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Vv14-3-3ω Is a Susceptible Factor for Grapevine Downy Mildew

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Abstract

14-3-3 proteins are highly conserved regulatory molecules in plants. In grapevine (*Vitis vinifera* L.), 14-3-3 proteins are studied under abiotic stress. However, the role of 14-3-3 proteins in the interaction between grapevine and downy mildew is yet to be studied. In this study, we identified a highly conserved 14-3-3 protein in grapevine and performed a phylogenetic analysis, revealing a close relationship between one of its homologs, 14-3-3ω proteins from *Arabidopsis thaliana* and *Nicotiana benthamiana*. We designated this homolog as Vv14-3-3ω. Subcellular localization studies showed that Vv14-3-3ω resides in the plasma membrane and cytoplasm. Expression analysis revealed a strong induction of Vv14-3-3ω at early time points following *Plasmopara viticola* infection, correlating with enhanced pathogen sporulation in grapevine. Furthermore, transient overexpression of Vv14-3-3ω in *N. benthamiana* increased susceptibility to the *Phytophthora capsici* pathogen and suppressed Flg22-induced pattern-triggered immunity (PTI) responses. Overexpression of Vv14-3-3ω in *Nb14-3-3*-silenced *N. benthamiana* plants resulted in increased susceptibility to *P. capsici*, suggesting functional conservation of this isoform. These findings indicate that Vv14-3-3ω functions as a susceptibility factor, facilitating pathogen infection and disease progression in grapevine, and highlight its potential role for improving resistance against downy mildew.

Keywords: *Vitis vinifera*; *Plasmopara viticola*; plant immunity; pathogenicity

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1. Introduction

Grapevine downy mildew causes substantial economic losses to the grape industry globally. Among the most severe and notorious crop epidemics of downy mildew of grapes occurred in the late 1800s [1]. Grapes are globally recognized as economically significant fruit trees. Among grape varieties, Thompson Seedless is a highly valued cultivar due to its widespread use in the fresh fruit market, as well as in raisin production, juice processing, and winemaking. It ranks as one of the most extensively cultivated seedless grape varieties, serving as a key genetic resource in grape breeding and the study of gene function [2]. Furthermore, this cultivar is highly susceptible to grapevine downy mildew. *Plasmopara viticola* is the causal agent of grapevine downy mildew. It is considered the most notorious plant pathogen [3] among obligate biotrophs, second only to hemibiotrophs [4,5]. Although downy mildews cause substantial economic losses, the underlying mechanism by which this group of plant pathogens causes diseases is yet to be fully understood.

In the long evolutionary history of plant pathogen interactions, host susceptibility (S) factors play a critical yet often overlooked role in determining the outcome of infection. Unlike resistance (R) genes, which activate immune responses to restrict pathogen growth, *S* genes facilitate pathogen colonization either actively or passively [6]. Many *S* genes act

as negative regulators of plant immunity, dampening immune signaling pathways such as PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI) [7]. Pathogens often exploit these host *S* genes to establish a conducive environment for their proliferation. For example, it has been reported that the *P. capsici* effector PcAvh103 targets the lipase domain of the defense regulator Enhanced Disease Susceptibility 1 (EDS1). This interaction disrupts the formation of the EDS1-PAD4 complex, thereby disrupting the downstream signaling of this complex [8]. Avr3a, a conserved effector of the *Phytophthora* pathogen, targets CAD7, a conserved protein both in *N. benthamiana* and *A. thaliana*, to promote virulence [9]. Understanding the identity and function of *S* genes may provide a solid basis and open new avenues for improving crop resistance by removing or modifying these negative regulators.

Fourteen three three (14-3-3) proteins are a class of highly conserved eukaryotic proteins isolated from mammalian brain tissues. They are named because of their elution pattern in the 14th fraction at the 3.3 position during gel electrophoresis and cellulose chromatography [10]. These proteins are conserved eukaryotic phospho-sensors that play an important role in metabolism, growth, development, transport, and stress response [11]. The structural information of 14-3-3 proteins reveals that these proteins can dimerize easily and form flattened, horseshoe-like structures. This allows 14-3-3 proteins to be involved in numerous biological pathways through targeting specific proteins in animals and plants [12–14]. To date, multiple isoforms of 14-3-3 proteins have been reported. For example, seven isoforms of 14-3-3 are known in humans, which are encoded by different genes, two isoforms in budding yeast, and up to fifteen isoforms of 14-3-3s are reported in plants [15,16]. Phylogenetic analysis of protein sequences provides insights into the evolutionary history by revealing relationships among homologous proteins and highlighting patterns of functional divergence. Such trees are essential for classifying proteins into different groups. For example, a phylogenetic analysis of plant 14-3-3 proteins generally classified these proteins into two major phylogenetic groups known as ε (epsilon) and non-ε (non-epsilon). The ε group is considered more ancestral and conserved, with members implicated in core cellular processes, while the non-ε group has diversified and is thought to contribute to species or lineage-specific regulatory roles [17–19].

In animals, the role of 14-3-3 protein in cancer biology, cell proliferation, and apoptosis has been reported [20]. Several 14-3-3 isoforms have been implicated in plant immunity, where they regulate defense-related kinases, transcription factors, and enzymes in response to pathogen attack. Specifically, in plant pathogen interaction, 14-3-3 proteins are found to play a dual role. For example, in tomato, the TFT7 14-3-3 protein mediates the signal transduction with mitogen-activated protein kinases (MAPKKKα and MKK2) to induce a hypersensitive response [21]. While in plant *Xanthomonas campestris* interaction, 14-3-3 protein TFT1, upon its interaction with a bacterial effector XopN, suppresses PTI and promotes virulence [22]. In large-scale studies of phytopathology [23], 14-3-3 proteins were not identified as a common host protein. However, recent studies in plant hemibiotrophs have revealed that a RxLR effector PITG06478 of *P. infestans* interacts with Nb14-3-3 protein to suppress plasma membrane H+-ATPases and induce PCD [24]. *Phytophtora palmivora* 14-3-3 interacting RxLR effector (FIRE) was found to phosphorylate and hijack the 14-3-3 and negatively regulate plant immunity [25]. However, in obligate biotrophs, especially during grapevine downy mildew, the role of 14-3-3 in regulating grapevine immunity is yet to be unraveled. The objective of this study is to identify and functionally characterize the 14-3-3 protein in *V. vinifera* grapevine to understand its role in regulating grapevine immunity during *P. viticola* infection.

2. Materials and Methods

2.1. Phylogenetic Analysis

The protein sequences of the 14-3-3 proteins from *Vitis vinifera*, *Nicotiana benthamiana*, and *Glycine max* were retrieved from previously published studies [26–28], whereas the sequences of *Arabidopsis thaliana* and *Oryza sativa* 14-3-3 proteins were obtained from the TAIR database (https://www.arabidopsis.org/, accessed on 6 September 2025). The phylogenetic tree was constructed using IQ-TREE 2 with the maximum likelihood method (ML, Q.plant + R4). Bootstrap analysis was performed with 1000 replicates, and only values greater than 50 were displayed in the corresponding figure. Protein sequence alignment was performed using the cobalt alignment tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi, accessed on 6 September 2025).

2.2. Bacterial Strains and Plasmids

Characteristics of all strains and plasmids used in this study are listed in Supplemental Table S2, and the primers used are shown in Supplemental Table S3. Bacterial growth conditions are described in Supplemental Text S1. Corresponding plasmids were transformed into *agrobacteria* by electroporation. *Agrobacterium tumefaciens* strain GV3101 carrying a given binary vector was used for transient gene expression in *N. benthamiana* and in *V. vinifera*. For protein expression and subcellular localization in plants, the coding region of Vv14-3-3ω was amplified from their corresponding cDNA. The amplified fragments were introduced into pHB-GFP. For the VIGS assay, a 300 bp fragment of *Nb14-3-3ω* was first amplified from cDNA of *N. benthamiana* and then cloned into the TRV2 vector. All DNA constructs inserted into plasmids were confirmed by sequencing.

2.3. Plant Materials, Growth Conditions, and Transient Gene Expression in Plants

N. benthamiana, and grapevine (*V. vinifera* cv. Thompson Seedless) were grown in a growth room at 22 °C under 14 h and 10 h light and dark conditions, respectively. For protein expression in plants, the *A. tumefaciens* strain GV3101, carrying the pHB-Vv14-3-3ω, was grown on Luria–Bertani (LB) medium containing appropriate antibiotics at 28 °C. Overnight cultures were resuspended in 10 mM $MgCl_2$ (OD_{600} to 0.6). Bacterial suspensions were then supplemented with 100 µM acetosyringone and incubated for 3 h at room temperature. Leaves from 3- to 4-week-old *N. benthamiana* plants were used for infiltration.

2.4. Quantitative Reverse Transcription PCR

To check the expression pattern of corresponding genes (*Vv14-3-3ω*, *NbFls2*, *NbBKI1*, *NbAcre31,* and *NbAcre132*, *Nb14-3-3ω*), total RNA was extracted from plant material using an RNA extraction kit (Omega Bio-Tek Inc., Norcross, GA, USA). After extraction, RNA samples were quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and reverse-transcribed into cDNA using a Hifair III 1st Strand cDNA synthesis superMix kit (YEASEN Laboratories, Shanghai, China) according to the manufacturer's instructions. Quantitative PCR was performed on a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA, USA) with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. ROS Production Assay

To check the effect of Vv14-3-3ω on Flg22-induced ROS production in *N. benthamiana*, a ROS assay was performed as described previously [29] with some modifications. *N. benthamiana* leaves expressing proteins of interest were collected 24 h post-infiltration. The leaf disks (diameter 4 mm) corresponding to the infiltrated leaf area were incubated in

200 µL of ddH_2O in a 96-well plate in the dark for 12 h. Leaf disks were then treated with 1 µM Flg22 or water in a ROS detection buffer (100 µM luminol and 10 µg/mL horseradish peroxidase) in a multiplate reader (BioTek, Winooski, VT, USA) for 59 min.

2.6. Pathogenicity Assay

P. capsici colonization assay was performed according to a previous study [30]. Briefly, *N. benthamiana* leaves were agroinfiltrated with Vv14-3-3ω. After 24 h, leaves were inoculated with *P. capsici*, and the lesion area corresponding to *P. capsici* colonization was photographed under UV light at 55 h post-inoculation (hpi).

P. viticola infection assay was performed as described previously [31]. Briefly, *V. vinifera* 'Thompson Seedless' leaves were agroinfiltrated with Vv14-3-3ω or GFP. After 3 days, 20 µL of *P. vitcola* from a freshly prepared suspension (10^5 spores/mL) was applied to the leaf disk prepared from the agroinfiltrated leaves. Leaf disks were then incubated at 25 °C, and spores were counted after 5 days of infection under a microscope using a haemocytometer. Spore counts were performed using a Leica DM2500 microscope (Leica Microsystems, Mannheim, Germany).

2.7. Western Blot Analysis

For the Western blot assay, *agrobacteria* carrying Vv14-3-3ω-GFP or GFP were infiltrated into *N. benthamiana* leaves. Total proteins were extracted from the infiltrated leaves after 48 h of infiltration using ice-cold protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton-X-100, 1% protease inhibitor cocktail; pH 7.5). The obtained supernatants were then suspended in 5× SDS sample buffer and incubated at 100 °C for 10 min. Immunocomplexes were then separated on SDS-PAGE, followed by Western blot using corresponding antibodies.

2.8. Subcellular Localization Assay

For the subcellular localization assay, pHB-Vv14-3-3ω-GFP was agroinfiltrated in *N. benthamiana* leaves. The infiltrated leaves were analyzed for fluorescent signals under a confocal microscope (Leica TCS SP5II, Wetzlar, Germany) 48 h after inoculation.

2.9. VIGS Assay in N. benthamiana

For VIGS assays, *agrobacteria* carrying pTRV1 empty vector and pTRV2-*Nb14-3-3ω* were infiltrated in leaves of four-leaf-stage *N. benthamiana* plants. The agroinfiltrated plants were then grown for 3–4 weeks, and the silencing level of *Nb14-3-3ω* was confirmed through real-time PCR [31]. Silenced leaves were then used for different assays.

3. Results

3.1. Phylogenetic Analysis of Vv14-3-3 Isoforms

To understand the diversity and evolutionary relationships of 14-3-3 proteins in *V. vinifera* across different species, we performed a phylogenetic analysis of *V. vinifera* 14-3-3 proteins in comparison with those of *A. thaliana*, *N. benthamiana*, *O. sativa*, and *G. max*. The sequences of 14-3-3 proteins in corresponding species were obtained from available resources (see Section 2) (Supplemental Table S1) and were used to construct a phylogenetic tree to assess their sequence conservation and divergence across Vv14-3-3 accessions. Among the 11 *V. vinifera* 14-3-3 proteins, 3 isoforms of *V. vinifera* were categorized in the epsilon group while 8 out of 11 were categorized in the non-epsilon group, owing to their high conservation among the selected species (Figure 1a). In Arabidopsis, At14-3-3ω has been shown to play a role in cold tolerance [32] while in *N. benthamiana*, a closely related homolog has been found to play a role in suppression of plant immunity [25]. Among the identified *V. vinifera* 14-3-3 isoforms,

one candidate Vv14-3-3 protein (Vitvi033133) was selected for further study as its closest homologs in *A. thaliana* (AT1G78300.1) and *N. benthamiana* (Nb06g00434) are functionally characterized with distinct biological roles [25,32]. We refer to this *V. vinifera* 14-3-3 protein as Vv14-3-3ω based on its high sequence similarity with the Arabidopsis 14-3-3ω isoform (Figure 1b). These findings provide valuable insights into the evolutionary conservation of the Vv14-3-3 proteins across different species and suggest that 14-3-3 proteins, like in other important plant species, may also play an important role in *V. vinifera.*

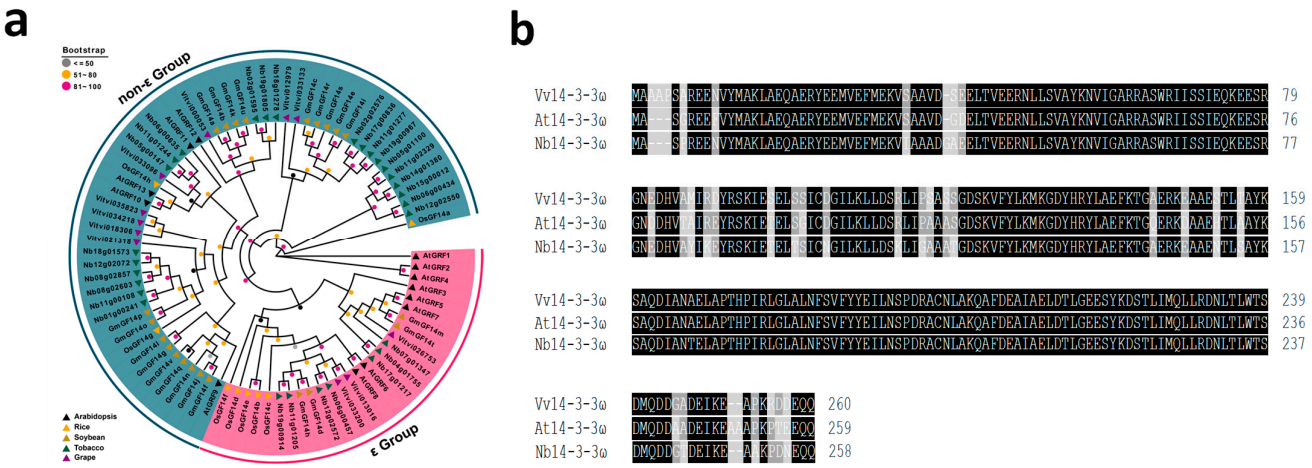


Figure 1. Evolutionary analysis of 14-3-3 proteins (**a**) Phylogenetic tree of 14-3-3 proteins from *V. vinifera*, *N. benthamiana*, *A. thaliana*, *O. sativa,* and *G. max*. Protein sequences were categorized into relatively conserved epsilon and diversified species-specific non-epsilon groups. A phylogenetic tree was constructed by the IQ-TREE 2 software using the maximum likelihood (ML, Q.plant + R4) option with 1000 bootstrap replicates. (**b**) Protein sequence alignment of Vv14-3-3ω, At14-3-3ω, and Nb14-3-3ω. Identical amino acids in these proteins are indicated by black color. Alignment was performed using the NCBI COBALT alignment tool.

3.2. Vv14-3-3ω Promotes P. viticola Sporulation on Grape Leaves

To investigate the expression pattern of *Vv14-3-3ω* in grapevines during *P. viticola* infection, susceptible *V. vinifera* 'Thompson Seedless' leaves were inoculated with *P. viticola*. The expression level of *Vv14-3-3ω* was analyzed at different time points post-inoculation. The transcript level of *Vv14-3-3ω* was low at early time points, strongly up-regulated at 24 hpi, and then decreased, indicating that *Vv14-3-3ω* is highly expressed during *P. viticola* infection (Figure 2a) and may play an important role in grapevine downy mildew interaction. We next transiently expressed GFP-tagged Vv14-3-3ω or GFP alone in leaves of 'Thompson Seedless' grapes to understand its role during *P. viticola* infection. Leaf disks prepared from the agroinfiltrated leaves were inoculated with *P. viticola* spores at 3 days post-agroinfiltration, and spores were counted after 5 days of infection. The results indicated that spores corresponding to *P. viticola* infection were significantly higher on leaves infiltrated with Vv14-3-3ω compared to control (GFP) (Figure 2b,c). These results indicated that Vv14-3-3ω negatively regulated grape immunity and promoted grapevine *P. viticola* infection.

3.3. Vv14-3-3ω Is Localized to the Plasma Membrane and Cytoplasm and Enhances P. capsici Pathogenicity in N. benthamiana

Since Vv14-3-3ω was found to enhance *P. viticola* sporulation, we next investigated its subcellular localization in *N. benthamiana* to better understand its site of action within plant cells. Vv14-3-3ω tagged with GFP was transiently expressed in *N. benthamiana* through agroinfiltration. The agroinfiltrated leaves were kept in the dark for 48 h and then checked under a fluorescence confocal microscope for the subcellular location of Vv14-3-3ω. The

GFP signals fused with Vv14-3-3ω were clearly visible outlining the cell periphery, consistent with plasma membrane and cytoplasmic association, indicating Vv14-3-3ω is localized to both the cytoplasm and the plasma membrane (Figure 3). By using the *N. benthamiana* and *P. capsici* system, Vv14-3-3ω could increase plant susceptibility to pathogen invasion. When Vv14-3-3ω was heterologously expressed in *N. benthamiana* leaves and inoculated with *P. capsici* at 24 h thereafter. A larger lesion area corresponding to *P. capsici* infection was observed in leaves expressing Vv14-3-3ω compared to the control (Figure 4a,b). Protein accumulation in the corresponding leaves was confirmed through Western blot (Figure 4c). These results suggest that Vv14-3-3ω may function at the membrane cytoplasm interface, potentially interacting with signaling proteins in multiple compartments, and may aid the pathogenicity of a pathogen by negatively regulating plant immunity.

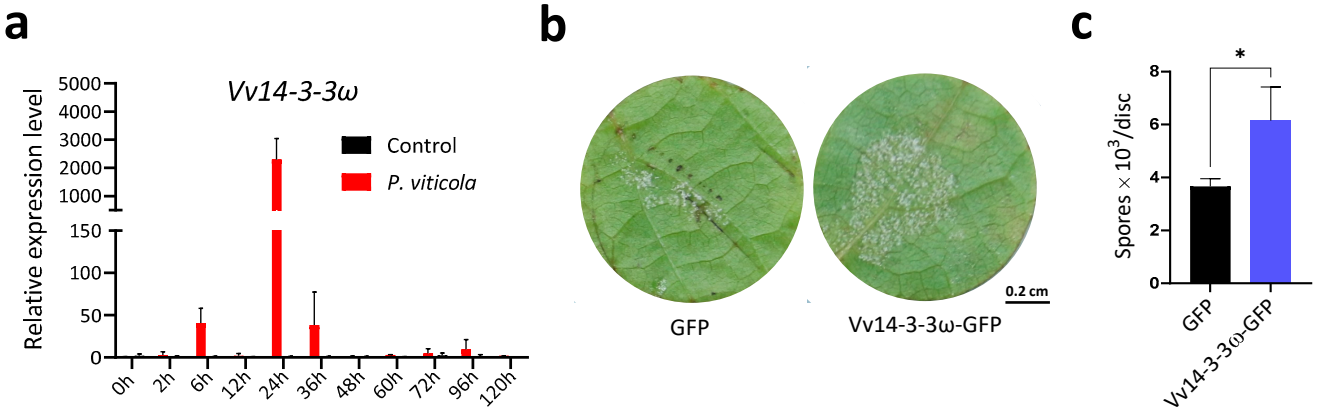


Figure 2. Vv14-3-3ω promotes grapevine downy mildew. (**a**) Expression pattern of *Vv14-3-3ω* during *P. viticola* infection in *V. vinifera*. Leaf disks of *V. vinifera* susceptible cultivar Thomson Seedless were inoculated with 20 µL of *P. vitcola* from a freshly prepared suspension (10^5 spores/mL) for RNA isolation, and the expression level of *Vv14-3-3ω* was measured by qRT-PCR. Water was used as a control. *VvActin* was used as an internal control. Error bar indicates standard error (SE). (**b**,**c**) *P. viticola* sporulation test. *Agrobacterium tumefaciens* cells carrying a construct encoding Vv14-3-3ω or GFP were infiltrated into *V. vinifera*, and *P. viticola*-infected water was applied on the same infiltrated leaves at 3 dpi. Spores were counted at 5 dpi of *P. viticola* infection using a hemocytometer. Data are the mean of three independent replicates. Asterisks represent the significantly different values at $p < 0.05$ (Student's *t*-test). Error bars represent SE.

3.4. Vv14-3-3ω Suppresses Flg22-Induced Immune Responses in N. benthamiana

Studies have indicated that many host proteins act as negative regulators of plant immunity and play an important role in virulence [7]. Moreover, our previous studies showed that conserved *V. vinifera* proteins are known to exhibit virulent behavior, primarily by suppressing plant innate immunity [30,31]. Thus, we investigated whether Vv14-3-3ω could suppress PTI responses. Flg22, a well-studied PAMP that has been reported to increase reactive oxygen species (ROS) production and induce the expression of PTI marker genes as an early PTI response, was used to check the role of Vv14-3-3ω on ROS production in *N. benthamiana*. pHB carrying either Vv14-3-3ω or GFP was agroinfiltrated in leaves of *N. benthamiana*, and the leaves were harvested 24 h post-infiltration. The infiltrated leaves were either treated with Flg22 or water (mock), and the ROS response was then estimated. ROS production in the leaves infiltrated with Vv14-3-3ω was suppressed compared to GFP control (Figure 4d). We also checked the expression pattern of Flg22-induced PTI marker genes upon agroinfiltration of Vv14-3-3ω-GFP in *N. benthamiana*. Transcript levels of *NbFLS2*, *NbBKI1*, *NbACRE31*, and *NbACRE132* were strongly reduced in leaves expressing Vv14-3-3ω-GFP compared to GFP control (Figure 4e). These results indicated that Vv14-3-3ω impairs Flg22-induced responses in *N. benthamiana*.

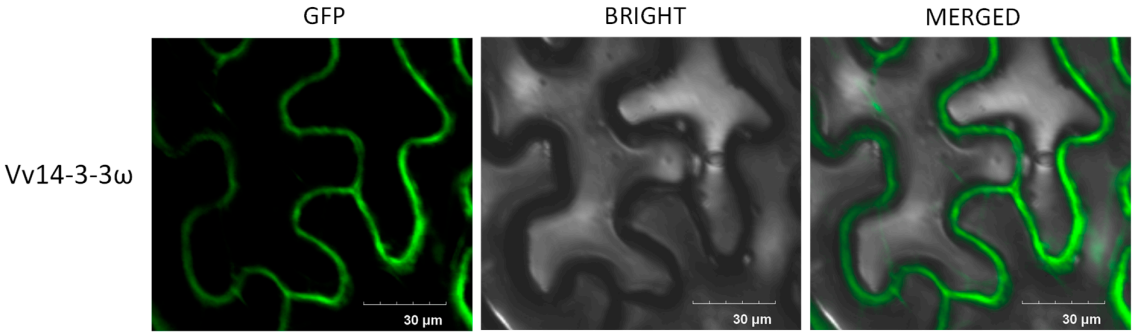


Figure 3. Sub-cellular localization of Vv14-3-3ω-GFP. Leaves of *N. benthamiana* were agroinfiltrated with Vv14-3-3ω-GFP, and their sub-cellular localization was observed after 48 h of infiltration under a confocal microscope. Scale bars, 30 µm.

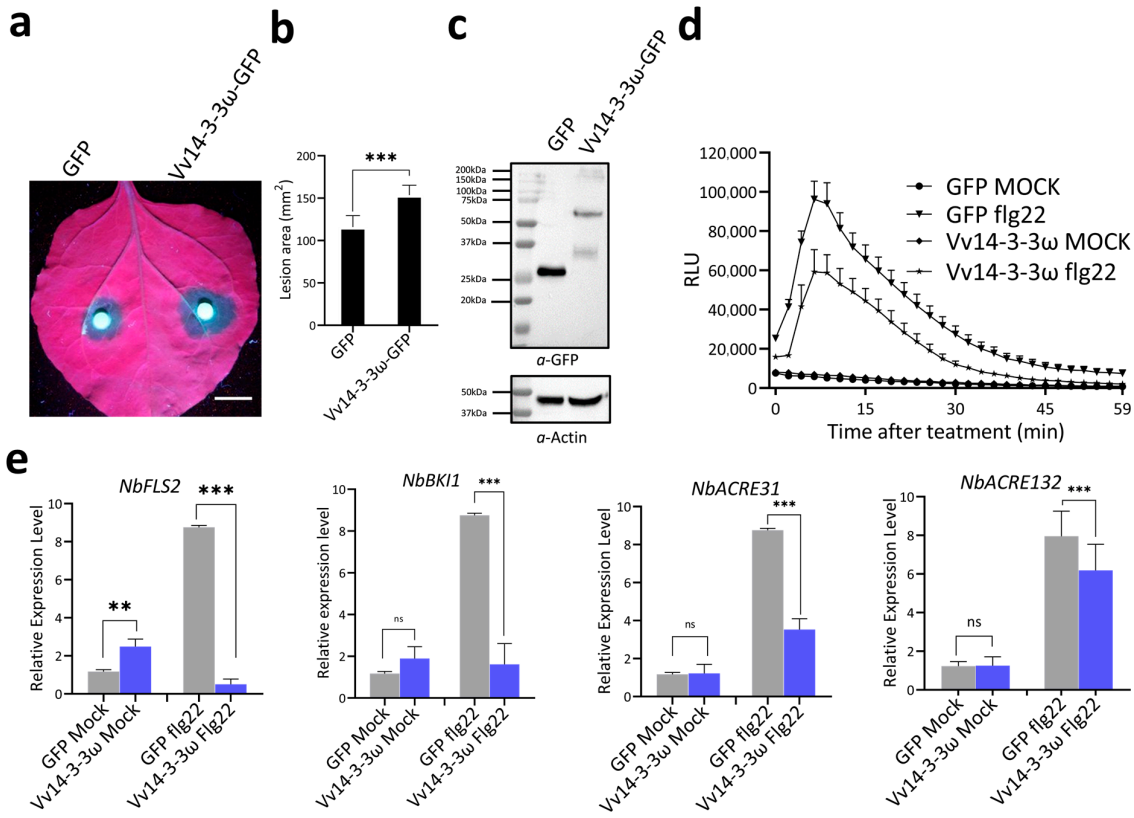


Figure 4. Vv14-3-3ω enhances *P. capsici* pathogenicity and impairs Flg22-induced PTI responses in *N. benthamiana*. (**a**,**b**) *P. capsici* colonization assay in *N. benthamiana* leaves transiently expressing Vv14-3-3ω-GFP or GFP. *P. capsici* was inoculated after 24 h of infiltration. Pictures were taken at 55 hpi under UV light, and, at the same time, the lesion area was measured. Data are the mean of six independent replicates. Asterisks show highly significantly different values at $p < 0.05$ (Student's *t*-test). Error bars indicate SE. Scale bar is 1 cm. (**c**) Western blot analysis from the *N. benthamiana* leaves transiently expressing with Vv14-3-3ω-GFP or GFP at 24 hpi. (**d**) ROS production induced by 1 µM Flg22 or water (mock) was determined in leaf disks of *N. benthamiana* transiently expressing either Vv14-3-3ω-GFP or GFP at 24 hpi. The results shown are representative of three independent experiments. Each data point consists of six replicates. Error bars indicate the standard error of the mean (SEM). (**e**) Vv-14-3-3ω suppresses the induction of PTI marker genes. The transcript accumulation of important PTI marker genes (*NbFls2*, *NbBKI1*, *NbAcre31*, and *NbAcre132*) in *N. benthamiana* leaves transiently expressing Vv14-3-3ω-GFP or GFP was assayed by qRT-PCR at 1 h after treatment with 1 µM Flg22 or water. *NbActin* was set as an internal reference. Mean and standard errors were calculated from three biological replicates (Student's *t*-test, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.5. Overexpression of *Vv14-3-3ω* in *Nb14-3-3ω* Silenced Leaves Suppress Immunity

High sequence similarity between *Vv14-3-3ω* and *Nb14-3-3ω* genes suggests that these genes are highly conserved and may perform similar functions (Supplementary Figure S1). Therefore, to explore whether Vv14-3-3ω can functionally replace Nb14-3-3ω, we transiently silenced *Nb-14-3-3ω* in *N. benthamiana* through tobacco rattle virus (TRV) based virus-induced gene silencing (VIGS) approach. Real-time PCR indicated that the relative mRNA expression level of *Nb14-3-3ω* in silenced leaves was significantly reduced (70%) compared to non-silenced leaves, confirming the successful silencing of *Nb14-3-3ω* (Figure 5a). GFP-tagged Vv14-3-3ω or GFP was agroinfiltrated in Nb14-3-3ω-silenced plants, and the leaves were assayed for flg22-induced ROS and *P. capsici* pathogen responses. Overexpression of Vv14-3-3ω reduced Flg22-triggered ROS production in Nb14-3-3ω-silenced leaves, indicating interference with pattern-triggered immunity (Figure 5b). Furthermore, transient expression of Vv14-3-3ω in Nb14-3-3ω-silenced leaves exhibited a noticeably larger lesion area following *P. capsici* infection, consistent with increased susceptibility (Figure 5c,d). Collectively, these observations support the role of Vv14-3-3ω as a negative regulator of plant immunity, and the resemblance of phenotype associated with Nb14-3-3ω suggests functional conservation between the grapevine and *N. benthamiana* 14-3-3 isoforms.

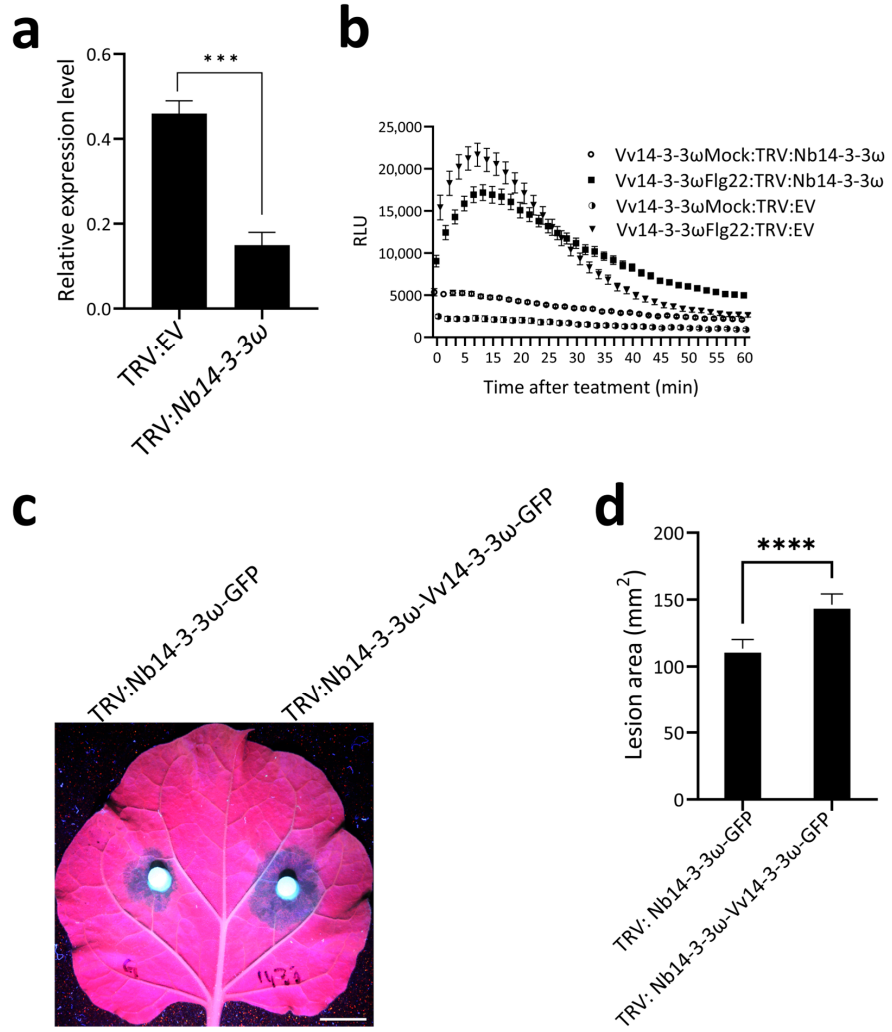


Figure 5. Overexpression of Vv14-3-3ω in *Nb14-3-3ω*-silenced leaves. (**a**) Analysis of the transcript level of *Nb-14-3-3ω* in plants undergoing VIGS. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium* culture carrying TRV: Nb14-3-3 or TRV: EV, and the relative mRNA expression level of *Nb14-3-3ω* was

analyzed in both silenced and non-silenced leaves on 9 dpi by qRT-PCR. *NbActin* was used as an internal reference. Data are the mean of three independent biological replicates. Error bars represent SE. (Student's *t*-test, *** $p < 0.001$, **** $p < 0.0001$). (**b**) Flg22-induced ROS burst by Vv14-3-3ω in *Nb14-3-3ω*-silenced leaves. Vv14-3-3ω-GFP or GFP was transiently expressed in *Nb14-3-3ω*-silenced *N. benthamiana* leaves, and ROS production induced by 1 μM Flg22 in the injected region was measured after 24 h. The results shown are representative of three independent experiments. Each data point consists of six replicates. The error bars indicate SEM. (**c**,**d**) *P. capsici* colonization assay in *Nb14-3-3* silenced *N. benthamiana* leaves transiently expressing Vv14-3-3ω-GFP or GFP. *P. capsici* was inoculated on corresponding leaves after 24 h of Vv14-3-3ω-GFP or GFP infiltration. Pictures were taken at 55 hpi under UV light, and, at the same time, the lesion area was measured. Data are the mean of six independent replicates. Error bars represent SE. Scale bar is 1 cm.

4. Discussion

To sense and respond to complex environmental changes, plants rely on a range of regulatory factors, among which the abundant 14-3-3 protein family plays a crucial role. The presence of multiple 14-3-3 isoforms across nearly all eukaryotes adds to the functional diversity and complexity of this regulatory protein family. In Arabidopsis, soybean, tomato, and rice, the number of 14-3-3 genes have been reported [33–37]. Studies have revealed that 14-3-3 proteins play an important role during the development of multiple organs in a number of plant species, including Arabidopsis, rice, soybean, rapeseed, and castor [38], but the role of 14-3-3, especially in grapevine downy mildew, is not well documented. In this study, our phylogenetic analysis revealed the presence of 11 isoforms of *V. vinifera* 14-3-3 proteins. We chose Vv14-3-3ω for further study based on its sequence similarity with already functionally characterized homologs in *A. thaliana* and *N. benthamiana*.

We found that *Vv14-3-3ω* is highly expressed at early time points when grapevines are infected with *P. viticola*. This suggests that Vv14-3-3 plays a role in the interaction between grapevine and downy mildew. Studies have shown that pathogens recruit 14-3-3 proteins to evade immune responses and promote virulence [39,40]. We also observed a similar role of Vv14-3-3*ω* during grapevine downy mildew interaction, as indicated by a significant increase in spore formation when grapevine leaves transiently expressing Vv14-3-3*ω* were infected with *P. viticola*. These observations were further supported by *P. capsici* infection assays performed on *N. benthamiana* leaves transiently expressing Vv14-3-3*ω*, as the lesion area corresponding to *P. capsici* infection was significantly larger than that of the GFP control. Enhanced pathogen virulence suggests that the immune system of the plant has been somehow suppressed, which can be witnessed through analyzing plant basal defense responses, such as Flg22-induced PTI responses like ROS burst and induction of PTI marker genes. To know if enhanced pathogen virulence correlated with Vv14-3-3 is the result of plant immune suppression, we checked Flg22-induced PTI responses in *N. benthamiana* leaves transiently expressing Vv14-3-3*ω* and found that Vv14-3-3*ω* can effectively suppress Flg22-induced ROS and the expression of PTI marker genes like *NbFLS2*, *NbBKI1*, *NbACRE131,* and *NbACRE132*. These observations are reminiscent of those observed for VvWRKY40 and AtERF019, in which pathogenesis was correlated with the suppression of such PTI responses [30,41].

Pathogens secrete effectors to suppress PTI and colonize their hosts [42,43]. Likewise, oomycete pathogens also secrete an arsenal of effectors at all stages of infection [44]. Some of these effectors are either secreted directly into the extracellular space or are translocated into plant cells to target various subcellular compartments where they target important host genes that can result in plant susceptibility to pathogens [45]. During pathogen attack, some host genes are highly upregulated, suggesting their important role in promoting pathogen infection. Some of these genes are preferentially negative regulators of plant immunity [9]. This could be that the induction of a negative regulator could interfere with

the proper functioning of important host genes that are important for defense activation. For example, EIJ1 negatively regulates plant immunity by binding to EDS1 in the cytoplasm, preventing its nuclear translocation and delaying defense activation. Upon infection, EIJ1 is degraded, allowing EDS1 to enter the nucleus and trigger immune responses [46]. The FITNESS protein negatively regulates plant immunity by modulating the jasmonic acid (JA) pathway [47]. Furthermore, VvWRKY40 was also found to negatively regulate plant immunity and promote infection in *N. benthamaiana* [30], and 14-3-3 was recently found to promote plant susceptibility to *Pythopthora palmivora*, suggesting it negatively regulates plant immunity [25]. We show that Vv14-3-3ω is expressed at early stages of infection and promotes plant susceptibility to the pathogen *P. viticola*. These findings are reminiscent of a previous study on 14-3-3 [25]. Pathogens exploit 14-3-3 proteins to suppress host defenses and enhance infection. For instance, the *Pseudomonas aeruginosa* type III effector ExoS requires interaction with host 14-3-3 proteins to boost its ADP-ribosyltransferase activity on small GTPases, thereby disrupting cytoskeletal dynamics during invasion [39]. Similarly, the fungal toxin fusicoccin strengthens the association between 14-3-3 proteins and the plant plasma membrane H^+-ATPase, resulting in increased proton pump activity [40]. Future work is required to identify the underlying mechanism by which Vv14-3-3ω promotes grapevine downy mildew infection, including the identification of potential interacting partners both within the plant and those delivered by *P. viticola*.

5. Conclusions

As a globally important fruit crop, the grapevine *V. vinifera* cv. Thompson Seedless is highly susceptible to downy mildew, underscoring the need to identify its key host factors involved in disease progression. Conserved host proteins play essential roles in shaping plant responses to pathogen invasion, making them valuable targets for functional investigation. In this study, we found that Vv14-3-3ω is a conserved host protein of *V. vinifera.* We choose Vv14-3-3ω from *V. vinifera* cv. Thompson Seedless for further study. Vv14-3-3ω is highly expressed at early time points during *P. viticola* infection and can increase sporulation on grapevines. Transient expression assays revealed that Vv14-3-3ω suppresses Flg22-induced plant immune responses and can promote *P. capsici* infection of *N. benthamiana* leaves. Further tests revealed that the resemblance of phenotype associated with Nb14-3-3ω suggests functional conservation of this isoform. The present study contributes to our understanding that Vv14-3-3ω negatively regulates plant immunity and promotes *P. viticola* infection in grapevines. Future work is required to unravel the downstream mechanism of Vv14-3-3ω-dependent pathogenicity during grapevine downy mildew interaction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11101199/s1, Text S1: Supplemental Materials and Methods. Figure S1: DNA sequence alignment of Vv14-3-3ω and Nb14-3-3ω. DNA sequence (coding region) of Vv13-3-3ω and Nb14-3-3ω were aligned using Megalign tool of DNASTAR Lasergene. Similar nucleotides are indicated by red color.; Table S1: 14-3-3 isoforms in *Vitis vinifera*, *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Oryza sativa* and *Glycine max*. Table S2: Strains and plasmids used in this study. Table S3: Primers used in this study.

Author Contributions: Z.B., A.K., and J.L. (Jiang Lu) designed the experiments, analyzed the data, and wrote the manuscript. Z.B. performed experiments. A.K. and J.L. (Jiang Lu). supervised the project. J.L. (Jiaqi Liu) and P.F. helped in proofreading and editing. All authors have read and agreed to the published version of the manuscript.

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