Transient Silence of VvCSN5 Enhances Powdery Mildew Resistance in Grapevine (*Vitis Vinifera*)

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Research Article

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Abstract

As one of the most economically important fruit crops in the world, grapevine (*Vitis vinifera*) suffers significant yield losses from many pathogens including powdery mildew caused by *Erysiphe necator*. By contrast, several wild Chinese grapevines including *Vitis pseudoreticulata* accession 'Baihe-35-1' exhibit a high resistance to powdery mildew pathogen. Here, we identified a grapevine gene *CSN5* (*COP9 signalosome complex subunit 5*), designated *VvCSN5*, which showed different expression patterns in 'Baihe-35-1' and in susceptible cultivar *V. vinifera* 'Thompson Seedless' during powdery mildew isolate *En* NAFU1 infection. Moreover, transient silence of *VvCSN5* in 'Thompson Seedless' leaves enhanced resistance to *En* NAFU1, which is accompanied by cell wall deposition at the attempt sites, and hypersensitive response-like cell death of penetrated epidermal cells. Several defense-related marker genes (*VvPR1*, *VvPR3*, *VvPAD4*, and *VvRBOHD*) had higher basal expression levels in *VvCSN5*-silenced leaves. In addition, we found the structure and activity of *CSN5* promoters in 'Thompson Seedless' and 'Baihe-35-1' were discrepant, which may be one of the reasons for their different resistance to powdery mildew infection. Taken together, these results imply that grapevine CSN5 plays an important role in the responses to powdery mildew.

Introduction

Grapevine is one of the most important fruit crops in the world, with over 7000 years cultivation history (Jaillon et al. 2007). Among all grapevine species, Eurasian grapevine species *Vitis vinifera* is the main cultivar for viticulture and wine production on account of its superior aroma and flavor characteristics. However, *V. vinifera* is vulnerable to various diseases, including powdery mildew caused by an obligate biotrophic fungus *Erysiphe necator*, which infects all green tissues of grapevine, causes a widespread and devastating disease (Gadoury et al. 2012; Qiu et al. 2015). In contrast, several wild Chinese grapevines species have a strong powdery mildew resistance, such as *Vitis pseudoreticulata* accession Baihe-35-1 (Gao et al. 2016; Hu et al. 2019), which is therefore an important germplasm for enhancing powdery mildew resistance of *V. vinifera* through genetic improvement.

To date, several powdery mildew resistance gene loci, including *RUN1*, *RUN2*, *REN5* in *Muscadinia rotundifolia* (Barker et al. 2005; Riaz et al. 2011; Blanc et al. 2012), and *REN4*, *REN6*, *REN7* in *V. romanetii* and *V. piasezkii* (Ramming et al. 2011; Pap et al. 2016; Mahanil et al. 2012) had been identified through forward genetic approaches. However, the molecular mechanisms research of grapevine resistance (R) genes is difficult because of its characteristic of perennial woody plants with a heterozygous genome (Feechan et al. 2013).

Plants have an innate immune system to avoid pathogen infection, and the two major branches of which are PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). The first branch of PTI uses transmembrane pattern recognition receptors (PRRs) to recognize multiple conserved microbial- or pathogen-associated molecular patterns (MAMPs and PAMPs) and cause penetration resistance eventually (Zipfel and Felix 2005). The second branch of ETI relies on a large

amount of nucleotide-binding leucine-rich-repeat (NB-LRR) proteins encoded by R-genes, which can identify the effectors released from a pathogen and then lead to hypersensitive response (HR) (Jones and Dangl 2006; Dangl and Jones 2001). As described above, the powdery mildew resistance in grapevine is also composed of penetration resistance and hypersensitive response (Douchkov et al. 2016; Wang et al. 2016). These two processes are interacted and impede the growth of powdery mildew by cell wall deposition and programmed cell death (Jones and Dangl 2006; Boutrot and Zipfel 2017; Hu et al. 2019).

COP9 signalosome complex subunit 5 (CSN5) is one of the eight subunits of COP9 (constitutive photomorphogenesis 9) signalosome (CSN) in *Arabidopsis*. It participated in regulating CULLIN-RING E3 ubiquitin ligases (CRLs) activity as the catalytic center of the complex (Wei and Deng 2003; Cope et al. 2002; Gusmaroli et al. 2007). As the most conservative subunit in CSN, CSN5 has become a common target of pathogenic effectors (Echalier et al. 2013; Jin et al. 2014). In *Arabidopsis*, AtCSN5a interacts with 29 distinct effectors from *Hyaloperonospora arabidopsidis* (*Hpa*) and *Pseudomonas syringae* (*Psy*), and AtCSN5a mutations increase resistance to both *Hpa* and *Psy* (Mukhtar et al. 2011). The role of CSN5 in biotic stress responses has also been reported in tobacco (Liu et al. 2002), tomato (Shang et al. 2019), wheat (Zhang et al. 2017), and rice (He et al. 2020). However, there is no report focus on CSN5 regulating defense response in grapevine.

In the present study, we identified a grapevine gene *CSN5* which showed different expression patterns in susceptible and resistant grapevine during powdery mildew infection. Through transient transformation, we discovered that silencing *VvCSN5* enhanced powdery mildew resistance in susceptible cultivar *V. vinifera* 'Thomspon Seedless' leaves. Further analysis of the promoter structure and function indicated that the differences in *CSN5* promoter between 'Thomspon Seedless' and 'Baihe-35-1' may be one of the reasons for their different resistance to powdery mildew. Our findings suggested that grapevine CSN5 negatively regulated powdery mildew resistance.

Materials And Methods

Plant materials and growth conditions

The tissue culture seedlings of *V. vinifera* cv. Thompson Seedless and *V. pseudoreticulata* accession Baihe-35-1 were transplanted in individual pots containing soil mix (peat: perlite: vermiculite, 4:1:1, v:v:v), and grown in a illumination incubator for two weeks with temperatures ranging from 22°C to 26°C, under a 14 h/10 h (light/dark) light cycle.

Cloning of grapevine CSN5 genes

The grapevine *CSN5* genes from 'Thompson Seedless' (*VvCSN5*) and 'Baihe-35-1' (*VpCSN5*) were amplified from corresponding leaves cDNA by Planta Max Super-Fidelity DNA Polymerase (Vazyme Bio Co., Nanjing, China), respectively. The amplified primers were derived from the *CSN5* gene sequence we uploaded to the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR products were cloned into the pMD19-T vector (Takara

Bio Inc., Dalian, China) and sequenced at the Beijing AuGCT Biotech, Yangling, Sequencing Department, then aligned with the genome of *V. vinifera* PN40024 (https://www.ncbi.nlm.nih.gov/genome). The sequence of amplified primers and related GeneBank accession number were shown in Table S1 and Table S2, respectively.

Plasmid construction

To obtain over-expression recombinant vector, full length *VpCSN5* gene was amplified from the pMD19-T-VpCSN5 fusion plasmid, and inserted into *Bam*HI and *Kpn*I sites in the pCAMBIA2300 vector containing the CaMV 35S promoter and green fluorescent protein (GFP) coding sequence by homologous recombination to generate 35S::VpCSN5-GFP.

To silence *VvCSN5* in 'Thompson Seedless', the vector pK7WIWG2D was used as the RNA interference vector. A 358 bp length fragment from pMD19-T-VvCSN5 was selected and amplified for constructing pK7WIWG2D-*VvCSN5* vector through Invitrogen Gateway recombination cloning technology (Thermo Fisher Inc., Carlsbad, USA).

To analyze the promoters of *VvCSN5* and *VpCSN5*, we designed the primers according to the genome of *V. vinifera* PN40024, and amplified about 1500 bp promoter sequences upstream the initiation codon of *CSN5* gene from 'Thompson Seedless' and 'Baihe-35-1', respectively. The PCR products were then cloned into the pMD19-T vector and sequenced. To construct the reporter plasmids for GUS activity analysis, we cloned the *VvCSN5* promoter (ProTS) and *VpCSN5* promoter (ProBH) sequences into pBI121 vector to drive encoding β-glucuronidase (GUS) gene, generated construct *ProTS:GUS* and *ProBH:GUS*. The pBI121 vector was pre-digested by *Hind*III and *Bam*HI enzyme. The sequence of related primers was shown in Table S1.

Subcellular location assay

To explore the subcellular localization of grapevine CSN5, we used the 35S::VpCSN5-GFP plasmid that mentioned above, and transiently expressed in grapevine mesophyll protoplasts was performed as previous study (Zhao et al. 2016; Liu et al. 2019), the *35S:GFP* as a control. The transformed protoplasts were incubated for 20–25 h at room temperature in the dark, then observed by Olympus BX51 fluorescence microscopy (Japan).

Agrobacterium **-mediated transient transformation and inoculation**

Agrobacterium tumefaciens strain GV3101 was used for the transient transformation of related plasmids. Firstly, centrifuged the shaken bacterial solution (OD600, 0.5–0.6) and resuspensed it with an equal volume of induction buffer (3mM Na_2HPO_4, 50mM MES (pH 5.6), 0.5% (w/v) Glucose, 100μM Acetosyringone), 28°C, 180 rpm shaking for one hour. Then, chose the third or fourth fully expanded leaves, carefully injected the suspending liquid into back of leaves with a disposable sterile syringe. The method of transient transformation referred to previous studies (Kurth et al. 2012; Krenek et al. 2015; Bertazzon et al. 2012; Urso et al. 2013; Xie et al. 2020). Three days later, transformed leaves were

inoculated with fresh conidia of powdery mildew isolate *En* NAFU1, which through touching the epidermis of the transformed leaves using heavily infected 'Thompson Seedless' leaves (Gao et al. 2016).

Evaluation of resistance to powdery mildew

Three groups of transformed leaves, *pK7WIWG2D-VvCSN5* (RNAi), *35S::VpCSN5-GFP* (OE) and *35S::GFP* (as a control, EV) were assessed for resistance to *En* NAFU1 (Gao et al. 2016). 3,3'-diaminobenzidine (DAB) and trypan blue (TB) stained the epidermal cells of transformed leaves were observed at 2 and 5 days post inoculation (dpi) to identify the accumulation of H_2O_2, fungal structures and dead host cells. Aniline blue-staining was used to visualize the accumulation of callose at 5 dpi (Gao et al. 2016; Hu et al. 2018; Wan et al. 2020). All the samples were then examined using an Olympus BX51 microscope (Japan). Hyphal length of *En* NAFU1 and frequencies of the invasion induced hypersensitive cell death (full of H_2O_2) were directly measured under Olympus BX51 using the CellSens operation software (Hu et al. 2018).

Quantitative real-time PCR (RT-qPCR) analysis

To explore the expression patterns of *VvCSN5* and *VpCSN5* during the powdery mildew infection, we selected disease-free leaves from 'Thompson Seedless' and 'Baihe-35-1' for powdery mildew isolate *En* NAFU1 inoculation. Then collected leaves with 0, 12, 24, 48, 72, 96, 120 hours post inoculation (hpi) in turn, samples were promptly stored in -80℃ refrigerator after quick freezing by liquid nitrogen. The sampling method of RT-qPCR for defense-related genes was the same as above.

Total RNA was extracted from collected leaves using E.Z.N.A. Plant RNA Kit (Omega, Guangzhou, China). The cDNA was obtained by reverse transcription using HiScript Q Select RT SuperMix (Vazyme, Nanjing, China). RT-qRCR assays were refer to previous study (Zhang et al. 2015). Grapevine *ACTIN7* (GenBank accession number is XM_002282480.4) was used as an internal control (Wang et al. 2017; Gutha et al. 2010; Reid et al. 2006). The relative transcript levels of the genes were calculated using the $2^{-\triangle\triangle Ct}$ and Normalized Expression method. Each sample was analyzed in three biological replications. The statistical significance was evaluated by Student's *t*-test. The sequence of RT-qPCR primers and gene accession number were shown in Table S1 and S2, respectively.

Histochemical and fluorometric assays for GUS activity

The histochemical GUS assay of leaves was carried out as previously described (Jefferson 1987). The *VvCSN5* promoter:*GUS* (*ProTS:GUS*) and *VpCSN5* promoter:*GUS* (*ProBH:GUS*) vector were transformed into fully unfolded healthy 'Thomspon Seedless' or tobacco leaves by vacuumizing and *35S:GUS* was used as a control. Three days after transformation, the transformed leaves were inoculated s by spraying the *En* NAFU1 spore suspension with a concentration of 5×10^5 sporangia ml^{-1}. Mock-inoculated leaves were sprayed with sterile water only. Collected 2 dpi leaves, placed in GUS dye and incubated at 37℃ for 24 hours. Then, 70% and 100% ethanol were successively used for decoloring at 37℃ for 10 hours. The method of GUS quantitative analysis was referred to previous study (Yu et al. 2013).

Statistical analysis

The relevant data were analyzed through Student's *t*-test or Tukey's HSD test using the statistics software tool IBM SPSS v26.0.0. The mean values ± standard deviation of the mean (SD) was calculated based on the results of at least three independent experiments, and significant differences compared with controls are represented by $p < 0.05$ and $p < 0.01$.

Results

Bioinformatics analysis and subcellular localization of grapevine CSN5

Although there are two *CSN5* homologous genes in *Arabidopsis thaliana* (*AtCSN5a* and *AtCSN5b*), only one *CSN5* gene in *V. vinifera* genome (Jin et al. 2014), we named *VvCSN5*. It's located on chromosome 3, the full length of genomic DNA is 8398 bp with six exons and five introns (Fig. 1a).

To understand the association of CSN5 in various plants, we firstly chosen nine homologous CSN5 proteins from seven species which functions had been well studied, and used them to build a phylogenetic tree with VvCSN5 from 'Thompson Seedless' and VpCSN5 from 'Baihe-35-1'. We found VvCSN5 and VpCSN5 were highly homologous with their homologous proteins (Fig. 1b). VvCSN5, VpCSN5 and other CSN5 proteins were also highly conserved at their functional region (MPN domain) (Fig. 1c). These results indicated that grapevine *CSN5* may play similar roles to those homologous genes.

It was reported that CSN5 is localized in cytoplasm and nucleus in most plants (Li et al. 2018; Wang et al. 2013). In order to explore the intracellular localization of grapevine CSN5, we transiently expressed VpCSN5-GFP fusion protein in 'Baihe-35-1' grapevine mesophyll protoplasts. As shown in Fig. 1d, the GFP expressed from the control construct was dispersed throughout the whole cell, while VpCSN5-GFP fusion protein localized to both cytoplasm and nucleus.

The expression patterns of VpCSN5 and VvCSN5 are different during powdery mildew infection

To determine whether there is any difference in expression patterns of *VvCSN5* and *VpCSN5* after powdery mildew infection, we inoculated the susceptible grapevine 'Thompson Seedless' and resistant grapevine 'Baihe-35-1' using isolate *En* NAFU1, respectively. RT-qPCR results showed that the *VvCSN5* transcript lightly increased in abundance and reached a peak of ~ 1.4-fold at 24 hpi, while *VpCSN5* transcript decreased rapidly at 24 hpi (0.4-fold) and then returned to a normal level at 48 hpi (Fig. 2). This suggested that grapevine CSN5 may be involved in the defense responses to powdery mildew.

Transient silence of VvCSN5 **enhances resistance to powdery mildew isolate** En **NAFU1 in grapevine**

As above mentioned, we have known that the expression patterns of CSN5 in susceptible and resistant grapevines were discrepant (Fig. 2). Whereupon, we wondered whether altering the expression levels of *CSN5* in 'Thompson Seedless' leaves could affect their resistance to powdery mildew. We thus obtained

VvCSN5-RNAi (RNAi) and 35S::VpCSN5-GFP (OE) transient transformed leaves through injection, construct *35S::GFP* (EV) was injected as control (Fig. 3a). As shown in Fig. 3b, most epidermal cells of injection range of 'Thompson Seedless' leaf for three constructs injected showed clear GFP signals. Similarly, the western blot analysis of GFP (~29.9 kDa) or VpCSN5-GFP fusion protein (~70.2 kDa) also confirmed the normal expression of related GFP protein (Fig. 3c), which means that the transient transformation was successful. Subsequently, we detected the transcript levels of *CSN5* in each transformed leaf or uninjected leaf (as control) by RT-qPCR. The results showed that *CSN5* transcript levels were up-regulated over 20-fold in OE leaves and down-regulated to 0.5-fold in RNAi leaves compared with control (Fig. 3d).

To assess the resistance to powdery mildew in leaves with different CSN5 levels,we collected transformed 'Thompson Seedless' leaves stained with trypan blue (TB) and 3,3'-diaminobenzidine (DAB), and measured the total hyphal length from isolated fungal colonies. As shown in Fig. 4a and b, the total hyphal length per colony in the leaves of RNAi was ~600 μm at 2 dpi, while the length in the leaves of OE and EV were ~950 and 840 μm, respectively. Moreover, at 5 dpi, nearly 20% of invaded cells showed whole-cell H_2O_2-staining in RNAi leaves, while both the leaves of EV and OE were less than 5% (Fig. 4a and c). In addition, cell wall deposition by callose accumulation is also an important indicator of *Vitis* resistance to powdery mildew (Consonni et al. 2006). The aniline blue-staining results showed that around the necrotic epidermal cells, major callose accumulated in RNAi leaves at 5 dpi. In contrast, there was no obviously callose accumulated in the leaves of EV and OE (Fig. 4d). These results suggested that silencing *VvCSN5* could enhance the resistance to powdery mildew in grapevine.

Grapevine CSN5 involve in multiple defenses signaling pathways

To investigate which defense-related genes were affected by CSN5, we selected several typical defense-related genes to analyze their expression patterns in transformed leaves of 'Thompson Seedless'. As shown in Fig. 5, for SA-associated genes, *VvPR1* (pathogenesis-related gene 1) showed a higher expression levels (over 2-fold) in RNAi leaves than that in EV at 0 dpi and remained high levels at 2 and 5 dpi, while the *VvPR1* level in OE was lower (~ 0.5-fold) than EV in each time point. *VvPR3* (pathogenesis-related gene 3) and *VvPAD4* (phytoalexin deficient 4) transcript levels in RNAi leaves was both slightly higher (~ 1.5-fold) than that in EV at 0 dpi, except that *VvPR3* levels at 5 dpi was not obviously differ from EV. However, the JA-associated gene *VvJAR1* (jasmonate resistant 1) showed a stronger expression (1.3 ~ 2.5-fold) in OE leaves than that in EV and RNAi. For hydrogen peroxide-associated gene *VvRBOHD* (respiratory burst oxidase homologue D), the increasing expression trend between RNAi and EV was similar, but the transcript levels at 0, 2, 5 dpi in RNAi was all significantly higher (1.5 ~ 2-fold) than that in EV, which implied hydrogen peroxide-pathway had a stronger activation in RNAi leaves. In addition, the ethylene-associated gene *VvACS2* (1-amino-cyclopropane-1-carboxylate synthase 2) transcript level in RNAi was lightly higher than EV at 2 dpi. These results indicated that grapevine CSN5 was associated with multiple defense-related genes during powdery mildew infection.

The promoters of VvCSN5 **and** VpCSN5 **are different in structure and activity**

According to the sequence alignment, we found there are only two different amino acid residues between VvCSN5 and VpCSN5 (Fig. S1). Thus, we wonder whether the different expression patterns between VvCSN5 and VpCSN5 due to the differences of their promoters. We firstly compared the promoters of *VvCSN5* (ProTS) and *VpCSN5* (ProBH) by sequence alignment. As shown in Fig. 6a, there were masses of differences between these two promoter sequences, including a number of base pair substitutions and a 132 bp fragment deletion in the *VpCSN5* promoter. To further analyze these two sequences, we forecasted the cis-regulatory elements (CREs) of the two promoters by PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and markered out the discrepant elements (Fig. 6a). Some of them, such as MYB-, MYC- and ABI3-elements, had been reported to be responsive to various stresses (Dubos et al. 2010; Abe et al. 2003; Tamminen et al. 2001)..

To verified the function of promoters, we used histochemical staining and fluorometric assays to examine expression of the GUS genes which respectively driven by ProTS or ProBH after inoculation with powdery mildew isolate *En* NAFU1. As shown in Fig. 6b and c, the *35S:GUS* control leaves were strongly stained by GUS dye, and the quantitative GUS assay showed similar activities in *En* NAFU1-inoculated and mock conditions. Furthermore, the *ProTS:GUS* in mock condition showed a very weak expression, while in *En* NAFU1-inoculated leaves showed a much strong activity which was 3.5-fold higher than in mock (Fig. 6b and c). However, there was no significant difference in *ProBH:GUS* activity between mock and *En* NAFU1-inoculated leaves (Fig. 6b and c). We also expressed *ProTS:GUS* and *ProBH:GUS* in tobacco leaves (for a higher transformation efficiency) with mock condition to confirm the reliability of GUS staining results of grapevine leaves. We discovered stronger staining in tobacco leaves than that in corresponding grapevine leaves (Fig. 6b), and similar to mock in grapevine leaves, the *ProBH:GUS* activity in tobacco was also higher than *ProTS:GUS* (Fig. 6c). These results implied that the differences in expression patterns of CSN5 between susceptible and resistant grapevine may cause by different promoter activation.

Discussion

As the catalytic center of COP9 signalosome (CSN), CSN5 plays an essential role for normal working of complex (Stratmann and Gusmaroli 2012; Jin et al. 2014). Thus, CSN5 is quite conserved in eukaryotes (Cope et al. 2002). We already know that CSN5 is involved in the process of pathogen defense in many plants, including *Arabidopsis* (Mukhtar et al. 2011), tobacco (Liu et al. 2002), wheat (Zhang et al. 2017), rice (He et al. 2020), etc. However, there was no related research on grapevine CSN5. In this study, we demonstrated the conservation of CSN5, and further discovered that it was associated with grapevine resistance to powdery mildew.

According to the expression analysis of grapevine CSN5 during powdery mildew infection, we found that the *VpCSN5* expression reached a minimum at 24 hpi, whereas *VvCSN5* reached a maximum at 24 hpi (Fig. 2), which was consistent with the timing of haustorial formation (Hu et al. 2019). This result implies that the changes of CSN5 expression are pathogen induced. Further combining with the results of

stronger powdery mildew resistance of CSN5-silenced leaves (Fig. 3), which both supporting a negative role of CSN5 on powdery mildew infection.

Previous studies showed CSN5 was mainly involved in plant pathogen-defense through JA- or SA-signaling pathways (Feng et al. 2003; Hind et al. 2011; Spoel et al. 2009). Interestingly, the effects of CSN5 to the two pathways are opposite. For example, the over-expression of *OsCSN5a* inhibits rice black-streaked dwarf virus (RBSDV) infection through activating JA-signaling pathway in rice (He et al. 2020). Whereas silencing *TaCSN5* in wheat improves wheat leaf rust resistance by activation of SA-signaling pathway (Zhang et al. 2017). In our study, a stronger powdery mildew resistance showed in CSN5-RNAi leaves and three SA-associated marker genes (*VvPR1*, *VvPR3*, *VvPAD4*) had higher basal expression levels (Figs. 4 and 5), which suggested that the role of grapevine CSN5 in pathogen defense is probably similar to *TaCSN5* in wheat. In addition, the JA-associated marker gene *VvJAR1* showed stronger expression in CSN5 over-expression leaves (Fig. 5), which suggested grapevine CSN5 may be positively correlated with JA-signaling pathway, like its homologous genes in *Arabidopsis* (Feng et al. 2003).

Interestingly, there is only one different amino acid residue in the MPN domain of VvCSN5 and VpCSN5 (Fig. S1), however, there were significant differences in their expression patterns during powdery mildew infection (Fig. 2). We know that gene expression depends on its promoter, in which pathogen-inducible gene expression requires the interaction between defense-related transcription factors (TFs) and cis-regulatory elements (CREs) in the promoter (Amorim et al. 2017; Rushton et al. 2002). For example, promoter mutations in rice *Xa13* gene cause down-regulation of expression during host–pathogen interaction, resulting in the race-specific resistance to *Xanthomonas oryzae* pv. *Oryzae* (Chu et al. 2006). In grapevine, 'Baihe-35-1' *VpRFP1* gene expressed under its own promoter activated a stronger disease resistance than driven by *V. vinifera* cv. Carignane *VvRFP1* promoter (Yu et al. 2013). In this study, we found that there were many base pair substitutions and a 132 bp fragment deletion in *VpCSN5* promoter that resulted in more CAAT-box and stress-related CREs existed in *VvCSN5* promoter sequence than that in *VpCSN5* promoter (Fig. 6a), including MYB-elements and ERE-elements, which were reported to be responsive to salicylic acid and ethylene (Yang and Klessig 1996; Fujimoto et al. 2000), respectively. This explained the reason of *VvCSN5* promoter displayed a strong inducible GUS activity in response to powdery mildew (Fig. 6b and c).

Our study expounded the effect of CSN5 on grapevine resistance to powdery mildew, confirmed the functional consistence between grapevine *CSN5* and its homologous genes. Further demonstrate the different structures of *CSN5* promoters between susceptible and resistant grapevines caused *CSN5* gene differently expressed during powdery mildew infection. Taken together, our results suggested grapevine *CSN5* was a negative regulator gene for grapevine powdery mildew resistance.

Declarations

Author contribution statement

CKC and YQW conceived and designed the research. CKC and ML conducted most of the experiments. GHK, XYZ, BM and YH participated in the experiments. CKC wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

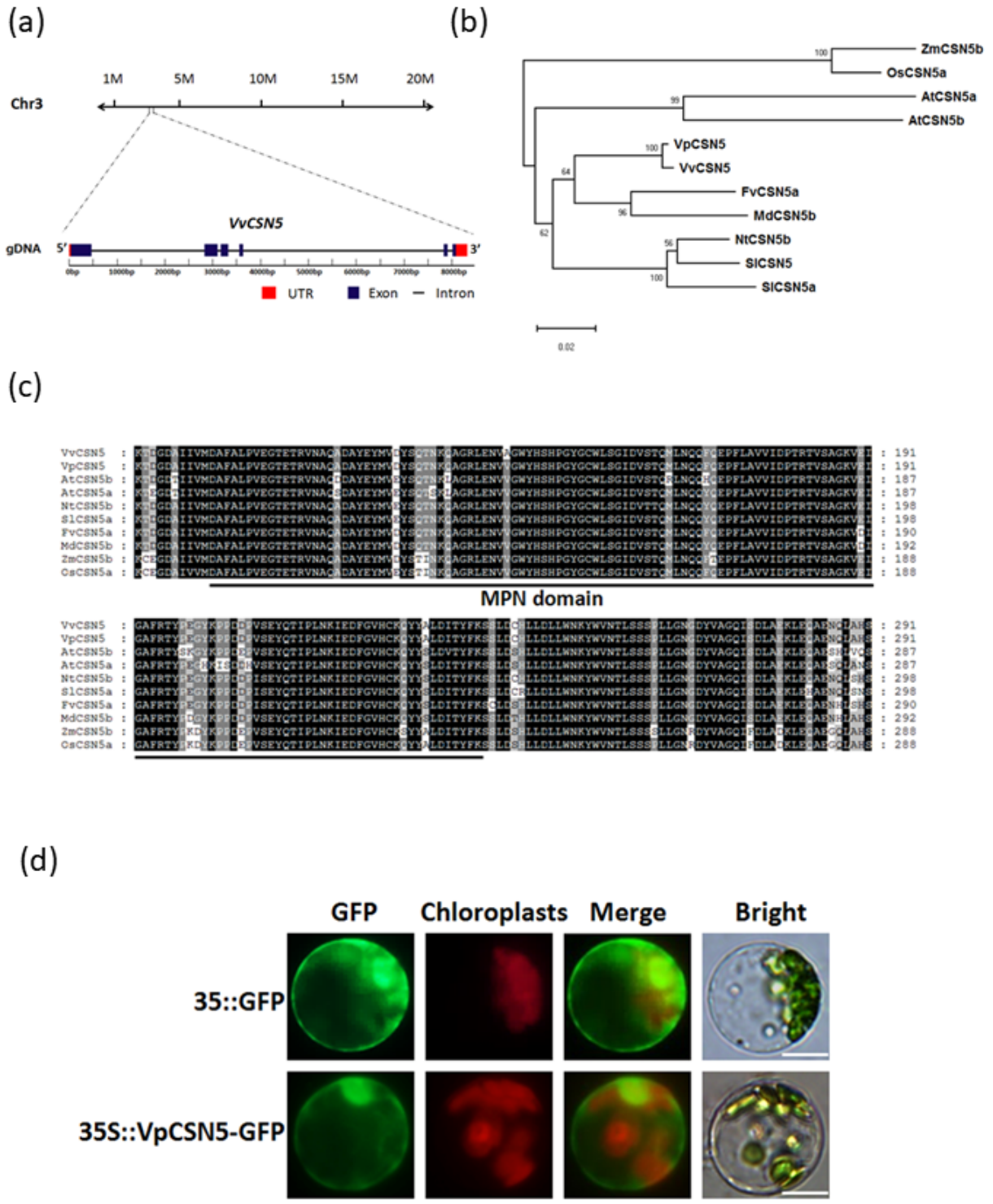

Figure 1

Sequence analysis and localization of grapevine CSN5. (a) Location of VvCSN5 on chromosomes and its basic structure. (b) Phylogenetic analysis to ‘Thomspon Seedless’ VvCSN5, ‘Baihe-35-1’ VpCSN5 and several well-studied homologous proteins. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA-X. (c) Alignment of the conserved core region (MPN domain) for the CSN5 proteins. Fully or partially conserved amino acid residues are shaded in

black or gray, respectively. The MPN domain is marked by black underline. The multiple sequence alignment was made by ClustalX. (d) Subcellular localization of VpCSN5. The VpCSN5 gene were cloned from ‘Baihe-35-1’ and used to construct 35S::VpCSN5-GFP vector in which GFP was fused at the C terminus. The VpCSN5 fused proteins and GFP control were transiently expressed in grape mesophyll protoplasts and observed by fluorescence microscopy. Individual and merged images of GFP (first panel column) and chlorophyll auto-fluorescence (second column) as well as bright field images (column on the right) of protoplasts are shown (bars = 10 μm). The GenBank accession numbers of the genes mentioned in figure are presented at the Table S2.

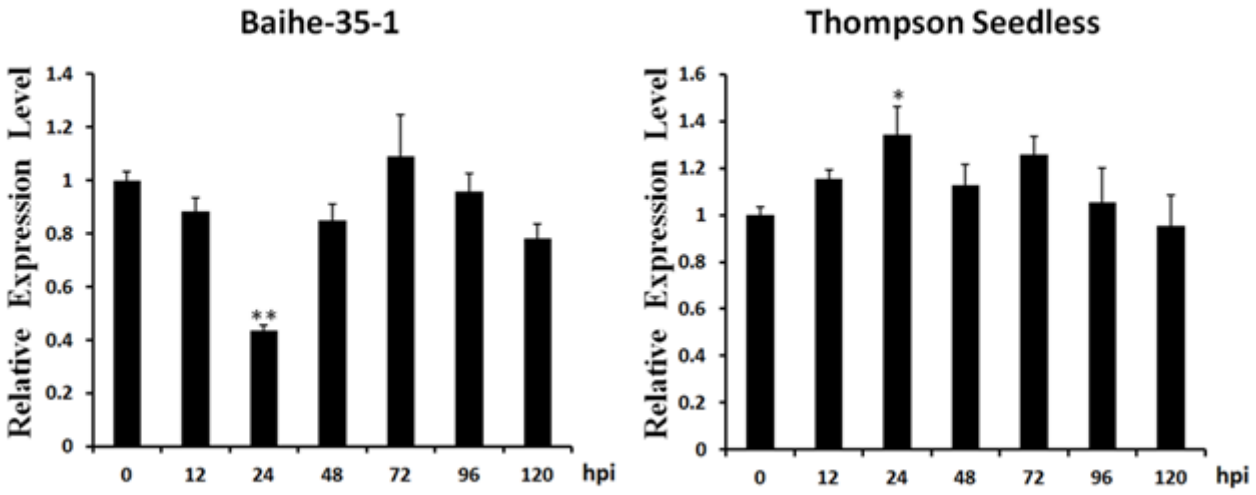

Figure 2

The different expression patterns of CSN5 gene in susceptible cultivar ‘Thompson Seedless’ and resistance grapevine ‘Baihe-35-1’ after En NAFU1 inoculation. Relative mRNA expression levels were measured by RT-qPCR. Grapevine ACTIN7 gene was used as an endogenous control. Each data point represents the mean ± standard deviation of triple biological replicates. Asterisks indicate that the transcript at each timepoint was significantly up- or down-regulated in comparison to 0 hpi (** and * = $P \leq 0.01$ and 0.05, respectively; Student's t-test).

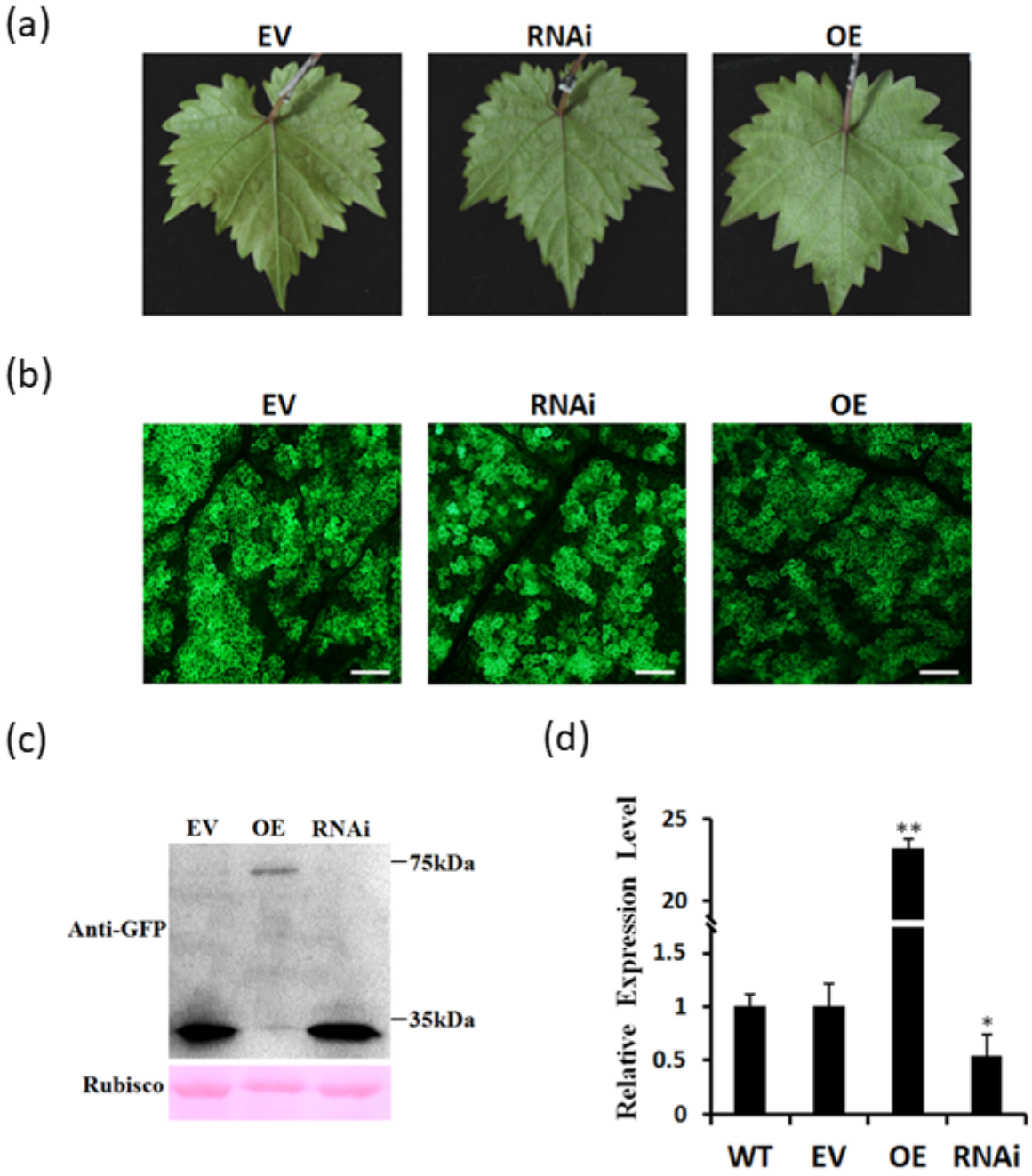

Figure 3

Detection of transient transformation efficiency of 'Thomspon Seedless' leaves. (a) Comparison of the leaves 3 days after injection. From left to right is 35S::GFP (EV), pK7WIWG2D-CSN5 (RNAi) and 35S::CSN5-GFP (OE), respectively. (b) GFP fluorescence detection of transformed leaves (bars = 50 μm). (c) Western blot detection for protein expression levels of GFP (~29.9kDa) or CSN5-GFP fusion protein (~70.2kDa) in different transformed leaves. (d) The mRNA transcript levels of grapevine CSN5 in each transformed leaf. WT represents non-injected 'Thomspon Seedless' leaves under the same growth conditions. Asterisks indicate that the transcript of CSN5 at each group was significantly up- or down-regulated in comparison to WT (** = P≤0.01; Student's t-test). Samples of (b)(c)(d) were taken from the leaves 3 days after injection.

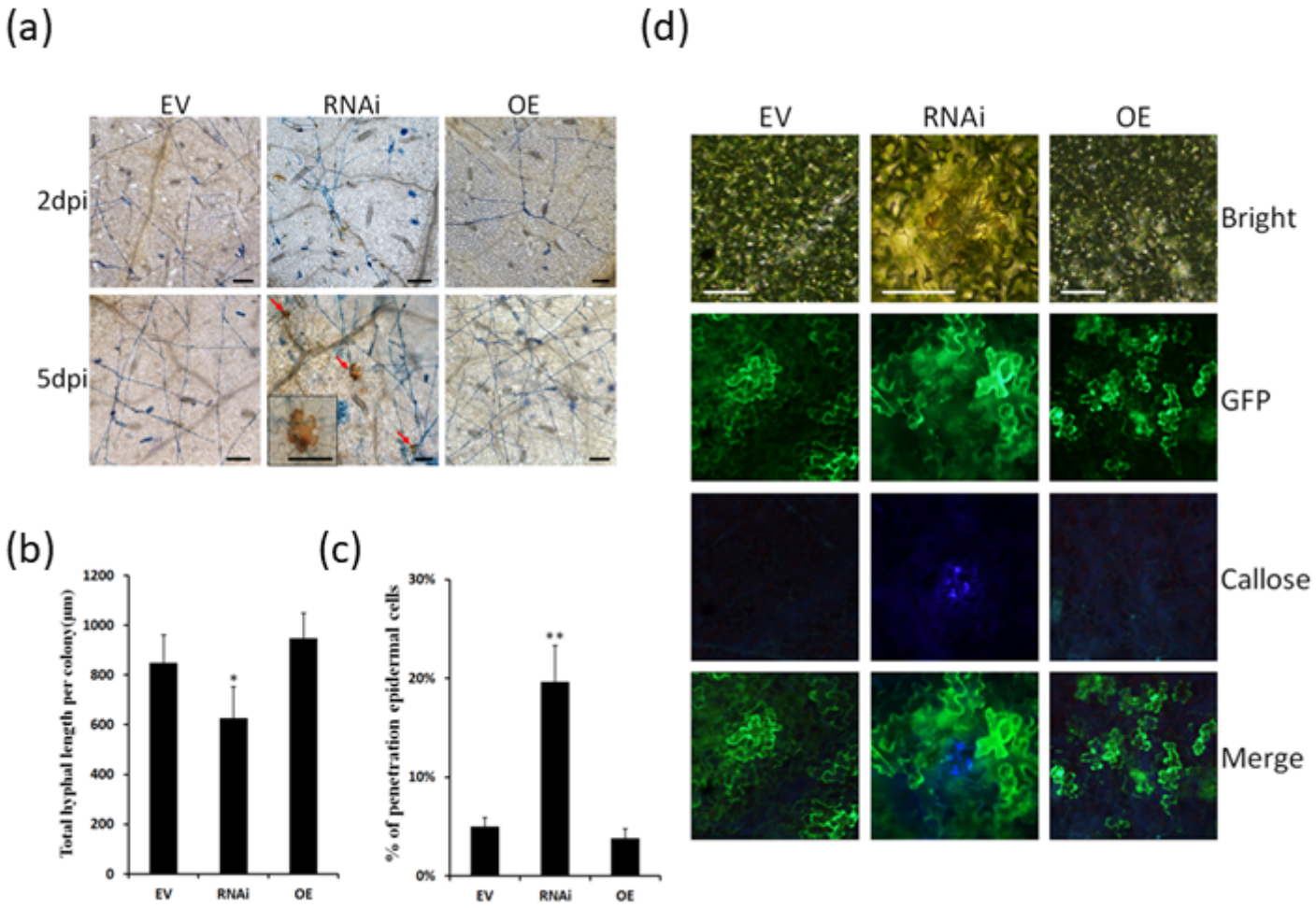

Figure 4

Transient silence of CSN5 improved ‘Thompson Seedless’ resistance to En NAFU1. (a) Representative micrographs showing DAB- and trypan blue-stained epidermal cells of the RNAi, OE and EV leaves at 2 or 5 dpi. Red arrowheads indicate H2O2 accumulation (bars = 50 μm). (b) Average hyphal length per colony of En NAFU1 inoculated on different groups leaves at 2 dpi. (c) Frequencies of the invasion induced hypersensitive cell death (full of H2O2) at 5 dpi. Data are means ± SE, calculated from three duplicated experiments. Asterisks indicate values significantly difference from EV (** and * = P≤0.01 and 0.05, respectively; Student's t-test). (d) Aniline blue-staining visualized the accumulation of callose in transformed leaves inoculated with En NAFU1 at 5 dpi. The images are respectively bright field, GFP, callose fluorescence and row 2 and row 3 merge image from the top to bottom (bars = 50 μm).

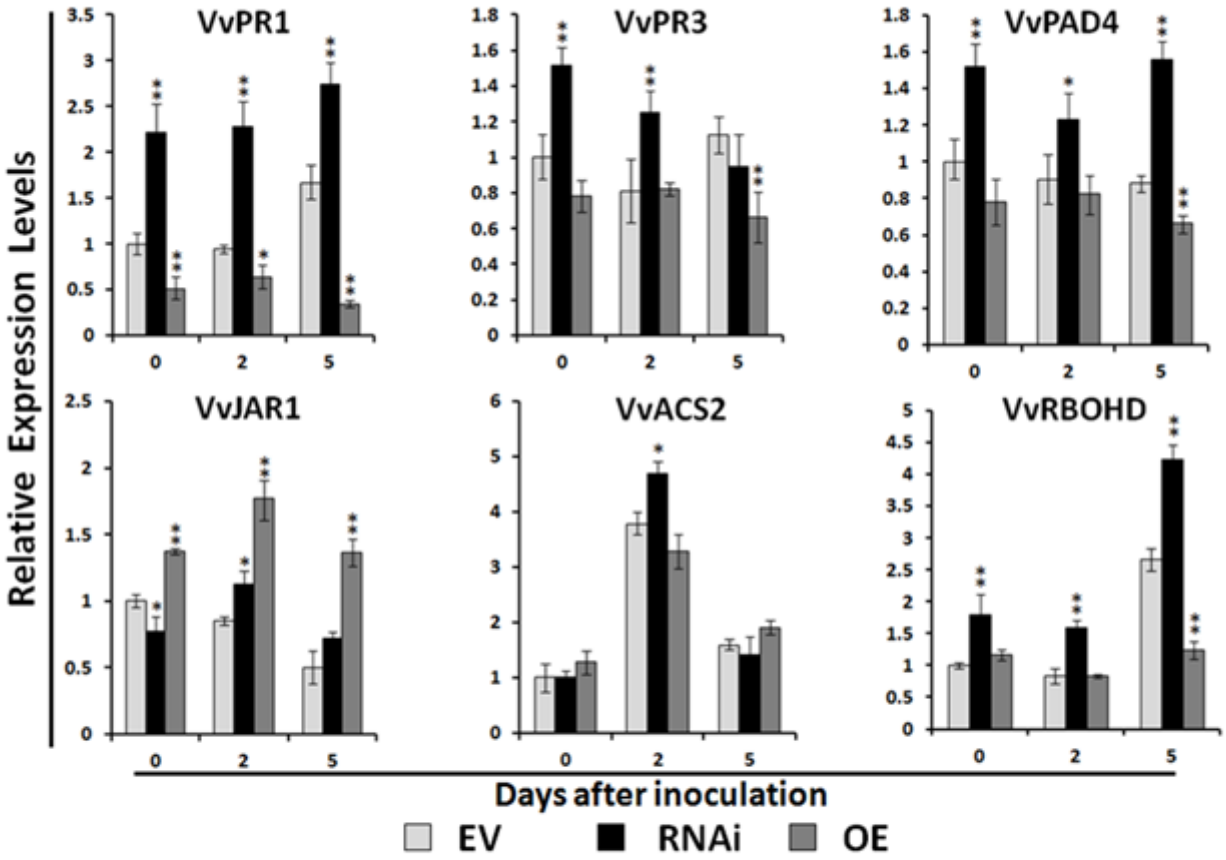

Figure 5

The expression levels of several defense-associated genes in RNAi, OE and EV leaves post En NAFU1 inoculation. Each data point represents the mean ± standard deviation of triple biological replicates. Grapevine ACTIN7 was used as an endogenous control. The GenBank accession numbers of the genes mentioned in figure are presented at the Table S2. Asterisks indicate that the mRNA expression of corresponding gene at each timepoint was significantly up- or down-regulated in comparison to EV (** and * = P≤0.01 and 0.05, respectively; Student's t-test).

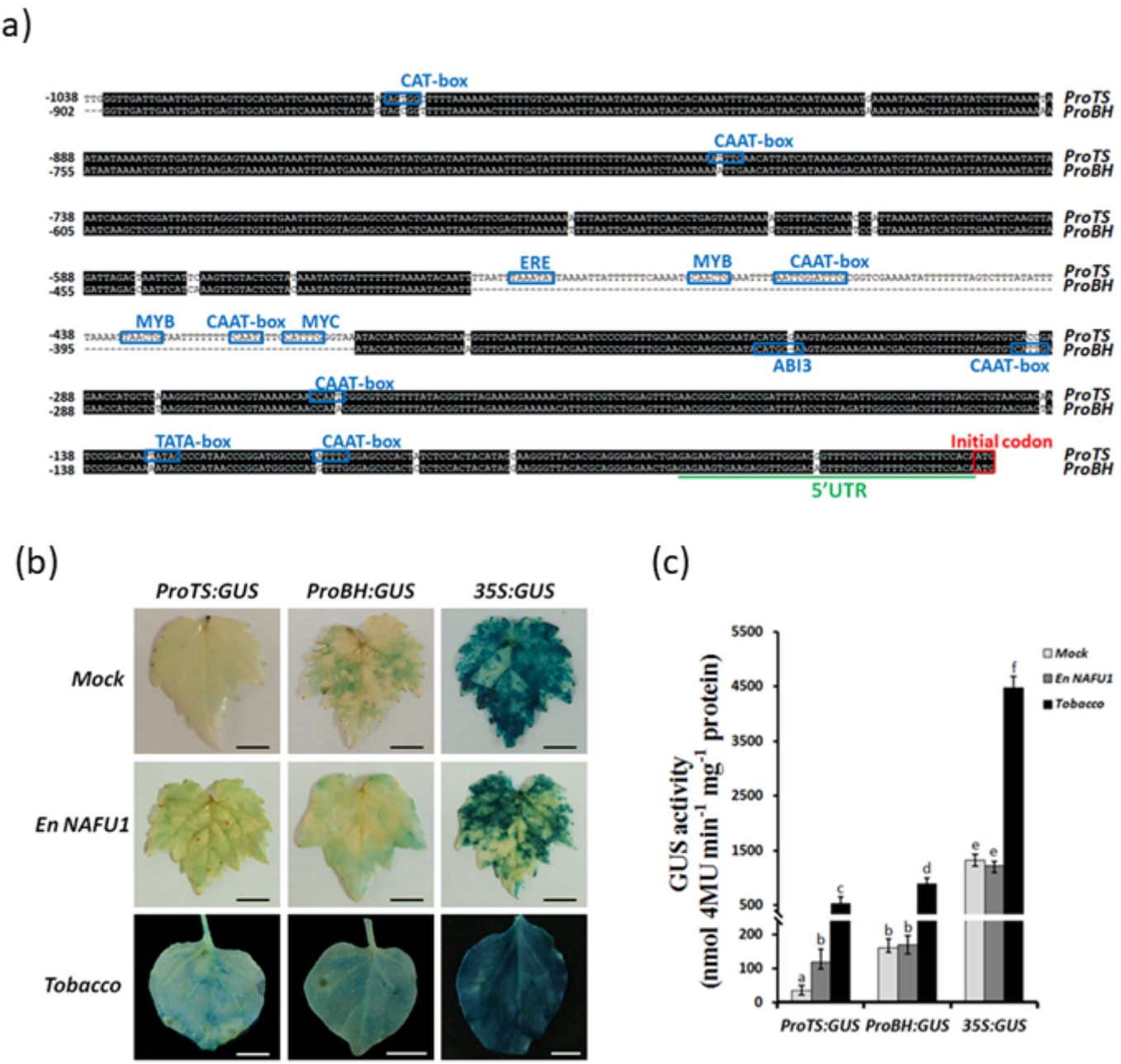

Figure 6

Sequence alignment and activity analysis of the two CSN5 promoters. (a) Sequence alignment of VvCSN5 promoter (ProTS) and VpCSN5 promoter (ProBH) , the differential cis-regulatory elements (CREs) were markered with blue boxes. The red box indicates the start codon. The sequence alignment was made by ClustalX and CREs was predicted by PlantCare. (b) GUS staining identifies activities of two promoters and the 35S:GUS as a control. Three leaves were stained each group. The present results indicate at least two closely stained leaves (bars = 1cm). (c) Fluorometric quantitative analysis of GUS activity in each transformed leaves. GUS activity was analyzed fluorometrically and expressed as nmol 4-methylumbelliferone (MU)/mg protein min-1. Different letters denote statistically significant differences ($P < 0.05$, ANOVA and post-hoc Tukey's HSD test).

Supplementary Files

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