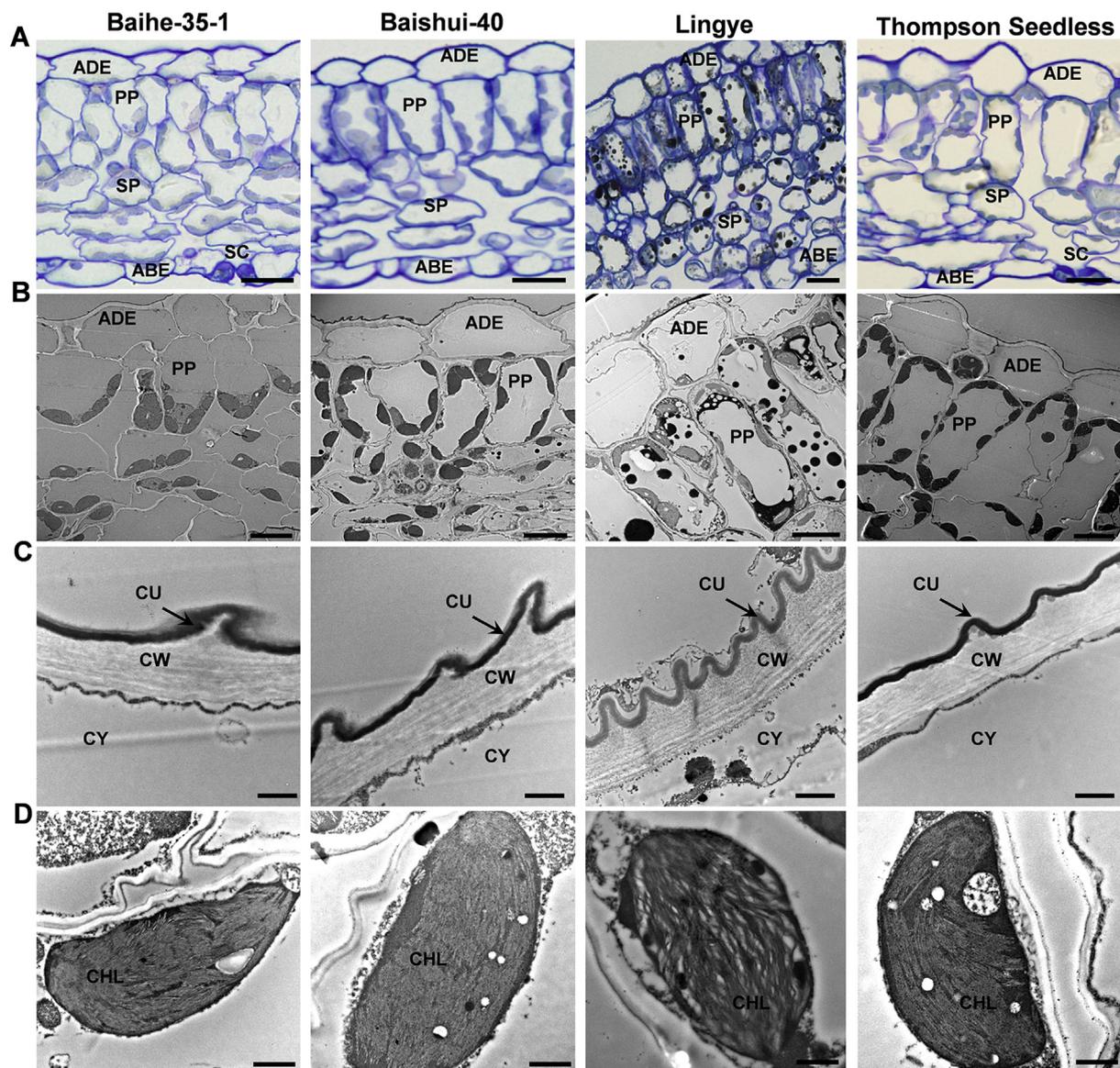


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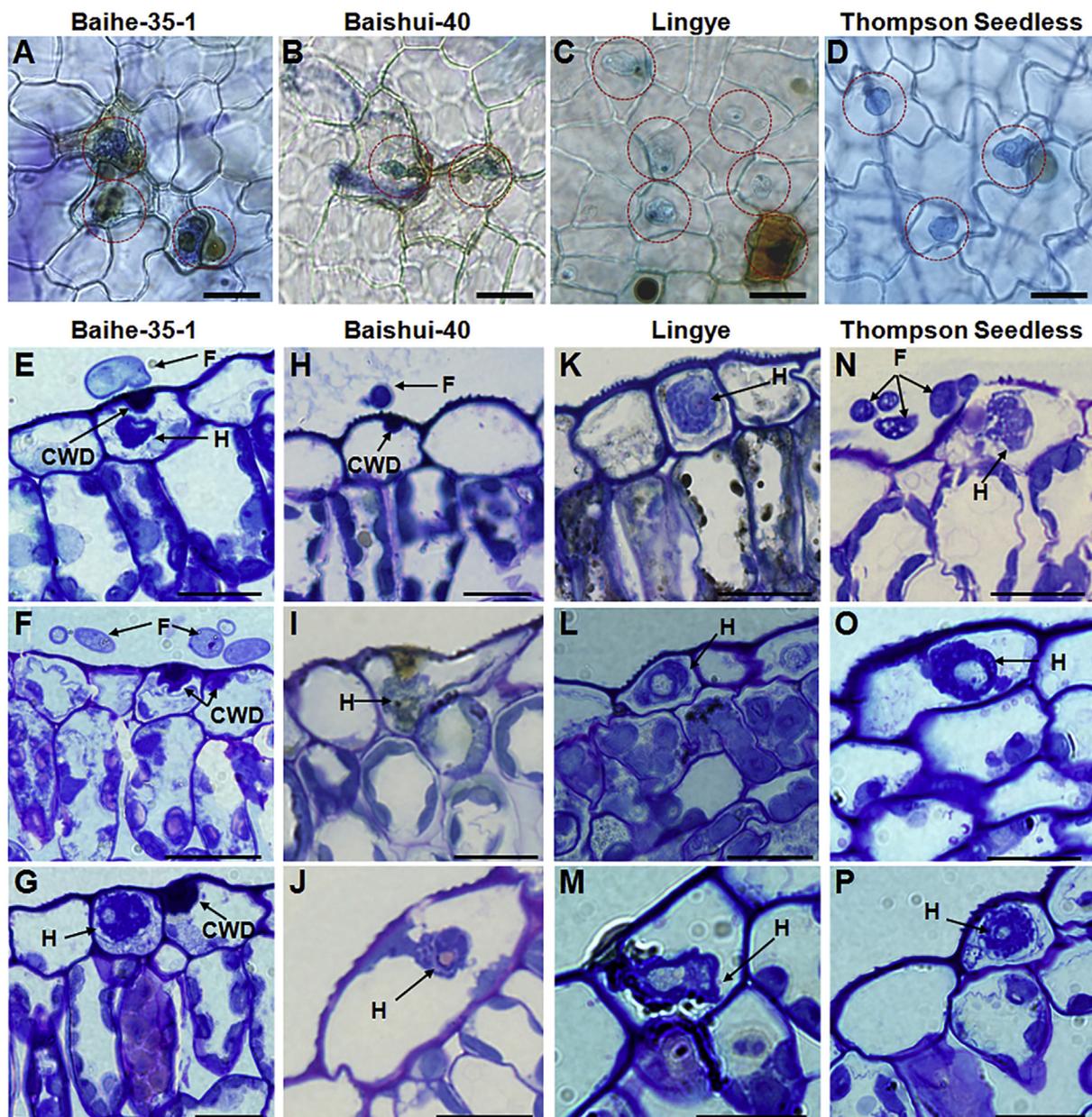
**Fig. 1.** Interactions between *En* NAFU1 and epidermal cells on the adaxial side of leaves in wild Chinese *Vitis* species and *V. vinifera* cv. ‘Thompson Seedless’. A: Representative leaves of five grapevines showing macroscopical sporulation of *En* NAFU1 at 20 dpi. Bars = 1 cm; B: Representative images showing hyphal growth and sporulation of *En* NAFU1 in five grapevines at 1, 3, 5, and 7 dpi, respectively. The red arrows denote the hypersensitive response-like cell death observed in trypan blue-stained sections, and the black arrows indicate conidiophores. Bars = 25  $\mu$ m, except the first line Bars = 10  $\mu$ m. C: Average hyphal length per colony of *En* NAFU1 inoculated on different grapevines at 3 dpi and 5 dpi. D: Quantification of spores per mg fresh leaf tissues infected with *En* NAFU1 in four grapevines at 20 dpi. The hyphal length was calculated from 30 independent colonies, while the amount of spores was obtained from 10 infected leaves. Each data point is the mean of three independent experiments ( $\pm$  standard deviation), and values followed by the same letter are not significantly different according to Duncan's multiple range test at  $P = 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

during powdery mildew infection in the four grapevines, we measured the SA and SAG content during *En* NAFU1 colonization by UPLC/ESI-MS/MS. Notably, SA and SAG were present at elevated levels in wild Chinese grapevines in comparison to ‘Thompson Seedless’ at 0 dpi, indicating more active SA metabolism exists in wild Chinese *Vitis* species (Fig. 5A). During the *En* NAFU1 infection process, free SA content slightly increased in *En* NAFU1-inoculated leaf tissues of four grapevines and reached top level at 1 dpi to 3 dpi, then declined gradually and remained nearly constant at 7 dpi (Fig. 5A). By contrast, the

difference of endogenous SA content among the four grapevines was more manifested on the level of SAG (Fig. 5B). We found that the SAG content in the three wild Chinese grapevine accessions were significantly higher than that in ‘Thompson Seedless’ at 0 dpi, 1 dpi, 3 dpi, 5 dpi, and 7 dpi (Fig. 5B). Particularly, ‘Baihe-35-1’ had two-fold higher of SAG content than ‘Thompson Seedless’ at 0 dpi, and the levels of SAG rapidly increased two fold at 1 dpi, then declined gradually and remained nearly constant at 7 dpi. The content of SAG in ‘Baishui-40’ and ‘Lingye’ gradually increased and reached top level at 5 dpi, and then



**Fig. 2.** Cytological study of the uninfected grapevine leaves. A: The transverse sections stained by toluidine blue and observed in microscope under bright light. Bars = 10  $\mu$ m. B: The transverse sections observed by transmission electron microscopy. Bars = 5  $\mu$ m. C: The close-up of cuticle. Bars = 0.25  $\mu$ m. D: The close-up of chloroplasts. Bars = 1  $\mu$ m. Abbreviation: ABE, abaxial epidermal cell; ADE, adaxial epidermal cell; CHL, chloroplast; CU, cuticle; CW, cell wall; CY, cytosol; PP, palisade parenchyma; SC, stomata guard cells; SP, spongy parenchyma. Leaf samples were collected from three independent plants for each grapevine species, and similar results were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Light micrographs of samples from grapevine leaves infected by *En* NAFU1. A to D: Trypan blue staining show the haustorium of *En* NAFU1 developed in the epidermal cells of ‘Baihe-35-1’, ‘Baishui-40’, ‘Lingye’, and ‘Thompson Seedless’, respectively. Red circles marked the haustoria of *En* NAFU1. Bars = 10  $\mu$ m. E to P: The transverse sections stained by toluidine blue showing the morphology and structure of haustorium developed in ‘Baihe-35-1’, ‘Baishui-40’, ‘Lingye’, and ‘Thompson Seedless’, respectively. CWD, cell wall deposition; F, fungal mycelium; H, haustorium. Bars = 10  $\mu$ m. All samples were collected from three independent plants in triplicate inoculation assays, and consistent results were obtained. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

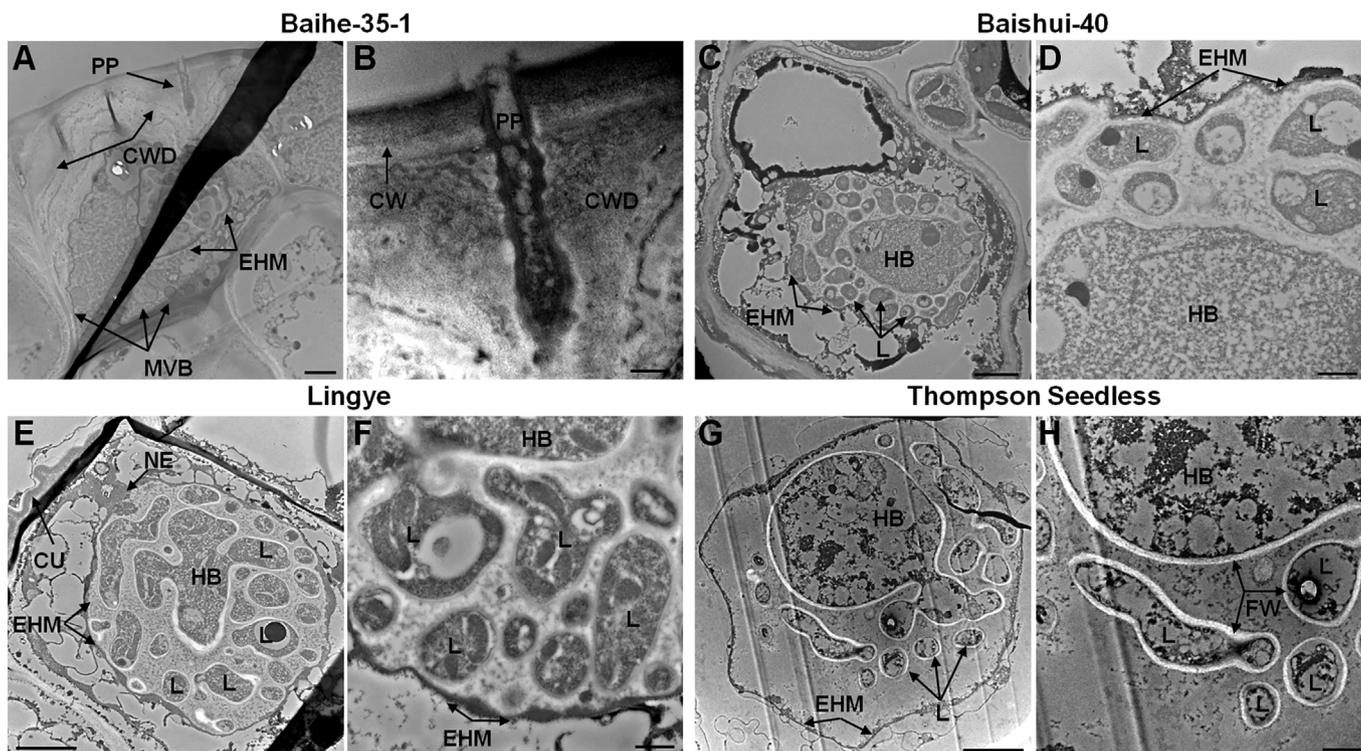
declined at 7 dpi (Fig. 5B). These results suggested that the enhanced disease resistance to powdery mildew in wild Chinese grapevines may be associated with the activation of the SA-dependent basal defense.

### 3.6. Defense-associated genes were differently expressed in wild Chinese *Vitis* species

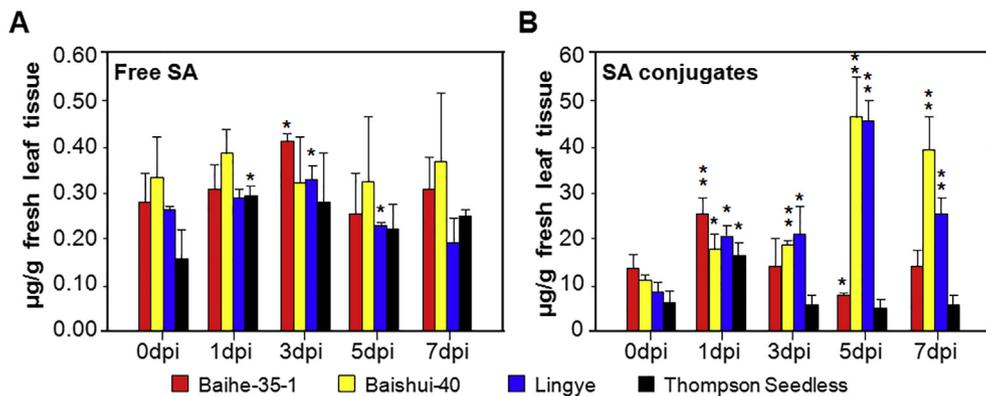
To seek more molecular cues of powdery mildew resistance in wild Chinese grapevines, we examined the transcript levels of defense-associated genes. As shown in Fig. 6, though *EDS1* (*enhanced disease susceptibility 1*) and *PAD4* (*phytoalexin deficient 4*), two core genes of SA-dependent defense pathway, exhibited elevated transcript level in wild Chinese grapevines in comparison to ‘Thompson Seedless’ at the early stage (0 hpi to 72 hpi) of *En* NAFU1 infection, their relative transcript

level in wild Chinese grapevines declined even lower than those in ‘Thompson Seedless’ at the late stage. The SA-induced genes, including *PR1* (*pathogenesis-related gene 1*), *PR2* (*pathogenesis-related gene 2*), *PR3* (*pathogenesis-related gene 3*), *PR5* (*pathogenesis-related gene 5*), and *NPR1* (*nonexpresser of PR genes 1*), maintain high level expression and their transcript level were gradually up-regulated in ‘Baishui-40’ and ‘Thompson Seedless’ during the powdery mildew colonization. However, in ‘Baihe-35-1’ and ‘Lingye’, the expression of above genes apparently was not (or only slightly) induced by *En* NAFU1. In addition, the *PDF1.2* (*plant defensin 1.2*) was gradually down-regulated in four grapevines, indicating the powdery mildew infection has a negative correlation with the jasmonic acid signaling (Fig. 6).

Moreover, we examined the transcript levels of several structural genes, such as *CHIT1* (*basic endochitinase 1*), *PAL1* (*phenylalanine*



**Fig. 4.** Transmission electron micrographs of the interaction between the haustorium of *En NAFU1* and four grapevines at 7 dpi. A to H: Close-up of a haustorium developed in ‘Baihe-35-1’, ‘Baishui-40’, ‘Lingye’, and ‘Thompson Seedless’, respectively. CU, cuticle; CW, cell wall; CWD, cell wall deposition; EHM, extra-haustorial membrane; FW, fungal cell wall; HB, haustorial main body; L, haustorial lobes; MVB, multivesicular bodies; NE, haustorial neck; PP, penetration pore. Bars = 2 μm in A, C, E, and G, bars = 500 nm in B, D, F, and H. Similar structural details were observed in all samples collected from three independent plants in triplicate inoculation assays.



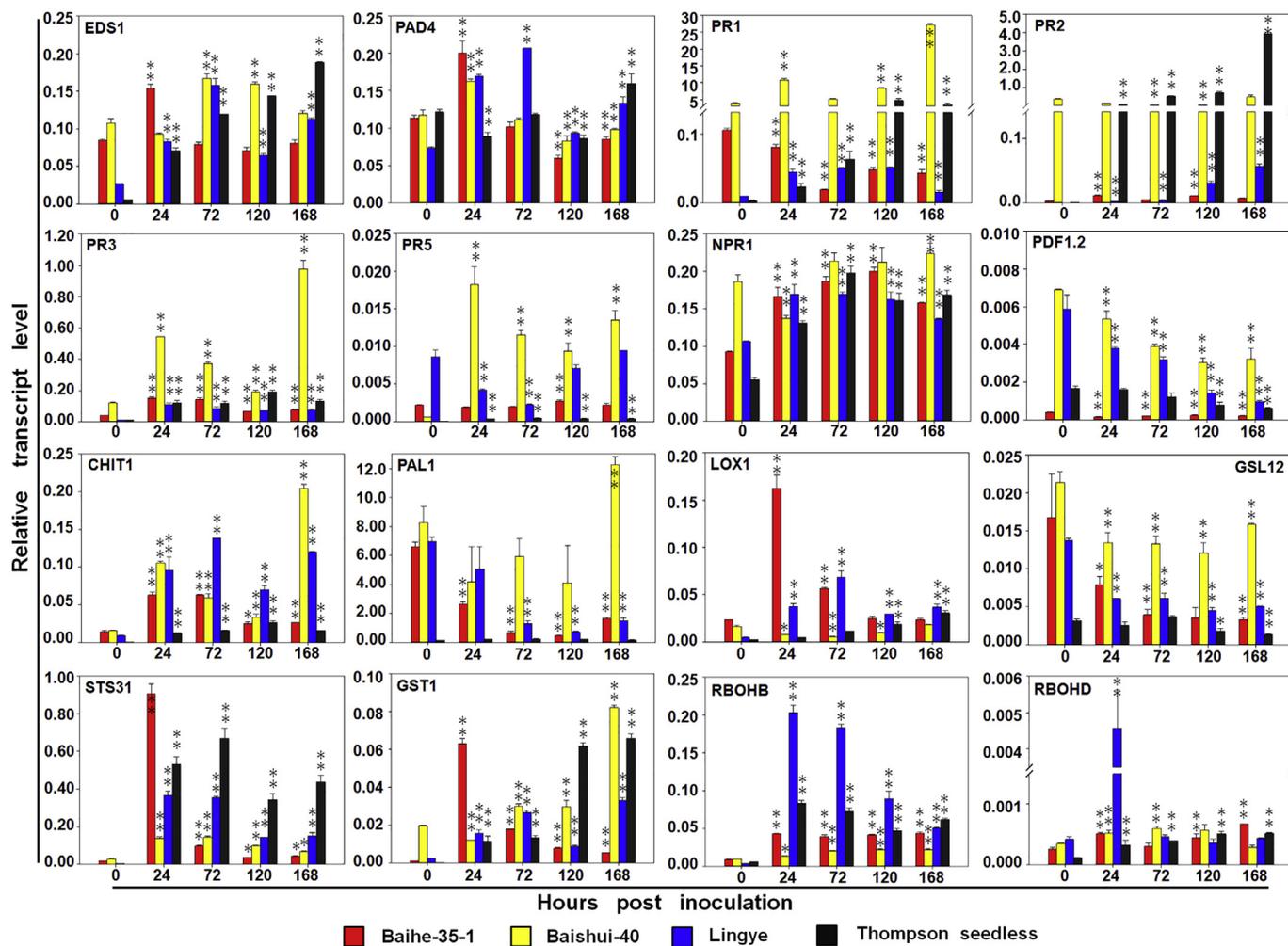
**Fig. 5.** Assays of endogenous SA content at five time points during *En NAFU1* infection in four grapevines. A: Changes of free SA content. B: Changes of SA conjugates content. Each data point represents the mean ± standard deviation of three independent experiments. Dark asterisks indicate that SA or SAG content at each timepoint was significantly elevated or reduced in comparison to 0 dpi (\*\* and \* =  $P \leq 0.01$  and  $0.05$ , respectively; Student's *t*-test).

ammonia-lyase 1), *LOX1* (lipoxygenase 1), and *GSL12* (callose synthase like 12), which are responsible for the biosynthesis of defense-associated molecules, including chitinase, phytoalexins, and callose. Obviously, these genes exhibited higher transcript level in wild Chinese grapevines than those in ‘Thompson Seedless’ during the whole detection process, and their expression were down-regulated after 24 hpi (Fig. 6). To investigate the roles of reactive oxygen species (ROS) in the defense strategies of wild Chinese grapevine in response to powdery mildew, we analyzed the relative transcript level of *RBOHB* (respiratory burst oxidase homologue B), *RBOHD* (respiratory burst oxidase homologue D), *STS31* (stilbene synthase 31), and *GST1* (glutathione S-transferase 1), which are involved in ROS generation and elimination during pathogen infections, respectively. Interestingly, in ‘Baihe-35-1’, both *STS31* and *GST1* showed transient elevation at transcription level at 24 hpi, but their transcript level were gradually up-regulated and reached the peak at the late stage in other three grapevines, especially in ‘Thompson Seedless’. *RBOHB* and *RBOHD* displayed similar expression patterns

during the *En NAFU1* colonization, they were strongly up-regulated in ‘Lingye’ and ‘Thompson Seedless’ but only slightly up-regulated in ‘Baihe-35-1’ and ‘Baishui-40’.

**4. Discussion**

Grape and wine productions in the world rely mainly upon the cultivars derived from the Eurasian grape species *V. vinifera* on account of its favored aroma and flavor. However, this species (*V. vinifera*) is highly susceptible to the powdery mildew pathogen *E. necator* (Gadoury et al., 2012). After all, *E. necator* originates on the *Vitis* species that were native to North America and has undergone long-term co-evolution with grapevines, leading to the conserved defense strategies developed in *Vitis* species failed to function properly (Brewer and Milgroom, 2010; Feechan et al., 2011). As another presumption, cultivated grapevines were clonally propagated, which might limit the generation of new *R*-genes due to the lack of effective genome



**Fig. 6.** The relative transcript level of defense-associated genes in four grapevine leaves post *En* NAFU1 inoculation. Each data point represents the mean  $\pm$  standard deviation of triple biological replicates. *EF1* (*elongation factor 1*, EC959059) and *Actin1* (AY847627) were used as reference genes. *EDS1* (*enhanced disease susceptibility 1*), *PAD4* (*phytoalexin deficient 4*), *PR1* (*pathogenesis-related gene 1*), *PR2* (*pathogenesis-related gene 2*), *PR3* (*pathogenesis-related gene 3*), *PR5* (*pathogenesis-related gene 5*), *NPR1* (*nonexpresser of PR genes 1*), *PDF1.2* (*plant defensin 1.2*), *CHIT1* (*basic endochitinase 1*), *PAL1* (*phenylalanine ammonia-lyase 1*), *LOX1* (*lipoxygenase 1*), *GSL12* (*callose synthase like 12*), *RBOHB* (*respiratory burst oxidase homologue B*), *RBOHD* (*respiratory burst oxidase homologue D*), *STS31* (*stilbene synthase 31*), and *GST1* (*glutathione S-transferase 1*). Asterisks indicate that the transcript of corresponding gene at each timepoint was significantly up- or down-regulated in comparison to 0 dpi (\*\* and \* =  $P \leq 0.01$  and  $0.05$ , respectively; Student's *t*-test).

recombination (Choi et al., 2016). To improve the powdery mildew resistance of *V. vinifera* via genetic improvement, attentions had been paid to the wild *Vitis* species native to North America and Eastern Asia (Wan et al., 2007; Ramming et al., 2012; Fung et al., 2008), and even to other genera (*Muscadinia*, *Ampelopsis*, *Cissus*, and *Parthenocissus*) within the Vitaceae family (Feechan et al., 2011). Generally speaking, an *E. necator* isolate induces penetration resistance or PCD-associated resistance during incompatible interactions while it colonizes above host plants (Feechan et al., 2011; Qiu et al., 2015). In this study, three representative wild grapevines were used as materials to elucidate the cytological and molecular mechanisms employed by wild Chinese *Vitis* species to defend against powdery mildew. Among them, 'Baishui-40' and 'Baihe-35-1' have always been resistant to powdery mildew in field since 1980, and 'Lingye' was highly resistant to powdery mildew in 1990s but it became susceptible at least since 2007 (He, 2012; Gao et al., 2016; Wan et al., 2007).

Cytological responses showed that cell wall deposition, encasement of haustorium, and hypersensitive response-like cell death could contribute to the two typical types of powdery mildew resistance in the wild relatives of grapevine (Figs. 1, 3 and 4). Cell wall deposition or papillae formation could be the consequence of pattern-triggered immunity (PTI), which is mediated by membrane localized receptor-like

kinases (RLKs) or receptor-like proteins (RLPs) through successful recognition of conserved pathogen-associated patterns or damage-associated molecules, such as chitin and oligosaccharide (Brule et al., 2018; Boutrot and Zipfel, 2017). Here, massive papillae formed at the *En* NAFU1 attempt sites in 'Baihe-35-1' and 'Baishui-40' (Figs. 3 and 4), indicating that sensitive and intensive PTI exists in these two grapevines. By contrast, though encasement of haustorium also occurred in 'Lingye' but cell wall deposition and papillae were rarely observed (Figs. 3 and 4), suggesting that PTI alone could not exert efficient defense against adaptive pathogens. In addition, hypersensitive response-like cell death is activated by nucleotide-binding domain, leucine-rich repeat proteins (NB-LRRs), which could directly or indirectly recognize the pathogen secreted effectors to elicit cell death (Jones et al., 2016; Feechan et al., 2013). In this study, in 'Baihe-35-1' and 'Baishui-40', we found that hypersensitive response-like cell death was a common phenomenon occurred in the penetrated cells with deformed haustoria (Figs. 1B–3A), suggesting that NB-LRR-mediated effector-triggered immunity (ETI) function well in these two grapevines.

Grapevines in vineyard were challenged with various diseases, which could be caused by fungi, oomycetes, bacteria, and viruses. In addition, while strong selection pressure exerted on these organisms from single dominant resistance genes (*R*-genes) or frequent spray of

chemical agent often result in rapid emergence of new virulent isolates, generation of new *R*-genes capable of recognizing new virulent pathogens in grapevines is relatively slow (Jones et al., 2014; Hu et al., 2018). Therefore, broad-spectrum genetic resistance is required for breeding grape cultivars. Through long-term co-evolution, the powdery mildew resistance in the wild North America grapevines always is pathogen race-specific resistance, which only manifested as hypersensitive response-like cell death (Blanc et al., 2012; Feechan et al., 2013; Ramming et al., 2012). As a consequence, the resistance mediated by the *NLR* loci isolated from the wild North America grapevines could be rapidly overcome by new evolved *E. necator* strains (Qiu et al., 2015; Feechan et al., 2013). The wild Chinese grapevines showed broad-spectrum resistance to powdery mildew isolates, and which could be associated with the callose-enriched encasement of haustorium (Ramming et al., 2011; Gao et al., 2016; Pap et al., 2016). Recently, Yin et al. reported that ‘Baihe-35-1’ and *V. piasezkii* accession ‘Liuba-8’ showed genetic resistance to *Plasmopara viticola*, the causal agent of grapevine downy mildew, and the cytological basis of downy mildew resistance also was attributed to the tight encasement of haustorium (Yin et al., 2017). These results are expected, since the obligate biotrophic fungus or oomycetes need to invade host cells and form a feeding structure, called haustorium, to steal nutrition from the host cell to complete their life cycle (Glawe, 2008; Koh et al., 2005). Thus, we speculated that cell wall deposition and encasement of haustorium are essential to the broad-spectrum disease resistance of wild Chinese grapevines.

Plants, including grapevine, protect themselves from pathogens using metabolite-based defense strategy, which consist of multilayer, independent but interacting signaling pathways (Alonso-Villaverde et al., 2011). Here, we found that higher level of endogenous free SA and SAG were present in wild Chinese *Vitis* species than ‘Thompson Seedless’ under both pathogen-free and *En* NAFU1-inoculated conditions (Fig. 5A and B), indicating the importance of SA signaling in grapevine defense responses. However, in ‘Baihe-35-1’, the transcript level of SA-responsive genes, such as *PR1*, *PR2*, and *PR5*, only slightly up-regulated or did not change during *En* NAFU1 infection (Fig. 6), which means the SA signaling was not activated in ‘Baihe-35-1’ in response to powdery mildew. Interestingly, a similar result was obtained from the transcriptome analysis of *Vitis aestivalis* ‘Norton’ post *E. necator* inoculation, which manifested as ‘endogenous salicylic acid levels were higher in *V. aestivalis* than in *V. vinifera* in the absence of the fungus’ and ‘only three PM-responsive transcripts in *V. aestivalis* and 625 in *V. vinifera*’ (Fung et al., 2008). Based on these results, we speculated that a SA-dependent defense strategy, which was not associated with pathogen induced reprogramming of the transcriptome, had been developed both in wild Chinese *V. pseudoreticulata* ‘Baihe-35-1’ and North American *V. aestivalis* ‘Norton’.

Notably, *E. necator* is an adapted pathogen for *Vitis* species, and it could secrete massive effector proteins into the epidermal cells via its haustorium and largely suppress the PTI of grapevines (Gadoury et al., 2012; Wu et al., 2018). Here, we found that positive defense-associated genes, such as *CHIT1* (chitinase), *PAL1* (phenylalanine ammonia-lyase), *LOX1* (lipoxygenase), and *GSL12* (Glucan synthase), were significantly down-regulated in all grapevines after 24 hpi (Fig. 6), which overlaps the time window of the generation of the first haustorium (Gao et al., 2016). These results might emphasize the importance of effector proteins for the successful colonization of *En* NAFU1 in grapevines. Other defense-related genes, such as *PR2* ( $\beta$ -1,3-glucanase), *STS31* (stilbene synthase), and *GST1* (glutathione S-transferase), could also be employed by pathogens to destroy the papillae and eliminate the excessive ROS in grapevine cells (Oide et al., 2013; Hatmi et al., 2014). As expected, the transcript level of these genes was up-regulated by *En* NAFU1 after 24 hpi, especially in susceptible ‘Lingye’ and ‘Thompson Seedless’ (Fig. 6).

In conclusion, we documented that the early events of grapevine defense response, including cell wall deposition at the attempt sites,

tight encasement of haustoria, and rapid activation of defense-associated signaling pathways, are critical for its resistance to powdery mildew. On the one hand, in addition to the *R*-genes-mediated HR-like responses, the penetration resistance also well function in ‘Baihe-35-1’ and ‘Baishui-40’ in responses to powdery mildew. On the other hand, some anomalies found in ‘Lingye’ urge us to make further efforts to understand effector-triggered susceptibility (ETS) or resistance (ETI) in future studies. These results help understand the genetic basis that confers high resistance in wild Chinese *Vitis* species against powdery mildew.

## Contributions

YQW conceived the study. YRG, YH, LSY, and WW performed the experiments. YH wrote the manuscript. YJW contributed to the study via consultation. YQW and YH interpreted the experimental data and revised the manuscript. All of the authors read and approved the final manuscript.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.09.049>.

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