Analysis of the grape (Vitis vinifera L.) thaumatin-like protein (TLP) gene family and demonstration that TLP29 contributes to disease resistance

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Thaumatin-like protein (TLP) is present as a large family in plants, and individual members play different roles in various responses to biotic and abiotic stresses. Here we studied the role of 33 putative grape (Vitis vinifera L.) TLP genes (VvTLP) in grape disease resistance. Heat maps analysis compared the expression profiles of 33 genes in disease resistant and susceptible grape species infected with anthracnose (Elsinoe ampelina), powdery mildew (Erysiphe necator) or Botrytis cinerea. Among these 33 genes, the expression level of TLP29 increased following the three pathogens inoculations, and its homolog from the disease resistant Chinese wild grape V. quinquangularis cv. ‘Shang-24’, was focused for functional studies. Over-expression of TLP29 from grape ‘Shang-24’ (VqTLP29) in Arabidopsis thaliana enhanced its resistance to powdery mildew and the bacterium Pseudomonas syringae pv. tomato DC3000, but decreased resistance to B. cinerea. Moreover, the stomatal closure immunity response to pathogen associated molecular patterns was strengthened in the transgenic lines. A comparison of the expression profiles of various resistance-related genes after infection with different pathogens indicated that VqTLP29 may be involved in the salicylic acid and jasmonic acid/ethylene signaling pathways.

Thaumatin is a sweet-tasting protein that was identified in fruits of Thaumatococcus daniellii Benth, a plant native to tropical West Africa1. It contains a characteristic thaumatin domain common to osmotin-like protein and the PR5-like protein kinase receptor, which are collectively grouped into the thaumatin-like protein (TLP) family2. Thaumatin is synthesized first as a precursor protein that is then processed to remove 6 and 22 amino acids at C and N termini, respectively3. Most TLP proteins contain the consensus sequence G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-[GQ]-x(2,3)-C4. They also contain 16 conserved Cys residues and a REDDD structure, where eight disulfide bonds contribute to maintaining the stability of the protein structure5.

TLP proteins are functionally diverse, with proteins from the PR5 subgroup being known for their involvement in biotic and abiotic stress responses6, while some TLP genes have been shown to participate in cold, salt and drought stress responses7-9. Others are responsible for a broad-spectrum of resistance to multiple pathogens, including Elsinoe ampelina, Verticillium dahliae, and some filamentous fungi, such as Botrytis cinerea and Fusarium oxysporum10-12. Thaumatin-like proteins have also been found to combine with G-protein-coupled receptors and their over-expression can confer enhanced resistance to pathogens13, 14. Therefore it is needed to understand the role of TLP genes in crop plants in order to enhance disease resistance.

Grape (Vitis vinifera) anthracnose (E. ampelina) and powdery mildew (E. necator) are some of the most globally widespread fungal diseases15, 16. Wild Chinese grape (V. quinquangularis) exhibits high resistance to a variety of pathogens, and it is an important source of disease resistance genes16. The grape TLP gene has previously been

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found to increase host resistance to pathogens\textsuperscript{17}, and the grape PR5 protein, VVTL, was reported to inhibit \textit{E. ampelina} spore germination and mycelium growth \textit{in vitro}\textsuperscript{15,18}. It has been shown that the expression of grape TLP genes increased after \textit{E. ampelina} inoculation, as does the expression of genes encoding a range of antimicrobial proteins, including chitinase and β-1,3 glucanase\textsuperscript{20}, PR1/PR1α\textsuperscript{21}, stilbene and chalcone synthase\textsuperscript{22}, polysaccharide-inhibitor proteins\textsuperscript{23} and lipid-transfer proteins\textsuperscript{24}.

In the current study we extended these earlier studies and more broadly investigated the regulation and potential functions of the grape TLP (VvTLP) gene family by evaluating the expression patterns of the different genes in response to different pathogen treatments. These results together with a functional analysis of one TLP genes, VqTL29, demonstrated that the grape TLP family is involved in pathogen resistance.

Results

Identification of grape TLP genes. A total of 33 TLP genes were identified in the grape genome sequence. They were named VvTLPl to VvTL33 based on their distribution and relative linear order on the chromosomes (Table 1). Sixteen of these genes (VvTL3, VvTL8, VvTL11, VvTL12, VvTL15, VvTL16, VvTL18, VvTL20, VvTL22, VvTL23, VvTL26, VvTL28, VvTL29, VvTL31 and VvTL32) were predicted to contain both Thaumatin_1 (PS00316) and Thaumatin_2 (PS1367) domain. Eight genes (VvTL6, VvTL7, VvTL9, VvTL10, VvTL19, VvTL24, VvTL30 and VvTL33) only contained a Thaumatin_2 domain and 6 genes (VvTL1, VvTL2, VvTL4, VvTL14, VvTL25 and VvTL27) had an incomplete Thaumatin_1 structure. A sequence alignment revealed a 9 amino acids difference between the proteins encoded by VvTL4 and VvTL3 in one literature\textsuperscript{25}, however since two genes have been assigned to the same chromosomal position, it is likely that they correspond to a single gene. Similarly, VvTL10 gene was also confirmed to correspond to VVTL1 in another literature\textsuperscript{26}. Detailed information about each VvTL gene is shown in Table 1.

Phylogenetic analysis and exon-intron organization. A phylogenetic tree was constructed using the protein sequences of all the 33 VvTL genes. The grape VvTL family was divided into 4 subfamilies (Type I, II, III and IV, Fig. 1a). The Type II subfamily colored in yellow (54.55%) contained the most members, followed by Type I colored in red (27.27%) and Type IV colored in green (15.15%). The least represented subfamily was Type III colored in blue (3.03%) with only VvTL14. Besides, thirty-one VvTL genes shown in gene structure analysis had less than 4 exons, while VvTL1 had 9 and VvTL18 had 10 exons. Four genes (VvTL4, VvTL5, VvTL14 and VvTL26) had no introns (Fig. 1b). Analysis of the protein domain organization showed that the thaumatin domain was present in 27 of the 33 grape TLP genes from Type I and II subfamily (Fig. 1c). In addition, VvTL1 was predicted to contain three ARM_REPEAT (Armadillo/plakoglobin repeat) domains functioned as the cell–contact and cytoskeleton-associated proteins\textsuperscript{26}, and a HEAT_REPEAT domain associated with chromosomal dynamics and functions, including the transcription factors and microtubule-associated proteins\textsuperscript{27}. VvTL18 contained two FE2OG_OXY (Fe 2+ -oxoglutarate dioxygenase) domains involved in the oxidation of the organic substrate using a dioxygen molecule\textsuperscript{28}. While both VvTL21 and VvTL23 had a PROKAR (Prokaryotic membrane lipoprotein lipid attachment) domain functioned as the signal peptidase\textsuperscript{29}.

Tandem duplication and synteny analysis. Tandem duplication events associated with the 29 thaumatin domain containing VvTL genes were analyzed next except for four genes (VvTL4, VvTL5, VvTL14 and VvTL21). A total of 18 genes (VvTL1, VvTL2, VvTL3, VvTL6, VvTL7, VvTL8, VvTL9, VvTL10, VvTL11, VvTL12, VvTL13, VvTL15, VvTL16, VvTL26, VvTL27, VvTL31 and VvTL32) clustered into 6 tandem duplication event regions on grape chromosome 1, 2, 3, 4, 15 and 18, indicating that more than half of the VvTL genes were generated by gene duplication (Fig. 2a). Tandem duplication was also found between VvTL18 and VvTL23, VvTL20 and VvTL33, but was not shown in 7 genes (VvTL17, VvTL19, VvTL22, VvTL24, VvTL28, VvTL29 and VvTL30). A synteny analysis of the \textit{Arabidopsis thaliana} and grape TLP genes further revealed the 20 syntinic relations that contain 15 \textit{AtTL} genes and 12 VvTL genes (Fig. 2b).

VvTL expression profiles. We conducted a systematic expression analysis of all the 33 VvTL genes in grape plants following inoculation with three pathogens. Antharcnice (\textit{E. ampelina}), powdery mildew (\textit{E. necator}) and \textit{B. cinerea} were used to infect the anthracnice-resistant grape ‘Shang-24’ (\textit{V. quinquangularis}) and anthracnine-susceptible grape Red Globe (\textit{V. vinifera})\textsuperscript{15,30}, powdery mildew-resistant grape ‘Shang-24’ and powdery mildew-susceptible grape ‘Hunan-1’ (\textit{V. pseudocerricata})\textsuperscript{31}, and \textit{B. cinerea}-resistant ‘Shuangyou’ (\textit{V. amurenis}) and \textit{B. cinerea}-susceptible grape Red Globe\textsuperscript{32}, respectively. The heat maps of resulting expression profiles were shown in Fig. 3 and the semi-quantitative RT-PCR and real-time quantitative PCR expression data were shown in Supplementary Figure S1. The expression levels of 23 genes (VvTL2, VvTL3, VvTL5, VvTL6, VvTL7, VvTL8, VvTL9, VvTL10, VvTL11, VvTL12, VvTL13, VvTL15, VvTL18, VvTL19, VvTL22, VvTL23, VvTL24, VvTL25, VvTL27, VvTL28, VvTL29, VvTL31 and VvTL33) increased following the anthracnice inoculation. The expression levels of 14 genes (VvTL1, VvTL3, VvTL6, VvTL7, VvTL8, VvTL12, VvTL13, VvTL15, VvTL16, VvTL17, VvTL19, VvTL20, VvTL26 and VvTL29) increased following the powdery mildew inoculation. And the expression levels of 19 genes (VvTL2, VvTL3, VvTL5, VvTL6, VvTL7, VvTL8, VvTL9, VvTL10, VvTL12, VvTL17, VvTL20, VvTL21, VvTL22, VvTL24, VvTL26, VvTL28, VvTL30, VvTL31 and VvTL33) increased following the \textit{B. cinerea} inoculation. Among these genes, the expression levels of 6 genes (VvTL3, VvTL6, VvTL7, VvTL8, VvTL12 and VvTL29) were simultaneously up-regulated following the three pathogens inoculations.

Response of VqTL29 over-expressing \textit{A. thaliana} lines to powdery mildew (\textit{Golovinomyces cichoracearum UCSCL}) challenge. Based on its strong induction by pathogen infection (Fig. 3), VqTL29 was selected for further functional analysis by constitutive over-expression in \textit{A. thaliana}. Sequence homology between VTL29 from ‘Shang-24’ and Red Globe and the analysis of the thaumatin domain in all TLP29 proteins
were shown in Supplementary Datas S1 and S2. Three verified transgenic A. thaliana line L1, L2 and L3 were inoculated with the causal agent of powdery mildew, Golovinomyces cichoracearum UCSC1, and were found with enhanced resistance, compared to wild type Col-0 at 7 days post-inoculation (Fig. 4a). Spores were eluted from infected leaves of the transgenic lines and Col-0, counted and significantly lower concentrations of spores were enhanced resistance, compared to wild type Col-0 at 7 days post-inoculation (Fig. 4a). Spores were eluted from over-expressing lines and Col-0. (Fig. 5d). The expression levels of

Table 1. Grape TLP genes and accession numbers. CDS: coding sequence, ORF: open reading frame.

Response of VqTLP29 over-expressing A. thaliana lines to B. cinerea inoculation. Detached leaves from the VqTLP29 over-expressing line L1, L2 and L3 inoculated with B. cinerea had more disease symptoms than those from Col-0 (Fig. 5a). Three days after inoculation, B. cinerea induced necrotic lesions were evident on the entire leaf of transgenic lines and were larger than those found on Col-0 (Fig. 5b). Symptoms were scored by defining three lesion classes (<40%, 40–80%, >80%). Percentages of lesion sizes over 40% in white and grey parts were shown in the transgenic line L1, L2 and L3 with 92%, 88% and 86%, respectively, while minimum 44% of lesion sizes over 40% were observed in Col-0 (Fig. 5c). A histochemical staining assay also showed that the extent of cell death, and levels of H2O2 and O2− were higher in the three transgenic lines than in Col-0 (Fig. 5d). The expression levels of VqTLP29 following B. cinerea inoculation decreased in the three transgenic.
lines (Supplementary Figure S2c), while the expression levels of PR1 and NPR1 were on peak at 48 hpi, and the expression of ICS1 began to increase gradually after the lowest value at 12 hpi. The expression levels of PR1, NPR1 and ICS1 in the transgenic lines were consistently higher than in Col-0. The expression levels of PDF1.2, a downstream gene in the JA/ET signaling pathway35, gradually increased in the transgenic lines following infection.

Figure 1. Genome wide organization of grape TLP (VvTLP) genes. (a) Phylogenetic tree based on the protein sequences of 33 VvTLP genes. Phylogenetic tree was constructed using the neighbor-joining method with MEGA5. Four subfamilies (Type I, II, III and IV) were analyzed and colored in red, yellow, blue and green, respectively. Bootstrap values at the nodes from 1000 replicates were used to assess the robustness of the tree. The scale is in amino acid substitutions per site. (b) Exon-intron structure of VvTLP genes: yellow indicates coding sequence (CDS), blue indicates untranslated 5′- and 3′- regions, black indicates introns. (c) Structures of VvTLP proteins: brown indicates Thaumatin_2 domain, blue rectangle indicates Thaumatin_1 domain, green ellipse in VvTLP21 and VvTLP23 indicates a PROKAR-lipoprotein domain, green ellipse in VvTLP18 indicates a FE2OG_OXY domain, blue pentagon indicates an ARM_REPEAT domain, blue ellipse indicates a HEAT_REPEAT domain, gray line indicates a disulfide bridge, gray icon indicates active sites, green line indicates undefined bridge/range.

Figure 2. Chromosome distribution and synteny analysis of grape and Arabidopsis thaliana TLP genes. (a) Chromosomes 1–19 are shown in different colors in a circular diagram. The approximate distribution of each VvTLP gene is marked with a short red line on the circle. Colored curves denote the details of syntenic regions between grape TLP genes. (b) The chromosomes of grape and A. thaliana are depicted as a circle. The approximate distribution of each AtTLP and VvTLP gene is marked with a short red line on the circle. Colored curves denote the details of syntenic regions between grape and A. thaliana TLP genes.
with the highest value measured at 48 hpi, and the expression levels of LOX3 decreased slightly during the onset of disease, but always remained higher than in Col-0 (Fig. 5e).

**Response of VqTLP29 over-expressing A. thaliana lines to Pst DC3000 inoculation.** To elucidate the role of VqTLP29 in bacterial resistance, the three VqTLP29 over-expressing line L1, L2 and L3 were inoculated with the bacterium Pseudomonas syringae pv. DC3000. Three days after inoculation, disease symptoms were less apparent in the transgenic lines than in Col-0 (Fig. 6a), which exhibited yellow spots at 24 hpi and dry leaves were curled and brittle after 3 days. Moreover, the quantities of bacteria were lower in the three transgenic lines than in Col-0 (Fig. 6b), while the frequency of cell death and the degree of $\text{O}_2^-$ accumulation were both higher (Fig. 6c). The expression levels of VqTLP29 following DC3000 inoculation increased in the three transgenic lines (Supplementary Figure S2b), and PR1 expression was higher in the transgenic lines than in Col-0, peaking at 48 hpi. The transcriptional regulators WRKY53 and NHL10 (NDR1/HIN1-like 10) are known to play roles in the A. thaliana SA pathway. The expression levels of WRKY53 were lower in the transgenic lines from 24–72 hpi than in Col-0, and the expression levels of NHL10 were higher in the transgenic lines, peaking at 24 hpi. The expression levels of PDF1.2 involved in the JA/ET signaling pathway were higher in the transgenic lines than in Col-0, with a peak at 24 hpi (Fig. 6d).

**Stomatal closure immunity response.** Stomatal closure is known to be part of the induced plant innate immunity response and serves to limit pathogen infection. In this study, we observed a marked reduction in leaf stomatal aperture in both the VqTLP29 over-expressing A. thaliana line L1, L2 and L3 and Col-0 during the first hour after DC3000 inoculation. However, after 2 hours of continued incubation, stomata reopened in Col-0, but not in the transgenic lines (Fig. 7a). It has been shown that the bacterium derived molecules, flg22 and LPS, act as pathogen-associated molecular patterns (PAMPs) to stimulate/induce the innate immunity in plants. We observed that within the first hour of incubation with flg22 or LPS, marked reduction in stomatal aperture were observed in all transgenic lines and Col-0. During the subsequent hours, the stomata in Col-0 reopened, while stomatal apertures still decreased in the transgenic lines (Fig. 7b).

Next we measured the expression of genes known to be involved in modulating stomatal guard cell movement or associated with signal transduction pathways in VqTLP29 over-expressing line L1 (Fig. 7c,d). FRK1 is flg22-induced receptor kinase involved in the SA pathway, and COI1 can activate the JA signaling pathway, and is used as an inhibitor of stomatal closure. ATPPC2 acts directly upon stomatal closure in the abscisic acid (ABA) pathway. FLS2 is a flagellin receptor. OST1 is a guard-cell-specific kinase. FLS2 and OST1 are required
for perception of bacterial surface molecules in *A. thaliana* stomatal guard cells. The expression levels of *FRK1* were higher in L1 than in Col-0 under normal condition. *FRK1* expressions decreased in L1 from 30–60 min post DC3000 inoculation and remained higher than in Col-0. The expression levels of *COI1* increased in both L1 and Col-0 following DC3000 inoculation. The expression levels of *ATPPC2* were higher in L1 than in Col-0 under normal condition. *ATPPC2* expression decreased in L1 after infection but still higher than in Col-0. The expression levels of *FLS2* and *OST1* increased in L1 after DC3000 inoculation, with *FLS2* attained its peak at 60 min and *OST1* attained its peak at 30 min. After treated with flg22 or LPS, the expression levels of *FRK1* increased in L1 and Col-0. The expression levels of *ATPPC2* decreased gradually in L1 after inoculation and still higher than in Col-0. The expression levels of *FLS2* increased in L1 peaking at the 30 min after inoculation. And the expression of *OST1* also increased in L1 and Col-0, with the maximum values at 30 min after flg22 or LPS inoculation.

Staining the leaves of different genotypes with aniline blue indicated that more callose were produced in response to DC3000 inoculation, and treatments with flg22 or LPS in the transgenic line L1, L2 and L3 than in Col-0 (Fig. 8).

**Discussion**

The TLP family has been extensively studied in animals and fungi and some TLP genes in plants are known to be involved in defense against pathogens. In the current study, 33 grape *VvTLP* genes were identified (Table 1) and analyzed into four subfamilies through the phylogenetic analysis (Fig. 1). The thaumatin domain was present in 27 of the 33 grape TLP genes from Type I and II subfamily, while was not in 6 genes (*VvTLP1*, *VvTLP2*, *VvTLP14*, *VvTLP17*, *VvTLP25* and *VvTLP27*) from Type III and IV subfamily. Four of the 33 grape TLP genes (*VvTLP4*, *VvTLP5*, *VvTLP14* and *VvTLP21*) did not have accession numbers in support of the expressed sequence tag (EST) data. However, structural analyses indicated that they contain the conserved thaumatin domain coding sequence, with the exception of *VvTLP14*, and they were included into the TLP family. Gene duplication events play a major role in grape genome rearrangement and expansion (Fig. 2), and segregation duplication events...
have been shown to provide a reference for the evolutionary relationship between TLP genes, thereby enabling functional predictions\(^46\). To verification the roles of grape TLP genes in signaling pathways related to pathogen induced stress\(^22\), we evaluated their expression in grape cultivars that had been infected with the biotrophic mycoparasite \textit{E. ampelina} and the hemi-biotrophic mycoparasite \textit{B. cinerea}, which has saprophytic growth (Fig. 3). The expression of cisgenic \textit{VVTL1} proved as a secreted protein in grape was shown to significantly inhibit the growth of hyphae of both \textit{E. ampelina} and \textit{E. necator}\(^18,19\), which was supported in this study with the increased expression after pathogens treatments. Other TLP genes have also been shown to increase resistance to plant diseases\(^10-12\), indicating a substantial role for the TLP genes in the regulatory networks involved in pathogen infection. The analysis of gene expression profiles often provides useful clues for functional assessment. The homolog of TLP29 from the wild grape species \textit{V. quinquangularis} 'Shang-24' was aimed for cloning and for additional functional studies. Analysis of \textit{VqTLP29} protein sequence showed that \textit{VqTLP29} protein was a secreted protein, and its signal peptide cleavage site was located between 23rd and 24th amino acid. Although the protein sequence from 172nd to 204th amino acid were defined as the nuclear localization signal assessed as three points with cNLS on line shown in Supplementary Data S2, the subcellular localization experiment was analyzed and confirmed that \textit{VqTLP29} protein was located in the cytoplasm in grape mesophyll protoplasts shown in Supplementary Figure S4 with percentages of the protoplast transfection shown in Supplementary Table S3.
Figure 6. Response of VqTLP29 over-expressing Arabidopsis thaliana lines to Pst DC3000 inoculation. (a) Disease symptoms in representative rosettes of the 4-week old VqTLP29 transgenic line L1, L2, L3 and Col-0 after DC3000 inoculation for 3 days. Leaves were injected with the DC3000 suspension using 1 mL needless syringes and kept highly moisturizing until the lesion shown. (b) The number of bacteria of the VqTLP29 transgenic lines and Col-0 3 days post DC3000 inoculation. The colony counting assay of the transgenic lines and Col-0 was carried out in three independent experiments. Asterisks indicate statistical significance (*P < 0.01, one-way ANOVA). (c) Cell death and O2 accumulation in the VqTLP29 transgenic lines and Col-0 72 h post DC3000 inoculation. Three infected leaves of the transgenic lines and Col-0 were required for each stain in three independent experiments. (d) Expression levels of disease resistance genes in the VqTLP29 transgenic lines and Col-0 at 0, 24, 48 and 72 hpi following DC3000 inoculation. Asterisks indicate statistical significance (*P < 0.01, **P < 0.01, one-way ANOVA). The experiments were repeated three times with consistent results.

Besides, over-expression of VqTLP29 in Arabidopsis thaliana enhanced the resistance to powdery mildew and the bacterium Pseudomonas syringae pv. tomato DC3000, but decreased resistance to B. cinerea.

The antifungal protein VqTLP29 encoded the thaumatin-like protein similar with PR5 protein mainly acted on the late stage of defensive reaction. Over-expression of PRs would reduce only a limited number of diseases, depending on the nature of the protein, plant species, and pathogen involved[29]. Over-expression of PR5 could enhance resistance to biotic and abiotic response with activating many defense genes in SA or JA/ET signaling pathway. The genotypes of VqTLP29 were shown higher expression values in transgenic lines under normal growth with the increased expression levels of SA-defense genes (PR1, NPR1) in Figs 4d, 5e and 6d) and SA-synthesis gene (ICS1 in Figs 4d and 5e). Higher expression levels of PR1 were reported and shown in over-expression of transgenic factor Di19 or CPK11 (Ca2+-dependent protein kinase 11) plants under normal condition, respectively[21]. Higher expression levels of NPR1 were also shown in over-expression of GhMKK5 plants under normal conditions, which belonged to MAPK kinase (MAPKKs)[26]. And the transcripts of ICS1 were repressed 8-fold in GmMPK4-silenced plants with GmMPK4 negatively controlling SA[21]. All above results were similar in this study and the data proved that over-expression of VqTLP29 had already activated SA signaling pathway under normal condition. The genotypes of VqTLP29 were shown higher expression values in transgenic lines under normal condition with the expression levels of JA-synthesis gene (LOX3 in Figs 4d and 5e) being enhanced over 10-fold, while that of JA/ET-defense downstream gene (PDF1.2 in Figs 5e and 6d,c) had no significant change. Activated NPR1 could ultimately lead to the activation of some SA-responsive genes but would act as a cytosolic function of inhibition of JA signaling pathway, which lead to lose the ability of activating JA-responsive genes[12]. Over-expression of VqTLP29 with higher NPR1 could not activate JA-responsive genes like PDF1.2 as referred by these studies[15, 56]. It is evident that LOX3 was a component in JA synthesis and used as an important lipoxygenase in JA synthesis[54, 51]. Although the expression level of LOX3 was over 10-fold higher in 35S:MYC3 transgenic lines than wild type under normal condition, the expression of PDF1.2 was not changed[57]. These data proved that over-expression of VqTLP29 had already activated JA synthesis while had no effect on JA signaling pathway in plants under normal growth.

We assessed the VqTLP29 transgenic lines with responses to two fungal pathogens and a bacterium. The phenotypes of transgenic and wild type plants were similar. VqTLP29 transgenic lines clearly improved resistance to powdery mildew and DC3000 with increased VqTLP29 expression, but increased susceptibility to B. cinerea with decreased VqTLP29 expression. The phytohormone SA plays an important role in limiting the invasion of powdery mildew and B. cinerea[13–58]. Studies showed that the expression level of PR5 increased post powdery mildew inoculation with increased expressions of PRI and NPR1[21, 56–58]. We observed that the expressions of PRI and...
*experiments were repeated three times with consistent results.

transgenic line L1 and Col-0 after 30 min and 60 min of inoculation (Fig. 4d). The immune response against powdery mildew has been shown to activate resistance to DC3000, and over-expression of NHL10 has also been shown to be involved in pathogen-triggered SA signaling. We noted an up-regulation of both genes after DC3000 inoculation in parallel with increased expression of defense related genes. We observed that the expression of PDF1.2 increased after bacterial inoculation, revealing that the JA/ET pathway was induced in the transgenic lines (Fig. 5e), which is supported by two studies with the same result of PDF1.2 expression. The expression level of LOX3 was higher in the transgenic lines, indicating over-expression of VqTLP29 could promote JA biosynthesis following powdery mildew inoculation (Fig. 4d). ICS1 expression appears to decrease at 12–24 hpi post powdery mildew and bacterial inoculation, which showed that ICS1 was cycling in response to the over-expression of VqTLP29. ICS1 is a member of the WRKY transcription factor family, and has been shown to be involved in pathogen-triggered SA signaling, as has NHL10. We noted an up-regulation of both genes after DC3000 inoculation in parallel with increased expression of PDF1.2, but the expression levels of WRKY53 were lower in the transgenic lines than in Col-0 (Fig. 6), which implied that over-expression of VqTLP29 has effects on SA or JA/ET pathway with DC3000 inoculation. Taken together, the expression data suggest that over-expression of VqTLP29 acts as a regulator that differentially modulates immunity against powdery mildew, B. cinerea and DC3000 via the SA or JA/ET signaling pathway.

Stomatal defense against bacterial invasion is an important component of the innate immunity, and is a target of virulence factors produced by DC3000. Here we used several marker genes involved in this process to investigate the role of VqTLP29 in bacterial resistance. FRK1 transcript levels were higher in the transgenic line L1 than Col-0 (Fig. 7a), which is consistent with the innate immune response in A. thaliana leaves being activated via an SA-dependent mechanism. The expression levels of both FLS2 and OST1 increased in L1 after different treatments, while ATPPC2 expression decreased. FLS2 has been shown to activate resistance to DC3000, and involved in ABA signaling pathway to act directly on guard cells inducing stomatal closure by promoting the efflux of potassium and anions and the removal of organic osmolytes. The mechanism by which VqTLP29 regulates stomatal response to PAMPs can be explored with the characterization that how it mediates gene regulation.

In conclusion, TLP gene expression is broadly influenced by E. ampelina, E. necator and B. cinerea inoculations, indicating the existence of a complex regulatory network that responds to biotic stress. In addition, we identified the role of VqTLP29 from disease resistant grape V. quinquangularis cv. ‘Shang-24’ in responses to various pathogens. Over-expression of VqTLP29 in A. thaliana had already activated SA signaling pathway, JA synthesis...
and had no effect on JA signaling pathway under normal condition. VqTLP29 over-expressing lines enhanced resistance to the powdery mildew and DC3000 but increased susceptibility to *B. cinerea*, with up-regulating the SA and JA/ET signaling. Data from these analyses will be useful in defining the transcriptional networks that are regulated by VqTLP29 during immune responses against pathogens.

**Methods**

**Plant materials.** In this study, grape genotype ‘Shang-24’ (*V. quinquangularis*), ‘Hunan-1’ (*V. pseudoreticulata*), ‘Shuangyou’ (*V. amurensis*) and Red Globe (*V. vinifera*) were used for analysis of disease resistance in the grape germplasm resources orchard of Northwest A&F University, Yangling, Shaanxi, China. Samples of grape organs were obtained from ‘Shang-24’. *A. thaliana* (transgenic lines and wild type Col-0) plants were grown at 21–22°C, and 70% relative humidity under long day (8 h dark, 16 h light) conditions. For all experiments, 4-week old plants were used. All experiments were repeated in triplicate and all samples were immediately frozen in liquid nitrogen and stored at −80°C until further use.

**Identification and annotation of grape TLP genes.** A profile of the TLP DNA-binding domain (PF00314) was downloaded from the Pfam protein family database (http://pfam.sanger.ac.uk/) and used to identify putative TLP genes from the grape genome sequence (http://www.genoscope.cns.fr/)65. The deduced TLP genes were annotated based on their respective chromosome distribution66 and their sequences were confirmed using an in-house transcriptome database.
Bioinformatic analysis of grape TLP genes. A phylogenetic tree of the 33 predicted VvTLP genes was constructed with MEGA5 software using the neighbor-joining method, and their exon-intron structures were determined based on alignments of the coding regions and full-length sequences (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Diagrams of the gene structures were generated using the Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn), and protein structures using PROSITE (http://prosite.expasy.org/). TLP gene duplications were identified as previously described, and syntenic blocks were used to construct a synteny analysis map of the VvTLP genes from the Plant Genome Duplication Database. Diagrams were generated using Circos version 0.63 (http://circos.ca/). The results of semi-quantitative RT-PCR were analyzed and quantified using the Gene Tools software, and the relative expression levels of VvTLP genes under different treatments compared to the controls were used for hierarchical cluster analysis with MeV 4.8.1.

Grape disease assays. The anthracnose (E. ampelinum) was isolated and sporulated on potato dextrose agar (PDA) at 25 °C. Spores were suspended in sterile water and 0.5 ml of the suspension (2.0 × 10⁶ spores ml⁻¹) was sprayed onto each side of the young leaves from three vines of ‘Shang-24’ and Red Globe. Sterile water was used as a control at the same time points. Samples were collected at 0, 6, 12, 24, 48, 72 and 120 hours post inoculation (hpi). Powdery mildew (E. necator) was used to inoculate young leaves of ‘Shang-24’ and ‘Hunan-1’, and sterile water was used as a control at the same time points. Samples were collected at 0, 6, 12, 24, 48, 72, 96, and 120 hpi.

A. thaliana disease assays. A. thaliana powdery mildew (Golovinomyces cichoracearum UCSC1) was preserved by growing it in the phytoalexin deficient 4 mutant. Sterile water was sprayed on the surface of the VqTLP29 transgenic line L1, L2, L3 and Col-0 before inoculations using the leaf pressing method. Samples were collected at 0, 24, 48, 76, 120, 144, and 168 hpi. A spore counting assay was carried out 7 days post powdery mildew inoculation. Spore suspensions were extracted and diluted in sterile water with 10 infected leaves and then counted using a hemocytometer under the microscope. B. cinerea was sporulated on PDA at 25 °C for 3 weeks and then spores were suspended in sterile water with 4% maltose and 1% peptone to a concentration of 2.0 × 10⁶ spores ml⁻¹. Leaves were detached and inoculated by dropping 10 μl of spore suspension onto the adaxial surface. Samples were collected at 0, 12, 24, 48 and 72 hpi, and the percentage of lesion areas with respect to the whole leaf was determined using grid statistics 3 days after infection. DC3000 was cultured overnight at 28 °C in LB medium with 1/2 salt concentration (pH 7.0, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract powder and 5 g L⁻¹ NaCl). Bacterium cultures with an OD₆₀₀ of 0.6 were collected by centrifugation (12,000 g, 10 min) and resuspended in 10 mM MgCl₂, containing 0.005% Silwet L-77 (OSI Specialties, Sigma) to a final OD of 0.02. Leaves were injected with the DC3000 suspension using 1 mL needless syringes, and samples were collected at 0, 24, 48, 72 and 96 hpi. A colony counting assay was carried out 3 days post DC3000 inoculation.

Histochemical staining assay was conducted with leaves 7 days post powdery mildew infection, 3 days post DC3000 inoculation and 3 days post B. cinerea inoculation. Nine susceptible leaves from the VqTLP29 transgenic lines and Col-0 were collected to visualize callose deposition by staining with 1% (w/v) aniline blue dissolved in 150 mM K₂HPO₄ (pH 9.5); cell death with trypan blue; O₂ accumulation with 6 mM nitro blue tetrazolium (NBT); and H₂O₂ with 1 mg ml⁻¹ lactic acid and 10 mg ml⁻¹ trypan blue. Infected leaves were boiled in trypan blue solution for 5 min and then bleached in 2.5 mg ml⁻¹ chloral hydrate for 48 h. NBT was dissolved in HEPES buffer (pH 7.5). Infected leaves were soaked in NBT solution for 2 h and DAB for 8 h, and then transferred into 80% ethanol at 60 °C for 2 h and finally held at room temperature for 48 h. Disease related genes used to assess the response to these treatments and the corresponding gene specific primers used for semi-quantitative PCR primers are listed in Supplementary Table S2.

Response of stomata to different treatments. To ensure that 80% of the stomata were open at the onset of the experiments, A. thaliana plants were placed in the light (100 μmol m⁻² s⁻¹) for 3 h. The epidermis of 3 fully expanded young leaves from the VqTLP29 transgenic lines and Col-0 was peeled off manually and immediately immersed in 10 mM MgCl₂ (mock treatment), DC3000 suspension (OD₆₀₀ of 0.02 in MgCl₂), 5 μM flg22 (Flagellin Fragment, peak area by HPLC ≥95%, Anaspec, USA) in MES buffer (25 mM MES-KOH, pH 6.15 and 10 mM KCl) and 100 μg ml⁻¹ LPS (lipopolysaccharide, Sigma-Aldrich, dissolved in MgCl₂). At 1 h and 3 h time points, treated epidermal peel samples were observed under a microscope (Olympus BX53). Stomatal transverse length and longitudinal width were measured using Image-J. Four treatments were conducted for the transgenic lines and Col-0 using 1 mL needle syringes. Treated leaves were sampled separately at 30 and 60 min and frozen in liquid nitrogen. Leaves of the transgenic lines and Col-0 18 h post treatments were stained with aniline blue to detect callose deposition.

Vector construction. Total ‘Shang-24’ RNA and cDNA was isolated as described below. The VqTLP29 coding sequence fragment (735 bp, Supplementary Data S1) was amplified by PCR using the primers VqTLP29-F (5'-GCJCTAGAATGGGATGCTGCT-3') and VqTLP29-R (5'-GGGGTGCACTTAGTGGTGAGG-3') with XbaI and KpnI sites (underlined) included. The PCR reactions were carried out as follows: 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 2 min, and the PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The resulting plasmids were sequenced by Sunny Biotechnology Co.
Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA) with a final volume of 20 μl mixture consisted of 10.0 μl cDNA, 0.8 μl each primer (10 μM), and 7.4 μl sterile H2O. Cycling parameters were: 95 °C for 30 s; 40 cycles at 95 °C for 5 s, 60 °C for 30 s. Melt-curve analyses was performed using the CFX Manager was used to analyze the relative expression levels with significance analysis of 2ΔΔCt.

RNA isolation, sqRT-PCR and qRT-PCR. Total RNA was extracted using an EZNAP Plant RNA Kit (R6827-01, Omega Bio-tek, USA). First-strand cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) and diluted 12-fold. VvActin (R6827-01, Omega Bio-tek, USA). First-strand cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) and diluted 12-fold. VvActin (R6827-01, Omega Bio-tek, USA). First-strand cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) and diluted 12-fold. VvActin (R6827-01, Omega Bio-tek, USA).

Statistical analysis. Data are presented as means and standard errors using Microsoft Excel and SigmaPlot 10.0. One-way ANOVA analysis was performed using the SPSS Statistics 17.0 software (IBM China Company Ltd., Beijing, China) to assess significant differences.

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Author Contributions
X.W. and X.Y. designed the study. H.Q., X.Z., C.G. and M.W. contributed to the experiments. X.Y. performed data analysis and assisted with the interpretation of the results. X.W. and Y.W. provided guidance throughout the study. X.Y. and X.W. wrote and revised the manuscript.

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