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ORIGINAL ARTICLE

Expression of callose synthase genes and its connection with *Npr1* signaling pathway during pathogen infection

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Abstract Callose synthesis occurs at specific stages of plant cell wall development in all cell types, and in response to pathogen attack, wounding and physiological stresses. We determined the expression pattern of "*upstream regulatory sequence*" of 12 *Arabidopsis* callose synthase genes (*CalS1–12*) genes and demonstrated that different callose synthases are expressed specifically in different tissues during plant development. That multiple *CalS* genes are expressed in the same cell type suggests the possibility that CalS complex may be constituted by heteromeric subunits. Five *CalS* genes were induced by pathogen (*Hyaloperonospora arabidopsis*, previously known as *Peronospora parasitica*, the causal agent of downy mildew) or salicylic acid (SA), while the other seven *CalS* genes were not affected by these treatments. Among the genes that are induced, *CalS1* and *CalS12* showed the highest responses. In *Arabidopsis npr1* mutant, impaired in response of pathogenesis related (*PR*) genes to SA, the induction of *CalS1* and *CalS12* genes by the SA or pathogen treatments was significantly reduced. The patterns of expression of the other three *CalS* genes were not changed significantly in the *npr1* mutant. These results suggest that the high induction observed of *CalS1* and *CalS12* is *Npr1* dependent while the weak induction of five *CalS* genes is *Npr1* independent. In a T-DNA knockout mutant of *CalS12*, callose encasement around the haustoria on the infected leaves was reduced and the mutant was found to be more resistant to downy mildew as compared to the wild type plants.

Keywords Callose · Downy mildew · Gene expression · Tissue specific promoter · Salicylic acid · Plant pathogen · Cell wall

Abbreviations

as-1	Activating sequence 1
BTH	Benzothiadiazole
CalS	Callose synthase
Gsl	Glucan synthase-like
GUS	*β*-glucuronidase
JA	Jasmonic acid
MeJA	Methyl jasmonic acid
NahG	Naphthalene (salicylate) hydroxylase G
Npr1	Nonexpresser of PR genes
pmr4	Powdery mildew resistant 4
SA	Salicylic acid
SAR	Systemic acquired resistance
TGA	*cis*-acting element TGACG

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TGA-Bzip	The basic leucine zipper transcription factors that recognize *cis*-acting element TGACG
W-box	*cis*-acting element (TTGAC) that is recognized by WRKY proteins
WRKY proteins	DNA-binding proteins containing a highly conserved WRKY sequence

Introduction

During plant development, callose is found at many locations, e.g. the forming cell plate, pollen, pollen tube, seeds, leaf and stem hairs, sieve plates, plasmodesmata, transient walls of the microsporogenic and megasporogenic tissues, secondary walls and at the developing xylem. Other biotic (pathogen infection and wounding) and abiotic (desiccation, treatment with heavy metals and environmental stresses) factors also induce the synthesis of callose (Stone and Clarke 1992; Verma and Hong 2001; Gregory et al. 2002). The *Arabidopsis* genome contains 12 callose synthase (*CalS*) genes (Verma and Hong 2001), also referred as glucan synthase-like (*Gsl*) genes (Richmond and Somerville 2001). *CalS* genes encode large trans-membrane proteins (Hong et al. 2001a), contain 1–49 introns and located on different chromosomes indicating divergence of this gene family (Hong et al. 2001b). Overexpression of *Arabidopsis CalS1* cDNA in tobacco BY-2 cells enhances callose synthesis at the forming cell plate (Hong et al. 2001b), while disruption of the *CalS12* gene renderes callose-less encasements of papillae in *Arabidopsis* upon pathogen infection (Jacobs et al. 2003; Nishimura et al. 2003). A mutation in *CalS5* gene renders pollen sterile (Dong et al. 2005). Apparently multiple *CalS* genes have evolved in plants to meet the need of callose synthesis in different locations and in response to different physiological and developmental signals (Verma and Hong 2001). Little is known about the regulation of expression of *CalS* genes either during development or due to external signals. The composition of the callose synthase complex is also not known (Verma and Hong 2001) and it is possible that this complex is composed of multiple homologous or heterologous CalS subunits. Callose synthase complex interacts with phragmoplastin, UDP-glucose transferase (Ugt1), Rho1 like protein (Rop1) and possibly annexin, depositing callose in different locations in response to specific developmental, biotic and abiotic signals. *CalS9* and *CalS10* genes (also referred to as *Gsl10* and *Gsl8*, respectively) are important for gametophyte development in plants (Toller et al. 2008).

Callose production is associated with defenses against fungal and oomycete pathogens, as well as the hypersensitive response elicited by diverse pathogens on nonhost species (Stone and Clarke 1992). *Hyaloperonospora arabidopsis* (previously known as *Peronospora parasitica*) is a naturally occurring oomycete parasite of *Arabidopsis*. It spreads *via* the production of sexual oospores and vegetative conidiospores. Upon contact with young leaf surface, conidiospores germinate within few hours, develop hyphal networks within leaf tissues and produce haustoria that invade the host cells. In response, the host cells synthesize and deposit callose around the haustoria (Donofrio and Delaney 2001).

Plants have adopted a variety of induced defense systems for protection against the invading pathogens and insects. These defense responses are regulated by cross communicating signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA) and ethylene play key roles (Thomma et al. 1998; van Wees et al. 2003). Upon pathogen infection, plants increase SA level which induces systemic acquired resistance (SAR). Treatment with the synthetic compound benzothiadiazole (BTH) enhances the expression of several resistance genes, as well as increases callose deposition (Kohler et al. 2002).

Little is known about the signal transduction pathway leading to callose synthesis during plant–pathogen interactions. *Npr1* gene functions in a signaling pathway leading to the induction of pathogenesis related (PR) genes and the onset of SAR (Ryals et al. 1996; Cao et al. 1997). Npr1 protein interacts with the transcription factors Tga2, Tga3, Tga5, and Tga6 (Zhang et al. 1999; Després et al. 2000; Zhou et al. 2000; Fan and Dong 2002), which bind to the *as-1 cis*-element (TGACG motifs) in the cognate gene (Lam et al. 1989). It has been demonstrated that NPR1 plays an important regulatory role in plant defense (Cao et al. 1994, 1997; Zhang et al. 1999; Despres et al. 2000; Zhou et al. 2000; Fan and Dong 2002; Pieterse and Van Loon 2004). However, it is not known if Npr1 is required for the induction of callose synthesis.

We report here that different *CalS* genes are expressed in a tissue-specific manner, and five *CalS* genes are induced by the treatment with SA or *H. arabidopsis*. Furthermore, expression analysis of the *CalS* "upstream regulatory sequence" (henceforth, it is termed as promoter) in the WT and *npr1* backgrounds revealed an Npr1 dependent *CalS1* and *CalS12* induction and an Npr1 independent induction of the three other *CalS* genes. These results suggest that there are at least two SA signaling pathways involved in the induction of different *CalS* genes during pathogen infection.

Materials and methods

Promoter–reporter constructs and plant transformations

Promoter elements of the 12 *CalS* genes were amplified by PCR using genomic DNA from *Arabidopsis thaliana*, ecotype Columbia. The forward primers for each *CalS* gene

(see Supplemental Table I) were designed to start from the end of the immediate upstream gene, or 2 kb upstream from the translational start codons, except for *CalS1* promoter for which about 2.5 kb upstream sequence was used. The reverse primers were designed to amplify part of the coding regions of each *CalS* gene (20 amino acid residues from the N-termini for proper membrane localization of the reporter protein). PCR-amplified fragments were cloned in pCR2.1 vector (Invitrogen, Carlsbad, CA), verified by DNA sequencing, and subcloned into pBI101.2 vector (DB Biosciences, Palo Alto, CA) as a translational fusion in frame with the β-glucuronidase (GUS) coding region.

Oomycete infection

The oomycete *H. arabidopsis* isolate Emco5 was maintained on *Arabidopsis* Ws-0 through weekly culture onto 2-week-old plants (Donofrio and Delaney 2001). One-week-old seedlings of wild type (Col-0), *npr1-3*, *nahG*, *calS1*, *calS12* and transgenic plants containing *CalS::GUS* constructs were spray-inoculated with a conidiospore suspension of approximately $5–8 \times 10^4$ conidiospores per milliliter of water. Plants were harvested 4 days post inoculation for GUS activity assay, RNA extraction and microscope analysis.

Histochemical staining and GUS activity assay

Histochemical staining of GUS expression was performed as described (Jefferson et al. 1987). After staining, chlorophyll was extracted from photosynthetic tissues with 70% ethanol. For each of the *CalS::GUS* constructs, 15 transgenic lines were analyzed and one representative line was selected for further experiments. For activity assay, 4-week-old plants were sprayed with 2 mM SA, 50 μM MeJA (in 0.1% ethanol), or 20 μM H_2O_2. Salicylic acid was dissolved in water as 2 mM solution and adjusted to pH 7.0 with KOH. Leaves were collected 24 h after the treatment and homogenized in extraction buffer (50 mM NaH_2PO_4 pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM β-mercaptoethanol). The GUS activity in the supernatant was measured (Jefferson et al. 1987) and expressed as nanomoles of 4-methylumbelliferone produced per minute per milligram total protein.

RNA extraction and RT-PCR

Rosette leaves were harvested 6 h after treatment with 2 mM SA or 4 days after conidiospore inoculation. Total RNA was isolated using the Trizol kit (Invitrogen). Reverse transcription of RNA (50 ng) was carried out using SuperScript II (Invitrogen) and PCR was performed for 25 cycles (94°C-1 min, 55°C-1 min, 72°C-1 min). Primers (see Supplemental Table II) specific to each *CalS* gene were designed to amplify PCR products of approximately 800–1,000 bp. RT-PCR of *actin-2* gene served as an internal control.

Genomic DNA extraction and isolation of a T-DNA insertion line

Seedlings of T-DNA insertion lines Salk_142792 for *cals1* and Salk_002911 for *cals12* were grown. One fully expanded leaf of a 4-week-old plant was used to extract genomic DNA. Two *CalS* gene-specific primers (CS1-LP 5′-AGAAGATCGCAAAGGTCAAACCAAT-3′ and CS1-RP 5′-AGGAAAGTCAAAGCATTCTGTGTGG-3′ for *CalS1* or CS12-LP 5′-CGCTTGACTTGACTGTACAAG CTG-3′ and CS12-RP 5′-AAGAAAGCAATCCGCCGT-C TC-3′ for *CalS12*) and one T-DNA primer (LBb1 5′-GC GTGGACCGCTTGCTGCAACT-3′) were used in PCR using genomic DNA as template (Siebert et al. 1995). Genomic DNA from wild type plants produced a PCR product of approximate 900 bp, whereas homozygous plants produced a 650 bp PCR band for *cals1* and 690 bp for *cals12*. Both PCR bands were present using genomic DNA from heterozygous plants. Seeds from a homozygous mutant lines, designated as *cals1* and *cals12*, were collected and used for further experiments.

Callose staining

Rosette leaves from wild type, *cals1*, *cals12*, *npr1* and *nahG* plants were fixed in ethanol/acetic acid (3:1, v/v) and stained with 0.01% aniline-blue (Hong et al. 2001b). The tissue was viewed in a fluorescent microscope using a UV filter.

Results

Tissue-specific expression of *CalS* genes

To localize the expression of different *CalS* genes at the cellular level, we cloned promoters of all 12 *CalS* genes of *Arabidopsis* and fused them with the *UidA* gene (GUS, Fig. 1). *Arabidopsis* plants expressing different *CalS::GUS* constructs were produced and used for histochemical analysis of GUS activity in various plant tissues. The expression of *CalS::GUS* in different tissues is summarized in Table I. All *CalS* promoters, except *CalS4*, were found to be expressed in the root (Table 1; Fig. 2c). *CalS1*, *2*, *3*, *5*, *9*, *10* and *11* were expressed in the cells of the entire root tip including root meristematic zone and vasculature. In contrast, *CalS6*, *7* and *8* were expressed only in the vasculature of the elongation zone. *CalS12* was expressed strongly at the root tip and weakly in the vasculature of elongation zone. *CalS1*, *3* and *9* were also expressed in root hairs (see

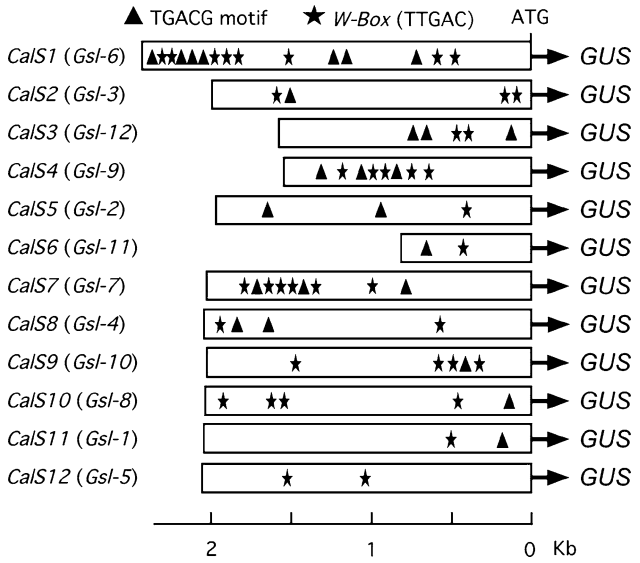

Fig. 1 *CalS::GUS* constructs and the presence of *cis*-elements in various *CalS* promoters. *CalS* promoters amplified from *Arabidopsis* genomic DNA were ligated in frame with the *UidA* gene (GUS coding region) and used for plant transformation. For the *CalS3*, *4* and *6* genes, the promoters were defined to start from the end of the immediate upstream gene. For the rest of the genes, a 2 kb fragment was used, except for the *CalS1* promoter (2.5 kb). The presence of TGACG motif and Wbox are marked by *solid triangle* and *star*, respectively. Alternative names of each *CalS* gene are included in *parenthesis*

Table 1 Expression of *CalS:GUS* in different tissues of transgenic plants

Tissue	*CalS* genes expression											
	1	2	3	4	5	6	7	8	9	10	11	12
Root tip	+	+	+	–	+	–	–	–	+	+	+	+
Root hair	+	–	+	–	–	–	–	–	+	–	–	–
Root elongation	+	+	+	–	+	+	+	+	+	+	+	+
Stem	+	+	+	–	+	+	+	+	+	+	+	+
Cotyledon	+	+	+	–	+	+	+	+	+	+	+	–
Leaf	+	+	+	–	+	+	+	+	+	+	+	–
Vascular	+	+	+	–	+	+	+	+	+	+	+	–
SAM	+	+	+	–	+	–	–	–	+	–	–	–
Pollen	–	–	–	–	+	–	–	–	+	+	+	+
Embryo	–	+	+	–	+	–	–	–	+	+	–	+
Silique	+	+	+	–	+	+	+	+	+	+	+	–
Petal	–	–	–	–	–	–	–	–	–	–	+	–

Transgenic plants expressing *CalS:GUS* were incubated in GUS staining solution overnight and GUS expression was scored as detected (+) and not detected (–)

Table 1; Fig. 2c). Except *CalS4*, all other promoters seem to be active in quiescent center and vasculature tissue of the roots.

Expression of all *CalS* promoters, except *CalS4* and *12* were detected in the cotyledons and primary leaves of the seedling (Fig. 2a, b). The expression of *CalS12* promoter was undetectable in uninfected leaves, but became intensive around the infection sites when the leaves were challenged with the pathogen *H. arabidopsis* (Fig. 2b, CS12), indicating that the expression of this gene is induced by pathogen infection. The expression of *CalS4* promoter was confined specifically within the axillary meristem (Fig. 2a), whereas *CalS 1*, *2*, *3,* 5, and *9* were expressed in the entire shoot meristem region.

In flowers, the expression of *CalS1* and *4* was undetectable, while *CalS2*, *3*, *6*, *7*, *8* and *11* were found to be co-expressed in anthers and filaments, the connective tissue (Fig. 3a). Co-expression of multiple *CalS* genes in the same tissue suggests that these genes may be functionally redundant or their gene products may form a heteromeric complex. Similarly, *CalS5*, *9*, *10*, and *12* promoters were co-expressed in the pollen grains and embryos (Table 1). The expression of *CalS2*, *3*, *9* and *10* was found in both carpels and embryos, whereas *CalS1 and CalS11* were expressed only weakly in the carpels but not in embryos. *CalS6*, *7* and *8* were expressed in the funicular and vascular tissue of the carpel, while weak expression of the *CalS6* promoter was observed in these tissues. No activity of *CalS4::GUS* was detected in the pollen grains, carpel and embryo, and this gene was used as a negative control (Fig. 3b–d).

Induction of *CalS* genes by SA

To understand the signal transduction pathway leading to the induction of *CalS* genes in response to pathogen attacks and physical wounding, 4-week-old transgenic plants containing different *CalS::GUS* constructs were treated with various chemicals (SA, JA or H_2O_2) and GUS activities of the leaf extracts were measured after 24 h of treatment. As shown in Fig. 4, five *CalS* promoters (*CalS1*, *5*, *9*, *10*, *12*) were found to be induced by the SA treatment as compared to the mock (water) control. The most profound induction was observed for *CalS1* and *CalS12* genes, with a more than two-fold increase over the control (Student's *t* test, $P < 0.05$). A moderate induction (ranging from 50 to 200% increase) was observed for *CalS5*, *9 and 10*. SA treatment did not have any apparent effect on the rest of the *CalS* genes (*CalS2*, *3*, *4*, *6*, *7*, *8 and 11*). Treatment with methyl-jasmonic acid (MeJA), a JA derivative, did not show any significant effect on the expression of any of the *CalS* promoters. SA- and JA dependent defense pathways have been shown to cross-communicate, providing the plant with a regulatory potential to fine tune the defense reaction depending on the type of infection or physical wound (Pieterse and van Loon 1999; Felton and Korth 2000; Feys and Parker 2000; Pieterse and Dicke 2007).

The results obtained using GUS induction was verified independently by RT-PCR. Total RNA isolated from 4-

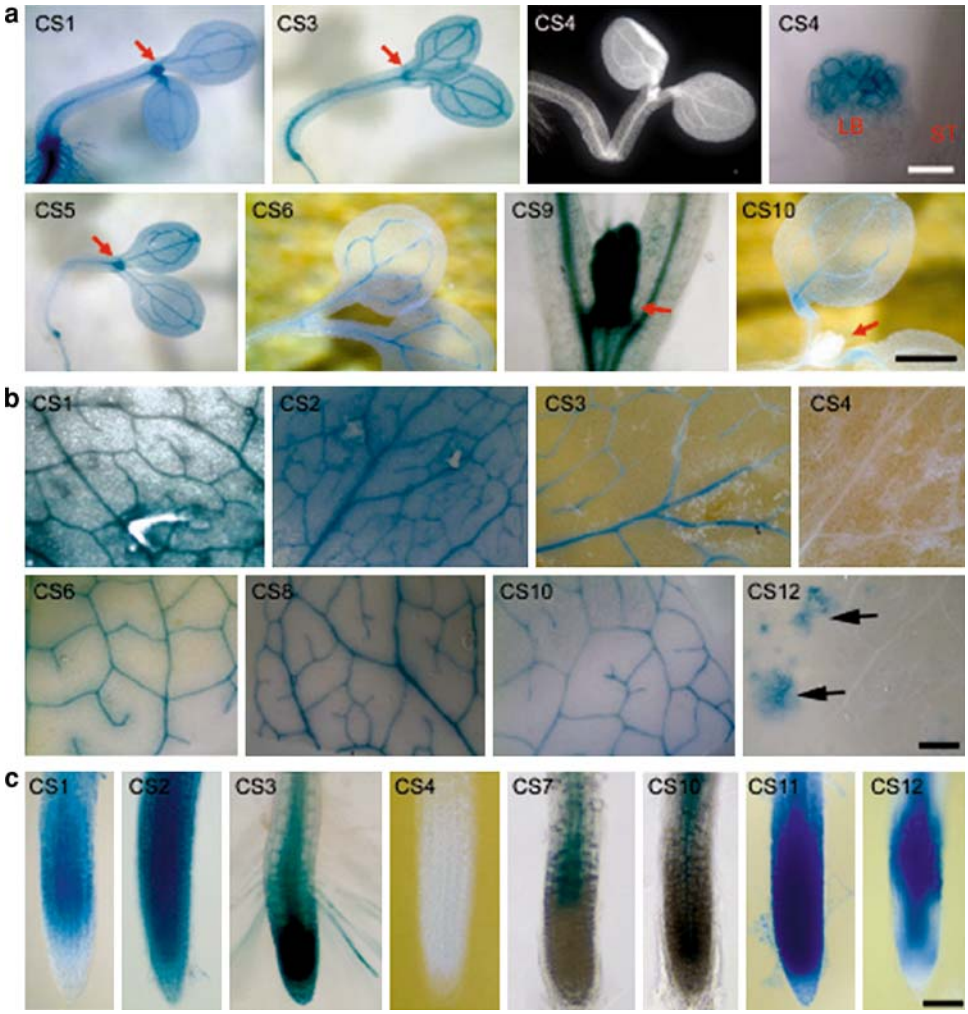

Fig. 2 *CalS::GUS* expression in vegetative tissues of transgenic plants. **a** Seedlings. *Arrows* indicate shoot apical meristems. *CalS4::GUS* (CS4) expression was observed only at the lateral bud (*LB*) but not in the stem (*ST*) or other parts of the plant. CS, *CalS::GUS*. *Bar* 2 mm except in CS4 where *bar* 0.03 mm. **b** Leaves. *CalS12::GUS* (CS12) was not expressed in the leaf, but its expression was induced by pathogen infection (*arrows* indicate infection sites). *Bar* 1 mm. **c** Roots. *Bar* 0.1 mm. *CalS* = CS

week-old wild type plants treated with water (mock control) or SA for 6 h was used in RT-PCR with *CalS* specific primers (Supplemental Table II). *CalS1* and *CalS12* transcripts were up-regulated 6 h after SA treatment (Fig. 5a left panel). These transcripts continued to increase till 24 h after the treatment (data not shown). No significant induction upon SA treatment was detected by RT-PCR for the rest of the *CalS* genes, including *CalS5*, *9* and *10*, which showed 50 to 200% increases in the GUS activity. This may be due to stability of GUS protein or actual promoter sequence in plants may be different as compared to the 5′upstream regulatory sequences we have cloned and used as putative promoters. The RT-PCR results on the induction of *CalS1* and *CalS12* by SA treatment are consistent with those observed using the GUS activity assay (Fig. 4).

Npr1 requirement for the induction of *CalS* genes by SA treatment

We searched the *CalS* promoters for the presence of the TGACG motif and *W-box* (TTGAC), the two most important regulatory motifs implicated in pathogen-induced gene expression in plants (Zhang et al. 1999; Després et al. 2000; Zhou et al. 2000). One or more copies of TGACG motif and *W-box* were found in all *CalS* promoters, except *CalS12* which contains two *W-box*es but no TGACG motif (Fig. 1). The TGACG motif of the *Arabidopsis PR-1* gene has been shown to serve as a binding site for TGA-bZIP transcription factor, which interacts with the NPR1 protein and mediates *PR-1* gene expression upon SA treatment (Zhang et al. 1999; Després et al. 2000; Zhou et al. 2000; Fan and Dong 2002). Because the TGACG motif is absent in the *CalS12* promoter, we investigated if the induction of *CalS* genes by SA treatment is dependent on the *Npr1*-pathway.

CalS::GUS constructs were used to transform *Arabidopsis npr1* mutant plants. GUS activity measured in transgenic *npr1* plants expressing *CalS1*, *5*, *9*, *10 and 12* were found to be increased (less than two-fold; Fig. 5b) by the SA treatment as compared to that in plants treated with water (Student's *t* test, $P < 0.05$), suggesting that the weak induction of these genes by SA is *Npr1* independent. Interestingly, the

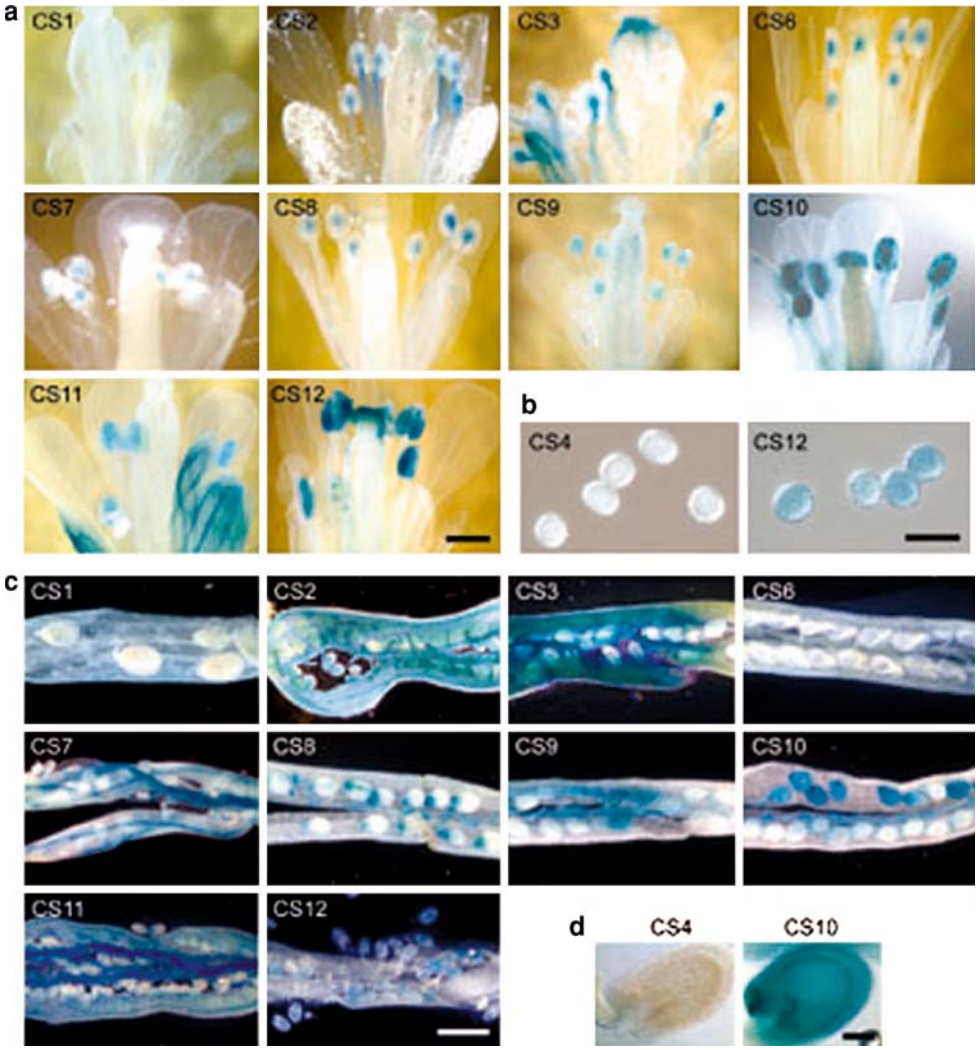

Fig. 3 *CalS::GUS* expression in different reproductive tissues of transgenic plants. **a** Flowers. *Bar* 0.2 mm. **b** Pollen grains. *Bar* 0.05 mm. **c** Siliques. *Bar* 0.2 mm. **d** Developing seeds. *Bar* 0.03 mm

high induction of *CalS1* and *CalS12* promoter by the SA treatment was not observed in the *npr1* mutant background (Fig. 5b), suggesting that the SA signaling pathway leading to the high induction of the *CalS1* and *CalS12* genes is *Npr1* dependent. We also verified these results using RT-PCR approach. The RT-PCR signals for *CalS1* and *CalS12* were increased after SA treatment as compared to the mock control (Fig. 5a right panel). No significant differences in the amount of RT-PCR products before and after SA treatment were found for the rest of the *CalS* genes. Taken together, these data indicate that the high induction of *CalS1* and *CalS12* requires *Npr1*, whereas the weak induction of other *CalS* genes (*CalS1*, *5*, *9*, *10 and 12*) is *Npr1* independent in the SAR signaling pathway.

Induction of *CalS* genes by pathogen infection

The Emco5 isolate of *H. arabidopsis* is capable to infect *Arabidopsis,* Col-0 ecotype (Holub and Beynon 1997; McDowell et al. 1998). One-week-old Col-0 seedlings expressing *CalS::GUS* constructs were challenged with *H. arabidopsis* isolate Emco5. GUS activity was measured 4 days after inoculation. Five *CalS* promoters, *CalS1*, *5*, *9*, *10*, *and 12* were found to be induced by this pathogen treatment (Fig. 6a). These are the promoters of the same five genes that were induced by the SA treatment (Student's *t* test, $P < 0.05$, Fig. 4). The most significant induction was observed in *CalS1* and *12*, which is consistent with our results obtained with the SA treatment (Fig. 4). We also used the RT-PCR approach to verify whether the *CalS1* and *12* transcripts were increased in wild type plants after pathogen infection. Primers specific to each of the 12 *CalS* genes were used to amplify PCR products using RNA samples isolated before or after pathogen infection. As shown in Fig. 6b, *CalS1*, and *12* transcripts were up-regulated significantly 4 days after *H. arabidopsis* inoculation. No significant differences were detected by RT-PCR for the mRNA levels of the rest of the *CalS* genes, which is consistent with results obtained after SA treatment (Fig. 5a, left panel).

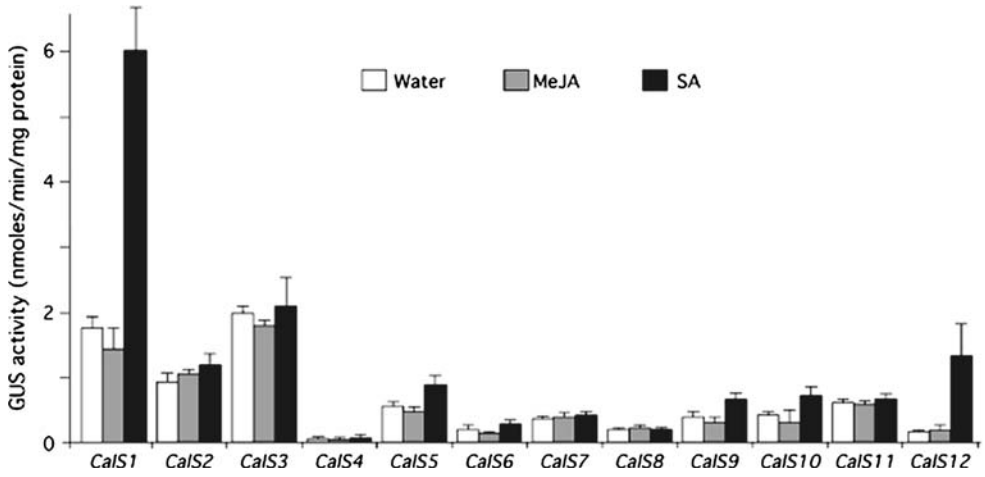

Fig. 4 Induction of *CalS* genes by SA and MeJA. GUS activity in plants expressing *CalS::GUS* constructs in response to treatments with 2 mM SA or 50 μM MeJA for 24 h. Mock treatment with water served as controls. Each value represents the mean of four replicates. The *error bars* correspond to the SD of four replicates

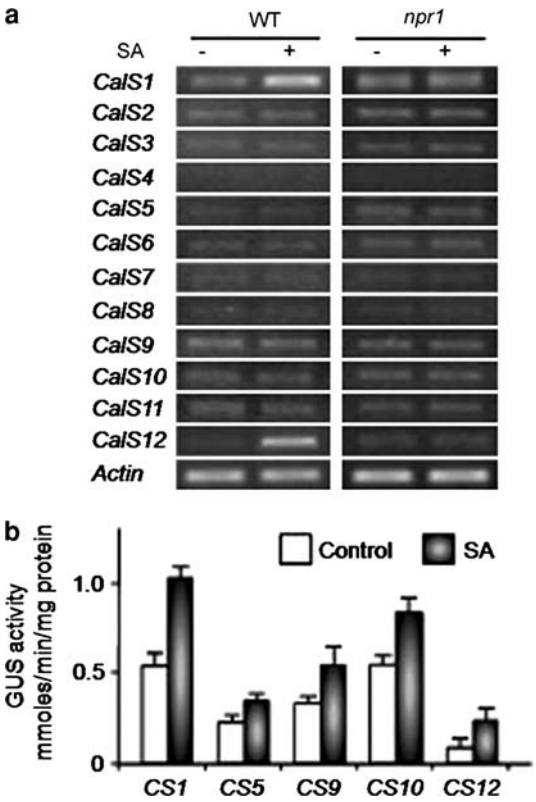

Fig. 5 Induction of *CalS* genes in *npr1* mutant by SA treatment. **a** RT-PCR of *CalS* genes in wild type (*WT*) and *npr1* plants treated with water (−) or 2 mM SA (+) for 6 h. RNA transcript levels of *CalS* genes were measured by RT-PCR using total RNA from leaves. Actin transcript was used as an internal control. **b** GUS activity in *npr1* plants expressing *CalS::GUS* constructs (CS1, 5, 9, 10, 12). GUS activity was measured in protein extracts prepared from leaves 24 h after treatment with 2 mM SA or water (*control*). Each value represents the mean of four replicates. The *error bars* correspond to the SD of four replicate measurements for each line

To test if NPR1 plays a role in pathogen signaling leading to the *CalS* gene expression, *H. arabidopsis* was inoculated onto *npr1* plants containing *CalS1::GUS* and *CalS12::GUS* transgenes. As shown in Fig. 6c, the increase in GUS activity in *npr1* expressing *CalS1::GUS* and *CalS12::GUS* was low after pathogen infection, suggesting that *Npr1* is required for the high level induction of *CalS1* and *CalS12* genes by pathogen infection. This data is consistent with that obtained for the SA treatment (Fig. 5b).

Haustorial callose deposition following infection of downy mildew in *cals1* and *cals12* mutants

One of the significant host responses to a pathogen (*H. arabidopsis)* invasion is the production of callose around the haustoria invaginating the host plasma membrane. To investigate possible roles of pathogen-induced SA signaling and the expression of *CalS1* and *CalS12* genes in the formation of callose encasements around the haustoria, we identified *cals1* and *cals12* T-DNA knockout mutant lines from the Salk *Arabidopsis* T-DNA lines collection. Using genomic DNA from Salk_142792 for *cals1* and Salk_002911 for *cals12* genes and specific primers (Fig. 7a, d), PCR fragments of 937 and 908 bp for *cals1* and *cals12* respectively, were obtained (Fig. 7b, e) while the genomic DNA from homozygous T-DNA mutant plants generated fragments of about 650 bp for *cals1* and 690 bp for *cals12* (Fig. 7c, f). Homozygous T-DNA lines for *cals1* and *cals12* were verified for the absence of *CalS1* or *CalS12* transcripts using RT-PCR. RNA from wild type plants produced a band of 900 bp, whereas the same from the T-DNA lines failed to produce that (Fig. 7c, f). *cals1* and *cals12* mutant lines showed no observable phenotype.

The callose encasements and number of conidiophores in *cals1* and *cals12* mutant lines were compared with that formed in the wild type, *npr1* and *nahG* (transgenic salicylate hydroxylase over-expressing) plants. The *npr1* mutant is known to be defective in the response to SA treatment (Cao et al. 1994; Dong 1998), whereas *nahG* plants are unable to accumulate SA (Ryals et al. 1996). Conidio-

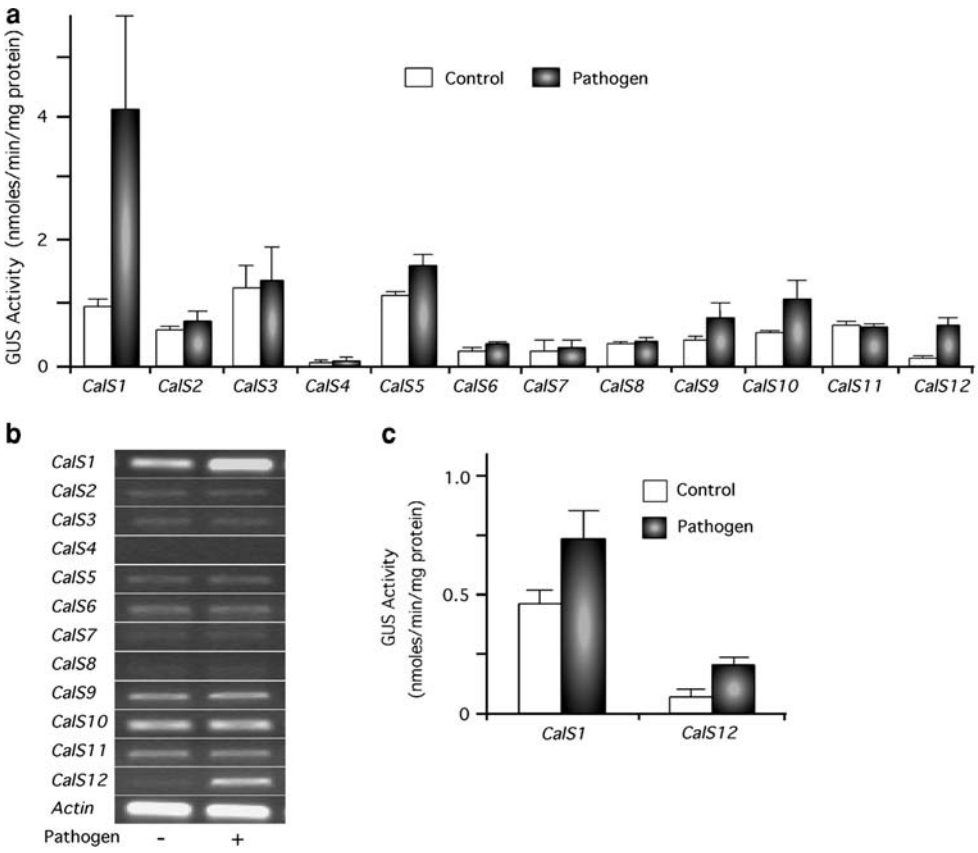

Fig. 6 Induction of *CalS* genes by *Hyaloperonospora arabidopsis* infection. **a** GUS activity in transgenic plants expressing *CalS::GUS* constructs (*CalS1–CalS12*) 4 days post inoculation with water (*control*) *or H. arabidopsis* (*pathogen*). Each value represents the mean of four replicates. The *error bars* correspond to the SD of four replicate measurements for each line. **b** Expression levels of *CalS* transcripts measured by RT-PCR using total RNA from leaves 4 days post inoculation with water (−) or *H. arabidopsis* (*pathogen* +). Actin transcript was used as an internal control. **c** GUS activity in *npr1* plants expressing *CalS::GUS* (*CalS1 and CalS12*) 4 days post inoculation with water (*control*) or *H. arabidopsis* (*pathogen*). Each value represents the mean of four replicates. The *error bars* correspond to the SD of four replicate measurements for each line

phores were produced 6 days after infection. The number of conidiophores per leaf in *cals12* plants was lower than in wild type plants (Student's *t* test, $P < 0.05$, Fig. 8a). There was no significant difference in the number of conidiophores formed per leaf between the *cals1* mutant and the wild type plants (Student's *t* test, $P < 0.05$, Fig. 8a). The *npr1* and *nahG* plants, which are compromised in SA signaling or SA accumulation, were more prolific in conidiophores production per leaf (having 51 ± 7.4 and 81 ± 6.5 conidiophores, respectively; Fig. 8a).

We stained leaves of wild type, *cals1*, *cals12*, *npr1* and *nahG* plants with aniline blue for detecting the presence of callose in infected tissue 6 days post-infection. Three patterns of callose distribution around the haustoria were observed, (a) full encasement by callose; (b) callose deposited in a collar-like form around the base or neck of the haustorium; (c) minimal callose deposition (see also Donofrio and Delaney 2001). All three types of callose distribution were detected in the leaves of the five genotypes studied, but the percentage of each type differed among the genotypes. The frequency of collar-like callose distribution around the haustoria in *cals1* and *cals12* mutants (25%) was not different from that in wild type (25%), but was much lower than that in *npr1* mutant (30%) or *nahG* plants (50%). Callose deposition was reduced significantly around the haustoria in *cals12,* as compared with that in wild type, *cals1*, *npr1* and *nahG* plants. Typical distribution of callose around the haustoria in these genotypes is shown in Fig. 8. It is possible that this different distribution patterns is due to the expression of different CalS isoforms.

Discussion

Expression of different *CalS* genes is regulated in a tissue-specific manner

There are 12 *CalS* genes in *Arabidopsis* distributed over its five chromosomes (Hong et al. 2001a). These *CalS* genes fall into two groups, one (*CalS11* and *CalS12*) containing 2–3 exons and the other (*CalS1–10*) having up to 50 exons. It has been suggested that multiple *CalS* genes may have evolved in higher plants for the synthesis of callose in different locations and in response to different physiological and developmental signals (Verma and Hong 2001).

Our data on *CalS::GUS* transgene analyses have indicated that all *CalS* genes (except *CalS4*) are expressed in the growing root. This may be a combination of several phenomena such as cell division, cell elongation, root hair emergence, vascularization, or constitutive expression to encounter soil pathogens. *CalS4* expression is confined to the primordia of branching axial buds (Fig. 2a). *CalS1*, *2*, *3*,

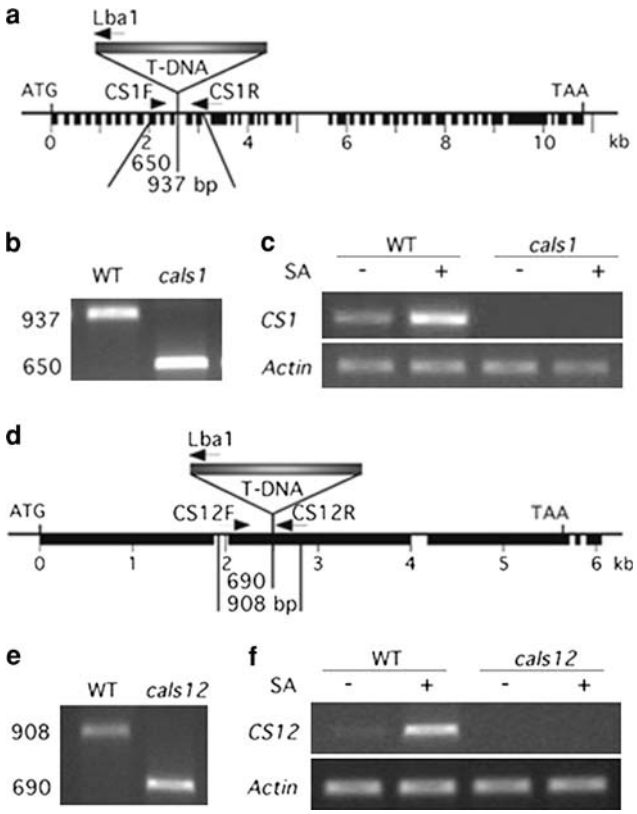

Fig. 7 Characterization of T-DNA lines for *cals1* and *cals12*. **a**, **d** *CalS1* and *CalS12* genes (*thin line*) contains 42 and 5 exons (*thick lines*), respectively. Salk_142792 and Salk_002911 lines have a T-DNA inserted in the *CalS1* and *CalS12* genes. Primers used to identify the homozygous line were marked by *arrows*. PCR products (937 and 650 bp for *CalS1*, and 908 and 690 bp for *CalS12*) of genomic DNA from wild type (*WT*) and homozygous T-DNA lines, respectively, were indicated. **b**, **e** PCR products of genomic DNA from WT and homozygous T-DNA lines. **c**, **f** RT-PCR of total RNA from WT and T-DNA lines prepared 6 h after treatment with water (−) or 2 mM SA (SA+). No RT-PCR products were detected in the T-DNA lines

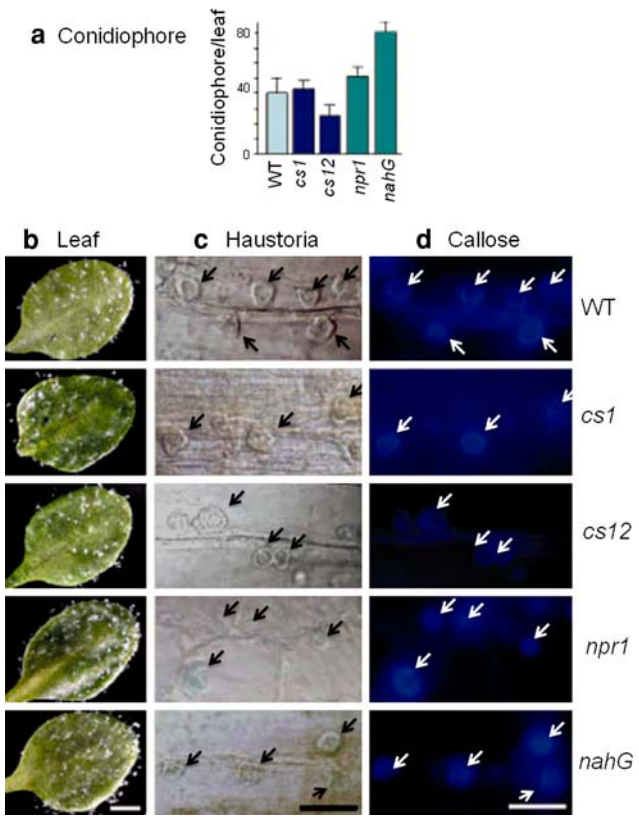

Fig. 8 Conidiophore production and callose deposition in *cals1* and *cals12* plants. **a** Number of conidiophores per leaf in WT plants, *cals1* (cs1), *cals12* (cs12) and plants of two other genotypes defective in SA response (*npr1*) or SA accumulation (*nahG*). The value represents the mean of conidiophores counted on five leaves, each from an individual plant of the same genotype. The *error bars* correspond to the SD of five replicate measurements for each line. **b** Leaf phenotypes and (*cd*) haustoria developed on leaves of WT, *cals1*, *cals12, npr1,* and *nahG* plants 6 days post inoculation with *H. arabidopsis*. Leaves were stained with aniline blue and photographs were taken in bright field (**c**) or with UV light (**d**). *Bar* 1 mm in **b** and 0.025 mm in **c**, **d**

5, *6*, *7*, *8*, *9*, *10*, and *11* were expressed in leaves. Interestingly, *CalS1*, *3* and *9* were also found to be expressed in root hairs. These data show that multiple *CalS* genes are expressed in the same cell-type. *CalS5*, *9*, *10 and 12* exhibited a very similar pattern of expression in pollen grains and developing embryos. These four genes may be responsible for the deposition of callose during reproductive events in *Arabidopsis*. Callose accumulation is important during megasporogenesis, microsporogenesis and pollen tube growth (Tucker et al. 2001; Worrall et al. 1992). *CalS2*, *3*, *6*, *7* and *8* were expressed at the junction of filaments and anthers. Four *CalS* genes are expressed in pollen at a low level while *CalS5* is expressed at a very high level. Mutation in this gene causes partial male sterility (Dong et al. 2005). These results indicate that several CalS isoforms may be required for callose production in a specific tissue. It remains to be tested if different CalS isoforms interact with each other to form heteromeric complexes (Verma and Hong 2001), or they form homomeric complexes that are co-localized. Such an analysis will await dissection of the CalS complexe(s).

We have compared the expression profile of all *CalS* genes in different tissues with some of the gene expression databases like Genvestigator (https://www.genevestigator.ethz.ch/gv/index.jsp) and AtGenExpress (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp). There are some similarities as well as differences among our observed expression profile of *CalS* promoters that we used and the native gene expression profiles. The differences between our observed expression profiles of GUS, driven by promoters of corresponding *CalS* genes and those reported in microarray/gene chip experiments may arise due to several reasons. First the "upstream regulatory elements", we used, may not be the complete promoter

sequence for the cognate *CalS* gene. Second, post-transcriptional regulations of *CalS* transcripts might influence its translation, which is not present for GUS expression. Moreover, GUS protein is very stable and the product of X-Gluc can diffuse in the tissues. These data provide a relative picture of *CalS* gene expression during development as well as during external biotic and abiotic stimuli (Verma and Hong 2001).

Multiple roles of callose in plant development and in response to pathogen attack

Callose encasements have been implicated in the host defense against pathogens by providing a physical barrier that impedes nutrient transfer from the host to the pathogen or possibly delay of pathogen growth long enough for the other host defenses to become active (Allen and Friend 1983). It has also been suggested that callose may reinforce the plant cell wall against pathogen penetration/infection or may provide a mechanical block against toxic compounds or toxins secreted by the pathogen (Kovats et al. 1991; Skou et al. 1984; Stone and Clarke 1992). However, *Arabidopsis Pmr4* and *Gsl5* (*CalS12*) mutant, failed to produce pathogen-induced callose (Jacobs et al. 2003; Nishimura et al. 2003), but was surprisingly more resistant to powdery mildew and downy mildew, rather than being more susceptible as observed by Vogel and Somerville (2000) using *Erysiphe cichoracearum*. It indicates that factor(s) other than callose deposition might also be involved in this phenomenon.

In this study, we identified a T-DNA insertional mutant of *cals12* (Fig. 7) and demonstrated that callose deposition around haustoria was reduced and the plants were more resistant to *H. arabidopsis* (Fig. 8). This result is consistent with the two other recent reports (Jacobs et al. 2003; Nishimura et al. 2003) and suggests a negative role of callose in plant defense against pathogen infection. Callose in papillae may in fact act as a protection layer for the fungus during pathogenesis, and lack of callose may expose the pathogen to the defense machinery of the host plant (Jacobs et al. 2003). It is also possible that pathogen-induced callose may negatively regulate the SA signaling pathway of the plant, and lack of callose in *cals12* mutant may thus enhance the SA signaling leading to the increased resistance to pathogen infection (Nishimura et al. 2003). Therefore, callose may play multiple roles in the interaction between a pathogen and host. SA treatment does not significantly increase expression of any of the *CalS* genes, as per Genvestigator and AtGenExpress databases. All these gene-chip experiments used 10 μM concentration of SA while we used 2 mM in our experiments. Besides the difference in SA concentration, several other reasons, as stated above, might have caused the differences between our GUS expression observations and the data reported in those transcript profiles.

Requirement of NPR1 in the induction of *CalS1* and *CalS12* by SA and pathogens

We demonstrated that five *CalS* genes could be induced by either SA or pathogen treatments of *Arabidopsis*. Among them, *CalS1* and *12* were increased up to fivefold by these treatments. Weak induction of *CalS5*, *9* and *10* by SA and pathogen treatments was detected in transgenic plants expressing *CalS::GUS* constructs. Ostergaard et al. also provided the evidence that *CalS12* (*Gsl5*) is under the control of the SA pathway using northern blot analysis (Ostergaard et al. 2002). Our results suggest that the high induction of *CalS1* and *CalS12* is *Npr1* dependent whereas the weak induction of *CalS1*, *5*, *9*, *10* and *12* genes is *Npr1*independent. The *Arabidopsis* NPR1 plays an important role in inducible plant disease resistance (Johnson et al. 2003; Kinkema et al. 2000; Li et al. 1999; Ryals et al. 1996). The expression of *Npr1* is also induced by SA and pathogen (Cao et al. 1997). *CalS1* promoter contains seven TGACG motif and eight *W-box cis*-elements, and *CalS12* promoter has only two *W-box* elements (Fig. 1). The TGACG motif is recognized by TGA-bZIP transcription factors, and the *Arabidopsis* genome contains 10 *TGA-bZIP* genes. *W-box* elements are recognized by WRKY transcription factors and the *Arabidopsis* genome has 74 *WRKY* genes. A group of *WRKY* genes is known to be induced by SA and pathogen in a Npr1 dependent manner (Yu et al. 2001; Kalde et al. 2003). Therefore, it is likely that Npr1 and distinct members of the *TGA-bZIP* and *WRKY* transcription factor families may function together to mediate the induction of *CalS1* and *CalS12* genes (Fig. 9). A WRKY binding site in the *PR-1* promoter has been shown to be a negative regulatory element in response to SA (Eulgem et al. 2000),

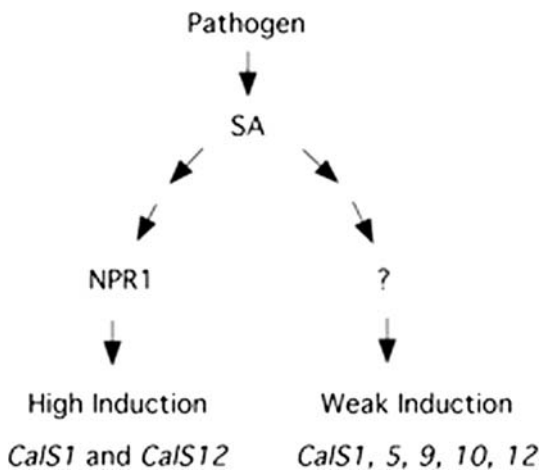

Fig. 9 A model for the regulation of *CalS1* and *CalS12* gene expression. The high induction of *CalS1 and CalS12* genes is regulated in an *Npr1*-dependent manner, whereas the weak induction of *CalS1*, *5*, *9*, *10 and 12* genes may involve unidentified factors and is independent of *Npr1*

whereas the parsley WRKY1 acts as a positive regulator of defense gene expression (Rushton et al. 1996).

The weak induction of *CalS* genes by pathogen infection is *Npr1* independent

Because a subset of *WRKY* genes are known to be induced by pathogen infection independently of the *Npr1* gene function (Yu et al. 2001; Dong et al. 2003; Kalde et al. 2003), the weak induction of *CalS* genes (*CALS1*, *5*, *9*, *10* and *12*) by SA/pathogen may be mediated via the interaction with TGA-bZIP and WRKY transcription factors (Fig. 9). Although a direct interaction between NPR1 and members of the TGA-bZIP family is required for the SA-mediated activation of *PR* genes (Zhang et al. 1999; Després et al. 2000; Subramaniam et al. 2001; Fan and Dong 2002), such an interaction may not be needed for the weak induction of the five *CalS* genes observed here. It is interesting to note that the expression of *Npr1* is induced by SA and pathogen, but such an induction is not abolished in the *npr1* mutant (Cao et al. 1997). This suggests that the induction of *Npr1* itself is Npr1 independent and a separate pathway other than the Npr1 dependent, must be involved in this process. It is possible that the pathway that controls *Npr1* induction is also involved in the weak induction of the *CalS* genes. Other promoter elements besides TGACG motif and *W-box*, and other transcription factors besides TGA-bZIP and WRKY may also be required to achieve precise regulation of different *CalS* genes expressed during SAR.

Transgenic plants expressing inducible *CalS* promoters may be used for the dissection of the signal transduction pathways leading to the induction of *CalS* gene expression in response to pathogen attacks, especially the *Npr1* independent pathway in the SA signaling. It could become a unique model system to study plant–pathogen interaction, since extrahaustorial callose could be visualized under UV after aniline staining which should facilitate screening of the mutants defective in the induction/activation of callose synthases following fungal infection. This system would also allow the identification of other *cis*-regulatory element(s) that are present in the *CalS12* gene that makes it Npr1 independent.

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