Transient silencing of VvCSN5 enhances powdery mildew resistance in grapevine (Vitis vinifera)

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Received: 1 March 2021 / Accepted: 2 May 2021 / Published online: 6 May 2021
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Abstract
As one of the most economically important fruit crops in the world, the grapevine (Vitis vinifera) suffers significant yield losses from various pathogens including powdery mildew caused by Erysiphe necator. In contrast, several wild Chinese grapevines, including Vitis pseudoreticulata accession Baihe-35-1, are highly resistant to powdery mildew pathogens. Here, we identified a grapevine gene CSN5 (COP9 signalosome complex subunit 5), designated VvCSN5, that was differentially expressed between the resistant ‘Baihe-35-1’ and susceptible ‘Thompson Seedless’ during powdery mildew isolate Erysiphe necator NAFU1 infection. Moreover, transient silencing of VvCSN5 in ‘Thompson Seedless’ leaves enhanced resistance to E. NAFU1. This resistance manifested in cell wall callose deposition at attempted infection 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 different, which may have been behind their different resistances to powdery mildew infection. Taken together, these results implied that grapevine CSN5 plays an important role in the response to powdery mildew infection.

Key message
Transient silencing of VvCSN5 in Vitis vinifera leaves enhances powdery mildew resistance. In addition, the CSN5 promoters in susceptible and resistant grapevines are different.

Keywords Grapevine · Powdery mildew (Erysiphe necator) · CSN5 · Transient transformation · Promoter

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

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

Plants have an innate immune system that resists pathogenic infections, the two major branches of which are PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006; Zhu 2016). The first branch (PTI) uses transmembrane pattern recognition receptors (PRRs) to recognize multiple conserved microbial- or pathogen-associated molecular patterns (MAMPs and PAMPs) and triggers penetration resistance mechanisms (Zipfel and Felix 2005). The second branch (ETI) relies on a large amount of nucleotide-binding leucine-rich-repeat (NB-LRR) proteins encoded by R-genes, which can identify effectors released from pathogens and initiate hypersensitive responses (Jones and Dangl 2006; Dangl and Jones 2001). Stemming from their innate immune systems, the resistance to powdery mildew in grapevines involves penetration resistance and hypersensitive responses (Douchkov et al. 2016; Wang et al. 2016). These two processes interact with and impede the growth of powdery mildew via 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 the COP9 (constitutive photomorphogenesis 9) signalosome (CSN) in Arabidopsis. It participates 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 conserved subunit in the CSN, CSN5 has become a common target of pathogenic effectors (Echallier et al. 2013; Jin et al. 2014). In Arabidopsis, AtCSN5a interacts with 29 distinct effectors from Hyaloperonospora arabidopsidis (Hpa) and Pseudomonas syringae (Psy), and the csn5a mutant has a strong resistance to both Hpa and Psy (Mukhtar et al. 2011). CSN5 has also been reported to play roles in the biotic stress responses of tobacco (Liu et al. 2002), tomato (Shang et al. 2019), wheat (Zhang et al. 2017), rice (He et al. 2020), etc. However, there has been no report focusing on CSN5 regulated defense responses in grapevine.

In the present study, we identified a grapevine CSN5 gene that was differentially expressed between grapevines that were susceptible or resistant to powdery mildew infections. Through transient transformation, we discovered that silencing VvCSN5 enhanced powdery mildew resistance in leaves of the susceptible cultivar V. vinifera ‘Thompson Seedless’. Further analysis of the promoter structure and function indicated that the differences in the CSN5 promoter between ‘Thompson Seedless’ and ‘Baihe-35–1’ may contribute to the differences in their resistances to powdery mildew. Our findings suggested that grapevine CSN5 could negatively impact powdery mildew resistance in grapevine.

### Materials and methods

#### Plant materials and growth conditions

Tissue culture plantlets 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 at 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 leaf cDNA using Planta Max Super-Fidelity DNA Polymerase (Vazyme Bio Co., Nanjing, China). The amplified primers were derived from the mRNA sequence of V. vinifera cv. Pinot Noir CSN5, which was available on 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. The sequences of amplified primers and related GenBank accession numbers are shown in Table S1 and Table S2, respectively.

#### Plasmid construction

To detect the subcellular localization of ‘Baihe-35–1’ VpCSN5 and ‘Thompson Seedless’ VvCSN5, and obtain VpCSN5 over-expression leaves, full length VvCSN5 and VpCSN5 genes were amplified from their corresponding pMD19-T fusion plasmids. They were inserted into the pCAMBIA2300 vector containing the CaMV 35S promoter (Thermo Fisher Inc., Carlsbad, USA). As an RNA interference vector, the pK7WIWG2D vector was used as an RNA interference vector. The 35S::VpCSN5-GFP and 35S::VvCSN5-GFP, respectively. The pCAMBIA2300 vector was pre-digested by the KpnI and BamHI enzymes.

To silence VvCSN5 in ‘Thompson Seedless’, the pK7WIWG2D vector was used as an RNA interference vector. The 35S::VvCSN5-GFP and 35S::VpCSN5-GFP, respectively. The pCAMBIA2300 vector was pre-digested by the KpnI and BamHI enzymes. The silenced VvCSN5 was selected and amplified for constructing the pK7WIWG2D-VvCSN5 vector using the Invitrogen Gateway recombination cloning technology (Thermo Fisher Inc., Carlsbad, USA).

To analyze the promoter functions of VvCSN5 and VpCSN5, we designed primers according to the genome sequence of V. vinifera PN40024 (https://www.ncbi.nlm.nih.gov/ genome), and amplified 1500 bp (approximately)
The method of inoculation followed previously established procedures. Leaves with heavily infected ‘Thompson Seedless’ leaves were used as controls. The epidermal cells of transformed leaves were co-stained with 3,3′-diaminobenzidine (DAB) and trypan blue (TB) and observed 2 and 5 days post inoculation (dpi) to monitor the accumulation of H₂O₂, fungal structures, and dead host cells. Aniline blue-staining was used to visualize the accumulated callose at 5 dpi (Gao et al. 2016; Hu et al. 2018; Wan et al. 2020). All samples were then examined using an Olympus BX-63 microscope (Japan). Hyphal length of En NAFU1 and frequencies of invasion induced hypersensitive cell death (full of H₂O₂) were measured directly under the Olympus BX-63 using the CellSens 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 inoculation with powdery mildew isolate En NAFU1. Leaves were then collected 0, 12, 24, 48, 72, 96, and 120 h post inoculation (hpi), and samples were promptly stored at −80 °C after snap freezing in 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 the 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-qPCR assays were conducted referring to a previous study (Zhang et al. 2015). Grapevine ACTIN7 (GenBank accession number XM_002282480.4) was used as an internal control (Wang et al. 2017; Reid et al. 2006; Gutha et al. 2010). The relative transcription levels of the genes were calculated using the 2⁻△△Ct method and normalized expression methods. Three biological replicates were analyzed for each sample. The statistical significance was evaluated using Student’s t-test. The sequences of RT-qPCR primers and related GenBank accession numbers are shown in Tables 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) vectors were transformed into fully unfolded ‘Thompson Seedless’ grapevine or tobacco leaves by vacuum-inoculating, with 35S::GUS being used as a control. Three days after transformation, the transformed leaves were inoculated by spraying with an En NAFU1 sporangium suspension with a concentration of 5 × 10⁵ sporangia ml⁻¹, mock-inoculated leaves were sprayed with sterile water only. Leaves were collected 2 dpi, placed in GUS dye and incubated at 37 °C for 24 h. Then, 70% and 100% ethanol were successively used.
for decoloring at 37 °C for 10 h. The method of GUS quantitative analysis followed a previous study (Yu et al. 2013).

**Statistical analysis**

The relevant data were analyzed through the statistical software tool IBM SPSS v26.0.0 and were presented as mean values ± standard deviation of three independent
et al. (2013). In order to explore the intracellular localization of many species (Li et al. 2018; Wang et al. 2013), several well-studied homologous proteins. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates using MEGA-X. Fully and partially conserved amino acid residues are shaded in black and gray, respectively. The MPN domain is underlined in black. The multiple sequence alignment was conducted by ClustalX. Subcellular localization of VpCSN5 and VvCSN5. The VpCSN5 and VvCSN5 genes were cloned from ‘Baihe-35–1’ and ‘Thompson Seedless’, respectively, and used to construct the 35S::VpCSN5-GFP and 35S::VvCSN5-GFP vectors in which GFP was fused at the C terminus. The fused proteins and GFP control were transiently expressed in tobacco leaves and observed by laser scanning confocal microscope. Individual and merged images of GFP (first panel column), chlorophyll auto-fluorescence (second column), and DAPI fluorescence (third column) as well as bright field images (forth column) of epidermic cells (bars = 20 μm). The GenBank accession numbers of the genes mentioned in the figure are provided in Table S2. (Color figure online)

Results

Bioinformatics analysis and subcellular localization of grapevine CSN5

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

To understand the relationship of CSN5 in different plants, we first chose nine CSN5 protein homologs from seven species in which their functions had been well studied, and used them to build a phylogenetic tree that included VvCSN5 from ‘Thompson Seedless’ and VpCSN5 from ‘Baihe-35–1’. We found that VvCSN5 and VpCSN5 were highly homologous with the other CSN5 proteins (Fig. 1b). In addition, these CSN5 proteins were also highly conserved at their core functional region (MPN domain) (Fig. 1c).

These results indicated that grapevine CSN5 may play a similar role to those homologous genes.

It has been reported that CSN5 is localized in the cytoplasm and nuclei of many species (Li et al. 2018; Wang et al. 2013). In order to explore the intracellular localization of grapevine CSN5, we transiently expressed the VpCSN5-GFP and VvCSN5-GFP fusion proteins in tobacco leaves. As shown in Fig. 1d, the GFP fluorescence signal of the 35S::GFP control was present in both nucleus and cytoplasm. Similarly, the VpCSN5-GFP and VvCSN5-GFP fusion proteins were also localized in the cytoplasm and nucleus.

Expression patterns of VpCSN5 and VvCSN5 differed during powdery mildew infection

To determine whether the expression patterns of VvCSN5 and VpCSN5 differed after powdery mildew infection, we inoculated the susceptible grapevine ‘Thompson Seedless’ and resistant grapevine ‘Baihe-35–1’ using the isolate En NAFU1. RT-qPCR results showed that the ‘Thompson Seedless’ VvCSN5 transcription had slightly increased in abundance and peaked at a ~1.4-fold increase at 24 hpi, whereas ‘Baihe-35–1’ VpCSN5 transcript had decreased rapidly by 24 hpi (0.4-fold), but had returned to a normal level by 48 hpi (Fig. 2). This result suggested that CSN5 is involved in the grapevine defense responses to powdery mildew.

Transient silence of VvCSN5 enhances resistance to a powdery mildew isolate

The expression patterns shown above demonstrated that CSN5 levels in susceptible and resistant grapevines were different (Fig. 2). Based on this observation we hypothesized that altering the expression levels of CSN5 in ‘Thompson Seedless’ leaves would affect their resistance to powdery mildew. We thus obtained VvCSN5-RNAi (RNAi) and 35S::VpCSN5-GFP (OE) transient transformed leaves through injection, and the 35S::GFP (EV) construct was injected as a control (Fig. 3a). As shown in Fig. 3b, most epidermal cells of ‘Thompson Seedless’ leaves injected with the three constructs showed clear GFP signals. Similarly, the western blot analyses of GFP (~29.9 kDa) and VpCSN5-GFP fusion (~70.2 kDa) proteins also confirmed the normal expression of the GFP related protein (Fig. 3c), which meant that the transient transformation was successful. Subsequently, we detected the transcription levels of CSN5 in each transformed leaf or un-injected leaf (control) by RT-qPCR. The results showed that, compared to the control, CSN5 transcription was up-regulated by over 20-fold in OE leaves and down-regulated by 0.5-fold in RNAi leaves (Fig. 3d).

To assess the resistance to powdery mildew in leaves with different CSN5 levels, we collected transformed ‘Thompson Seedless’ leaves and co-stained them with trypan blue (TB) and 3,3′-diaminobenzidine (DAB), and measured the total hyphal length of isolated fungal colonies. As shown in Fig. 4a and b, the total hyphal length per colony in the RNAi leaves was about 600 μm at 2 dpi, while the length in OE and EV leaves were about 950 and 840 μm, respectively. Moreover, at 5 dpi in RNAi leaves, nearly 20% of invaded cells showed whole-cell H₂O₂, while it was less than 5% in the EV and OE leaves (Fig. 4a, c). In addition, the accumulation...
of callose in cell walls is also an important indicator of *Vitis* resistance to powdery mildew (Consonni et al. 2006). The aniline blue-staining results showed that large amounts of callose had accumulated around the necrotic epidermal cells in RNAi leaves at 5 dpi. In contrast, there was no obvious callose accumulation in the leaves of EV and OE (Fig. 4d). These results suggested that silencing VvCSN5 could enhance powdery mildew resistance in grapevines.

**Grapevine CSN5 involves in multiple defenses signaling pathways**

To investigate the effects of CSN5 on defense-related genes, we selected several typical defense-related genes and assessed their expression patterns in transformed leaves. As shown in Fig. 5, for three SA-associated genes, the expression level of *VvPR1* (pathogenesis-related gene 1) in RNAi leaves was more than twofold that in EV at 0 dpi, and its level gradually increased between 2 and 5 dpi, while the *VvPR1* expression in OE was lower (~0.5-fold) than EV. For *VvPR3* (pathogenesis-related gene 3) and *VvPAD4* (phytoalexin deficient 4) transcription levels in RNAi leaves both increased by more than 1.5-fold compared to EV at 0 dpi, while the *VvPR3* levels at 5 dpi were not obviously different. However, the JA-associated gene *VvJAR1* (jasmonate resistant 1) showed stronger expression (1.3~2.5-fold) in OE leaves than in EV and RNAi leaves. For the hydrogen peroxide-associated gene *VvRBOHD* (respiratory burst oxidase homologue D), while there was increasing trend in expression in both RNAi and EV leaves, the transcription levels at 0, 2, and 5 dpi in RNAi were all significantly higher (1.5~twofold) than those in EV, which implied that the hydrogen peroxide-pathway was more strongly activated in RNAi leaves. In addition, transcription of the ethylene-associated gene *VvACS2* (1-amino-cyclopropane-1-carboxylate synthase 2) (Ma et al. 2015; Poulaki et al. 2020) in RNAi was slightly higher than EV at 2 dpi. These results indicated that grapevine CSN5 was associated with multiple defense-related genes during powdery mildew infection.

**Promoters of VvCSN5 and VpCSN5 differ in structure and activity**

According to the sequence alignment, we found there are only two amino acid residues that differed between VvCSN5 and VpCSN5 (Fig. S1). Because of their similarity, we hypothesized that the differences in expression patterns between VvCSN5 and VpCSN5 were due to differences in their promoters. Therefore, we first compared the promoters of VvCSN5 (ProTS) and VpCSN5 (ProBH) using sequence alignment. As shown in Fig. 6a, there were many differences between the 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 using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and marked out the discrepant elements (Fig. 6a). There were more CAAT-box and defense-related CREs in ProTS than in ProBH, including two MYB-elements, one MYC-element, and one ERE-element (Fig. 6a), which have been reported to be responsive to various stresses (Dubos et al. 2010; Abe et al. 2003; Zhang et al. 2016; Ayadi et al. 2020).

To verify the promoter function, we used histochemical staining and fluorometric assays to examine the expression of the GUS genes that are driven by ProTS or ProBH after being inoculated with the powdery mildew of the 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 the *En NAFU1*-inoculated and mock conditions. Furthermore, the *ProTS:GUS* in mock conditions showed very weak expression, while in the *En NAFU1*-inoculated leaves it showed a much stronger activity, 3.5-fold higher than in the mock condition (Fig. 6b, c). However, there was no significant difference in *ProBH:GUS* activity between mock and *En NAFU1*-inoculated leaves (Fig. 6b, c). We also expressed *ProTS:GUS* and *ProBH:GUS* in tobacco leaves (to facilitate a higher transformation efficiency) in mock conditions to confirm the reliability of the GUS staining results in the grapevine leaves. We observed stronger staining in the tobacco leaves than in...
the corresponding grapevine leaves (Fig. 6b), but similar staining compared to the mock condition grapevine leaves, the ProBH:GUS activity in tobacco was also higher than ProTS:GUS (Fig. 6c). These results indicated that ProTS was more strongly induced by powdery mildew infection compared to ProBH.
Discussion

As the catalytic center of the COP9 signalosome (CSN), CSN5 plays an essential role in the normal function of the complex (Stratmann and Gusmaroli 2012; Jin et al. 2014). Thus, CSN5 is highly conserved among eukaryotes (Cope et al. 2002). Research has already shown that CSN5 is involved in the pathogen defense of 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 has been no such research on grapevine CSN5. In this study, we demonstrated that CSN5 is conserved in grapevines, and further discovered that CSN5 was associated with grapevine resistance to powdery mildew.

According to the expression analysis of grapevine CSN5 during powdery mildew infection, we saw that the VpCSN5 expression dropped to a minimum at 24 hpi, whereas VvCSN5 peaked at 24 hpi (Fig. 2), which was consistent with the timing of haustoria formation (Hu et al. 2019). This result implied that the changes in CSN5 expression were pathogen induced. Furthermore, when combined with the results showing stronger powdery mildew resistance in CSN5-silenced leaves (Fig. 3), we see that both indicate that CSN5 plays a negative role in powdery mildew infection in grapevines.

Previous studies have shown that CSN5 was able to regulate plant pathogen-defense responses by activating the JA pathway while inhibiting the SA pathway (Feng et al. 2003; Hind et al. 2011; Spoel et al. 2009). For example, the over-expression of OsCSN5a inhibited infection by the rice black-streaked dwarf virus (RBSDV) by activating the JA-signaling pathway in rice (He et al. 2020). Whereas silencing TaCSN5 in wheat improved wheat rust resistance by activation of the SA-signaling pathway in rice (He et al. 2020). Whereas silencing TaCSN5 in wheat improved wheat rust resistance by activation of the SA-signaling pathway in rice (He et al. 2020).

In our study, there was stronger powdery mildew resistance in CSN5-RNAi leaves wherein three SA-associated marker genes (VvPR1, VvPR3, VvPAD4) had higher basal expression levels (Figs. 4, 5), which suggested...
that the role of grapevine CSN5 in pathogen defense is probably similar to that of TaCSN5 in wheat. In addition, the JA-associated marker gene VvJAR1 had higher expression in CSN5 over-expression leaves (Fig. 5), which suggested that grapevine CSN5 may be positively correlated with the JA-signaling pathway, like its homologous genes in Arabidopsis and rice (Feng et al. 2003; He et al. 2020).

Defense responses of plants against biotrophic pathogens like powdery mildew are mainly mediated by the SA pathway (Loake and Grant 2007; Verma et al. 2016), whereas the JA pathway is responsible for defense against necrotrophic pathogens and herbivorous insects (Ku et al. 2018; Wasternack et al. 2007). Although the JA-associated marker gene VvJAR1 was upregulated in CSN5 over-expression leaves (Fig. 5), its resistance to powdery mildew was not enhanced (Fig. 4). The most likely reason was that the grapevine resistance to powdery mildew is primarily regulated by the SA pathway rather than the JA pathway (Hu et al. 2019; Qiu et al. 2015).

Interestingly, there were no differences in amino acid residues 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 a gene’s expression depends on its promoter, so pathogen-inducible gene expression requires that defense-related transcription factors (TFs) interact with the cis-regulatory elements (CREs) of the promoter (Amorim et al. 2017; Rushton et al. 2002). For example, promoter mutations in the rice Xa13 gene cause down-regulation of expression during host–pathogen interactions, resulting in the race-specific resistance to Xanthomonas oryzae pv. Oryzae (Chu et al. 2006). In grapevines, the ‘Baihe-35–1’ VpRFP1 promoter contains more SA responsive CREs, which result in the VpRFP1 gene being able to activate a stronger disease response than in V. vinifera cv. Carignane.
VvRFP1 (Yu et al. 2013). In this study, we found that there were many base pair substitutions and a 132 bp fragment deletion in the VpCSN5 promoter, which led to more stress-related CREs existing in the VvCSN5 promoter sequence than that in VpCSN5 promoter (Fig. 6a). These included MBS (MYB-binding site) and ERE (ethylene-responsive element), which were reported to be responsive to salicylic acid, ethylene and pathogen stress (Yang and Klessig 1996; Fujimoto et al. 2000; Yu et al. 2019; Ayadi et al. 2020). This not only explained why the VvCSN5 promoter displayed strong inducible GUS activity in response to powdery mildew (Fig. 6b, c), but also implied that the differences in expression of CSN5 between susceptible and resistant grapevines may be caused by differences in promoter function.

Our study expounded on the effects of CSN5 on grapevine resistance to powdery mildew, and confirmed the functional consistency between grapevine CSN5 and its homologous genes. Furthermore, we demonstrated that the differences in the structures of susceptible and resistant grapevine CSN5 promoters were the cause of the differential

![Sequence alignment and activity analysis of the two CSN5 promoters.](image-url)

**Fig. 6** Sequence alignment and activity analysis of the two CSN5 promoters. a Sequence alignment of the VvCSN5 (ProTS) and VpCSN5 (ProBH) promoters, the differential cis-regulatory elements (CREs) are marked with blue boxes. The red box indicates the start codon. The sequence alignment was conducted in ClustalX, and CREs were predicted by PlantCARE. b GUS staining reflecting the activities of the two promoters and the 35S:GUS as a control. Three leaves from each group were stained (bars = 1 cm). c Fluorometric quantitative analysis of GUS activity in transformed leaves. GUS activity was analyzed fluorometrically and expressed as nmol 4-methylumbelliferone (MU)/mg protein min⁻¹. Different letters denote significant differences between groups (P < 0.05, ANOVA and post-hoc Tukey’s HSD test). (Color figure online)
CSN5 gene expression during powdery mildew infection. Taken together, our results suggested grapevine the CSN5 gene was negatively associated with grapevine powdery mildew resistance.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02098-z.

Acknowledgements This study was funded by the National Natural Science Foundation of China (Grant No. 31972986, 31772264 to YQW).

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

Declarations

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

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