Callose Synthase Family Genes Involved in the Grapevine Defense Response to Downy Mildew Disease

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ABSTRACT

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The deposition of callose is a common plant defense response to intruding pathogens and part of the plant's innate immunity. In this study, eight grapevine callose synthase (*CalS*) genes were identified and characterized. To investigate biological function of *CalS* in grapevine against the infection of *Plasmopara viticola*, expression patterns of grapevine *CalS* family genes were analyzed among resistant/susceptible cultivars. After *P. viticola* infection, expression of *CalS1*, 3, 7, 8, 9, 10, and 11 were significantly modified among the grapevine cultivars. For

example, the expression of *CalS1* and *CalS10* were greatly increased in downy mildew (DM)-immune *Muscadinia rotundifolia* 'Carlos' and 'Noble'. Transient expression assay with promoters of the *CalS1* and *CalS10* genes confirmed that they were regulated by the oomycete pathogen *P. viticola*. *CalS1* promoter activity was also significantly up-regulated by ABA in DM-immune *M. rotundifolia* 'Noble', but down-regulated in DM-susceptible *Vitis vinifera* 'Chardonnay'. The *CalS1* promoter, however, was also down-regulated by GA in 'Chardonnay', but not affected in 'Noble'. The promoter activity of *CalS10* was significantly up-regulated by GA in 'Chardonnay', but not regulated by ABA at all. It is proposed that *CalS1* and *CalS10* were involved in grapevine defense against DM disease.

Callose, a β -1,3-glucan, exists in most plant tissues and plays an important role in plant defense response (Chen and Kim 2009; Eshraghi et al. 2014; Li et al. 2010). It has been reported that callose could be deposited between the plasma membrane and cell wall or around the stomata after pathogen attack (Brown et al. 1998; Gindro et al. 2003; Gomez-Gomez et al. 1999). Its deposition could impede the microbial penetration as a physical barrier or inhibit the development of pathogen haustoria by preventing it from obtaining nutrients of the host cells (Donofrio and Delaney 2001; Yang et al. 2014). The deposition of callose could be regulated by conserved pathogen-associated molecular patterns, hormone signals such as ABA, resistance-associated chemicals and secondary metabolites such as the nonprotein amino acid β -amino butyric acid (BABA) and the 4-methoxylated indole glucosinolates (Clay et al. 2009; Flors et al. 2008; Ton and Mauch-Mani 2004; Ton et al. 2009).

Callose is synthesized by callose synthases (*CalS*), which contains multiple transmembrane segments and a hydrophilic central loop (Kelly et al. 1996; Verma and Hong 2001). In *Arabidopsis thaliana*, 12 callose synthase genes were identified and characterized (Richmond and Somerville 2000). Among them, the biological role of *AtCalS12/GSL5* (first described as PMR4; POWDERY MILDEW RESISTANT4) was well studied in stress and pathogen response. This gene is required for wound and papillary callose formation in response to fungal pathogens and transported from the plasma membrane to the site of attempted

fungal penetration during powdery mildew infection (Ellinger et al. 2013; Luna et al. 2011; Nishimura et al. 2003). *AtCalS12/GSL5* gene could also be up-regulated by salicylic acid (SA) treatment and this induction is Npr1 (nonexpresser of PR genes) dependent (Dong et al. 2008). In addition, *AtCalS1*, 5, 9, and 10 (*GSL6*, 2, 10, and 8) were induced by the oomycete pathogen *Peronospora parasitica* that causes downy mildew (DM) disease of *A. thaliana*. Their expression levels were also affected by SA treatment and the most significant induction was observed in *AtCalS1/GSL6*. Promoter activity analysis indicated that the high induction of *AtCalS1/GSL6* by SA treatment requires Npr1 (Jacobs et al. 2003). In other plants, silencing of *CiCalS1* in *Citrus limon* increased plant susceptibility to *Xanthomonas citri* subsp. *citri*, suggesting that the plant cell wall associated defense is the principal initial barrier against *Xanthomonas* infection in citrus plants (Enrique et al. 2011). Similarly, in wheat spike, the suppression of callose synthase activity by free fatty acids could alter the resistance of wheat to *F. graminearum* (Blümke et al. 2014). In addition to the defense response, callose synthase genes were also reported to mediate the synthesis of callose during plant development. For example, *AtCalS5/GSL2* played an important role in the synthesis of exine of pollen wall and the viability of the pollen (Dong et al. 2005). *OsGSL5* from rice and *NaGSL1* from tobacco, orthologs of the *AtCalS5/GSL2* gene, were also highly expressed in developing anthers (Brownfield et al. 2007; Doblin et al. 2001; Yamaguchi et al. 2006).

To elucidate the molecular mechanism of functional genes in plants, transgenic studies are found as an efficient system, including model plant species *Arabidopsis* and tobacco (Liu et al. 2005; Malnoy et al. 2003), and crop species pepper and rice (Lee and Hwang 2006; Li et al. 2005). Compared with the stable genetic transformation, transient expression is a time saving approach. For instance, transient expression of *VvNPR1* in grapevine leaves could enhance plant resistance to fungal infection (Henanff et al. 2009). Transient expression of a *glyoxal*

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oxidase gene isolated from a disease resistant grape species *Vitis pseudoreticulata* in a susceptible genotype was found to enhance defense against powdery mildew (Guan et al. 2011). Similarly, the transient expression technology was used to study promoter activities. For example, studying the promoter activity of a stilbene synthase gene from *V. pseudoreticulata* (*VpSTS*) using *Agrobacterium*-mediated transient transformation system revealed that *VpSTS* was induced by infection, salicylic acid, and low temperature (Xu et al. 2010).

DM, caused by *Plasmopara viticola*, is the most destructive grapevine disease in viticulture areas with warm and humid climate conditions (Boso and Kassemeyer 2008). A previous study demonstrated that callose was mainly deposited in the leaf surface near stomata among DM-resistant grapevines, but less so in the DM-susceptible grapevines after *P. viticola* incubation (Yu et al. 2012). To further understand the mechanism of callose deposition in grapevine against DM disease, this study was designed to investigate the expression and regulation of grapevine callose synthase family genes in response to *P. viticola* pathogen infection and disease development.

MATERIALS AND METHODS

Plant materials and plant treatments. Two DM-immune *M. rotundifolia* cultivars ('Noble' and 'Carlos'), the DM-resistant and the partially resistant *V. amurensis* 'Shuanghong' and 'Zuoshanyi', and a DM-susceptible *V. vinifera* 'Chardonnay' were maintained in 1 gallon pots in glasshouse at China Agricultural University, Beijing. The potted grapevines were transferred to a big plant growth chamber (16 h light, 28°C/8 h dark, 25°C) for *P. viticola* inoculation and incubation.

P. viticola inoculum was collected from an experimental vineyard in Beijing. The third to fifth unfolded leaves from the apex were sprayed with a *P. viticola* suspension of 1×10^5 sporangia/ml. The inoculated leaves were covered with plastic bags to maintain high humidity. Control plants were inoculated with sterile distilled water and maintained under the same conditions. Samples were collected at 6 and 12 h postinoculation (hpi), 1, 3, 5, and 8 days postinoculation (dpi), and were immediately frozen in liquid nitrogen and stored at -80°C until utilization. All experiments were repeated three times.

For ABA treatment, the third to fifth unfolded leaves from the shoot apex were sprayed with 100 μM ABA and were harvested 12 h after treatment. Leaves sprayed with water at the same time point were used as control. The experiment was repeated three times.

DNA, RNA extraction, and real-time RT-PCR. Genomic DNA and total RNA were extracted from leaf tissues using the CTAB method (Murray and Thompson 1980). The first strand of cDNA was synthesized using M-MLV Reverse transcription (Promega). A grape elongation factor (*EF1- α*) gene was used as an internal control for normalization (Jiao et al. 2015). The expression levels of the *CalS* genes in different grape cultivars were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) using the specific primers shown in Supplementary Table S1. The specificity of each primer pair was evaluated through melting curve analysis and a single DNA fragment was amplified for each gene as only one melting peak was detected for each primer pair (Supplementary Fig. S1). For the pathogen treatment assay, data were quantified using $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001) and the level of the *CalS* transcript at 0 hpi was set at 1.0. The relative expression was represented as the fold changes by comparing *P. viticola*-treated sample and water-treated sample at the same time point. All the statistical analysis for the data were performed with one-way analysis of variance (ANOVA) or Student's *t* test using SPSS 16.0 (SPSS Inc.) at the 95% confidence level.

Identification of *CalS* sequences. The *CalS* family genes of grape were identified using BlastN queries of grape genome ([http://](http://www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html)

www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html). The sequences of different *CalS* family genes from *Arabidopsis*, cotton, and barley were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The multiple sequence alignment and phylogenetic tree construction were performed using ClustalX1.8 (Thompson et al. 1994) and MEGA4 (Tamura et al. 2007). The conserved domains within *CalS* proteins were online predicted using NCBI's Conserved Domain Database and SMART (<http://smart.embl-heidelberg.de/>).

Promoter-GUS constructs. Promoter regions of the two genes, *CalS1* and *CalS10*, were amplified by PCR using genomic DNA of grapevine leaves. The primers for each *CalS* gene promoter were designed based on the sequences 2-kb upstream from the translational start codons (Supplementary Table S2). PCR-amplified fragments were cloned into PGEM-T Easy vector, verified by DNA sequencing, and subcloned into binary expression vector pBI121 as a fusion with β -glucuronidase (GUS, as a reporter) coding region. The *cis*-acting elements prediction within the promoter region was analyzed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

GUS activity assay. The expression constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 and transformed into the first unfolded leaf of in vitro-grown *V. vinifera* 'Thompson Seedless' plantlets by the vacuum infiltration method (Santos-Rosa et al. 2008; Xu et al. 2010). After the vacuum infiltration, leaves were dried on sterile filter paper and transferred to 0.8% agar plates, which were incubated at 25°C in dark for 1 day, and then with a 16:8 h light/dark cycle for another day. After the incubation, leaves were inoculated with *P. viticola* suspension (1×10^5 sporangia/ml) or sprayed with 1 mM ABA/1 mM GA. The treated leaves were kept in the 0.8% agar plates (16 h light, 28°C/8 h dark, 25°C). Samples for histochemical staining and GUS quantification were collected 24 h after treatments.

Histochemical staining of GUS expression was performed as described by Jefferson (1987). After staining, the chlorophyll was completely removed from leaf tissues by successive washes with 75 to 100% ethanol. Finally, pictures for the treated leaf samples were taken with a SONY camera. The quantification of GUS activity was measured as described by Jefferson (1987) and expressed as nanomoles of 4-methylumbelliferone (4-MU) produced per minute per milligram of protein.

RESULTS

Isolation and characterization of *CalS* in grapevine. When callose synthase sequences of *A. thaliana* were used in BlastN queries against the grape genome sequences (<http://www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html>), eight putative callose synthase (*CalS*) genes were identified and were named according to the *CalS* nomenclature system used in Verma and Hong (2001). These *VvCalS* were designed as *VvCalS1*, *VvCalS3*, *VvCalS5*, *VvCalS7*, *VvCalS8*, *VvCalS9*, *VvCalS10*, and *VvCalS11* based on their sequence similarity to *AtCalS* (Supplementary Table S3). Except *VvCalS11* for which the chromosome location was unknown, the *VvCalS* genes were located on chromosomes 4, 6, 12, 13, 17, and 19. They contained 20 to 45 exons and 30 to 48 introns and their open reading frames (ORF) ranged from 4,908 to 6,159 bp, encoding 1,632 to 2,053 amino-acid polypeptides (Table 1).

One β -1,3-glucan synthase domain and several transmembrane domains which were conserved among *CalS* families were identified in *VvCalS*. The transmembrane domains were positioned in both the C-terminal and the N-terminal of the protein (Fig. 1A). The sequence similarity among the *VvCalS* genes varied from 30 to 80%. For example, *VvCalS1* shares 79.5% identity with *VvCalS3*, while *VvCalS5* only shares 33.3% identity with *VvCalS11*. Phylogenetic analysis among the grape (*Vitis vinifera*) *VvCalS*, *Arabidopsis* (*Arabidopsis thaliana*) *AtCalS*, barley

(*Hordeum vulgare*) HvCalS, and cotton (*Gossypium hirsutum*) GhCalS suggests that these *CalS* families could be divided into four groups and *VvCalS* genes were distributed among them all (Fig. 1B).

Expression of *CalS* genes in response to *P. viticola* infection. The expression patterns of grapevine *CalS* genes in response to *P. viticola* infection were analyzed among grapevines with different resistance levels, including the DM-immune *M. rotundifolia* ‘Noble’ and ‘Carlos’, the resistant *V. amurensis* ‘Shuanghong’, the partially resistant *V. amurensis* ‘Zuoshanyi’, and the susceptible *V. vinifera* ‘Chardonnay’. At early *P. viticola* infection, *CalS1* was significantly up-regulated in the DM-immune *M. rotundifolia* ‘Carlos’ (6 and 12 hpi) and ‘Noble’ (12 hpi), as well as the partially resistant *V. amurensis* ‘Zuoshanyi’ (12 hpi), but no significant changes were detected in other grape cultivars. With the exception of *M. rotundifolia* ‘Noble’, the expression level of *CalS3* was significantly increased in all the cultivars. *CalS7* was remarkably up-regulated in *M. rotundifolia* ‘Carlos’ (6 and 12 hpi) and the

resistant *V. amurensis* ‘Shuanghong’ (6 and 12 hpi). The expression of *CalS10* was significantly increased in the DM-immune muscadine cultivars at 6 and 12 hpi. Its expression was also up-regulated in *V. amurensis* ‘Zuoshanyi’ (12 hpi) and *V. vinifera* ‘Chardonnay’ (6 hpi) (Fig. 2).

The expression of other *CalS* genes, *CalS8*, *CalS9*, and *CalS11*, was not changed significantly in muscadine grapes at both 6 and 12 hpi. *CalS8* was only up-regulated in the susceptible *V. vinifera* ‘Chardonnay’ (6 and 12 hpi). *CalS9* was significantly up-regulated in resistant *V. amurensis* ‘Shuanghong’ at 12 hpi. Similarly, the expression of *CalS11* was increased in ‘Shuanghong’ (12 hpi) and ‘Chardonnay’ (6 hpi) (Fig. 2). The expression of *CalS5* was very low in all grape cultivars and no significant changes were found.

When *CalS* gene expressions of the grapevines were further analyzed from 1 to 8 days after *P. viticola* infection, *CalS1* was up-regulated in the *M. rotundifolia* ‘Carlos’ and ‘Noble’ at 1 dpi. The expression of the former continued to increase at 3 dpi and up to

TABLE 1. *CalS* genes in *Vitis vinifera* ‘Pinot noir’

Gene ^a	Accession number	Number of introns	Number of exons	ORF (bp)	Chromosome location
<i>VvCalS1</i>	GSVIVT01001361001	41	42	5,760	chr13:23971224.0.23998913
<i>VvCalS3</i>	GSVIVT01025362001	41	42	5,847	chr6:1470318.0.1508936
<i>VvCalS5</i>	GSVIVT01026489001	43	44	5,877	chr4:22776367.0.22794921
<i>VvCalS7</i>	GSVIVT01020854001	43	44	5,694	chr12:995099.0.1078999
<i>VvCalS8</i>	GSVIVT01000622001	44	45	6,159	chr19:13989275.0.14016964
<i>VvCalS9</i>	GSVIVT01025370001	30	29	5,715	chr6:1350308.0.1408792
<i>VvCalS10</i>	GSVIVT01007560001	48	49	5,085	chr17:12030876.0.12098518
<i>VvCalS11</i>	GSVIVT01005204001 XM_002263721.1 ^b	–	–	4,908	ChrUn

^a The number to each *VvCalS* gene was assigned on the basis of homology with *AtCalS*.

^b The open reading frame (ORF) of *VvCalS11* was obtained by splicing.

Fig. 1. Sequence analysis of grapevine callose synthase. **A**, Structure domain of *VvCalS* proteins. The deduced amino acid sequences of *Vitis CalS* genes were analyzed by using the SMART search. Transmembrane domains were indicated by the vertical gray bars and glucan synthase domains were indicated by the long black rectangle. **B**, Phylogenetic analysis of grapevine, *Arabidopsis*, barley, and cotton *CalS* proteins using MEGA neighbor-joining. Numbers at the branch points are bootstrap values. *VvCalS*: *Vitis vinifera* *CalS* protein; *AtCalS*: *Arabidopsis thaliana* *CalS* proteins, *AtCalS1* (AF237733.1), *AtCalS2* (NM_179847.1), *AtCalS3* (NM_121303.6), *AtCalS4* (NM_123045.2), *AtCalS5* (NM_179622.2), *AtCalS6* (NM_115772.2), *AtCalS7* (NM_100528.1), *AtCalS8* (NM_112317.1), *AtCalS9* (NM_111596.5), *AtCalS10* (GQ373182.1), *AtCalS11* (NM_116736.1), and *AtCalS12* (NM_116593.3); *HvCalS*: *Hordeum vulgare* *CalS* protein (AY177665); and *GhCalS*: *Gossypium hirsutum* *CalS* protein (AF085717).

sixfold at 5 and 8 dpi, while the later was returned to normal expression level after 1 dpi. The expression of *CalS1* was not changed significantly in other cultivars. *CalS7* was significantly up-regulated at 1, 3, and 5 dpi, but down-regulated at 8 dpi in the *M. rotundifolia* ‘Carlos’. Interestingly, it was down-regulated in another muscadine grape ‘Noble’ at all the sampling time points. In the partially resistant *V. amurensis* ‘Zuoshanyi’, the expression of *CalS7* was increased at 1 dpi and then decreased at 3 dpi. The expression of *CalS10* was dramatically increased in the DM-immune *M. rotundifolia* ‘Carlos’ at 1, 3, and 5 dpi and ‘Noble’ at 3, 5, and 8 dpi. In the DM-resistant *V. amurensis* ‘Shuanghong’, its expression was significantly up-regulated at 8 dpi. In the partially resistant *V. amurensis* ‘Zuoshanyi’, *CalS10* was up-regulated at 1

and 8 dpi. No changes of *CalS10* were found in the DM-susceptible Chardonnay (Fig. 3).

The other three grape *CalS* genes, *CalS3*, *CalS8*, and *CalS11*, were not regulated significantly by *P. viticola* in two immune muscadine grapes. *CalS3* was up-regulated in *V. amurensis* ‘Shuanghong’ (1 dpi), ‘Zuoshanyi’ (1 and 8 dpi) and *V. vinifera* ‘Chardonnay’ (1 and 3 dpi). The expression of *CalS8* was increased in ‘Zuoshanyi’ (1, 5, and 8 dpi) and *V. vinifera* ‘Chardonnay’ (3 and 5 dpi), but decreased in *V. amurensis* ‘Shuanghong’ (1, 5, and 8 dpi). *CalS11* was up-regulated in ‘Shuanghong’ (1 dpi), but down-regulated in ‘Zuoshanyi’ (3 dpi). However, the expression of *CalS9* increased at 3 and 5 dpi in the immune *M. rotundifolia* ‘Noble’, but decreased at 3 dpi in the partially resistant *V. amurensis* ‘Zuoshanyi’

Fig. 2. Expression analysis of *CalS* genes among resistant/susceptible cultivars during the early infection. Grapevine leaves were collected at 6 and 12 h post-*Plasmodiopsis viticola* inoculation. The mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. The relative expression was represented as the fold changes by comparing *P. viticola*-treated sample and water-treated sample at the same time point. ★ indicates a significant difference of gene expression between the *P. viticola*-treated sample and the water-treated sample at the same time point ($P = 0.05$). * indicates a significant difference between 6 h postinoculation (hpi) and 12 hpi at the 0.05 level.

(Fig. 3). *CalS5* was expressed in a very low level in these grape cultivars at all sampling time points.

Isolation and sequence analysis of *CalS1* and *CalS10* gene promoter. Promoters of *CalS1* and *CalS10* genes were chosen for investigating the regulation of gene expression patterns

associated with *P. viticola* infection. Sequences of 2-kb upstream from the translational start codons of *VvCalS1* and *VvCalS10* were amplified from the same five grape cultivars as above. As expected, promoter sequences within the same species are more similar than those amplified from different species (Supplementary Figs. S2 and

Fig. 3. Expression analysis of *CalS* genes among resistant/susceptible cultivars. Grapevine leaves were collected at 1, 3, 5, and 8 days post-*Plasmopara viticola* inoculation. The mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. The relative expression was represented as the fold changes by comparing *P. viticola*-treated sample and water-treated sample at the same time point. ★ indicates a significant difference of gene expression between the *P. viticola*-treated sample and the water-treated sample at the same time point ($P = 0.05$). Different letters in each cultivar are significantly different among different time points at the 0.05 level according to analysis of variance.

TABLE 2. *Cis*-elements associated with pathogen- and stress-responsiveness in *CalS1* promoter among five cultivars

<i>Cis</i> -element	Number of <i>cis</i> -elements					Characterization
	Noble	Carlos	Shuanghong	Zuoshanyi	Chardonnay	
ABRE	1	1	0	0	0	<i>Cis</i> -acting element involved in the abscisic acid responsiveness
ARE	1	1	2	2	0	<i>Cis</i> -acting regulatory element essential for the anaerobic induction
CGTCA-motif	1	1	1	1	1	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness
GARE-motif	2	2	4	4	4	Gibberellin-responsive element
HSE	0	0	0	1	1	<i>Cis</i> -acting element involved in heat stress responsiveness
LTR	0	0	1	1	0	<i>Cis</i> -acting element involved in low-temperature responsiveness
MBS	0	0	2	2	2	MYB binding site involved in drought-inducibility
P-box	2	2	2	2	2	Gibberellin-responsive element
TC-rich repeats	2	2	2	2	2	<i>Cis</i> -acting element involved in defense and stress responsiveness
TCA-element	3	3	3	4	4	<i>Cis</i> -acting element involved in salicylic acid responsiveness
TGA-element	1	1	1	1	1	Auxin-responsive element
TGACG-motif	1	1	1	1	1	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness
WUN-motif	1	1	1	2	1	Wound-responsive element

S3). When the *CalS1* promoter sequences of muscadine grapes were compared with those in other cultivars, several large fragment deletions, several small fragment insertions, and more than 50 SNPs were identified. The *cis*-acting elements analysis demonstrated that several stress- and hormone-regulated elements, such as the abscisic

acid-responsive element ABRE, the MeJA-responsive element CGTCA-motif, the gibberellin-responsive element GARE-motif and the salicylic acid responsive element TCA, were detected in the *CalS1* promoters. While the *cis*-elements were identified among all five cultivars studied, ABRE could only be found in DM-immune

TABLE 3. *Cis*-elements associated with pathogen- and stress-responsiveness in CalS10 promoter among five cultivars

<i>Cis</i> -element	Number of <i>cis</i> -elements					Characterization
	Noble	Carlos	Shuanghong	Zuoshanyi	Chardonnay	
ABRE	1	1	1	3	1	<i>Cis</i> -acting element involved in the abscisic acid responsiveness
ARE	3	4	3	2	3	<i>Cis</i> -acting regulatory element essential for the anaerobic induction
Box-W1	2	2	2	2	2	Fungal elicitor responsive element
CGTCA-motif	2	2	2	2	2	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness
ERE	2	2	2	2	2	Ethylene-responsive element
GARE-motif	0	0	1	1	1	Gibberellin-responsive element
HSE	4	4	5	5	4	<i>Cis</i> -acting element involved in heat stress responsiveness
LTR	2	1	1	1	1	<i>Cis</i> -acting element involved in low-temperature responsiveness
MBS	3	3	3	3	3	MYB binding site involved in drought-inducibility
TC-rich repeats	3	3	2	3	3	<i>Cis</i> -acting element involved in defense and stress responsiveness
TCA-element	1	1	0	1	1	<i>Cis</i> -acting element involved in salicylic acid responsiveness
TGACG-motif	2	2	2	2	2	<i>Cis</i> -acting regulatory element involved in the MeJA-responsiveness

Fig. 4. GUS activity analysis after *Plasmopara viticola* infection. **A**, Structural diagram of vectors used in this experiment. 35S::GUS, vector pBI121; PMrCalS1, *CalS1* promoter from *Muscadinia rotundifolia* 'Noble'; PVvCalS1, *CalS1* promoter from *Vitis vinifera* 'Chardonnay'; PMrCalS10, *CalS10* promoter from *M. rotundifolia* 'Noble'; PVvCalS10, *CalS10* promoter from *V. vinifera* 'Chardonnay'; and PN::GUS, nonpromoter vector pBI121. **B**, Histochemical assay for GUS expression. Grapevine leaves of *V. vinifera* 'Thompson seedless' were agro-infiltrated with each of the plasmids. GUS staining was performed after 24 h of inoculation with water or *P. viticola*. Water, control treatment; PV, infected by *P. viticola*. **C**, Quantitative analysis of GUS expression. Mean GUS activity $\pm$ standard deviation ($\pm$ S.D.) is averaged from three independent experiments ($n = 3$). S.D. is indicated on each bar. * indicates a significant difference ($P = 0.05$) of GUS activity.

muscadine grapes. However, the *cis*-acting element involved in heat stress responsiveness, the *cis*-acting element involved in low-temperature responsiveness, and MYB binding site were not found in the muscadine grapes and the number of GARE-motifs was less in the muscadine grapes than in other cultivars (Table 2).

Analyzing the promoter sequences of *CalS1* revealed that a small fragment deletion, three small fragment insertions, and more than 50 SNPs were found in DM-immune ‘Noble’ and ‘Carlos’, when they were compared with those in other cultivars. Although, hormone-regulated elements, such as the abscisic acid-responsive element ABRE, the MeJA-responsive element CGTCA-motif, the gibberellin-responsive element GARE-motif, and the salicylic acid-responsive element TCA, also existed in the *CalS10* promoters, the GARE-motif could only be found in non-muscadine grapes. In addition, the ethylene-responsive element ERE and fungal elicitor responsive element Box-W1 were found in the *CalS10* promoter region, while it did not appear in *CalS1* promoters (Table 3).

Regulatory analysis of *PCalS1* and *PCalS10* after inoculation by *P. viticola*. To further study the pathogen regulation pattern of *CalS1* and *CalS10*, the promoters which have been amplified from the DM-immune *M. rotundifolia* ‘Noble’ and the susceptible *V. vinifera* ‘Chardonnay’ were subcloned into a binary expression vector with β -glucuronidase (GUS) coding region (Fig. 4A) and transiently transformed into *V. vinifera* ‘Thompson Seedless’ leaves, respectively. Histochemical staining assay revealed that the GUS activity could be significantly observed in 35S positive control, while it was varied among different combinations of *CalS*

promoters. The expression of the *GUS* in *PMrCalS1* and *PMrCalS10* was induced by *P. viticola* inoculation, while no significant change was observed in *PVvCalS1* or *PVvCalS10* under the same treatment (Fig. 4B). The quantification analysis of GUS activity also clearly demonstrated that they were significantly up-regulated by *PMrCalS1* and *PMrCalS10* after *P. viticola* infection. However, there were no significant difference in GUS activities between water treatment and *P. viticola* inoculation in grapevine leaves with PN (nonpromoter), 35S, *PVvCalS1*, and *PVvCalS10* (Fig. 4C).

Regulation of *CalS1* and *CalS10* by ABA and GA. To further study the role of *CalS1* and *CalS10* in responding to ABA treatment, the immune *M. rotundifolia* ‘Noble’ and the susceptible *V. vinifera* ‘Chardonnay’ were treated with 100 μ M ABA. Twelve hours after ABA treatment, the expression of *CalS1* was significantly up-regulated (sixfold) in ‘Noble’, but significantly down-regulated in ‘Chardonnay’. However, the expression of *CalS10* was not affected by the ABA treatment in either cultivar (Fig. 5A). The histochemical staining assay and quantification analysis also showed that the expression of the *PMrCalS1*-*GUS* was increased, but the expression of the *PVvCalS1*-*GUS* was decreased after ABA treatment. No significant GUS changes were observed under the *CalS10* promoter after ABA treatment (Fig. 5B and C).

In terms of the GA treatment assay, the expression of *PVvCalS10*-*GUS* was induced, but the expression of the *PVvCalS1*-*GUS* was suppressed. No significant GUS changes were observed under the *PMrCalS1* and the *PMrCalS10* promoter (Fig. 5B and C).

Fig. 5. GUS activity analysis after ABA and GA treatment. **A**, Expression analysis of *CalS1* and *CalS10* in response to ABA treatment. Leaves sprayed with water were used as control. The expression level of *CalS* in the control leaves is set at 1.0 and the expression levels were normalized to that of *EF1- α* . Data are the mean $\pm$ standard deviation ($\pm$ S.D.) and error bars indicate standard error of means ($n = 3$). * indicates a significant difference ($P = 0.05$). **B**, Histochemical assay for GUS expression. GUS staining was performed after 24 h of inoculation with water, ABA or GA. Water, control treatment; ABA, treated by 1 mM ABA solution; GA, treated by 1 mM GA solution. The experiment was repeated three times. **C**, Quantitative analysis of GUS expression. Mean GUS activity $\pm$ S.D. is averaged from three independent experiments ($n = 3$). S.D. is indicated on each bar. * indicates a significant difference ($P = 0.05$) of GUS activity.

DISCUSSION

By comparing the sequence of *A. thaliana* callose synthase genes, eight *VvCalS* genes were identified in the *V. vinifera* 'Pinot Noir' genome. All grapevine callose synthases appear to be membrane proteins, with 7 to 10 transmembrane domains. Among the eight *CalS* genes tested in grapevine leaves, *CalS5* was expressed at a very low level. It is possible that *CalS5* plays a role in other tissues, as it has been reported that the expression of *AtCalS* genes was regulated in a tissue-specific manner (Dong et al. 2008).

In *A. thaliana*, *AtCalS 1, 5, 9, 10*, and *12* participate in defense response to pathogen infection (Dong et al. 2008). *AtCalS3, 7*, and *11* play key roles in plant growth and reproduction (Enns et al. 2005; Vatén et al. 2011; Xie et al. 2011). In this study, grapevine *CalS1, 3, 7, 8, 9, 10*, and *11* were all up-regulated by *P. viticola* in all the cultivars tested, suggesting that these *CalS* genes might be involved in defense responses against *P. viticola* attack.

It has been reported that callose deposition could be detected in the stomata (in the guard cell) around the germinating zoospores within 24 h after inoculation (Gindro et al. 2003), and the quantity of callose deposition was correlated to the resistance levels of grapevines (Yu et al. 2012). For example, the DM-immune *M. rotundifolia* showed stronger callose deposition than less resistant grape species 24 h after *P. viticola* inoculation. Gene expression analysis indicated that *CalS1* and *CalS10* were raised quickly in *M. rotundifolia* 'Carlos' and 'Noble' grapevines. *CalS3* may also play a role in the early callose deposition as its expression increased significantly in response to pathogen infection. In the previous study, no callose deposits were observed in the DM-susceptible *V. vinifera* grapevines (Yu et al. 2012). However, gene expression analysis showed a significant up-regulation of *CalS3* and *8* in susceptible *V. vinifera* 'Chardonnay' as early as 6 hpi, even though they did not lead to the callose deposition in the DM-susceptible grapevine.

Previous studies showed that the callose deposition could also be observed around the necrosis of cells surrounding and pathogen haustoria 24 hpi (Gindro et al. 2003; Yu et al. 2012). *CalS1* and *CalS10* were continuously induced after 24 hpi while significant down-regulations were detected in *CalS7, 8, 9*, and *11*. It is clear that the pathogen regulation of callose synthase genes in grapevine was complicated, which is consistent with other reports that callose synthesis played multiple roles in response to pathogen attack (Dong et al. 2008; Jacobs et al. 2003). Additionally, multiple callose synthase genes were up-regulated at the same time point in the same cultivar, suggesting a complementary function of these *CalS* proteins.

Callose deposition was also reported to be tightly correlated with the plant hormone abscisic acid (ABA) (Ton and Mauch-Mani 2004; Ton et al. 2005). A primed accumulation of callose was shown to be regulated by an ABA-dependent signaling pathway (Ton and Mauch-Mani 2004). Flors et al. (2008) found that the low ABA content in the wild-type *A. thaliana* led to a weak induction of callose deposition. Our results were consistent with those studies and suggested a possible connection between the expression of selected callose synthase genes and ABA level in grapevine. It has been reported that β -aminobutyric acid (BABA) could induce resistance against oomycetes in lettuce and *A. thaliana* (Pajot et al. 2001; Zimmerli et al. 2000), as well as in grapevine 'Chasselas' (Hamiduzzaman et al. 2005). This enhanced resistance in grapevine against *P. viticola* was associated with high callose deposition. In our study, a correlation between ABA treatment and *CalS1* expression was observed in the DM-immune muscadine grapes. A dual role of another plant growth regulator GA was reported in *A. thaliana*. The exogenous application of GA resulted in enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000 and susceptibility to *A. brassicicola* in *A. thaliana* (Bari R and Jones 2009). Our results suggested that both grapevine *CalS1* and *CalS10* were regulated by GA. To elucidate the regulation mechanism of

CalS, further studies are necessary to better understand the relationship between *CalS* expression and the hormone signals during *P. viticola* infection.

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