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ORIGINAL ARTICLE

VqDUF642, a gene isolated from the Chinese grape *Vitis quinquangularis*, is involved in berry development and pathogen resistance

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Abstract

Main conclusion **The DUF642 gene *VqDUF642*, isolated from the Chinese grape species *V. quinquangularis* accession Danfeng-2, participates in berry development and defense responses against *Erysiphe necator* and *Botrytis cinerea*.**

The proteins with domains of unknown function 642 (DUF642) comprise a large protein family according to cell wall proteomic analyses in plants. However, the works about functional characterization of DUF642s in plant development and resistance to pathogens are scarce. In this study, a gene encoding a DUF642 protein was isolated from Chinese grape *V. quinquangularis* accession Danfeng-2, and designated as *VqDUF642*. Its full-length cDNA contains a 1107-bp open reading frame corresponding to a deduced 368-amino acid protein. Multiple sequence alignments and phylogenetic analysis showed that VqDUF642 is highly homologous to one of the DUF642 proteins (VvDUF642) in *V. vinifera*. The VqDUF642 was localized to the cell wall of tobacco epidermal cells. Accumulation of VqDUF642 protein and *VqDUF642* transcript abundance increased at the later stage of grape berry development in Danfeng-2. Overexpression of *VqDUF642* in transgenic tomato plants accelerated plant growth and reduced susceptibility to *Botrytis cinerea*. Transgenic Thompson Seedless grapevine plants overexpressing *VqDUF642* exhibited enhanced resistance to *Erysiphe necator* and *B. cinerea*. Moreover, *VqDUF642* overexpression affected the expression of a couple of pathogenesis-related (PR) genes in transgenic tomato and grapevine upon pathogen inoculation. Taken together, these results suggest that VqDUF642 is involved in plant development and defense against pathogenic infections.

Keywords Grapevine · Chinese wild *Vitis* species · DUF642 · Berry development · Disease resistance

Introduction

Plant cell wall is an extracellular matrix for the protection of cells (Chen et al. 2009; Del Bem and Vincentz 2010; Graham et al. 2000; Popper et al. 2011; Sarkar et al. 2009; Vázquez-Lobo et al. 2012). It is composed of polysaccharides, glycosylated or non-glycosylated proteins, and lignin (Carpita and Gibeaut 1993; Fry 2004; McCann et al. 2001; Ridley et al. 2001; Somerville et al. 2004). Plant cell wall proteins are involved in cell wall signaling, intercellular communication, and cellular defense (Cassab 1998; Chen et al. 2009; Cosgrove 2005; Fry 2004; Hückelhoven 2007; Jamet et al. 2008; Vázquez-Lobo et al. 2012). Cell wall proteomic analyses have been conducted to identify the proteins with domains of unknown function (DUF)

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(Jamet et al. 2008). In spermatophyte plants, the DUF642 proteins constitute a novel family with yet unknown function (Vázquez-Lobo et al. 2012).

Grapevine (*Vitis* sp.) is a global fruit crop due to the economic and health benefits of its products (Bouquet et al. 2008). Pathogens are a major problem for grapevines worldwide, causing huge reduction in productivity and fruit quality (He et al. 1991). Interestingly, wild grape species are valuable source of resistance to crop pathogens (Pavloušek 2007). China is one of the origins of *Vitis* species, and a number of Chinese wild *Vitis* species show strong resistance to pathogens (Wang and He 1997; Wang et al. 1995). For example, *V. amurensis*, *V. romanetii*, *V. piasezkii*, *V. davidii*, *V. davidii* var. *cyanocarpa*, *V. liubanensis*, and *V. bashanica* are resistant to powdery mildew, while *V. yeshanensis*, *V. davidii* var. *cyanocarpa*, and *V. pseudoreticulata* are resistant to downy mildew (Wan et al. 2007; Wang et al. 1995). These Chinese grape species may contain certain resistance-related cell wall proteins.

Danfeng-2, an accession of Chinese grape species *V. quinquangularis*, has been reported to possess excellent resistance to pathogens (Wan et al. 2007; Wang et al. 1995). Our research group has proven that all genotypes of *V. quinquangularis*, particularly Danfeng-2, had significantly higher resveratrol contents than most of the tested *Vitis vinifera* cuitvars (Shi et al. 2014; Zhou et al. 2015). Resveratrol, which is considered to be a natural phenol and a phytoalexin, increasingly accumulates during the development of the Danfeng-2 berry (Shi et al. 2014). We recently characterized proteomic changes during berry development of Danfeng-2, and identified thousands of differentially expressed proteins, including DUF642s. However, there is limited information about the role of DUF642 proteins in grapes. Only one DUF642 protein has been identified in *V. vinifera* cv. Cabernet Sauvignon (Negri et al. 2008).

Development and ripening of the grape berry are closely related to changes in the metabolic activities of berry tissues and the composition of cell wall (Famiani et al. 2000; Negri et al. 2008; Nunan et al. 2001; Robinson and Davis 2000; Waters et al. 2005). Therefore, it is hypothesized that DUF642 accumulation during the berry ripening stages plays an important role in the development of the Danfeng-2 berry. Pectin methylesterases (PMEs) are crucial for monitoring the biomechanical characteristics of cell wall during different developmental stages (Hongo et al. 2012; Müller et al. 2013; Peaucelle et al. 2011). In this study, the full-length *VqDUF642* cDNA was isolated in Danfeng-2 and its expression at different developmental stages of grape berry was investigated. The involvement of VqDUF642 in plant development and pathogen susceptibility was demonstrated using a transgene assay in tomato or grapevine. Besides, transcript levels of the defense-related marker genes, *PR1* to *PR4*, were analyzed in pathogen-inoculated transgenic plants. The ultimate aim of this study was to investigate whether VqDUF642 participates in the regulation of plant development and defense against pathogen invasion.

Materials and methods

Plant materials

The Chinese grape species *V. quinquangularis* accession Danfeng-2 and *V. vinifera* cv. Cabernet Sauvignon plants used in this study were grown in the grape germplasm collection at Northwest A&F University, located in Yangling, People's Republic of China (34°20′N, 108°24′E). *Vitis vinifera* cv. Thompson Seedless and *Solanum lycopersicum* cv. Micro-Tom used in the transgenic experiments were grown in a growth chamber and purchased from the PanAmerican Seed website (http://www.panamseed.com/), respectively. The tobacco (*Nicotiana benthamiana*) plants used for subcellular localization were grown in the greenhouse.

Isobaric tags for relative and absolute quantitation (iTRAQ) based quantitative proteomic analysis

The iTRAQ assay was performed as described by Wang et al. (2013). Total proteins were extracted, digested, and iTRAQ labeled. Peptides from proteins were applied to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. Proteome Discoverer 1.4 (Thermo Fisher Scientific, Sunnyvale, CA, USA) was used for protein quantitation and STRAP was used for annotation of identified proteins (Bhatia et al. 2009). Differently expressed proteins were defined with p-values <0.05 and a ratio of ≥ 1.5 as significant.

RNA isolation and cDNA synthesis

Total RNA was isolated from 0.5 to 1.0 g grape berries or leaves using the RNeasy plant mini kit (Qiagen, Shanghai, China). Genomic DNA contamination was removed according to the manufacturer's instructions using the RNase-Free DNase Set (Qiagen, Shanghai, China). The purified RNA (3 μg) was reversely transcribed through the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for RT-PCR with the Oligo $(dT)_{20}$ primer following the manufacturer's protocol.

Full-length cloning and sequence analysis

The VqDUF642 amino acid sequence (GenBank accession no. AMB38759) was used for a BLAST search of the grape

genome on http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/. The cDNA obtained was used as a template for homology-based cloning of *VqDUF642* in Danfeng-2, using primers (Supplementary Table S1) based on the homologous sequences. The VqDUF642 protein sequence was presented as WebLogo sequences using WebLogo version 3.4 (http://weblogo.threeplusone.com/create.cgi). Phylogenetic tree was constructed using MEGA 5.0 software. The VqDUF642 was also analyzed on SignalP 4.1 to predict signal peptide, which is a short (5-30 amino acids long) peptide existing at the N-terminus of most of newly synthesized proteins that are associated with the secretory pathway (Blobel and Dobberstein 1975) (http://www.cbs.dtu.dk/services/SignalP/). Moreover, two GPI-AP prediction tools, the big-PI (http://mendel.imp.univie.ac.at/gpi/gpi_server.html) and DGPI (www.expasy.org/tools/), were used to predict the GPI-anchor of VqDUF642.

Quantitative real-time PCR

Prior to the qRT-PCR experiments, the cDNA reactions were diluted with ddH_2O to a concentration of 10 ng/μl. The specific primer pairs for qRT-PCR were designed using Primer Express 3.0 (Supplementary Table S1). The tomato *SlActin* (GenBank accession no. XM_002282480) and grapevine *GAPDH* (GenBank accession no. GR883080) were used as reference genes. PR genes were selected as defense-related markers for expression analysis after pathogen infection: *SlPR1* (GenBank accession no. EU589238), *SlPR2* (GenBank accession no. NM_001247876), *SlPR3* (GenBank accession no. NM_001247474), and *SlPR4* (GenBank accession no. NM_001247154); *VvPR1* (GenBank accession no. AY298726), *VvPR2* (GenBank accession no. AF239617), *VvPR3* (GenBank accession no. NM_001281146), and *VvPR4* (GenBank accession no. XM_002264684). The qRT-PCR analysis was conducted using a SYBR Green method in a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Localization of VqDUF642

The *VqDUF642* coding sequence without the termination codon was cloned into the pCAMBIA1302 vector (http://www.cambia.org) to generate 35S::*VqDUF642–GFP*. The sequenced plasmids were introduced into *N. benthamiana* leaves as described by Yuan et al. (2016), and then cultured in growth chamber for 48 h. After cultivation, GFP fluorescence was visualized at an excitation wavelength of 480 ± 20 nm and an emission wavelength of 510 ± 20 nm under a Zeiss confocal microscope LSM510 (Carl Zeiss Thornwood, NY, USA). For preparation of plasmolysed cells, transiently transformed tobacco leaves were incubated in 5 M NaCl solution for 10–20 min before examined by confocal microscopy.

Overexpression constructs and *Agrobacterium tumefaciens* strains

Genetic transformation assays using the ORF region of *VqDUF642* cDNA were performed with *A. tumefaciens* strain GV3101 harboring pCAMBIA1302 and pART-CAM-S binary vectors for tomato transformation and grape transformation, respectively. These two vectors contain *hptII* and *nptII* coding regions, which were used as selectable markers and confer hygromycin and kanamycin resistance, respectively.

Stable transformation and confirmation

The tomato seedlings with fully expanded cotyledons and meristematic bulks induced from Thompson Seedless both on pre-culture medium were used for the genetic transformation (Supplementary Table S2) (Mezzetti et al. 2002). *VqDUF642* fused with GFP-tag and FLAG-tag, under the control of CaMV35S promoter, were transformed into tomato and grape tissues via *Agrobacterium tumefaciens*-mediated transformation respectively, as previously described by our research group (Dai et al. 2015; Zhou et al. 2014).

DNA was isolated from 0.5 to 1.0 g leaf samples from plants that were regenerated in selection medium (Supplementary Table S2) as performed by Thomas et al. (1993). The insertion of *VqDUF642* into the transformed tomato and grape lines was verified by PCR using primers for *hptII* and *nptII*, respectively.

Total proteins were extracted from approximately 0.5 of fresh samples according to the method of Méchin et al. (2007). The expression of VqDUF642 protein in the transgenic tomato and grape lines was verified by western blotting, as described by Cheng et al. (2016). Transgenic tomato (GFP-tagged) and grape (FLAG-tagged) plants expressing VqDUF642 protein were recognized by anti-GFP and anti-DDDDK antibodies (abcam, English), respectively.

Germination testing and seedling development

Germination was tested as described by Zúñiga-Sánchez et al. (2014). Briefly, dry mature T3 seeds were stored at 4 °C for 1–2 months. The seeds were sown on Petri dishes containing MS media (Murashige and Skoog 1962) and cultured in the growth chamber under greenhouse conditions. Under microscopic observation, the seeds at the stages of testa rupture and endosperm rupture were counted periodically. The percentage of 4-leaf-stage transgenic and control seedlings was calculated at 2 weeks after sowing.

Measurement of the pectin methylesterase activity

Pectin methylesterase (PME) activity was measured according to the method of Zúñiga-Sánchez et al. (2014) from four types of samples: seedlings with four leaves (two expanded cotyledons and first two true leaves), shoot tips, leaves and fruit (harvest-ripe). The PME activities were calculated by measuring the stained areas using the ImageJ 1.34S software (National Institutes of Health, Bethesda, MD, USA).

Erysiphe necator infection assay

The young grape leaves were used for inoculation with powdery mildew *E. necator* (Cheng et al. 2016). Three plants per line were assayed for resistance to *E. necator*, as previously described by Fung et al. (2008) and Vidal et al. (2006). Susceptibility to *E. necator* infection was recorded at 24, 48, 72 and 168 h post inoculation (hpi). The infected leaves were fixed and preserved for microscopic evaluation according to a previous procedure (Vanacker et al. 2000). Western blotting was performed to analyze the expression of VqDUF642 protein in the inoculated leaves. Moreover, transcript abundances of the pathogenesis-related (PR) genes in the inoculated grape lines were also analyzed by qRT-PCR.

Botrytis cinerea infection assay

The wild-type (WT) and transgenic tomato and grape leaves were inoculated with *B. cinerea* as described by Zhao et al. (2012). Six leaves per line were used for inoculation. The decayed parts and conidiophores growth on the leaves were observed at 48 and 96 hpi. The inoculated leaves were fixed and preserved for microscopic evaluation according to a previous procedure (Vanacker et al. 2000). Transcript levels of the *B. cinerea* endogenous marker gene *Bcactin* (GenBank accession no. XM001553318) were examined by qRT-PCR to monitor the growth of this pathogen. The expression of VqDUF642 protein in the inoculated tomato and grape lines was also analyzed by western blotting. The qRT-PCR was applied to analyze expression of the pathogenesis-related (PR) genes in the inoculated leaves.

The inoculation of transgenic and non-transformed tomato fruit was performed as described by Cantu et al. (2008). After surface sterilization, fruit were inoculated with 10 µl *B. cinerea* suspension (500 conidia/µl) or the same volume of water at four sites per fruit. Inoculated fruit were then incubated in a growth chamber with high humidity at 20 °C. Susceptibility to *B. cinerea* infection was recorded periodically as described by Cantu et al. (2008).

Statistical analysis

The mean values of all experimental results were compared by one-way ANOVA. Significant differences between results were determined by a post hoc comparison test (Student–Newman–Keuls) at $p < 0.05$ using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Isolation and sequence analysis of a novel *VqDUF642* gene from Chinese wild *V. quinquangularis*

To clone the *VqDUF642* gene, VqDUF642 protein (GenBank accession no. AMB38759) was used in a BLAST search of a grape genome library. A highly homologous protein (VvDUF642, GenBank accession no. XP_002284962) was found in *V. vinifera*, and specific primers were designed based on its nucleotide sequence to amplify the full length of the *VqDUF642* cDNA in Danfeng-2. The cDNA clone of *VqDUF642* (GenBank accession no. KP899561) consists of a 1107 bp ORF predicted to encode 368 amino acids. The *VqDUF642* gene is located on chromosome 4 of the published Pinot Noir genome (Jaillon et al. 2007) (Fig. 1a).

A total of 67 of VqDUF642-homologous proteins from 13 plant species shared conserved DUF642 regions (Supplementary Fig. S1). Amino acid alignments and phylogenetic analysis showed that VqDUF642 is highly similar to VvDUF642 in *V. vinifera* (99 % identity) (Supplementary Fig. S1, Fig. 1b). Moreover, the prediction of the VqDUF642 sequence showed that it contains a signal peptide, which was 20 amino acids long, and none of predicted GPI-anchor sites were found.

Expression patterns of *VqDUF642* in the developing grape berry

Grape berry development was assessed and described by Coombe in 1995. In the present study, the development was divided into four stages: the berries were still hard and green (stage I—25 days after anthesis); the berries began to soften and the brix started to increase (stage II—40 days after anthesis); the berries began to change color and enlarge (stage III—50 days after anthesis); and the berries were harvest-ripe (stage IV—80 days after anthesis). Danfeng-2 berry samples were collected from these four developmental stages. Cabernet Sauvignon berry samples were collected from four developmental stages: stage I, 30 days after anthesis; stage II, 45 days after anthesis; stage III, 60 days after anthesis; and stage IV, 110 days

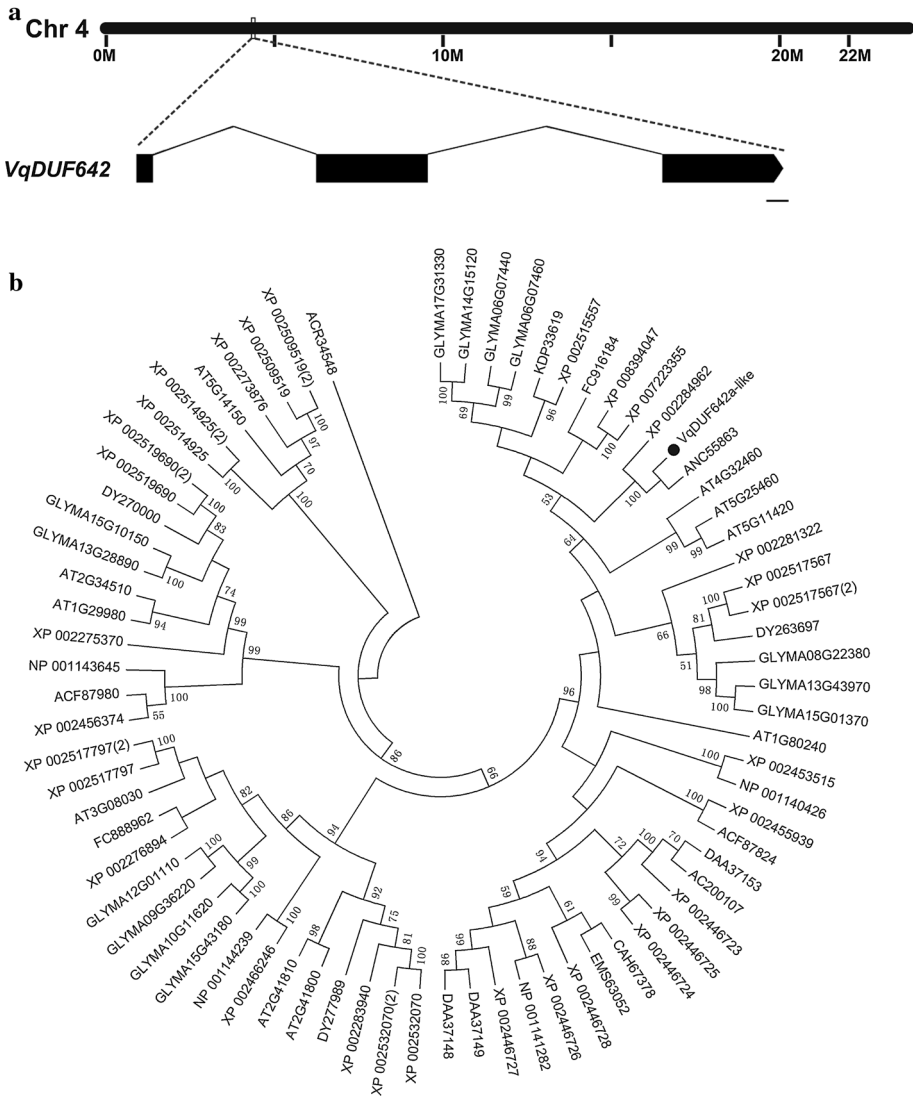


Fig. 1 Sequence analysis of the gene *VqDUF642*.
a Chromosomal location and schematic representation of the gene *VqDUF642*.
b Phylogenetic relationships between VqDUF642 and closely related proteins. VqDUF642 is highlighted with a *black-filled circle*. The tree was generated using the deduced amino acids from 13 plant species through the ClustalW method with MEGA 5.0. The branch support values are indicated

after anthesis. The proteomic changes were illustrated in Danfeng-2 during these four grape berry developmental stages. The iTRAQ analysis revealed that the expression of VqDUF642 protein rapidly increased at the last stage (stage IV) of berry development in Danfeng-2, compared to VvDUF642 protein in Cabernet Sauvignon (Fig. 2a).

To determine whether the *VqDUF642* gene is involved in berry development in grapes, its transcripts were analyzed during four stages of berry development in Danfeng-2. In Danfeng-2, transcript levels of *VqDUF642* remained stable from stage I to stage II and then substantially increased to reach a peak at stage III (Fig. 2b). By contrast, transcript levels of *VvDUF642* in Cabernet Sauvignon significantly increased from stage I to stage II, which displayed the highest level. Thereafter, transcript abundances of *VqDUF642* and *VvDUF642* decreased to the lowest level in Danfeng-2 and Cabernet Sauvignon, respectively (Fig. 2b).

VqDUF642 is localized to the cell wall

To investigate the subcellular localization of the VqDUF642–GFP protein, the 35S::*VqDUF642–GFP* and negative control 35S::*GFP* plasmids were transiently transformed into tobacco leaves via syringe infiltration. The control GFP protein was observed throughout the cell (Fig. 3). The VqDUF642–GFP fusion protein targeted the cell wall of plasmolysed epidermal cells (Fig. 3).

Molecular analysis of transformants

Genetic transformation assays were performed using *A. tumefaciens* strain GV3101 harboring the pCAM-35S::*VqDUF642–GFP* and pART-35S::*VqDUF642-FLAG* binary vectors for tomato and grape transformation, respectively (Fig. 4a, b).

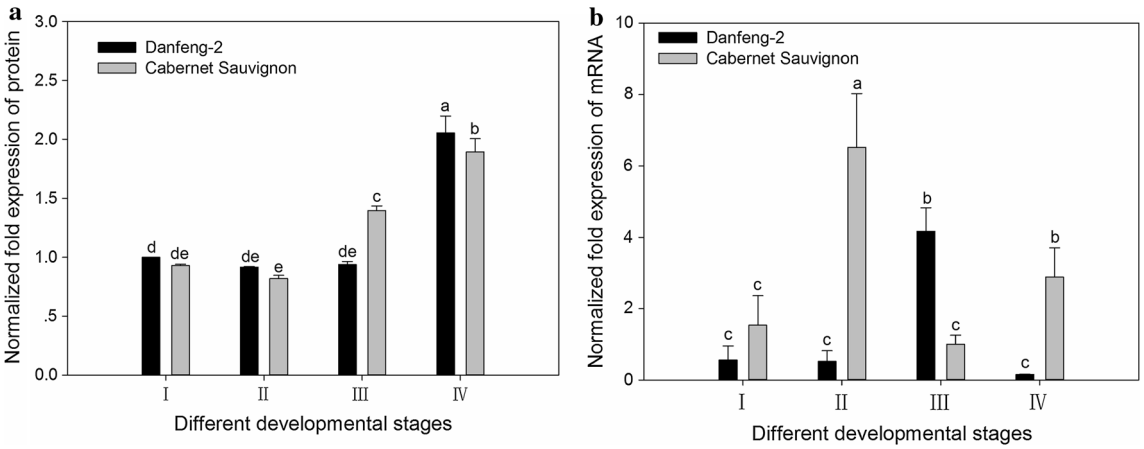


Fig. 2 Expression patterns of DUF642 gene in grape berries at different developmental stages: berries were still hard and green (stage I); berries began to soften and the brix started increasing (stage II); berries began to change color and enlarge (stage III); and berries were harvest-ripe (stage IV). **a** Expression levels of the DUF642 protein in grape berries from wild Chinese *V. quinquangularis* accession Danfeng-2 (VqDUF642) compared with *V. vinifera* cv. Cabernet Sauvignon (VvDUF642) at different developmental stages, as assessed by iTRAQ. **b** Transcript levels of the DUF642 gene in grape berries from wild Chinese *V. quinquangularis* accession Danfeng-2 (*VqDUF642*) compared with *V. vinifera* cv. Cabernet Sauvignon (*VvDUF642*) at different developmental stages, as indicated by qRT-PCR. Each value represents the mean ± SE of three different experiments. Means with *different letters* are significantly different at $p < 0.05$

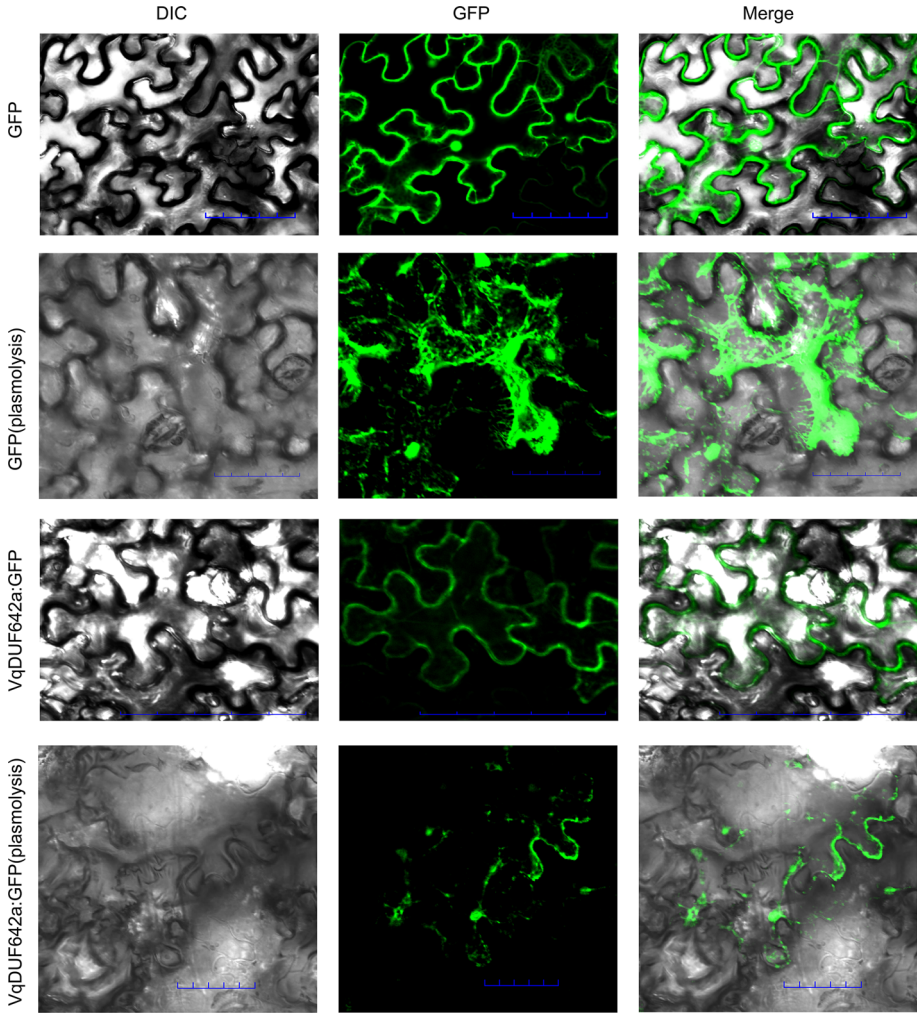


Fig. 3 Subcellular localization of VqDUF642. GFP alone was localized in the whole cells; VqDUF642-GFP was restricted to the surface of the tobacco epidermal cells, and VqDUF642-GFP was localized in the cell wall of the tobacco epidermal cells after plasmolysis. *Scale bar* 50 μm

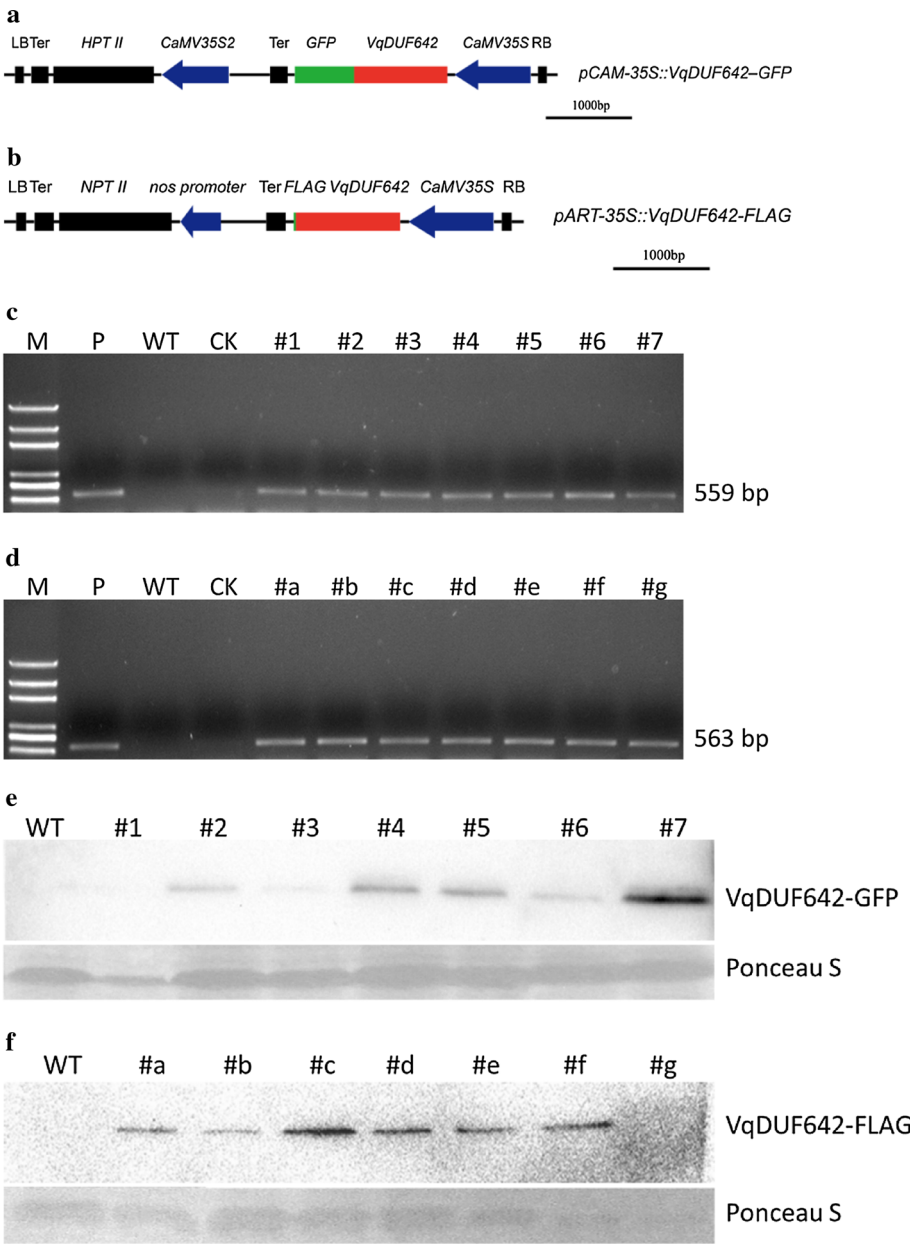
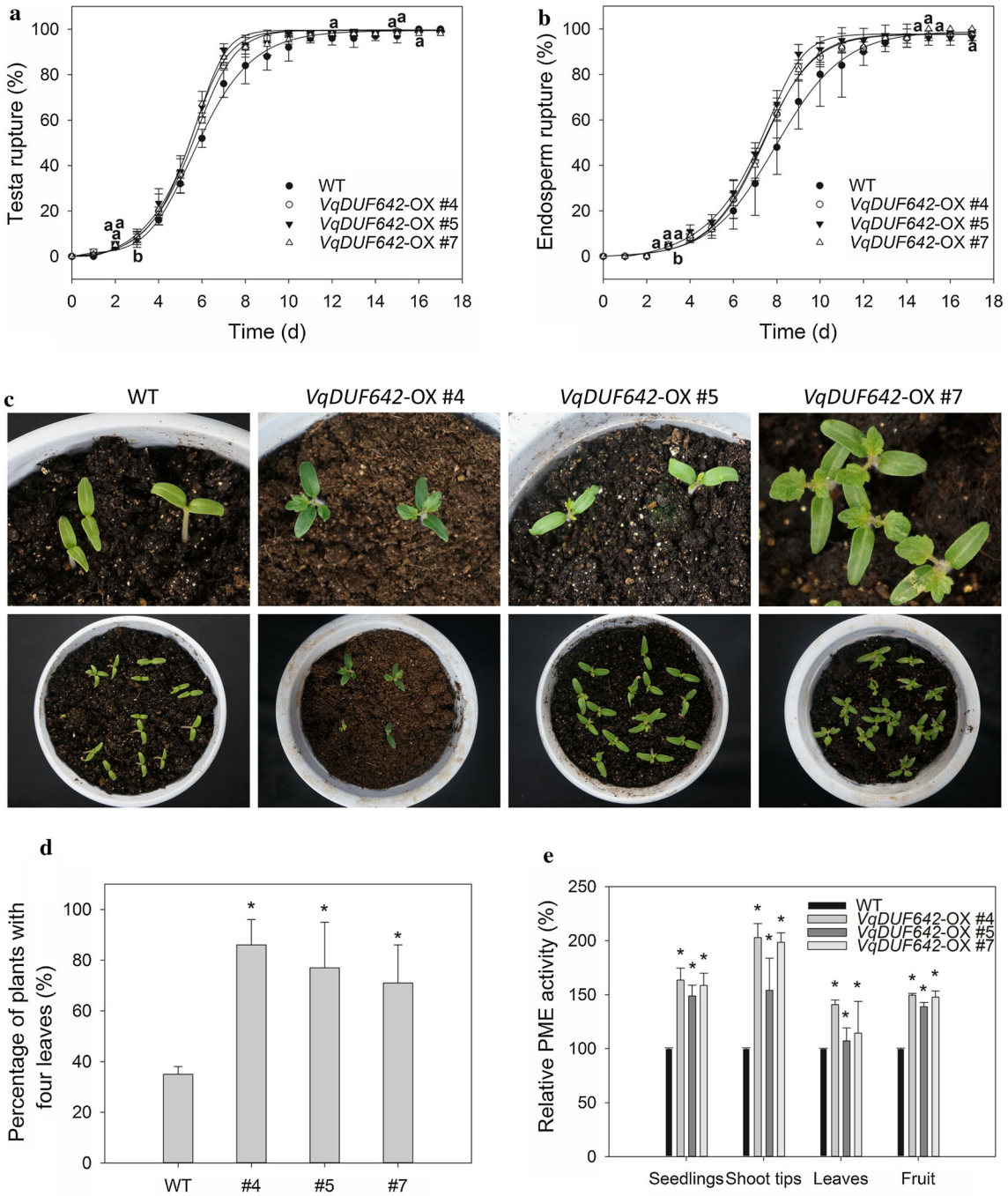


Fig. 4 Molecular analysis of the gene *VqDUF642*-overexpressing lines of transgenic Micro-Tom tomato and Thompson Seedless grape plants. **a** Schematic representation of the T-DNA region of the binary pCAMBIA1302 vector for subcellular localization and tomato transformation. *LB* left T-DNA border, *RB* right T-DNA border, *Ter* terminator, *CaMV35S* Cauliflower mosaic virus 35S promoter, *nos promoter* nopaline synthase promoter, *HYG* hygromycin phosphotransferase gene, *GFP* GFP protein tag used for western blotting. **b** Schematic representation of the T-DNA region of the binary pART-CAM-S vector for grape transformation. *LB* left T-DNA border, *RB* right T-DNA border, *Ter* terminator, *CaMV35S* Cauliflower mosaic virus 35S promoter, *nos promoter* nopaline synthase promoter, *NPTII* neomycin phosphotransferase gene, FLAG, a short, hydrophilic protein tag used for western blotting. **c** PCR analysis of the transgenic and wild-type tomato lines. *M* marker, *P* plasmid, *WT* wild-type, *CK* water control; *#1 to #7* different transgenic lines. **d** PCR analysis of the transgenic and wild-type Thompson Seedless lines. *M* marker, *P* plasmid, *WT* wild-type, *CK* water control, *#a to #g* different transgenic lines. **e** Western blot detection of the expression levels of the VqDUF642 protein in leaves of different transgenic tomato lines. **f** Western blot detection of the expression levels of the VqDUF642 protein in leaves of different transgenic Thompson Seedless lines

Gene introgression in the transgenic plants was confirmed by PCR analysis using specific primers for *hptII* or *nptII* (Fig. 4c, d). One expected band was amplified from the transgenic lines, while no PCR signals for foreign genes were detected in the water control (CK) and wild type (WT). The putative transgenic plants were transferred to a


a
Testa rupture (%)
Time (d)
WT
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7
b
Endosperm rupture (%)
Time (d)
c
WT
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7
d
Percentage of plants with four leaves (%)
WT
#4
#5
#7
e
Relative PME activity (%)
Seedlings
Shoot tips
Leaves
Fruit
f
Seedlings
Shoot tips
Leaves
Fruit
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7
VqDUF642-GFP
Ponceau S

◀**Fig. 5** Effect of the gene *VqDUF642* overexpression on the development of T3 transgenic Micro-Tom tomato lines. **a** Percentage of seeds at the stage of testa rupture at various time points after sowing. The seed germination assays were performed at 26 ± 1 °C. Time (d) represents days after sowing onto medium in Petri dish. **b** Percentage of seeds at the stage of endosperm rupture at various time points after sowing. **c** Effect of the gene *VqDUF642* overexpression on the development of T3 transgenic tomato seedlings compared with the wild-type (WT) control at 2 weeks after sowing. Five to ten fruits of each line and ten to 20 seeds of each fruit were analyzed. **d** Percentage of transgenic and control seedlings with four leaves (two expanded cotyledons and first two true leaves) at 2 weeks after sowing. Five to ten fruits of each line and ten to 20 seeds of each fruit were analyzed. The percentage was calculated based on the number of correspondingly germinated samples for each line. **e** PME activities in the seedlings (7 days post-germination), shoot tips and leaves of tomato plants (14 days after sowing), and fruit (ready to harvest). **f** Expression levels of the VqDUF642 protein were determined from seedlings (7 days post-germination), shoot tips and leaves of tomato plants (14 days after sowing), and fruit (ready to harvest) using western blotting. Western blot analysis was performed more than three times for each sample and representative data from single experiment is provided. Each value represents the mean ± SE of three different experiments (each experiment containing at least three lines and each line containing at least three plants). *Letters* or *asterisks* indicate significant differences between lines ($p < 0.05$)

greenhouse for further analysis (20 tomato lines and 7 grape lines).

Western blotting results demonstrated variable expression levels of VqDUF642 protein in the different transgenic lines (Fig. 4e, f). The transgenic plants with higher expression levels of VqDUF642 protein were selected for further analysis (tomato: lines 4, 5 and 7; grape: lines c, d and f).

Impact of *VqDUF642* overexpression on the development of tomato plants

Since the genetic transformation and production of fruit and seed in grapevines is time-consuming, an overexpression assay was performed in tomato to investigate the developmental role of VqDUF642. The seeds, plantlets, and fruit of the WT and transgenic tomato lines were observed periodically. No visible phenotypic differences in fruit size, seed number, and seed size were observed between the transgenic tomato plants and WT controls (Supplementary Fig. S2). To determine the effect of *VqDUF642* overexpression on seed germination, we tested the germination of T3 transgenic and control seeds on Petri dishes. Although the initial testa rupture and endosperm rupture times of the transgenic seeds were shorter than those of the WT seeds, the transgenic seeds did not show significantly different testa rupture rates or endosperm rupture rates compared to the WT seeds (Fig. 5a, b). Two weeks after sowing, some germinated seedlings generated four leaves (two expanded cotyledons and first two true leaves) (Fig. 5c). The percentage of four-leaf seedlings in each *VqDUF642*-overexpressing transgenic tomato line (#4, 86.44 %; #5, 77.26 % or #7, 71.38 %) was about two-fold higher than that of control plants (35.12 %) (Fig. 5d). Accordingly, the growth rate of the transgenic seedlings was markedly faster than the control seedlings.

To analyze the effect of *VqDUF642* overexpression on PME activity during plant development, we measured the PME activity of seedlings, shoot tips, leaves, and fruits from the transgenic and WT control lines. The PME activity was significantly higher in the *VqDUF642*-overexpressing transgenic tissues, particularly in the seedlings and shoot tips, than in the corresponding WT tissues (Fig. 5e). This result correlated well with the expression of VqDUF642 protein in different tissues of transgenic lines from western blotting (Fig. 5f).

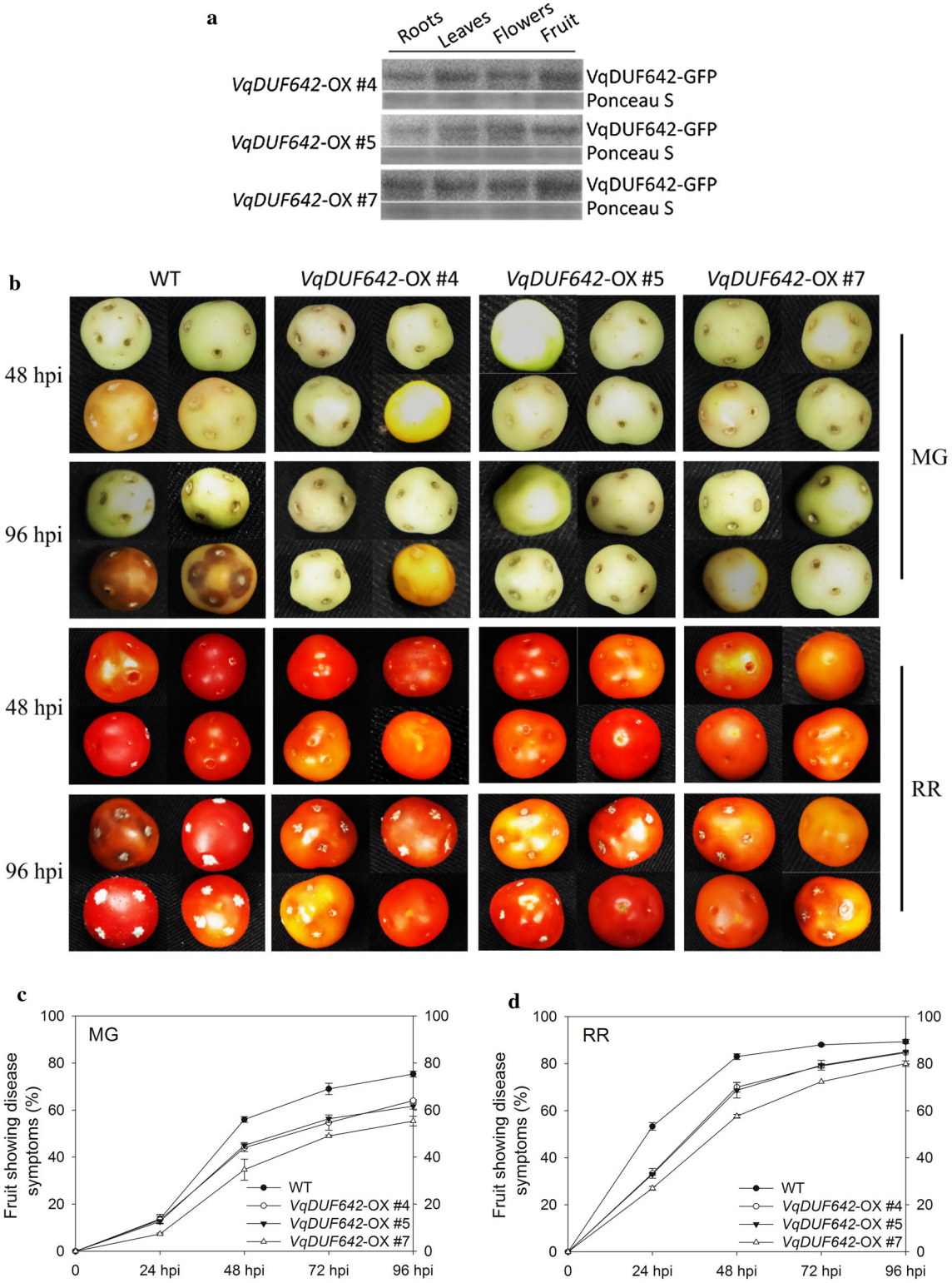
Impact of *VqDUF642* overexpression on susceptibility of tomato to *B. cinerea*

Fruits and young leaves of transgenic tomato plants with higher expression levels of VqDUF642 protein were selected for the *B. cinerea* susceptibility analysis (Fig. 6a). Most transgenic and WT tomato fruit showed *B. cinerea*-induced symptoms on both mature green (MG, 35 days post anthesis) and red ripe (RR, 45 days post anthesis) fruit at 48 and 96 hpi (Fig. 6b). At each time point, the percentage of MG and RR fruit showing disease symptoms in transgenic lines was lower than that in WT controls (Fig. 6c, d). Overexpression of *VqDUF642* led to a decreased susceptibility of tomato fruit to *B. cinerea*, especially of MG fruit (Fig. 6c, d).

However, no significant difference in number of decayed spots or conidiophores growth was observed on WT and *VqDUF642*-overexpressing leaves inoculated with *B. cinerea* (Fig. 6e, f). To investigate the fungal growth, we analyzed the transcript levels of the endogenous *Bcactin* gene of *B. cinerea* in the inoculated leaves. Decreased *Bcactin* transcript levels were detected in the transgenic tomato leaves compared to the WT leaves (Fig. 6g). Moreover, expression levels of VqDUF642 protein increased after inoculation in different transgenic tomato lines (Fig. 6h). Transcript abundances of PR genes (*SlPR1*, *SlPR2*, *SlPR3*, and *SlPR4*) in *VqDUF642*-overexpressing transgenic tomato lines increased at 48 and 96 hpi respectively, compared with the WT control (Fig. 6i).

Assessment of resistance to *E. necator* in *VqDUF642*-overexpressing transgenic grapevine plants

Leaves of transgenic Thompson Seedless grape plants with higher expression levels of VqDUF642 protein were


a
Roots
Leaves
Flowers
Fruit
VqDUF642-OX #4
VqDUF642-GFP
Ponceau S
VqDUF642-OX #5
VqDUF642-GFP
Ponceau S
VqDUF642-OX #7
VqDUF642-GFP
Ponceau S
b
WT
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7
48 hpi
96 hpi
MG
48 hpi
96 hpi
RR
c
MG
Fruit showing disease symptoms (%)
0
20
40
60
80
100
0
24 hpi
48 hpi
72 hpi
96 hpi
WT
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7
d
RR
Fruit showing disease symptoms (%)
0
20
40
60
80
100
0
24 hpi
48 hpi
72 hpi
96 hpi
WT
VqDUF642-OX #4
VqDUF642-OX #5
VqDUF642-OX #7

◀**Fig. 6** Impact of the gene *VqDUF642* overexpression on susceptibility of Micro-Tom tomato plants to *Botrytis cinerea*. **a** Western blotting detection of the expression levels of the VqDUF642 protein in different transgenic tomato tissues. **b** *B. cinerea*-induced symptoms on the transgenic tomato and wild-type (WT) fruit at 48 h and 96 h post-inoculation (hpi) under high humidity growth conditions. **c** Percentage of MG (*mature green*) fruit showing disease symptoms after inoculation with *B. cinerea*. Disease symptoms of four inoculated sites per fruit were observed from 50 fruits of each line at 0, 24, 48, 72, and 96 hpi. **d** Percentage of RR (*red ripe*) fruit showing disease symptoms after inoculation with *B. cinerea*. Disease symptoms of four inoculated sites per fruit were observed from 50 fruits of each line at 0, 24, 48, 72 and 96 hpi. **e** *B. cinerea*-induced symptoms on the transgenic and WT tomato leaves at 0, 48, and 96 hpi under natural growth conditions. The fourth leaf from the top of each plant and six plants from each line were analyzed. At least 180 inoculation drops were evaluated for each treatment. **f** Progression of *B. cinerea* on tomato leaves. Images of the stained transgenic and WT tomato leaves were captured at 48 and 96 hpi. *Scale bar* 50 μm. **g** Expression levels of the *Bcactin* gene at 48 and 96 hpi were determined from inoculated transgenic and WT tomato leaves using qRT-PCR. Tomato actin gene (*SlActin*) was used as an internal control. **h** Expression levels of the VqDUF642 protein at 0, 48, and 96 hpi were determined from inoculated tomato leaves using Western blotting. Western blot analysis was performed more than three times for each sample and representative data from single experiment is provided. **i** Expression levels of the selected pathogenesis-related (PR) genes (*SlPR1*, *SlPR2*, *SlPR3*, and *SlPR4*) at 48 and 96 hpi were determined from inoculated transgenic and WT tomato leaves using qRT-PCR. The tomato actin gene (*SlActin*) was used as an internal control. Each value represents the mean ± SE of three different experiments (each experiment containing at least three lines and each line containing at least three plants). *Asterisks* indicate significant differences between lines ($p < 0.05$)

selected for *E. necator* inoculation (Fig. 7a). Germination and development of conidia were tracked at 24, 48, 72, and 168 hpi to compare the characteristics of *E. necator*-induced symptoms on transgenic and WT control leaves (Fig. 7b, c). The infection was more severe in the control leaves than in transgenic line c and f at 48 hpi, since the number of hyphae was significantly greater on the control leaves than on the leaves of line c and f. The number of secondary hyphae and conidiophores growing on the leaves of the transgenic plants was significantly smaller than the control at 72 and 168 hpi, respectively (Fig. 7c). Additionally, expression levels of VqDUF642 protein were promoted after inoculation with *E. necator* in different transgenic grape lines (Fig. 7d). Also, qRT-PCR analysis suggested that transcript levels of *VvPR1*, *VvPR2*, *VvPR3*, and *VvPR4* dramatically increased at 48 and 96 hpi in the three *VqDUF642*-overexpressing transgenic grape lines compared with WT (Fig. 7e).

Assessment of resistance to *B. cinerea* in *VqDUF642*-overexpressing transgenic grapevine plants

The *VqDUF642*-overexpressing transgenic grapevine lines exhibited enhanced resistance to *B. cinerea*, which was demonstrated by the reduced number of necrotic lesions and ratio of conidiophores on the transgenic leaves compared to the WT controls (Fig. 8a, b). A significantly greater number of decayed spots or sectors and a faster conidiophores growth were observed on the WT leaves than the *VqDUF642*-overexpressing leaves upon *B. cinerea* infection. To investigate the fungal growth, we analyzed the transcript levels of *Bcactin* in the inoculated leaves. Reduced *Bcactin* transcript abundance was detected in the transgenic grapevine leaves compared to the control leaves (Fig. 8c). Expression levels of VqDUF642 protein increased following *B. cinerea* inoculation in different transgenic grape lines (Fig. 8d). Transcript levels of *VvPR1*, *VvPR2*, *VvPR3*, and *VvPR4* were up-regulated at 48 and 96 hpi in the three *VqDUF642*-overexpressing transgenic grape lines infected with *B. cinerea* (Fig. 8e). Thus, overexpression of *VqDUF642* appeared to have a positive influence on the grape plant defense against *B. cinerea*.

Discussion

The DUF642 family has been identified in a number of plant species, including *Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa* (Bayer et al. 2006; Chen et al. 2009; Irshad et al. 2008; Jamet et al. 2006; Minic et al. 2007; Negri et al. 2008). In grapes, 6 DUF642 proteins have been identified in the proteome of *V. vinifera*, but no grape DUF642 gene has been studied (Lorenzini et al. 2016; Vázquez-Lobo et al. 2012). In the present study, one DUF642 gene, annotated as *VqDUF642*, was isolated from the Chinese grape *V. quinquangularis* accession Danfeng-2. Amino acid sequence analysis suggested that VqDUF642 is highly homologous to VvDUF642 in *V. vinifera* (Fig. 1). Expression of *VqDUF642* seems to be different at the transcriptional and the post-transcriptional levels. It seems that a delay was observed between the highest level of accumulation of transcripts and that of protein. However, comparison of protein and mRNA expression patterns showed that *VqDUF642* gene, which increased at the later stage of berry development in Danfeng-2, was probably involved in berry development (Fig. 2).

The plant cell wall can invaginate around the feeding structures of pathogenic, symbiotic fungi, and oomycetes, forming an interface between the host cell and those feeding structures and stimulating the plant immune system. Cell wall proteins are indispensable in the innate immune system of wild plants (Jones and Dangl 2006). In our research, the evidence from the nuclear localization analyses suggested that VqDUF642, which was localized to the cell wall (Fig. 3), may contribute to grape plant resistance against pathogen invasion.

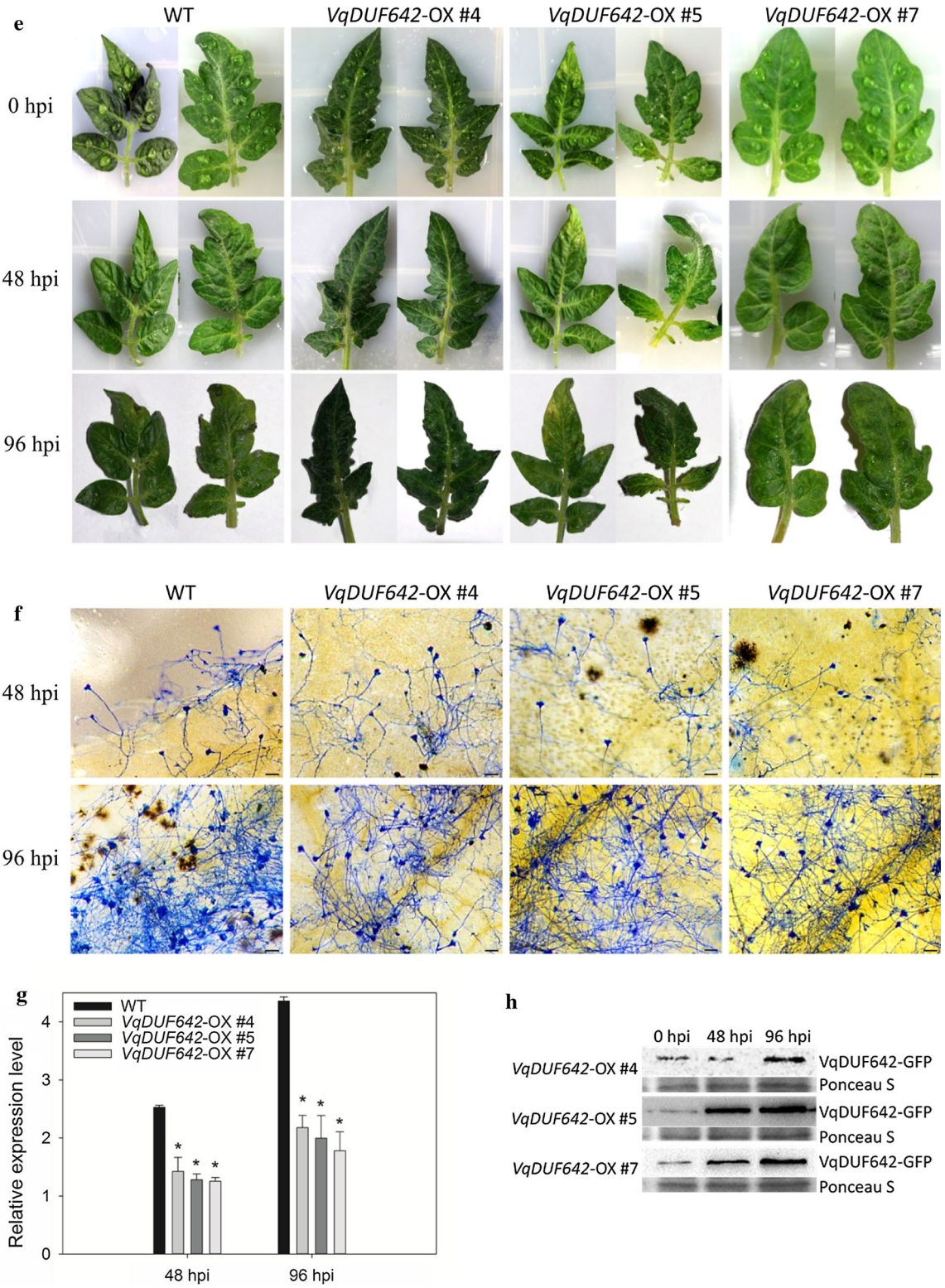


Fig. 6 continued

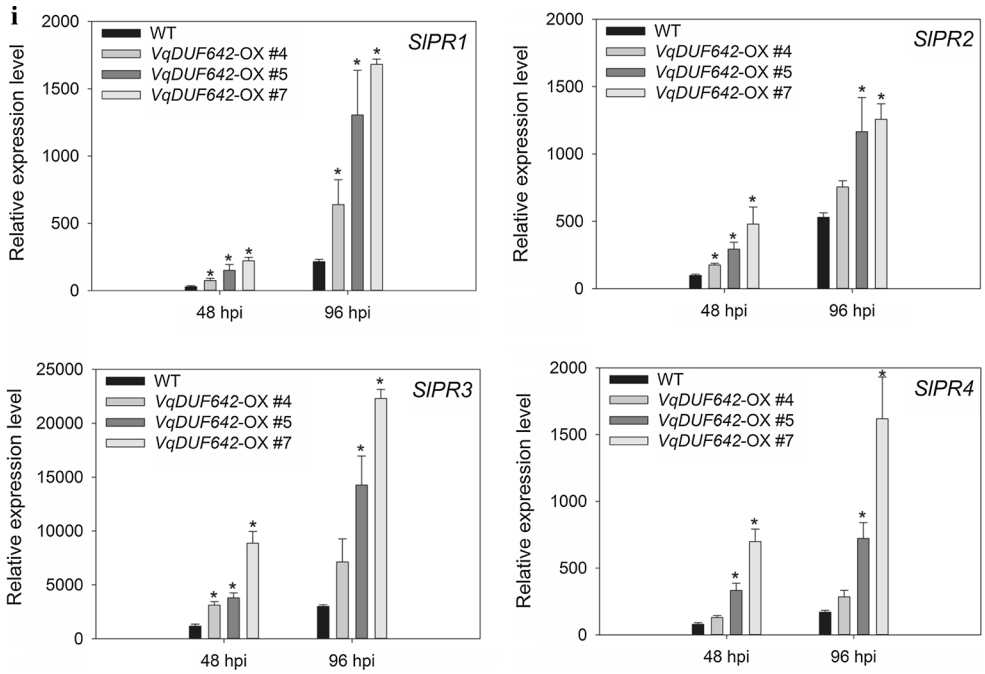
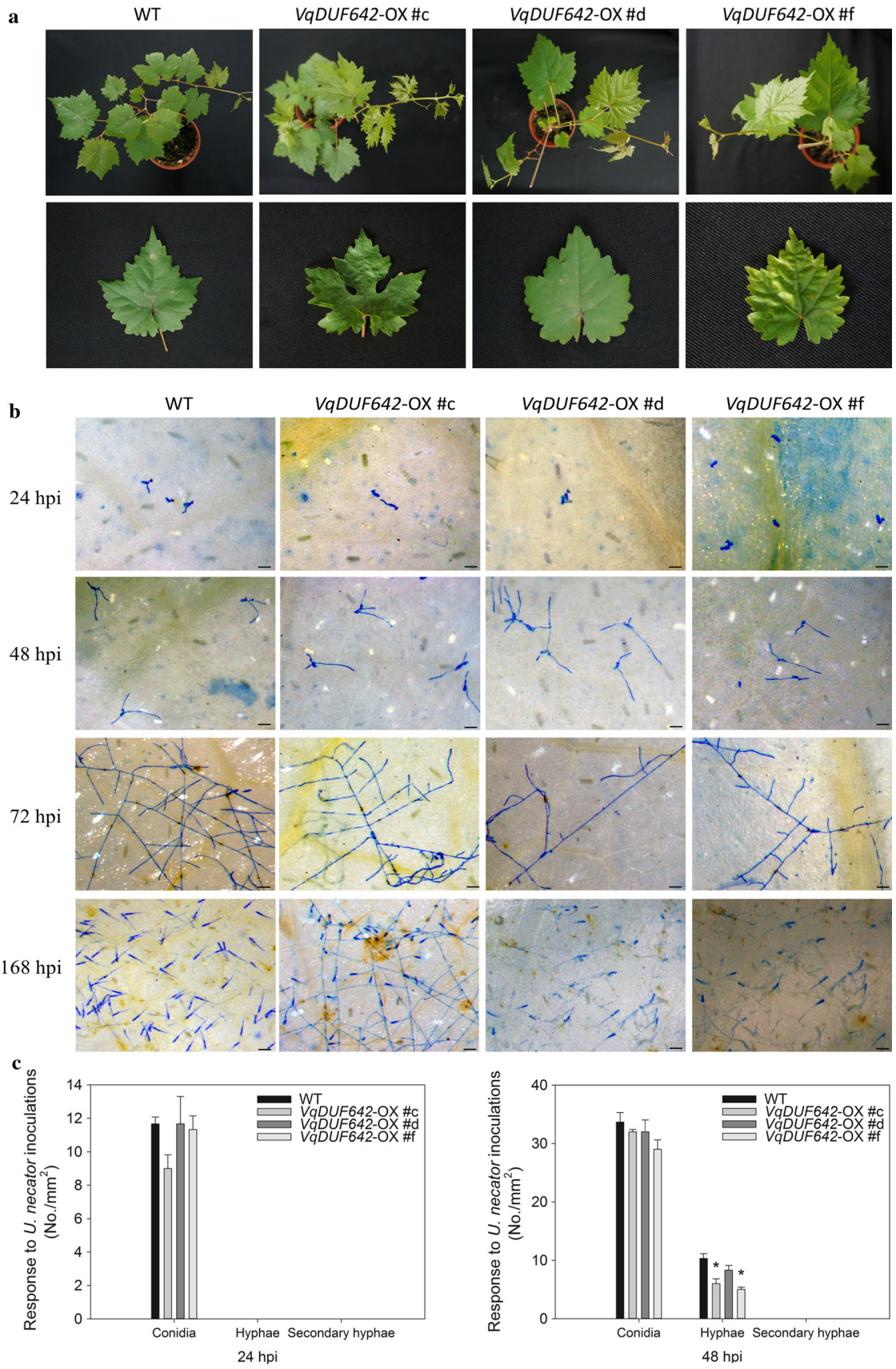


Fig. 6 continued

To dissect the role of VqDUF642 in berry development, the gene encoding VqDUF642 was overexpressed in T3 tomato lines. The rate of seed germination and the percentage of seedlings with four leaves showed that transgenic seedlings overexpressing *VqDUF642* grew faster than the control lines, suggesting that *VqDUF642* overexpression had a positive impact on the development of the tomato plants (Fig. 5a–d). In a previous study, transgenic Arabidopsis plants overexpressing DUF642 genes *At4g32460* and *At5g11420* exhibited reduced times of initial testa and endosperm rupture, but there were no morphological changes between the transgenic and control lines (Zúñiga-Sánchez et al. 2014). In our research, overexpression of *VqDUF642* did not cause any morphological changes during fruit and seed formation.

In this study, PME activity was increased in leaves, fruit, seedlings, and shoot tips of *VqDUF642*-overexpressing transgenic tomato plants. Fruit ripening is a process of cell wall loosening (Konozy et al. 2013), which is accompanied by the alteration of PME activity (Andrews and Li 1995; de Assis et al. 2001). PME activity is increased in banana during the color change stage from green to yellow (Hultin and Levine 1965), and in tomato fruit during the stage of veraison (Dennison et al. 1954; Hobson 1963; Prasanna et al. 2007). However, the level of PME activity decreases as grape berries develop (Nunan et al. 2001), and it varies across the stages of acerola fruit development (de Assis et al. 2001). Fruit ripening involved in the rearrangement of the cell wall architecture are affected by PME activity (Brummell and Harpster 2001; Frenkel et al. 1998; Micheli 2001; Phan et al. 2007; Tieman and Handa 1994). In our work, increased PME activity in the fruit of transgenic tomato lines indicates that VqDUF642 was involved in the fruit development.

In tomato, several cell wall proteins, which are up-regulated during fruit ripening, are involved in plant defense against pathogenic infections (Choi et al. 2008; Kim and Triplett 2004; Pressey 1997; Segura et al. 1999; Stotz et al. 2009). Fruit ripening occurs concomitantly with a significant increase in susceptibility to *B. cinerea* (Cantu et al. 2008). Similar results have also been observed in our research. RR fruits had greater susceptibility to *B. cinerea* than MG fruits of tomato. Suppression of tomato fruit ripening results in firmer fruit, but did not reduce the susceptibility to *B. cinerea* and *Alternaria alternata* according to a previous study (Powell et al. 2003). In our study, *VqDUF642*-overexpressing transgenic tomatoes exhibited faster growth rate and increased resistance to *B. cinerea*, compared with the WT plants.

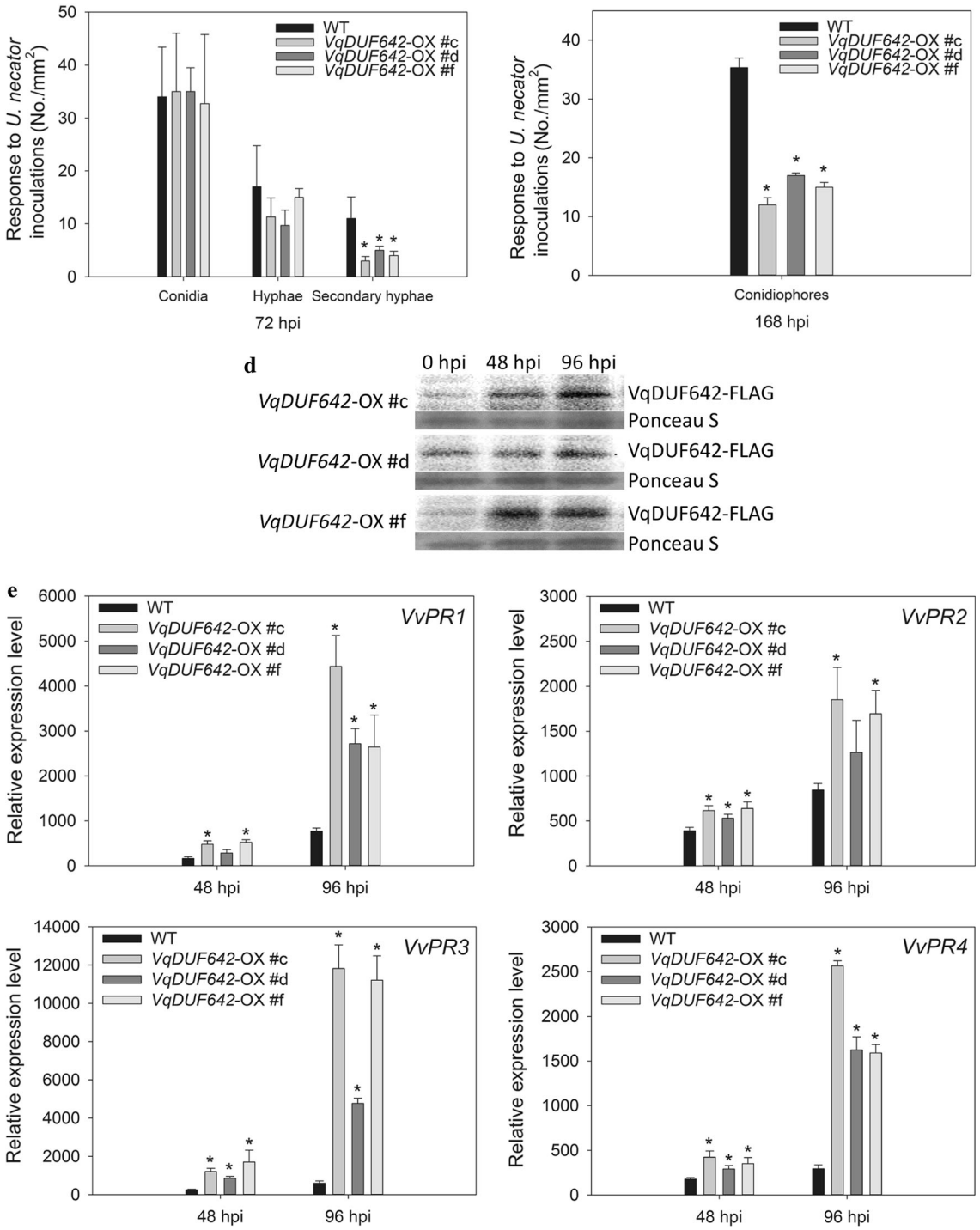

a
WT
VqDUF642-OX #c
VqDUF642-OX #d
VqDUF642-OX #f
b
WT
VqDUF642-OX #c
VqDUF642-OX #d
VqDUF642-OX #f
24 hpi
48 hpi
72 hpi
168 hpi
c
Response to U. necator inoculations (No./mm2)
WT
VqDUF642-OX #c
VqDUF642-OX #d
VqDUF642-OX #f
Conidia
Hyphae
Secondary hyphae
24 hpi
48 hpi

◀**Fig. 7** Assessment of resistance to *Uncinula necator* in *VqDUF642*-overexpressing *V. vinifera* cv. Thompsom Seedless grape plants. **a** *U. necator*-induced symptoms in transgenic Thompson Seedless plants and wild-type (WT) plants at 168 h post-inoculation (hpi) under natural growth conditions. The third and fourth leaves from the top of each plant and three plants from each line were analyzed. At least 108 inoculation positions were evaluated for each treatment. **b** Progression of *U. necator* on the Thompson Seedless leaves. The images of the stained transgenic and WT plants were captured at 24, 48, 72, and 168 hpi. *Scale bar* 50 μm. **c** Responses of the transgenic and WT plants to *U. necator* inoculations. *No.* number. **d** Expression levels of the VqDUF642 protein at 0, 48, and 96 hpi were determined from inoculated grape leaves using Western blotting. Western blot analysis was performed more than three times for each sample and representative data from single experiment is provided. **e** Expression levels of the selected pathogenesis-related (PR) genes (*VvPR1*, *VvPR2*, *VvPR3*, and *VvPR4*) at 48 and 96 hpi were determined from inoculated transgenic and WT grape leaves using qRT-PCR. The grapevine *GAPDH* gene was used as an internal control. Each value represents the mean ± SE of three different experiments (each experiment containing at least three lines and each line containing at least three plants). *Asterisks* indicate significant differences between lines ($p < 0.05$)

To determine the role of VqDUF642 in response to pathogenic infection, we also overexpressed this gene in Thompson Seedless grapevine plants. When we inoculated these transgenic plants with *E. necator* or *B. cinerea* (Figs. 6h, 7d, 8d, respectively), an elevated accumulation of VqDUF642 protein was observed. In *Arabidopsis*, expression of DUF642 genes *At5g25460* and *At3g08030* have been shown to correlate with bacterial invasion and insect attack (Depuydt et al. 2009; Hu et al. 2008). The expression of *At5g25460* was down-regulated after *Ralstonia solanacearum* inoculation (Hu et al. 2008), while the expression of the *At3g08030* was up-regulated upon inoculation with *Rhodococcus fascians* (Depuydt et al. 2009). In gymnosperms, transcript abundance of DUF642 gene increases in response to wounding or feeding by pathogens (Ralph et al. 2006). DUF642 is thought to play an essential role in the cellular defense mechanism against pathogen attack (Bayer et al. 2006; Konozy et al. 2013). Plant PR genes are induced by pathogen infection, which were represented through those genes' quantitative changes (Jacobs et al. 1999; Van Loon et al. 2006; Zhu et al. 2012). In this study, transcript levels of PR genes were higher in transgenic tomato and grapevine lines than in WT control lines inoculated with *B. cinerea* and *E. necator* (Figs. 6i, 7e and 8e). Overexpression of *VqDUF642* in transgenic grape plants enhanced pathogen defense, which suggests that VqDUF642 may be a positive regulator of resistance to pathogenic diseases.

Cell wall is an important part of the plant defense system and it works by presenting physical barriers to pathogen invasion (Asselbergh et al. 2007). Cell wall defense is a complicated process that involves the expression of defense-related and cross-linked cell wall proteins (Asselbergh et al. 2007; Bradley et al. 1992; Ribeiro et al. 2006; Showalter 1993). In the present research, the introduction of foreign *VqDUF642* into tomato and grape plants could be considered to be a cell wall modification. Cell wall modification is known to increase pathogen resistance through suppressing cell wall degradation, blocking pathogen feeding, and preventing the proliferation of toxins (Bestwick et al. 1998; Brisson et al. 1994; Ribeiro et al. 2006; van Kan 2006). One of the first responses after pathogen invasion of a cell is cell death, while neighboring cells perceive defense signals. Next, a series of specific cellular protection mechanisms are activated (Hammond-Kosack and Jones 1996). In our research, enhanced resistance to pathogens in transgenic plants could be due to the presence of larger amount of a protein possibly interacting with cellulose microfibrils. Some studies indicate the importance of cell wall modification in the defense against pathogens in tomato plants (Asselbergh et al. 2007), broad bean and tulip leaves (Mansfield and Hutson 1980), onions (McLusky et al. 1999), wheat leaves (Mitchell et al. 1994), and lily (Van Baarlen et al. 2004). Cell wall modification through introduction of the foreign cell wall protein enhanced the defense against *E. necator* and *B. cinerea* in grape plants, which to our knowledge, has not been presented before.

In conclusion, a *VqDUF642* gene, encoding DUF642 protein, isolated from the Chinese grape species *V. quinquangularis* accession Danfeng-2 was localized to the cell wall. Accumulation of VqDUF642 protein and transcript levels of *VqDUF642* increased at the later berry developmental stage in Danfeng-2. The overexpression experiments in tomato provided evidences that VqDUF642 was involved in the plant development and pathogen susceptibility. Furthermore, enhanced resistance to *E. necator* and *B. cinerea* was observed in *VqDUF642*-overexpressing transgenic Thomson Seedless plants. These results indicate that VqDUF642 probably participate in the regulation of plant development and defense against pathogen invasion. The function of this novel gene should be taken into account for future grapevine breeding.



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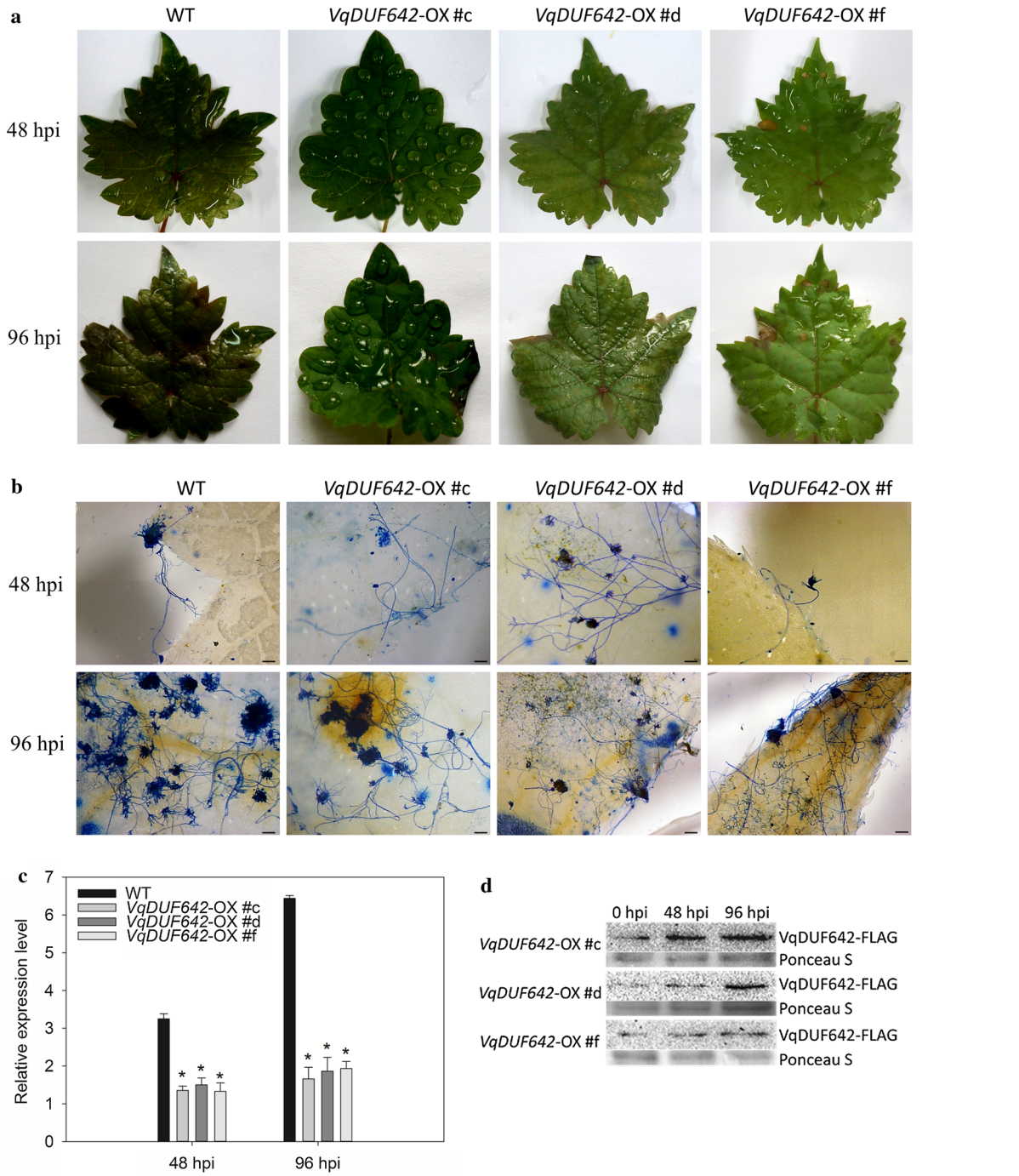
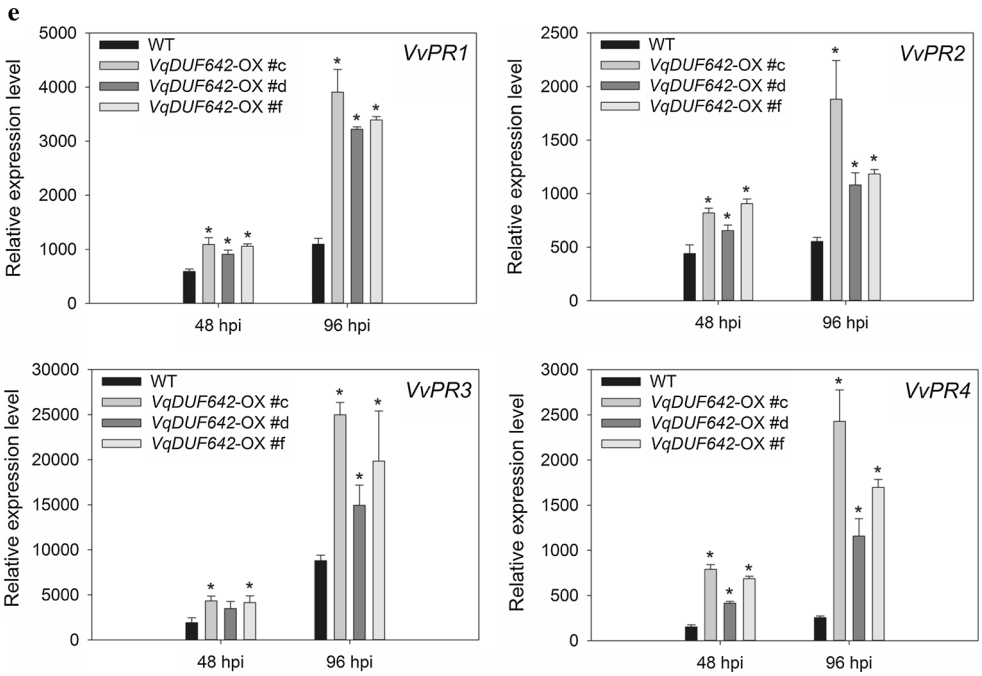


Fig. 8 Assessment of resistance to *Botrytis cinerea* in *VqDUF642*-overexpressing Thompson Seedless grape plants. **a** *B. cinerea*-induced symptoms in the transgenic Thompson Seedless and wild-type (WT) plants at 48 and 96 h post-inoculation (hpi) under natural growth conditions. The third and fourth leaves from the top of each plant and three plants from each line were analyzed. At least 360 inoculation drops were evaluated for each treatment. **b** Progression of *B. cinerea* on the Thompson Seedless leaves. Images of the stained transgenic and WT plants were captured at 48 and 96 hpi. *Scale bar* 50 µm. **c** Expression levels of the *Bcactin* gene were determined using qRT-PCR. The grapevine *GAPDH* gene was used as an internal control. **d** Expression levels of the VqDUF642 protein at 0, 48, and 96 hpi were determined from inoculated grape leaves using Western blotting. Western blot analysis was performed more than three times for each sample and representative data from single experiment is provided. **e** Expression levels of the selected pathogenesis-related (PR) genes (*VvPR1*, *VvPR2*, *VvPR3*, and *VvPR4*) at 48 and 96 hpi were determined from inoculated transgenic and WT tomato leaves using qRT-PCR. The grapevine *GAPDH* gene was used as an internal control. Each value represents the mean ± SE of three different experiments (each experiment containing at least three lines and each line containing at least three plants). *Asterisks* indicate significant differences between lines ($p < 0.05$)

Fig. 8 continued



Author contribution statement YW designed research, XX conceived and performed the experiments, XX analyzed the data, XX and YW wrote the paper, and YW revised the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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