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The *Arabidopsis* gene *SIGMA FACTOR-BINDING PROTEIN 1* plays a role in the salicylate- and jasmonate-mediated defence responses

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Abstract

The chloroplast-localized SIB1 protein was previously identified by its interaction with SIGMA FACTOR 1 (SIG1), a component of the RNA polymerase machinery responsible for transcription of plastid genes. The physiological function of SIB1 is little known. We found that expression of *SIB1* is induced by infection with *Pseudomonas syringae*, suggesting its possible involvement in the defence response. The *sib1* loss-of-function mutation compromises induction of some defence-related genes triggered by pathogen infection and the treatments with salicylic acid (SA) and jasmonic acid (JA), two key signalling molecules in the defence response. Conversely, constitutive over-expression of *SIB1* causes the plants to hyper-activate defence-related genes following pathogen infection or the SA and JA treatments, leading to enhanced resistance to infection by *P. syringae*. SIB1 is a member of the large plant-specific VQ motif-containing protein family, and might act as a link to connect defence signalling with chloroplast function.

Keywords

disease resistance; jasmonic acid; salicylic acid; SIB1; sigma factors

INTRODUCTION

Plants use multi-layered defence systems to protect themselves against pathogen attack. In addition to pre-formed physical and biochemical barriers, the induced defence response is triggered when a host receptor recognizes an invading pathogen through microbe-associated

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molecular patterns (MAMPs) (Ausubel 2005; Jones & Dangl 2006). Successful pathogens have evolved various mechanisms to counteract the first layer of host defence. One of the virulence mechanisms employed by pathogens is the delivery of a variety of effector proteins into host cells that suppress the defence response, and modify host physiology to favour pathogen propagation (Abramovitch & Martin 2004; Jones & Dangl 2006; da Cunha, Sreerekha & Mackey 2007). In turn, plants have evolved resistance (R) proteins, each of which directly or indirectly recognizes a cognate effector. This recognition triggers the hypersensitive response (HR) [also known as effector-triggered immunity (ETI)], which often leads to programmed cell death of plant tissue at the attempted invasion site to limit pathogen spread (Jones & Dangl 2006). A pathogen strain expressing a recognized effector, thus triggering the plant's HR, is often called an avirulent strain, whereas the strain that evades recognition, thus causing disease, is called a virulent strain.

Detection of an invading pathogen through its MAMPs and effectors triggers a series of signalling pathways leading to the expression of appropriate plant defence mechanisms. Salicylic acid (SA) and jasmonic acid (JA) are two of the best known defence signalling molecules that are accumulated in response to pathogen infection to activate different sets of defence-related genes (Glazebrook 2005). The SA response pathway is mediated by the protein NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1), a key regulator of both local and systemic acquired resistance (SAR) (Cao *et al.* 1994). SA accumulation alters the redox state of NPR1, which then shuttles from the cytosol to the nucleus where it activates transcription factors (Mou, Fan & Dong 2003; Tada *et al.* 2008). JA mediates a signalling pathway through COI1, an SKP/Cullin/F-box (SCF) E3 ubiquitin ligase (Xie *et al.* 1998), to regulate transcriptional repressors (JAZ proteins) and the transcriptional activator (MYC2), leading to transcriptional reprogramming (Boter *et al.* 2004; Chini *et al.* 2007; Thines *et al.* 2007).

Growing evidence indicates that the chloroplast plays an important role in host-pathogen interaction. Functional chloroplasts are essential for some effector-triggered defence pathways and for the SA-mediated induction of *PATHOGENESIS-RELATED* (*PR*) genes (Genoud *et al.* 2002; Karpinski *et al.* 2003; Zeier *et al.* 2004; Roberts & Paul 2006). Furthermore, it was found that the MAPKcascade mediates the defence response through alteration of chloroplast metabolic activities, leading to generation of reactive oxygen species (ROS) from the chloroplast source (Liu *et al.* 2007). Not surprisingly, pathogens have evolved mechanisms to manipulate chloroplast function to suppress host defence and promote virulence. Many effectors contain chloroplast localization signals (Greenberg & Vinatzer 2003). Several effectors have been reported to target chloroplast proteins (Abbink *et al.* 2002; Fu *et al.* 2007; Jelenska *et al.* 2007; Caplan *et al.* 2008).These findings underline the importance of the chloroplast as a battlefield in host-pathogen interaction.

The plastid genome of a higher plant generally contains 60-200 open reading frames (ORFs) (Leister 2003). Plastid genes are transcribed by either the plastid-encoded RNA polymerase (PEP) or the nucleus-encoded RNA polymerase (NEP) (Kanamaru & Tanaka 2004; Lysenko 2007). NEP is believed to be responsible for overall transcription of the plastid genome, whereas PEP is a prokaryotictype enzyme principally involved in transcription of photosynthesis-related genes. The function of PEP is regulated by the nuclear-encoded sigma subunits (SIGs), which confer promoter recognition specificity and are required for transcription initiation (Kanamaru & Tanaka 2004; Lysenko 2007). Six *SIG* genes (*SIG1-6*) have been identified from the *Arabidopsis* nuclear genome. The *sig2* mutation leads to chlorophyll deficiency (Shirano *et al.* 2000; Privat *et al.* 2003), possibly through its effects on transcription of *psbD, psbJ* and *D1*, as well as several tRNA-encoding plastid genes (Kanamaru *et al.* 2001; Nagashima *et al.* 2004a). SIG3 and SIG4 regulate transcription of *psbN* (Zghidi *et al.* 2007) and *ndhF* (Favory *et al.* 2005), respectively. *SIG5* is induced by

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various abiotic stresses, including blue light irradiation, and controls transcription of *psbD* (Tsunoyama *et al.* 2002, 2004; Nagashima *et al.* 2004b). SIG6 functions in light-dependent chloroplast development, and its loss-of-function mutation affects expression of several plastid genes (Ishizaki *et al.* 2005; Loschelder *et al.* 2006). SIG1 is one of the most abundant sigma factors in *Arabidopsis* (Tanaka *et al.* 1997), but its function has not been defined. The rice *sig1* mutant was found to have reduced chlorophyll content, an increase in transcript levels of at least 10 plastid genes and the reduction in transcript levels of at least 12 other plastid genes (Tozawa *et al.* 2007).

Arabidopsis SIGMA FACTOR-BINDING PROTEIN 1 (SIB1) was previously identified through yeast two-hybrid screening as an interacting protein of AtSIG1, and the SIG1-SIB1 interaction was further verified through a pulldown assay (Morikawa *et al.* 2002). SIB1 contains a putative plastid targeting signal, and the SIB1-GFP fusion was found to be localized in chloroplasts (Morikawa *et al.* 2002). The finding suggests that SIB1 may regulate the function of SIG1. Recently, the *Arabidopsis SIB1* gene was found to be induced by SA (Narusaka *et al.* 2008); however, its biological function remains unclear. Here, we report data suggesting a role of *SIB1* in the disease resistance pathway in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown in a growth room with the following conditions: 22 °C, 50% humidity, a 9/15 h day/night cycle at a light intensity of 125 mol m^{-2} s^{-1} provided by cool white fluorescent bulbs. The plants were fertilized every 2 weeks. Plants used for pathogen inoculation and chemical treatments were approximately 5 weeks old. For seed production, 5-week-old plants were transferred from the growth room to an air-conditioned greenhouse with the same growth conditions, except the light was provided by natural sunlight and supplemented by the fluorescent light bulbs at a 15/9 h day/night cycle.

Pathogens and plant inoculation

Culturing and preparation of *Pseudomonas syringae*, plant inoculation and the *in planta* bacterial growth assay were carried out as previously described (Ge *et al.* 2007). For *in planta* bacterial growth assay, leaves were infiltrated with a bacterial suspension of 5×10^4 cfu mL^{-1}. For preparing pathogen-challenged leaf tissues for RNA extraction, leaves were infiltrated with bacterial suspension of 1×10^7 cfu mL^{-1}.

Botrytis cinerea was maintained in the maltose agar medium (1.5% maltose, 0.3% peptone, 3% glucose and 12 g L^{-1} agar). For spore production, the potato dextrose medium was used (24 g potato dextrose broth and 12 g agar for 1 L medium) for culturing. Leaves were pricked with a needle, and the punched site of each leaf was inoculated by placing 20 μL of spore suspension (with a concentration of 5×10^5 spores mL^{-1}). After the inoculums were dry, the plants were covered for 3 d to maintain high humidity. Symptoms were scored 5-6 d post-inoculation. *Alternaria brassicicola* culturing and inoculation were performed in a similar way, except that the fungus was grown on the V8 medium (163 mL V8 juice, 1.63 g $CaCO_3$ and 12 g agar for 1 L medium) for spore production.

PCR analysis of the *sib1* T-DNA insertion alleles

The T-DNA flanking sequence of SALK_063337 was amplified using the primer pair LBb1 (5′-AGTTGCAGCAAGCGGTCCACGC-3′) and SIB1p1 (CTCTTAC AGGAACCGAACATGGAG). The wild-type (wt) fragment of this locus was amplified using SIB1p1 and SIB1p2r (TTACGATGAGAACTCGATAACCTGA).

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RNA isolation and RNA blot analysis

RNA was isolated using the TRIzol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). RNA electrophoresis, transfer onto nylon membrane and hybridization were performed according tostandard protocols (Sambrook, Fritsch & Maniatis 1989). The probes were labelled using the Ready-to-Go DNA labelling kit (Amersham Biosciences, Piscataway, NJ, USA). The following primers were used to amplify the DNA fragments from genomic DNA for using as the probes in the RNA blotting analysis: 5′-GGTACCGAACATGGAGTCATCATC-3′ and 5′-TCTAGACATAGAATCG ATGCTTCCA-3′ for *SIB1*; 5′-GATCAGTCATCATCAA CGTTGCTC-3′ and 5′-CAGAGAGAACCAATGCTTC CTAAAG-3′ for At2g41180 (T3K9.5). Hybridization and washes were carried out under the high stringent conditions at 65 °C.

Treatments with salicylate (SA) and jasmonate (JA)

SA and JA were purchased from Sigma-Aldrich (St Louis, MO, USA). Plants were sprayed with the SA solution at the concentration of 1 mm, and leaf samples were harvested at different time-points after the treatment. For the JA treatment, plants were sprayed with 100 μM methyl jasmonate.

Construction of 35S::SIB1 transgenic lines

To construct *35S::SIB1*, the *SIB1* genomic fragment without the putative promoter region was amplified through PCR with the primer pair SIB1Kpn (5′-GGTACCGAACATGGAGTCATCATCG-3′) and SIB1Pst (5′-CTGCAGC AGTAACGGGTACATTGGG-3′). The PCR product was cloned into pCR-BluntII-TOPO. The *KpnI*/*PstI* fragment was cut from the TOPO clone, and inserted into downstream of the 35S promoter in the binary vector pCHF3 to generate *35S::SIB1*.

Chlorophyll determination

The relative amount of chlorophyll in leaves was determined using Chlorophyll Meter SPAD-502 (Spectrum Technologies, Plainfield, IL, USA) according to the manufacturer's instruction. For each genotype, eight fully expanded leaves from four 6-week-old plants were measured.

Quantitative real-time reverse transcription PCR (qPCR) analysis

The primers used for the qPCR analysis were designed through Universal ProbeLibrary Assay Design Center, Roche Applied Science (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000). Transcript levels were normalized against constitutively expressed *AtACT2*. The primer sequences are listed in Supporting Information Table S1. Total RNA samples for the analysis were isolated using the TRIzol reagent as described earlier, and purified by RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA, cat. no. 74204), and treated with DNase. cDNA was synthesized with iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA, cat. no. 170-8891). PCR was performed in a 20 μL reaction mixture with SYBR GreenER (Invitrogen, cat. no. 11762) using the following program: 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by one cycle of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s.The instrument used for qPCR was the Mx3005P system from Stratagene (La Jolla, CA, USA).

Affymetrix GeneChip analysis

Leaves of 5-week-old wt plants were inoculated with *Pst* avr*Rpm1* (1×10^7 cfu mL^{-1} suspension), and collected for RNA isolation 6 h post-inoculation. RNA was isolated using the TRIzol extraction method followed by RNA purification using RNeasy MiniElute

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Cleanup kit (Qiagen). Six Affymetrix ATH1 arrays were used to hybridize the RNA samples including three biological replicates for the pathogen-treated wt and *sib1* samples. The original microarray data (.cel files) were background corrected, quantile normalized and summarized for each probe set using the *affy* package (Irizarry *et al.* 2003) in BioConductor with default settings. The summary scores were then analysed probe set by probe set using the MAANOVA package (Wu *et al.* 2003) in BioConductor. A shrinkage-based *t*-test was conducted to compare the two groups (Cui *et al.* 2005). An empirical *P* value was obtained for each probe set based on permutation of the observed data (Yang & Churchill 2007). The fold changes are shown to the genes only if the difference is statistically significant (with a *P* value of <0.05).

RESULTS

Pathogen-triggered induction of *SIB1* is partially dependent on NPR1 and SA accumulation

We have previously carried out a defence transcriptome analysis using the Affymetrix GeneChip to identify *Arabidopsis* genes that are quickly induced by infection with the bacterial pathogen *P. syringae* (Ge *et al.* 2007). One of the pathogen-responsive genes identified in the analysis is *At3g56710*, which encodes the nuclear-encoded and chloroplast-localized SIB1 (Morikawa *et al.* 2002). *SIB1*'s strong induction following pathogen infection suggests it could be a component in a defence response pathway. The result prompted us to examine the role of SIB1 in disease resistance.

We further examined expression patterns of *SIB1* during the host-pathogen interaction through RNA blot analysis. RNA samples were isolated from *Arabidopsis* wt plants (Col-1 ecotype) and the *sib1* mutant (see below) at different time-points following infection with either the virulent *P. syringae* strain DC3000 (*Pst*) or the avirulent *Pst* strain that expresses the type III effector gene *avrRpm1* (*Pst* avr*Rpm1*). A single 0.85 kb band was detected from the wt plants infected with *Pst* avr*Rpm1*, but not from the *sib1* mutant, indicating that the probe only detects the *SIB1*transcripts (Fig. 1a). As shown in Fig. 1b, *SIB1* displayed a similar induction pattern following infection by both *Pst* and *Pst* avr*Rpm1*. The *SIB1* transcripts were barely detectable in uninfected (M) tissues, but started to increase within 1.5 h (h) after infection. The *SIB1* transcripts reached a much higher level at 5 h post-infection (hpi), and its level remained elevated at 24 hpi.

To investigate whether the pathogen-triggered *SIB1* expression is dependent on SA and NPR1, we examined *SIB1*'s expression patterns in *npr1, enhanced disease susceptibility 5* (*eds5*) and *NahG* plants.The *eds5* is defective in SA accumulation (Nawrath *et al.* 2002). The *NahG* plants express the bacterial enzyme salicylate hydroxylase, which not only degrades SA, making the plants defective in disease resistance (Delaney *et al.* 1994), but also produces the degradation product catechol, causing a defect in basal resistance (van Wees & Glazebrook 2003). The RNA blot result showed that pathogen-triggered accumulation of the *SIB1* transcripts was compromised but not abolished in *npr1, eds5* and *NahG* plants (Fig. 1b), indicating that its induction is partially dependent on SA accumulation and NPR1. The result from qPCR analysis further confirmed that pathogen-triggered induction of *SIB1* was compromised by the *npr1* and *eds5* mutation, and by the *NahG* over-expression (Fig. 1c).

In a separate experiment, we compared induction of *SIB1* by *Pst* avr*Rpm1*, and by the *P. syringae* mutant *hrcC*. The strain *Pst hrcC* is defective in delivering type III effectors into host cells, and therefore does not cause disease (Boch *et al.* 2002). The *Pst hrcC* mutant strain was also able to induce *SIB1* expression (Fig. 1d). *SIB1* induction by *Pst hrcC* was slightly weaker than that by *Pst* avr*Rpm1* at 5 hpi; however, *SIB1* transcripts were accumulated at a higher level in the *Pst hrcC*-treated leaves than in the *Pst* avr*Rpm1*-treated

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leaves at 24 hpi, raising a possibility that some type III effector(s) might attenuate *SIB1* expression. The above result also suggests that the pathogen-mediated induction of *SIB1* does not require the effector-triggereddefence response, but is largely triggered by the MAMP-triggered defence response.

T3K9.5 (At2g41180) was previously identified as a homolog of SIB1 that shares over 50% sequence identity at the amino acid level with SIB1, and was also found to interact with SIG1 (Morikawa *et al.* 2002). Transcript levels of *At2g41180* were slightly higher following infection with *Pst* avr*Rpm1* in the wt plants as revealed by the RNA blotting and qPCR analysis (Fig. 1e,f). However, in the *sib1* mutant, the *At2g41180* transcript level was found to be more significantly increased than in the wt plants following the pathogen infection (Fig. 1e,f).

SIB1 is a member of the plant-specific VQ motif-containing protein family

The *SIB1* gene contains a single exon, and encodes a polypeptide with 151 residues. BLAST searches revealed that SIB1 does not share a significant sequence similarly to other proteins with known functions or to any non-plant proteins in the GenBank sequence databases, suggesting that it is a plant-specific protein. SIB1 contains the plant-specific VQ motif that is found in at least 34 predicted *Arabidopsis* proteins (Fig. 2 and Supporting Information Table S2). A group of VQ motif-containing proteins from a variety of plant species were described previously (Andreasson *et al.* 2005). The supplementary data section provides additional information on sequence analysis of VQ motif-containing proteins, and lists the 34 such proteins identified from the *Arabidopsis* sequence database. A large majority of these proteins are small (100-250 amino acids) and share little sequence similarity to other proteins in the GenBank databases. It remains to be determined whether the VQ motif-containing proteins have descended from a common ancestor. Interestingly, genes encoding VQ domain proteins are found in the genomes of higher plants and mosses, but not in the algal genomes, indicating that the VQ domain-containing proteins are unique to land plants.

Among the members of this VQ motif-containing family in *Arabidopsis*, At2g41010 was previously identified as a calmodulin-binding protein (AtCAMBP25) and functions as a negative regulator of osmotic stress tolerance (Perruc *et al.* 2004).Another known member of this family is MKS1 (At3g18690), which was originally identified as a substrate of *Arabidopsis* MAP kinase 4 (MAPK4) (Andreasson *et al.* 2005). MKS1 interacts with both MAPK4 and WRKY tran-scription factors WRKY25 and WRKY 33, and is implicated in the SA-dependent defence pathway by coupling MAPK4 to the WRKY transcription factors (Andreasson *et al.* 2005). In our defence transcriptome results, at least 14 of the *Arabidopsis* genes encoding members of this family were found significantly induced by pathogen infection, and onewas suppressed (Supporting Information Table S2), suggesting that many of them are likely involved in stress responses.

Figure 2 shows a sequence alignment of the VQ motif-containing regions from 25 plant proteins including 17 *Arabidopsis* proteins and two proteins each from rice, grape, moss (*Physcomitrella patens*) and spike moss (*Selaginella moellendorffii*). In the Conserved Domain Database (CDD) (Marchler-Bauer *et al.* 2007), the consensus sequence of the VQ motif is listed as FXhVQChTG (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam05678) where *X* is any amino acid and *h* is a hydrophobic amino acid. However, based on our sequence alignment results (Fig. 2 and data not shown), there are generally three residues between the F and VQ residues, and VQ is not often followed by a C residue. In addition, the two hydrophobic residues are not always conserved. Therefore, we suggest the consensus sequence for the VQ motif to be changed to FXXXVQXXTG.

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Over-expression of *SIB1* enhances resistance to *P. syringae*

We carried out mutational analyses to determine the role of SIB1 in plant defence.

A putative T-DNA insertion line (SALK_063337) for *SIB1* was obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH, USA). A homozygous line for the T-DNA insertion allele was identified from the pooled T4 seedlings through PCR analysis. This line has a single T-DNA insertion just upstream of the stop codon of the *SIB1* ORF. In the RNA blot analysis using RNA isolated from leaves with or without pathogen infection, the normal *SIB1* transcripts were not detected in the homozygous insertion line (Fig. 1a), although a weak band with an abnormal size (approximately 1.2 kb) was detected, indicating that the insertion resulted in knock-out or severe knock-down of the *SIB1* gene. We named this insertion line *sib1-1* (abbreviated as *sib1*). We later obtained another homozygous T-DNA insertion line (SALK_127478C) from the ARBC. SALK_127478C has a T-DNA insertion in the promoter region approximately −200 from the ATG start codon of the *SIB1* ORF. Similarly, *SIB1* transcripts were undetectable from this line (Fig. 3a). SALK_127478C was named *sib1-2*. The *sib1* mutant plants are morphologically indistinguishable from wt plants, although the mutant plants were slightly larger in size (Fig. 3b).

An *in planta* bacterial growth assay was performed to determine whether the *sib1* mutation results in any alteration in resistance to *Pst*. Leaves of 5-week-old plants were inoculated with *Pst*, and leaf discs were collected at 2 and 4 d post-infection (dpi) for counting bacterial numbers. In multiple independent assays, bacterial growth rates in the *sib1* mutant were 1.3- to 3.4-fold higher than in wt plants; however, the differences were found statistically insignificant in three out of four independent experiments (Fig. 3c and data not shown).

Transgenic *Arabidopsis* lines were generated that express *SIB1* under the control of the cauliflower mosaic virus 35S promoter (*35S::SIB1*). Among 20 independent *SIB1*over-expression lines generated for this study, at least 12 lines exhibited various degrees of growth retardation compared with wt plants (Fig. 3b). We choose two transgenic lines (*35S::SIB1-1* and *35S::SIB1-2*) for further analyses. *35S::SIB1-1* plants showed a more pronounced growth retardation, whereas *35S::SIB1-2* plants displayed a moderate growth retardation (Fig. 3b). The RNA blotting result revealed that these two lines constitutively accumulate high levels of *SIB1* transcripts with a higher transcript level in *35S::SIB1-1* than in *35S::SIB1-2* (Fig. 3d). The leaves of the over-expression lines appeared darker than wt leaves, and contained higher chlorophyll content (Fig. 3e).

The results from the *in planta* bacterial growth assay revealed that *SIB1* over-expression leads to enhanced resistance to *Pst* (Fig. 3c). In multiple independent assays, bacterial growth in the *35S::SIB1-1* plants were found to be 2.0- to 6.7-fold lower than in wt plants. The *35S::SIB1*-2 line was also found to be more resistant than wt plants with the bacterial growth rate reduced by 1.8- to 4.2-fold; however, in three out of five independent assays, the difference between *35S::SIB1* and two wt plants was not found to be statistically significant. We did not find any obvious difference between wt, *sib1* and the *SIB1* over-expression lines in appearance of hypersensitive cell death following inoculation with *Pst* avr*Rpm1*.

Mutations in *SIB1* alter induction of defence-related genes triggered by pathogen infection and the treatments with SA and JA

RNA blot analysis was carried out to examine whether the *SIB1* loss- and gain-of-function mutations cause alteration in pathogen-triggered induction of the defence-related genes, *PR1, PR2* and *avrRpt2-INDUCED GENE 1* (*AIG1*). RNA samples were isolated from leaves inoculated with *Pst* avr*Rpm1*, and collected at different time-points following the pathogen infection. We did not find any obvious difference in transcript profiles of *AIG1* between wt

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and the loss- and gain-of-function mutants of *SIB1* (data not shown). However, the two *PR* genes were induced more rapidly and more strongly in the *SIB1* over-expression lines than in wt plants, whereas in the *sib1* loss-of-function mutant, the transcript levels of *PR1* and *PR2* were slightly lower than in the wt plants at 24 hpi (Fig. 4a). Because *PR1* and *PR2* are SA-responsive genes, we further determined whether the mutations of *SIB1* alter the plant response to the SA treatment. As shown in Fig. 4b, the *PR1* gene was strongly induced within 4 h of SA application; however, the *sib1* mutant showed weaker *PR1* induction than wt. This result was confirmed from an independent qPCR analysis (Fig. 4c). Similarly, *sib1-2* was also compromised in SA-triggered *PR1* induction (Fig. 4d). Conversely, induction of *PR1* by SA was significantly enhanced in the *SIB1* over-expression line (Fig. 4b,c).Although *PR2* induction by SA was enhanced in the *SIB1* over-expression line, we did not detect a significant difference in SA-mediated induction patterns of *PR2* between the *sib1, sib1-2* and wt plants (data not shown).

We then carried out a transcriptome analysis to reveal genes whose pathogen-mediated induction might be affected by the *sib1* mutation. Leaves of wt and *sib1* plants were inoculated with *Pst* avr*Rpm1*, and the inoculated leaves were collected at 6 hpi. Total RNA extracted from these leaf samples was used for labelling and for hybridization of the Affymetrix's *Arabidopsis* ATH1 GeneChip. Three biological replicates were included in the experiment. The transcript profiles of the pathogen-infected leaves of wt and *sib1* plants were compared to identify differentially expressed genes between the wt and the *sib1* plants. *PR1* and *PR2* transcript levels were not found to be significantly different between the wt and *sib1* plant at 6 hpi (less than 1.3-fold with a P value of >0.05). However, 64 other genes were expressed over twofold lower in the *sib1* mutant than in the wt plants. Supporting Information Table S3 lists the genes whose transcript levels differ more than twofold between the wt and *sib1* mutant (with a P value of <0.05).

Although infection with biotrophic *Pst* strains generally triggers the SA signalling pathway, some well-known JA-responsive genes, such as *pdf1.2a* and *pdf1.2b*, are also induced by the *Pst* avr*Rpm1* infection. However, the result from the GeneChip analysis showed that the transcript levels of these two genes were 5.4- and 2.3-fold lower, respectively, in the *sib1* mutant than in the wt plants (Supporting Information Table S3). qPCR analysis further confirmed that the *sib1*, as well as *sib1-2*, mutations compromise the pathogen-mediated induction of *pdf1.2a* and *pdf1.2b* (Fig. 5a). In contrast, the *SIB1* over-expression line accumulated higher transcript levels of these two genes in both uninfected and pathogen-infected plants (Fig. 5a). To further reveal whether the loss- and gain-of-function mutants of *SIB1* affect the JA signalling pathway, we used qPCR analysis to compare induction patterns of *pdf1.2a* and *pdf1.2b* following the JA treatment. As shown in Fig. 5b, JA-mediated induction of these two genes was significantly reduced in the *sib1* and *sib1-2* mutants, but was stronger in the *SIB1* over-expression line. Because the JA signalling pathway plays a crucial role in a plant's resistance to necrotrophic pathogens, we challenged the *sib1* mutant, the *SIB1* over-expression line and wt plants with the necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola*; however, we did not find any obvious difference between the loss- and gain-of-function mutants of *SIB1* and wt plants in resistance to these fungal pathogens.

Does SIB1 regulate the function of SIG1?

Disruption of the *SIG1* gene in rice (*OsSIG1*) is not fatal, and the study on the rice *sig1* mutants has revealed that OsSIG1 regulates transcription of the plastid genes *psaA* and *psaB* (Tozawa *et al.* 2007). The specific function of SIG1 (At1G64860) in *Arabidopsis* remains unknown. We obtained two T-DNA insertion lines (Salk_000235 and Salk_147985) (AT1G64860) for the *AtSIG1* gene from the ABRC. Salk_147985 has a T-DNA insertion at 80 bp upstream of the stop codon. However, we could not obtain any homozygous insertion

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line from its progenies, suggesting that the insertion at this gene is lethal. Salk_000235 has a T-DNA insertion at 47 bp after the stop codon.A homozygous insertion line for Salk_000235 was obtained; however, this insertion apparently does not affect *AtSIG1* expression as RNA blot analysis showed that there was no detectable difference in *AtSIG1* transcript accumulation between wt and the homozygous insertion line.

Expression of *AtSIG1* was found to be suppressed following infection with *Pst* avr*Rpm1*: its transcript level was 3.3-fold lower at 6 hpi in the wt plants than in theuninfected plants (Fig. 6a). If *AtSIG1* is also a transcriptional regulator of *psaA* and *psaB* in *Arabidopsis*, we would expect that *psaA* and *psaB* transcript levels might also be reduced following the pathogen infection. However, the transcript levels of these two plastid genes were slightly higher in the infected tissues (Fig. 6b). A possible explanation for the inconsistence in the level of *AtSIG1*, and *psaA* and *psaB* is that the AtSIG1 protein level might not be reduced in the infected tissues, although we could not rule out the possibility that AtSIG1 might not control expression of *psaA* and *psaB* in *Arabidopsis*. The transcript levels of *AtSIG1, psaA* and *psaB* in the *sib1* mutant were not significantly different from those in the wt plants; however, in the *SIB1* over-expression line, the transcript levels of all these three genes were slightly reduced (Fig. 6a-c). It remains to be determined whether this reduction was directly or indirectly regulated by SIB1 over-expression.

DISCUSSION

SIB1 was initially identified by its interaction with SIG1, and was speculated to function as an inhibitor of SIG 1 (Morikawa *et al.* 2002). We found that the *SIB1* gene is up-regulated, but the *SIG1* gene is down-regulated by pathogen infection, suggesting that both SIG1 and SIB1 could be involved in host-pathogen interactions. The *sib1* mutation impairs pathogen-triggered induction of a subset of defence-related genes. Further analyses revealed that the *sib1* mutations cause a defect in the SA- and JA-mediated defence response. Conversely, *SIB1* constitutive over-expression makes the plants hyper-responsive to pathogen infection, and the SA and JA treatments, leading to enhanced disease resistance. However, the *sib1* mutant did not show a significant alteration in resistance to *P. syringae* or to the necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola*, which could be caused by functional redundancy with other defence pathways. The homolog of *SIB1, At2g41180,* could also have an overlapping function with *SIB1*. However, the transcript level of *At2g41180* was not significantly changed following pathogen infection. The attempt to obtain a knock-out line for *At2g41180* was not successful as the T-DNA insertion line from ABRC (Salk_152005C) which carries the insertion at approximately 90 bp upstream of the start codon does not appear to be a knock-out or knock-down mutant (data not shown).

SIB1 is a member of the plant-specific VQ domain-containing protein family. At least 34 predicted proteins in *Arabidopsis* contain the VQ motif. These proteins are generally small and share little significant sequence similarity to other proteins in the GenBank sequence databases. Other than the regions containing the conserved VQ motif, their primary structures are highly diverse. The land plant lineage-specific evolution and expansion of this family suggest that they might function in land plant-specific biological processes. Two other VQ motif-containing proteins have been characterized to date, and were found to be involved in signalling pathways in abiotic stress tolerance (Perruc *et al.* 2004) and in disease resistance (Andreasson *et al.* 2005). Interestingly, these two proteins and SIB1 were all identified initially by their interaction with other proteins. It is intriguing to speculate that many of the VQ domain-containing proteins might act as protein scaffolds that couple signalling components (such as calmodulin and MAPK4) to their downstream effectors (such as transcriptional regulators).

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Recently, *SIB1* was reported as an SA-induced gene (Narusaka *et al.* 2008). Narusaka *et al.* (2008) also generated SIB1 over-expression lines which were found to accumulate elevated transcript levels of ROS-related genes. However, the over-expression lines did not show any significant difference from wt plants in resistance to *P. syringae* (Narusaka *et al.* 2008). We found that, among the *35S::SIB1* lines, only those with higher levels of the *SIB1* transcripts exhibited moderately enhanced resistance to infection by *Pst*, whereas the other over-expression lines did not show any obvious difference from wt plants in resistance to *Pst*.

Although the *sib1* loss-of-function mutation does not lead to significant change in resistance to *Pst* or to *B. cinerea* and *A. brassicicola*, the transcriptome analysis revealed that expression of a subset of defence-related genes was affected by the *sib1* mutation. Many of the differentially expressed genes are known to be SA and/or JA responsive.Analysis of expression patterns of several SA- or JA-responsive genes following the treatments of SA and JA further indicated that both SA- and JA-responsive pathways are affected by the *sib1* mutations.

Chloroplasts play an important role in the defence response (see reviews by Karpinski *et al.* 2003; Roberts & Paul 2006). Changes in abundance of genes and proteins involved in photosynthesis and other chloroplast functions following pathogen infection have been well documented (Bunker *et al.* 1995; Lehto *et al.* 2003; Zou *et al.* 2005; Jones *et al.* 2006; Thilmony, Underwood & He 2006). Phytopathogens have evolved various mechanisms to manipulate chloroplast function. Ptr ToxA, a host-selective proteinaceous toxin from the wheat pathogen *Pyrenophora tritici-repentis*, targets a chloroplast protein with an undefined function (Manning, Hardison & Ciuffetti 2007). Several virulence effector proteins have been determined to target chloroplast proteins, including HopI1 (Jelenska *et al.* 2007), HopU1 (Fu *et al.* 2007), the TMV p50 helicase (Abbink *et al.* 2002) and NRIP1 (Caplan *et al.* 2008). In tobacco, NRIP1 and another chloroplast protein, an FtsH-like metalloprotease, are also required for the N-mediated HR (Seo *et al.* 2000; Caplan *et al.* 2008). The fact that pathogens target chloroplast proteins with different activities indicates that the virulence strategies used by pathogens to manipulate chloroplast are diverse.

We have not found obvious alteration in the transcript levels of *psaA* or *psaB* by the *sib1*mutation. *psaA* And *psaB* are two of the chloroplast genes regulated by SIG1 in rice; however, it is not clear whether these chloroplast genes are also regulated by SIG1 in *Arabidopsis*. We could not rule out the possibility that SIB1 might function in the defence response through a mechanism other than its binding to and regulating SIG1. Determination of the SIB1's protein levels, subcellular localization and its interaction with SIG1 during the defence response will help to define the precise mechanism by which *SIB1* regulates the defence pathway. Nevertheless, our study indicates that *SIB1* is involved in both the SA- and JA-mediated defence responses, and might act as a link between chloroplast function and defence signalling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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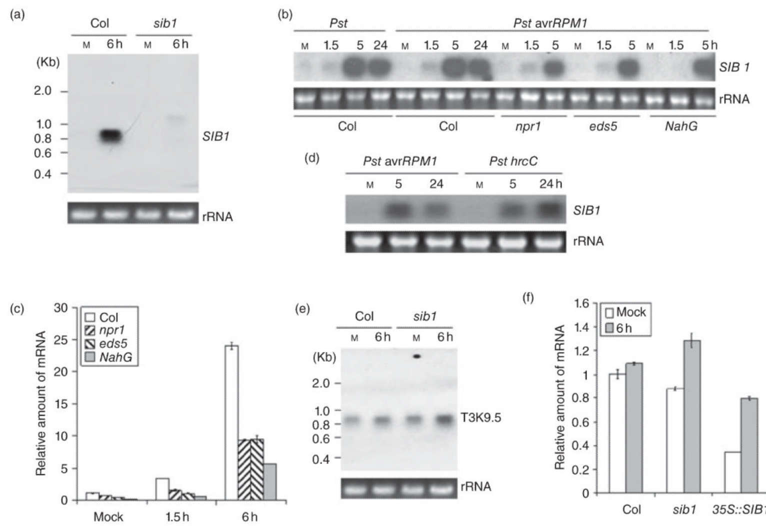

Figure 1.

Pathogen-triggered expression of the *SIB1* gene. (a) An RNA blot probed with the *SIB1* gene. RNA was isolated from the uninfected and *Pst* avr*Rpm1*-infected plants. The pathogen-infected wild-type (wt) plants accumulated a 0.85 kb SIB1 transcript, whereas the *sib1* mutant did not produce a detectable level of the normal *SIB1* transcripts. Col: the Columbia ecotype used as wt plants. A portion of the ethidium bromide-stained agarose gel is shown as an RNA loading control. (b) An RNA blot revealed that *SIB1* expression was induced by both *Pst* and *Pst* avr*Rpm1*, and its expression was compromised in *npr1, enhanced disease susceptibility 5* (*eds5*) and *NahG* plants. (c) The transcript levels of *SIB1* in uninfected and *Pst* avr*Rpm1*-infected wt, *npr1, eds5* and *NahG* plants determined by qPCR analysis. (d) An RNA blot showing *SIB1* transcript levels following infection with the *Pst hrcC* mutant strain and *Pst* avr*Rpm1*. (e and f) Transcript levels of *At2g41180* in wt, *sib1* and *SIB1* over-expression line (see below) determined by RNA blotting (e) and qPCR analysis (f). Leaves were inoculated with *Pst* avr*Rpm1*. Mock (M) represents RNA samples from leaves after mock (H_2O) inoculation.

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        SIB1 (41) KVRYISNPMRVQTCASKFRELVQELTGQD
   At1g21320 (61) VNIYTVTPRIIHTHPNNFMTLVQRLTGQT
   At3g18690 (66) VVIYAVSPKVVHATASEFMNVVQRLTGIS
   At1g78410 (12) MKVVFINTQYVETDARSFKTVVQELTGKN
   At2g41180 (38) KVRYISNPMRVETCPSKFRELVQELTGQD
    CAO63044  (1) KVVYIGNPRMVKVKESEFRALVQELTGQD
    CAN69541  (2) KVVYISSPMKVKTSASKFRALVQELTGRD
Os01g0808900 (40) KVVYISNPMRVKTSAAGFRALVQELTGRN
Os07g0687400 (40) RVVYIASPMKLTASPEEFRAVVQELTGRH
   At2g42140 (34) RIIHIFAPEIIKTDVANFREIVQNLTGKQ
      SmSeq1  (2) KVVQIFNPTVIRTDAASFRELVQELTGMN
      SmSeq2  (2) RVVHIFNPTVIKTDAANFRSLVQELTGRS
   At3g58000 (33) RIIHIFAPEIIKTDVANFRELVQSLTGKP
   At2g44340 (34) RIIHIFAPEVIKTDVKNFRSLVQSLTGKP
   At3g60090 (46) RIIHIFAPEIINTDVKNFRTLVQSLTGKP
   At1g28280 (50) ESGNPYPTTFVQADTSSFKQVVQMLTGSA
   At5g08480 (10) ATTCKPVTTFVQTDTNTFREIVQRLTGPT
   At5g53830 (42) HLSNPYPTTFVQADTSTFKQVVQMLTGSS
XP_001760459 (52) GQEFASITTFVQVDTSSFRELVQKLTGAS
XP_001771471 (16) SQEFASTTTFVQVDTSSFRELVQKLTGAS
   At4g15120 (55) RASRRTPTTLFNTDTANFRAMVQQFTGGP
   At4g20000 (55) ASRRAIPTTLLNANPSNFRALVQKFTGRS
   At1g35830(104) RASRRAPTTVLTTDTSNFRAMVQEFTGVP
   At4g39720(119) RASRRAPTTVLTTDTSNFRAMVQEFTGIP
   At5g65170(144) RVSRRAPTTVLTTDTSNFRAMVQEFTGNP
   Consensus                        FXXXVQXXTG
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Figure 2.

A sequence alignment of the VQ domain-containing regions from 25 plant proteins. Sequences were aligned using the ClustalW algorithm of the VectorNTI software. Identical residues are in black background, and similar/conserved residues are in grey background. Included in the alignment are 17 *Arabidopsis* proteins, two rice proteins, two grape proteins (CAO63044 and CAN64541), two moss proteins (XP_001760459 and XP_001771471) and two spike moss sequences (SmSeq1 and SmSeq2).

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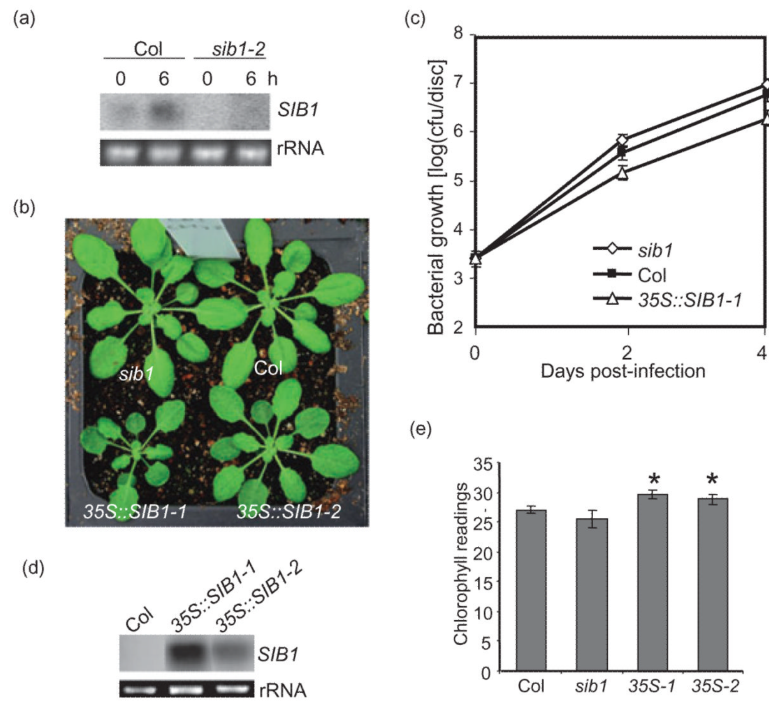

Figure 3.

Phenotype analyses of loss- and gain-of-function mutations of *SIB1*. (a) The RNA blot shows the *SIB1* transcript was undetectable from the *sib1-2* mutant. The RNA samples were extracted from uninfected leaves (0 h) and leaves inoculated with *Pst* avr*Rpm1* at 6 hpi. (b) Morphological phenotypes of 4-week-old plants. (c) *In planta* growth of *Pst* in wild-type (wt), *sib1* and *35S::SIB1-1* leaves. Each data point represents the average of three replicates ± SD. (d) The RNA blot result showing the *SIB1* transcript levels in uninfected leaves of the two *SIB1* over-expression lines and wt. (e) The relative amount of chlorophyll in leaves of wt, *sib1* and the *SIB1* over-expression lines. Values are the average of eight replicates ± SD.

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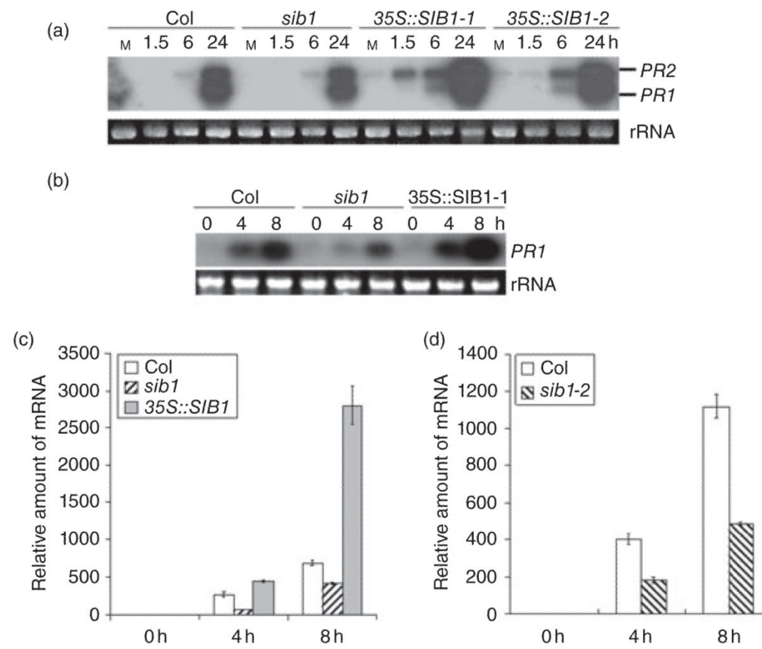

Figure 4.

Loss- and gain-of-function mutations of *SIB1* affect expression of defence-related genes triggered by pathogen infection and the SA treatment. (a) An RNA blot probed with *PR1* and *PR2* genes. Leaves were inoculated with *Pst* avr*Rpm1*. *SIB1* over-expression led to quicker and stronger induction of these genes. The *sib1* mutant accumulated a slightly lower level of these transcripts at 24 hpi. (b, c) *PR1* transcript levels in wild-type (wt), *sib1* and the *SIB1* over-expression lines following the SA treatment determined by RNA blot analysis (b) and qPCR analysis (c). (d) The qPCR result revealed that *sib1-2* was also defective in SA-mediated induction of *PR1*.

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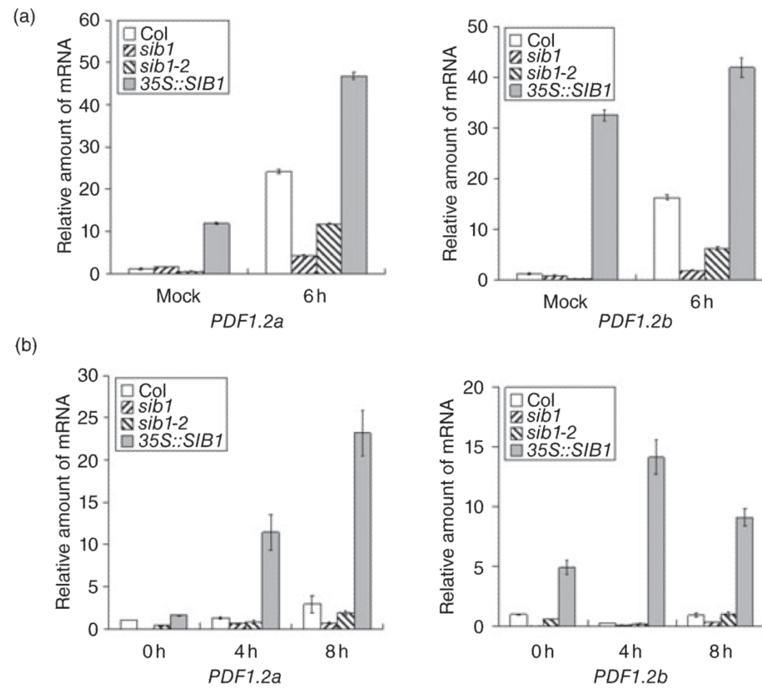

Figure 5.
Loss- and gain-of-function mutations of *SIB1* alter expression of jasmonic acid (JA)-responsive genes following pathogen infection and the JA treatment. RNA was extracted from leaves taken at different time-points after inoculation with *Pst* avr*Rpm1* (a) and the JA treatment (b), and the transcript levels of *pdf1.2a* and *pdf1.2b* were determined by qPCR analysis.

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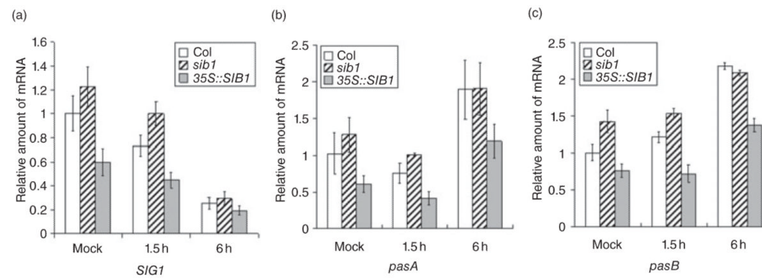

Figure 6.

Determination of transcript levels of *SIG1, psaA* and *psaB*. RNA samples were extracted from uninfected and *Pst* avr*Rpm1*-infected leaves (1.5 and 6 hpi) of wild-type (wt), *sib1* and the *SIB1* over-expression line. The transcript levels of *SIG1* (a), *psaA* (b) and *psaB* (c) were determined by qPCR.