

Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase

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Summary

Reversible modifications of target proteins by small ubiquitin-like modifier (SUMO) proteins are involved in many cellular processes in yeast and animals. Yet little is known about the function of sumoylation in plants. Here, we show that the *SIZ1* gene, which encodes an Arabidopsis SUMO E3 ligase, regulates innate immunity. Mutant *siz1* plants exhibit constitutive systemic-acquired resistance (SAR) characterized by elevated accumulation of salicylic acid (SA), increased expression of pathogenesis-related (*PR*) genes, and increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Transfer of the *NahG* gene to *siz1* plants results in reversal of these phenotypes back to wild-type. Analyses of the double mutants, *npr1 siz1*, *pad4 siz1* and *ndr1 siz1* revealed that *SIZ1* controls SA signalling. *SIZ1* interacts epistatically with *PAD4* to regulate PR expression and disease resistance. Consistent with these observations, *siz1* plants exhibited enhanced resistance to *Pst* DC3000 expressing *avrRps4*, a bacterial avirulence determinant that responds to the *EDS1/PAD4*-dependent TIR-NBS-type *R* gene. In contrast, *siz1* plants were not resistant to *Pst* DC3000 expressing *avrRpm1*, a bacterial avirulence determinant that responds to the NDR1-dependent CC-NBS-type *R* gene. Jasmonic acid (JA)-induced *PDF1.2* expression and susceptibility to *Botrytis cinerea* were unaltered in *siz1* plants. Taken together, these results demonstrate that *SIZ1* is required for SA and *PAD4*-mediated *R* gene signalling, which in turn confers innate immunity in Arabidopsis.

Keywords: CC-NBS-type *R* gene, plant innate immunity, salicylic acid, *SIZ1* SUMO E3 ligase, systemic-acquired resistance, TIR-NBS-type *R* gene.

Introduction

Plants protect themselves against pathogens through a variety of responses, including basal defence and gene-for-gene resistance mechanisms (Jones and Takemoto, 2004). Basal defence is activated by recognition of conserved pathogen-associated molecular patterns, such as those presented by bacterial flagellin or lipopolysaccharide. Suppression of basal defence appears to be essential for

pathogenicity, and contributes to increased virulence in susceptible interactions (Kim *et al.*, 2005). Gene-for-gene resistance, often accompanied by hypersensitive response (HR), is elicited when a product encoded by a plant resistance (*R*) gene recognizes a cognate pathogen-produced avirulence molecule that is either a direct or indirect product of an *avr* gene. This then leads to the formation of necrotic

lesions at the sites of infection. Often, HR-associated cell death lesions are attributed to an immune response known as systemic-acquired resistance (SAR; Ryals *et al.*, 1994). SAR confers resistance throughout the plant to a broad spectrum of pathogens and correlates with the expression of pathogenesis-related (PR) proteins, which are typical indicators of SAR (Yun *et al.*, 1997). SAR is preceded by accumulation of salicylic acid (SA), which is observed not only at the site of infection, but also in tissues remote from the infection (Durrant and Dong, 2004). SA is a necessary and effective signal for SAR induction, and application of exogenous SA induces the expression of PR genes and subsequent SAR.

Genetic approaches have been used to identify several regulatory proteins that control SA-dependent defence responses in Arabidopsis (Li *et al.*, 2001). The mutation *eds1* (enhanced disease susceptibility) suppresses both basal resistance and specific resistance controlled by a subset of R genes whose products share a common structural motif referred to as the toll-interleukin-1 receptor (TIR)-type nucleotide binding leucine-rich repeat (NB-LRR; Aarts *et al.*, 1998; Parker *et al.*, 1996). Mutation of *EDS1* reduces SA levels in infected leaves and enhances susceptibility to pathogens (Feys *et al.*, 2001; Zhou *et al.*, 1998). Many coiled-coil (CC)-NB-LRR proteins require the presence of a functional NDR1 protein. NDR1 is a plasma membrane-localized protein regulated by post-translational modifications that include C-terminal processing and N-linked glycosylation (Coppinger *et al.*, 2004). Mutant *ndr1-1* plants are susceptible to the bacterial strain *Pst* DC3000 expressing the effector genes *avrB*, *avrRpt2*, *avrRpm1* and *avrPphB*, but not *avrRps4* (Aarts *et al.*, 1998; Coppinger *et al.*, 2004). SA levels in *sid2* plants remain extremely low even after pathogen infection, and reach only 5–10% of wild-type (WT) levels, compromising both basal and systemic resistance (Wildermuth *et al.*, 2001). Mutation of *SID2* is thus believed to block SA synthesis (Nawrath and Metraux, 1999). *SID2/EDS16* encodes a pathogen-induced isochorismate synthase (Wildermuth *et al.*, 2001). The low SA level observed in *sid2* mutants after infection also indicates that the isochorismate pathway is the main source of SA synthesis during SAR. The *EDS5* gene exhibits sequence homology with members of the orphan multidrug and toxin extrusion transporter families (Nawrath *et al.*, 2002). Pathogen-induced *EDS5* expression precedes SA accumulation, and requires expression of *EDS1* and its partner *PAD4*, which encode proteins with sequence similarity to triacylglycerol (TAG) lipases. Thus, *EDS5* functions downstream of *EDS1* and *PAD4* and upstream of SA (Nawrath *et al.*, 2002). Following pathogen infection or application of SA, the allelic mutants *npr1*, *nim1* and *sai1* accumulate SA but fail to deploy SAR, implying that the product of *NPR1* acts downstream of SA (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). *NPR1* encodes an

ankyrin-repeat protein that interacts with a b-ZIP transcription factor (TGA2) that is required for activation of SA-regulated *NPR1* gene expression and disease resistance. This suggests that *NPR1* acts by altering the activity of downstream transcription factors (Fan and Dong, 2002).

Small ubiquitin-like modifier (SUMO) peptides are attached covalently to target proteins as post-translational modifications. In mammals, SUMO modifies proteins that participate in diverse cellular processes including transcriptional regulation, nuclear transport, maintenance of genome integrity and signal transduction (Hay, 2005). Although our understanding of sumoylation in plants remains limited, this process has been implicated in the heat stress response, pathogen defence, abscisic acid (ABA) signalling, phosphate deficiency responses and flowering time control (Hanania *et al.*, 1999; Hotson *et al.*, 2003; Kurepa *et al.*, 2003; Lois *et al.*, 2003; Miura *et al.*, 2005; Murtas *et al.*, 2003; Novatchkova *et al.*, 2004; Orth *et al.*, 2000). We determined previously that Arabidopsis *SIZ1* is a homologue of mammalian PIAS (protein inhibitor of activated signal transducer and activator of transcription, STAT) and yeast Siz (SAP and Miz) family SUMO E3 ligases. Arabidopsis *SIZ1* positively regulates *PHR1*-dependent phosphate-starvation-responsive genes and negatively regulates phosphate starvation morphological responses, including cessation of primary root elongation, increased lateral root and root hair development (Miura *et al.*, 2005).

Based on a number of observations (Hanania *et al.*, 1999; Hotson *et al.*, 2003; Orth *et al.*, 2000; Roden *et al.*, 2004) that implicate the SUMO pathway in plant–pathogen interactions, it is tempting to speculate that SUMO may play an important role in disease resistance. Here, we examined the role of Arabidopsis SUMO E3 ligase (*SIZ1*) in the pathogen defence response. The results identify *SIZ1* as a negative regulator of SA- and *PAD4*-mediated signalling in plants. Presumably by a feed-forward mechanism, activation of SA signalling by mutations in *SIZ1* leads to elevated accumulation of SA and constitutive defence responses, including PR gene expression, and pathogen resistance.

Results

Expression profiling establishes that pathogen defence genes are expressed constitutively in siz1 plants

Many proteins and transcription factors are regulated by SUMO modification, and these modifications affect several biological processes (Gill, 2005; Hay, 2005). Because *SIZ1* is an Arabidopsis SUMO E3 ligase (Miura *et al.*, 2005), we assumed that *SIZ1* may regulate plant growth and development through effects on gene expression. To help reveal the biological function of *SIZ1*, cDNA microarrays containing 25 425 Arabidopsis genes (Gong *et al.*, 2005) were used to compare gene expression patterns between *siz1* and WT

plants grown in MS media for 7 days without any exogenous treatment.

We identified genes with a ≥ 3 -fold difference in expression between *siz1* and WT plants (Table 1, Tables S1 and S2). Even in the absence of pathogen infection, several disease-response genes were expressed strongly in *siz1* seedlings (Table 1). For example, genes encoding pathogenesis-related PR1 protein (Glazebrook, 1999), chitinase, osmotin-like protein (Merkouropoulos *et al.*, 1999), glutathione-S-transferase, peroxidases (Brisson *et al.*, 1994) and lipid transfer protein (Molina *et al.*, 1993) were highly expressed in *siz1* plants. Interestingly, this phenomenon was observed in the *acd11* mutant that also displays an elevated pathogen defence response (Brodersen *et al.*, 2002). Microarray analysis also demonstrated that phosphate-starvation-responsive genes are slightly upregulated in *siz1* plants without phosphate deficiency, e.g. *AtPT2* (1.7-fold), *AtPS2* (2.0-fold) and *AtPS3* (1.2-fold), as previously reported (Miura *et al.*, 2005).

RNA blots confirmed the microarray data demonstrating that *PR1*, *PR2* (*BGL-2*) and *PR5*, which are induced during SAR (Glazebrook, 1999) in WT plants, are expressed constitutively in *siz1* plants (Figure 1a, Table 1). In contrast, JA-responsive genes such as *PDF1.2* are not constitutive in either *siz1* or WT plants (Figure 1a, Table 1).

An incompatible host-pathogen response will result in a greater accumulation of *PR1* gene product than a compatible response (Molina *et al.*, 1999). Thus, as expected, the incompatible pathogen (*Pst* DC3000 *avrRpm1*) induces a greater accumulation of PR1 in WT plants than does the compatible pathogen (*Pst* DC3000; Figure 1b). In contrast, PR1 is constitutively expressed in *siz1* plants to essentially the same levels in both the presence and absence of

pathogen infection (Figure 1b). Similarly, PR1 is induced by SA in WT, but is constitutively expressed in *siz1* plants (Figure 1c).

siz1 plants exhibit increased resistance to a virulent bacterial pathogen

Consistent with the increased expression of defence-related genes (Figure 1, Table 1), the *siz1* mutation confers increased resistance to the virulent bacterial pathogen *Pst* DC3000 (Figure 2a). A 100-fold lower number of *Pst* DC3000 bacteria resulted from infection of *siz1* compared to WT plants. Chlorosis (a typical disease symptom) is also evident on the leaves of WT plants by 5 days after inoculation, but is significantly reduced on the leaves of *siz1* plants (Figure 2b). Expression of the WT allele of *SIZ1*-GFP in *siz1* mutant plants complements the *siz1* dwarf-like phenotype and restores susceptibility to *Pst* DC3000 infection (Figure S1).

SA-dependent SAR signalling is negatively regulated by *SIZ1*

Given that SA is both necessary and sufficient for SAR, the levels of SA and its sugar conjugate, SA glucoside (SAG), were compared between WT and *siz1* plants. In the mutant *siz1-2*, the levels of SA and SAG are 28- and 15-fold higher than in WT, respectively (Figure 3). Over-expression of a gene encoding bacterial salicylate hydroxylase (*nahG*) in *siz1* plants substantially decreases the levels of SA, PR1 and resistance to *Pst* DC3000 (Figures 3 and 4b,c). Mature *siz1* plants exhibit a dwarf-like phenotype, as do other SA-accumulating mutants (Bowling *et al.*, 1994; Li *et al.*, 2001; Petersen *et al.*, 2000), and this phenotype is also suppressed

Table 1 Microarray analysis of mRNA levels in *siz1-2* versus wild-type (WT) plants

Locus	Gene description	Fold difference ^a (<i>siz1-2</i> /WT)
At2g14610	Pathogenesis-related PR-1 protein (PR1)	8.4
At4g33720	Pathogenesis-related protein 1 precursor	6.1
At5g37990	Carboxyl methyltransferase family protein	5.2
At1g17170	Glutathione transferase	4.4
At2g43590	Putative endochitinase	4.3
At2g18660	Expansin-related protein 3 precursor	4.2
At5g13900	Lipid transfer protein (LTP) family protein	4.1
At1g02850	Glycosyl hydrolase family 1	3.9
At2g35380	Peroxidase family	3.6
At2g02930	Glutathione transferase (GST6)	3.5
At3g12500	Basic endochitinase	3.5
At2g15490	Putative glucosyltransferase	3.5
At1g02920	Glutathione transferase (GST11)	3.4
At4g11650	Osmotin-like protein (OSM34)	3.4
At4g19880	Glutathione-S-transferase-related	3.3
At2g29480	Glutathione transferase, putative (GST20)	3.3
At5g66170	Senescence-associated protein	3.3
At3g04210	Disease resistance protein (TIR-NBS class)	3.3
At5g44420	Plant defensin protein, putative (PDF1.2)	1.5

^aRelative levels of mRNA accumulated in *siz1-2* versus wild-type plants.

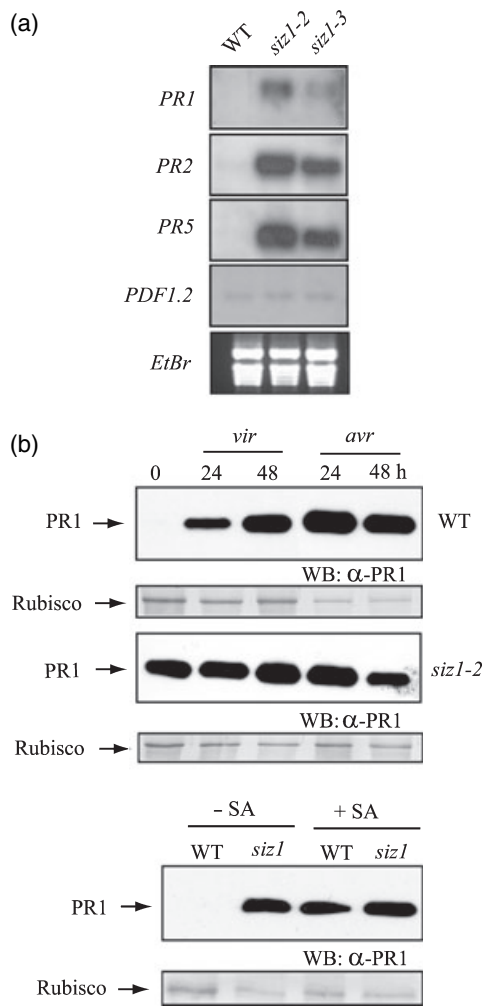


Figure 1. PR1 expression in wild-type and *siz1-2* plants. (a) Altered *PR* gene expression in *siz1* plants. Northern blots of total RNA (10 µg each) from wild-type (WT) and *siz1* plants were hybridized using the probes indicated. Ethidium bromide staining of the rRNA band was used as a loading control. (b) Plants were inoculated with 5×10^7 cfu ml⁻¹ (OD = 0.1) of *Pst*DC3000 containing *avrRpm1* (*avr*) or not containing (*vir*) the avirulence gene. Samples were harvested at the time points indicated, and PR1 protein expression was determined by Western blot analysis using a rabbit polyclonal anti-PR1 antibody (Wang *et al.*, 2005). Coomassie blue staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; bottom) was used as a loading control. (c) PR1 protein expression levels in WT and *siz1* plants 24 h after treatment with 2 mM SA containing 0.005% Silwet L-77. Controls were treated with H₂O containing 0.005% Silwet L-77.

by *nahG* (Figure 4a). Taken together, these data indicate that increased *PR* gene expression results from increased SA levels caused by the *siz1* mutation (Figures 3 and 4).

Examination of SAR-signalling genes in *siz1* plants indicated that the expression levels of *EDS1*, *PAD4*, *SID2* and *EDS5* are up-regulated, whereas the expression levels of *NDR1* and *NPR1* are not (Figure 5 and Table S3). These results suggest that *SIZ1* functions as an upstream negative

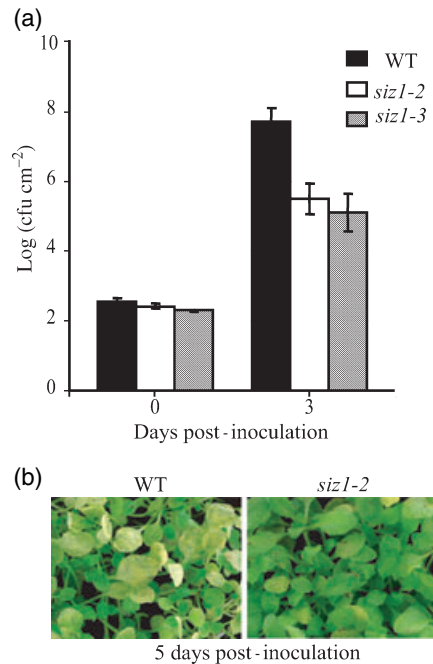


Figure 2. The loss-of-function mutant *siz1* exhibits enhanced bacterial resistance. (a) Resistance of *siz1-2* and *siz1-3* to a bacterial pathogen. Four-week-old wild-type (WT) and *siz1* (*siz1-2* and *siz1-3*) plants were inoculated with 1×10^5 cfu ml⁻¹ *Pst* DC3000, and the number of bacteria per area of leaf were plotted on a log₁₀ scale for days 0 and 3. Values represent the mean ± SD for colony-forming units extracted from three independently sampled leaf discs. (b) Representative 3-week-old wild-type or *siz1-2* plants 5 days after dipping into a solution containing 1×10^8 cfu ml⁻¹ *Pst* DC3000 with 0.005% Silwet L-77.

regulator of SA accumulation and subsequent to SA-mediated SAR signalling.

The roles of pad4, npr1 and ndr1 mutations in the siz1 phenotype

NPR1 is an essential regulatory component of SAR that is known to function downstream of SA (Cao *et al.*, 1994). To determine whether *siz1* is epistatic to *npr1*, we examined the phenotype of *npr1 siz1* double mutants. Although the double mutant retains bacterial resistance and the *siz1*-like dwarf stature (Figure 6a,c) that is associated with high SA concentrations (Zhang *et al.*, 2003), constitutive expression of PR1 is partially suppressed by the absence of functional *NPR1* (Figure 6b). These results suggest that *SIZ1* regulates SAR through both *NPR1*-dependent and -independent pathways. Remarkably, the level of SA was ninefold higher in *npr1 siz1* than in the *siz1* single mutant (Figure 3), a finding that is consistent with the role played by *NPR1* in SA signalling, as well as in negative feedback regulation of SA following SAR induction (Clarke *et al.*, 1998; Zhang *et al.*, 2003).

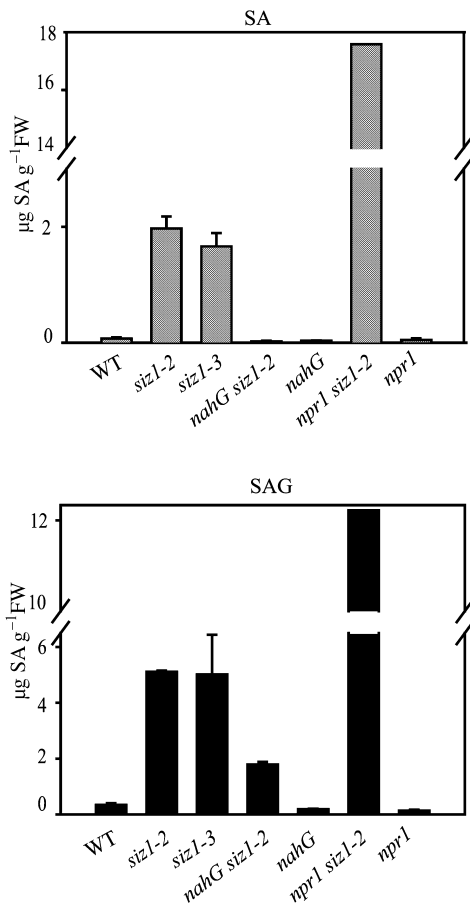


Figure 3. SA levels in wild-type (WT), *siz1-2*, *siz1-3*, *nahG siz1-2*, *nahG*, *npr1 siz1-2* and *npr1* plants. The levels of free SA and SA glucosides (SAG) in leaves from 4-week-old soil-grown plants were analysed using HPLC. Values represent the mean and standard deviations obtained from three replicates of each sample.

The *pad4* mutation substantially suppresses the dwarf morphology, the elevated PR1 expression and the pathogen resistance of *siz1* (Figure 6). This clear, but partial suppression of the *siz1* phenotype by *pad4* is consistent with the partial suppression by *pad4* of *dnd1*, *dnd2* and *snc1* mutants (Jirage *et al.*, 2001; Zhang and Li, 2005). In contrast, the *ndr1 siz1* double mutant exhibits a similar *siz1* dwarf-like phenotype (Figure 6a). PR1 expression and pathogen resistance in *siz1* are also not significantly suppressed by the *ndr1* mutation (Figure 6b,c). These results indicate that SIZ1 functions mainly through its effect on SA levels and subsequent to signalling through *PAD4* to induce an SAR response.

The siz1 mutation confers hyperactivation of EDS1/PAD4-mediated R-gene-specific resistance

In Arabidopsis, activation of *PAD4* and its partner *EDS1* are required for transduction of pathogen-induced signalling

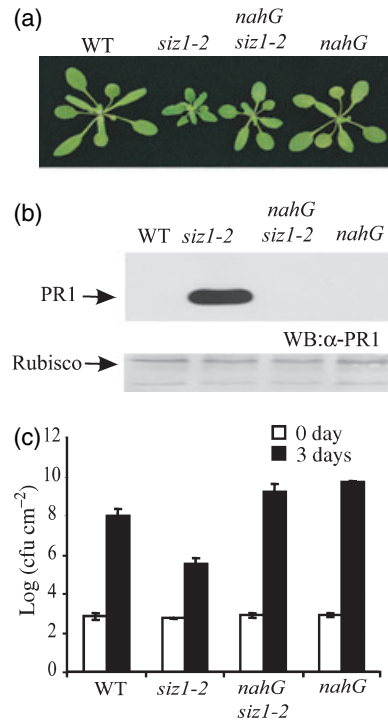


Figure 4. Degradation of SA by NahG leads to the suppression of *siz1* phenotypes. (a) Growth phenotypes of wild-type (WT), *siz1-2*, *nahG siz1-2* and *nahG* plants. Plants were grown in soil and photographed at 4 weeks. (b) Western blot of 2 µg total protein showing accumulation of PR1 in wild-type (WT), *siz1-2*, *nahG siz1-2* and *nahG* plants. Coomassie blue staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; bottom) was used as a loading control. (c) Growth of *Pst* DC3000 in wild-type (WT), *nahG siz1-2*, and the parental *nahG* and *siz1* lines. Experimental conditions were as described in Figure 2(a).

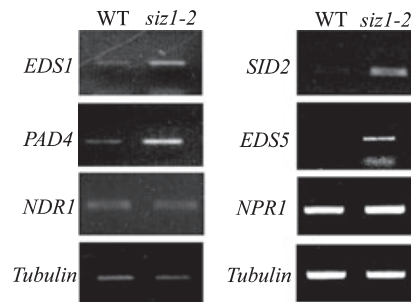


Figure 5. Expression of *EDS1*, *PAD4*, *NDR1*, *SID2*, *EDS5* and *NPR1* in wild-type (WT) and *siz1-2* plants. Total RNA was extracted from 4-week-old plants and RT-PCR was performed using gene-specific primers (Table S3). The level of tubulin was used as an internal control to normalize the amount of cDNA template.

through TIR-NBS-LRR *R* gene products, such as *RPS4*. However, *NDR1* is activated by pathogens through the function of CC-NBS-LRR class *R* genes, including *RPM1* and *RPS2* (Aarts *et al.*, 1998; Feys *et al.*, 2001; Rustérucchi *et al.*,

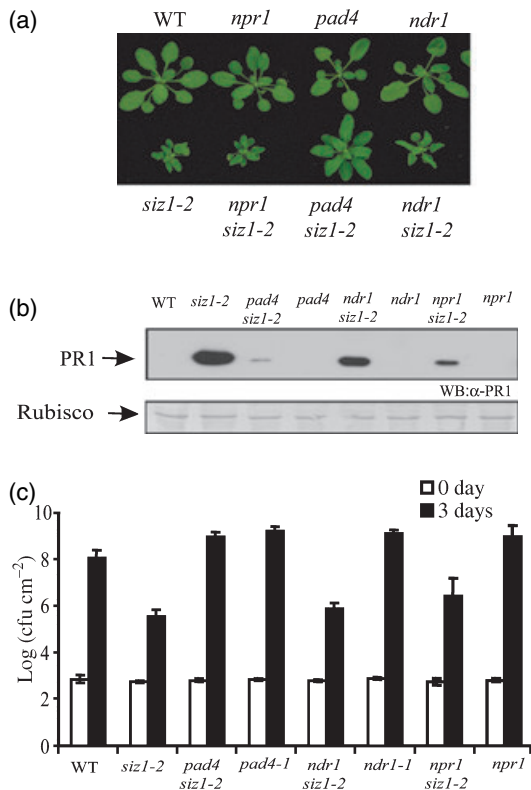


Figure 6. The *siz1* phenotype requires PAD4 function.

(a) The phenotype of 4-week-old soil-grown wild-type (WT), *npr1*, *pad4*, *ndr1*, *siz1-2*, *npr1 siz1-2*, *pad4 siz1-2* and *ndr1 siz1-2* plants. Representative plants are shown.

(b) Western blot analysis. Coomassie blue staining of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; bottom) was used as a loading control.

(c) Suppression of *siz1-2*-induced resistance to *Pst* DC3000 by *pad4*. Experimental conditions were as described in Figure 2(a).

2001; Tornero *et al.*, 2002; Wiermer *et al.*, 2005). If SIZ1 functions primarily in the EDS1/PAD4-mediated *R* gene signalling pathway (Figure 6), then virulence factors associated with bacterial type III effectors that interact with TIR-NBS-LRR R proteins could have altered functions in *siz1* mutants. To test this hypothesis, we monitored the growth of *Pst* DC3000 expressing three different type III effectors (*avrRpm1*, *avrRpt2* or *avrRps4*), in WT and *siz1* plants. Inhibition of growth of *Pst* DC3000 *avrRpm1* or *Pst* DC3000 *avrRpt2* was similar for the *siz1* and WT plants (Figure 7a, and data not shown). *Pst* DC3000 *avrRpm1* cells introduced into WT plants multiply slowly compared to strains harbouring other *avr* genes as described previously (Jurkowski *et al.*, 2004; Mackey *et al.*, 2002). However, relative to growth in WT plants, the growth of avirulent *Pst* DC3000 *avrRps4* was significantly reduced in *siz1* plants (Figure 7b). These results strongly indicate that PAD4-mediated SAR defence (gene-for-gene resistance) signalling is highly activated by the *siz1* mutation.

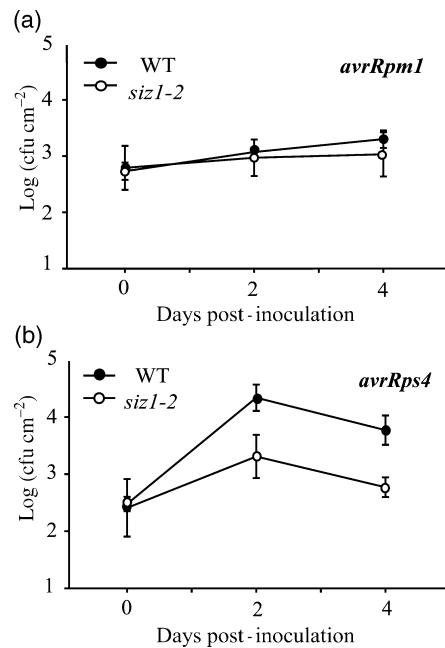


Figure 7. AvrRps4-mediated *R* gene signalling is altered in *siz1* plants.

Growth of *Pst* DC3000 expressing *avrRpm1* (a) or *avrRps4* (b) was assessed in wild-type and *siz1-2* plants as described in Figure 2(a). No significant difference was observed in the growth of *Pst* DC3000 expressing *avrRpm1* between *siz1-2* and wild-type plants on days 2 and 4 ($P = 0.13$ and 0.2 , respectively, $n = 3$). However, the growth of *Pst* DC3000 expressing *avrRps4* was significantly reduced in *siz1-2* plants compared to wild-type on days 2 and 4 ($P < 0.05$ and $P < 0.01$, respectively, $n = 3$). The experiments were repeated three times and similar results were observed.

Pre-existing SAR in *siz1* plants suppresses the hypersensitive response

Because the *siz1* mutation activates SAR defence signalling, SAR itself could be expected to be a constant condition in *siz1* plants. A constitutive SAR condition suppresses the cell death associated with HR that is caused by avirulent bacterial pathogens and oomycete pathogens (Devadas and Raina, 2002; Li *et al.*, 2001). We infiltrated the leaves of 4-week-old *siz1* plants with *Pst* DC3000 expressing either *avrRpm1* or *avrRps4* at a dose of $OD_{600} = 0.1$ (5×10^7 cfu ml⁻¹) to characterize the HR of SIZ1 plants. Infection of WT plants with strains harbouring *avrRpm1* or *avrRps4* resulted in a confluent collapse of tissue at the site of pathogen infiltration within 24 h (Figure S2a). This is the characteristic feature of host cell death associated with HR. However, *siz1* plants did not show any visible HR at 8 h and exhibited only a very weak HR after 24 h in response to infection with *Pst* DC3000 *avrRpm1* (Figure S2a). When we challenged plants with *Pst* DC3000 carrying *avrRps4*, WT plants showed cell death within 24 h after infection, whereas *siz1* plants exhibited only a weak HR even 24 h after infection (Figure S2a). We also measured the electrolyte leakage in *siz1* and WT plants after *Pst* DC3000 *avrRpm1* infection. The WT

plants infiltrated with $OD_{600} = 0.1$ (5×10^7 cfu ml⁻¹) *Pst* DC3000 *avrRpm1* showed significantly increased conductivity within 8 h. The *siz1* plants did not show any significant increase in ion leakage within 8 h, and the levels remained unchanged even after 24 h (Figure S2b). These results are consistent with phenotypes of other mutants such as *snc1*, *dnd1* or *hrl1*, which also exhibit constitutive SAR and suppress host cell death associated with HR (Devadas and Raina, 2002; Jurkowski *et al.*, 2004; Li *et al.*, 2001). It appears that release of *SIZ1* suppression of SA accumulation leads to hyperactivation of SAR through a PAD4/EDS1-mediated *R* gene resistance pathway and also suppresses HR cell death.

SIZ1 appears to specifically control SAR

Inoculation of Arabidopsis with the necrotrophic fungus *Botrytis cinerea* leads to a JA-mediated defence response (Ferrari *et al.*, 2003; Veronese *et al.*, 2006). To determine whether or not *SIZ1* functions in the JA-mediated resistance signalling pathway, we monitored *siz1* plants for resistance to *B. cinerea* and the expression of *PDF1.2*, a gene controlled by JA (Figures 8 and 9). The *bos1* control plants (*Botrytis*-susceptible mutant; Mengists *et al.*, 2003) exhibited highly necrotic and chlorotic leaves (Figure 8a), whereas *siz1* and WT plants both developed less necrosis following inoculation with *B. cinerea*. In addition, when *siz1* and WT plants were treated with methyl JA, they exhibited a similar pattern

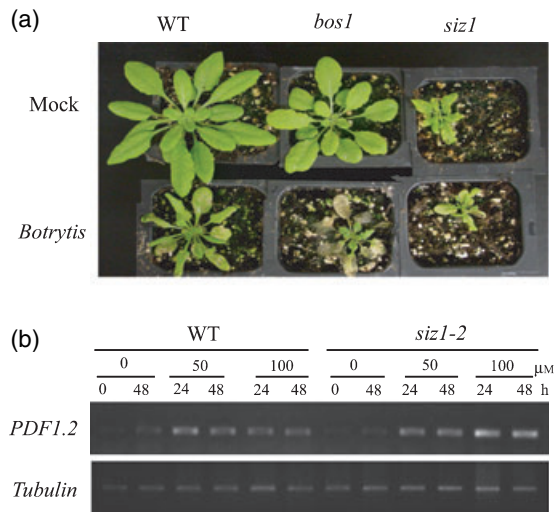


Figure 8. The *siz1* mutation does not alter sensitivity to *Botrytis cinerea* or *PDF1.2* expression. (a) Response to mock infection (top row) or *B. cinerea* (bottom row). The *Botrytis*-susceptible mutant *bos1-1* was included as a control and showed distinct disease symptoms 4 days after inoculation with *B. cinerea*. (b) Accumulation of *PDF1.2* in wild-type and *siz1-2* plants treated with 50 or 100 μM MeJA. Plants were sprayed with MeJA or mock-treated (0.005% Silwet L-77 in H₂O), and leaves were harvested at the times indicated. The expression level of tubulin was used as an internal control to normalize the amount of cDNA template.

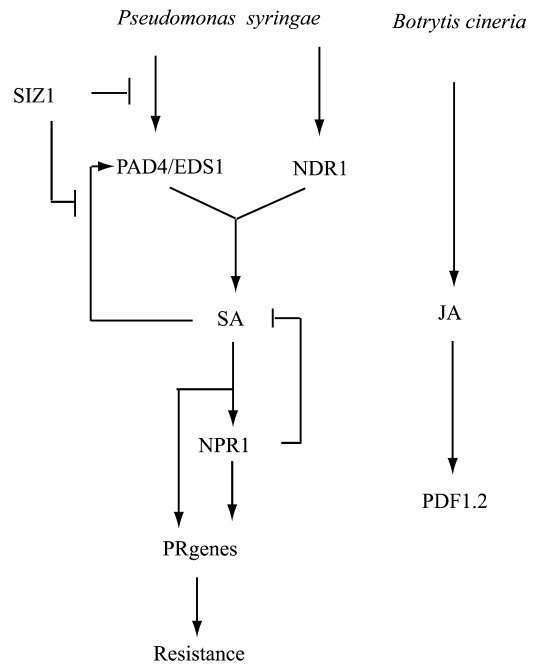


Figure 9. Model for basal defence controlled by *SIZ1*. The loss-of-function mutant *siz1* results in a SAR phenotype that includes constitutive *PR* gene expression, SA accumulation and disease resistance. Suppression of all the *siz1* phenotypes in *nahG* plants suggests that *SIZ1* functions upstream of SA. Positive feedback regulation exists between EDS1/PAD4 and SA, and the *pad4* mutation suppresses the *siz1* phenotype. *SIZ1* may negatively regulate SA feedback amplification and/or the *R* gene pathway(s) that require EDS1/PAD4 to function.

of *PDF1.2* expression (Figure 8b). These results suggest that *SIZ1* functions independently of the *B. cinerea*-induced JA-mediated defence signalling pathway.

Discussion

Function of SIZ1 in plant defence responses

Plant innate immunity requires a successful surveillance of bacterial invasion processes through both a basal defence system and *R*-gene-mediated signalling. Plant proteins involved in pathogen resistance have evolved the ability to recognize, either directly or indirectly, the products of pathogen *avr* genes. Recent studies have suggested that both SUMO and SUMO proteases are effective modifiers of type III secretion systems (TTSS) that facilitate the introduction of virulence factors into host cells (Orth *et al.*, 2000; Roden *et al.*, 2004). In resistant hosts, recognition of a virulence factor either directly or indirectly (guard hypothesis) by cognate *R* proteins results in the modification of non-*R* cellular factors that participate in the defence response (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002; Marathe and Dinesh-Kummar, 2003). It is also known that a diverse range of proteins become sumoylated or desumoylated in

response to stress (Kurepa *et al.*, 2003). Thus, post-translational modification of proteins by sumoylation/desumoylation represents an important regulatory mechanism in plant signalling pathways that mediate the response to environmental stimuli. In fact, previous studies have shown that disruption of SUMO-mediated signalling in both plant and animal hosts by pathogens is a highly conserved, universal mechanism that controls the interactions that affect the pathogenicity of potential pathogens (Hanania *et al.*, 1999; Hotson *et al.*, 2003; Orth *et al.*, 2000; Roden *et al.*, 2004).

Regulation of SA-mediated plant defence signalling by SIZ1

SA-mediated plant immunity plays a central role in the plant defence response. Loss of SIZ1 function leads to increased SA levels and constitutive *PR* gene expression (Figures 1a and 3), and, as with other SA-accumulating mutants, *siz1* plants exhibit enhanced disease resistance to bacterial pathogens (Figure 2; Bowling *et al.*, 1994; Li *et al.*, 2001; Petersen *et al.*, 2000). SA enhances the expression of both *EDS1* and *PAD4* as part of a positive feedback loop that amplifies disease resistance. SA-dependent signalling is highly activated in *siz1* plants, as evidenced by the increased expression of *EDS1*, *PAD4*, *SID2*, *EDS5* and *PR* genes (Figures 1 and 5). Expression of *PR1* is reduced in *pad4 siz1* and *nahG siz1* plants, and is not significantly changed in the *ndr1 siz1* mutant background (Figures 4 and 6). In addition, we observed hyperactivation of the RPS4-mediated *R* gene pathway by mutation of *SIZ1* (Figure 7). *SIZ1* appears to repress SA signalling upstream of *PAD4* through an SA feed-forward amplification loop involved in basal defence, or through an *EDS1/PAD4*-mediated gene-for-gene resistance pathway (Figure 9). Furthermore, *siz1*-mediated immunity appears to require both *NPR1*-independent and -dependent pathways downstream of SA.

Potential direct targets of sumoylation by SIZ1

Given that *SIZ1* has been identified as a SUMO E3 ligase (Miura *et al.*, 2005), it is highly likely that some components in SA signalling are direct targets of sumoylation/desumoylation. The SUMOplot prediction programme (<http://www.abgent.com/doc>) has established that *EDS1*, *PAD4*, *SAG101* and *NPR1* have conserved SUMO attachment motifs (Ψ KXE: Ψ , large hydrophobic amino acid; K, lysine; X, any amino acid; E, glutamate; Johnson, 2004). Data from mammalian studies indicate that sumoylation occurs preferentially in the nucleus (Jackson, 2001), although there are exceptions such as sumoylation of the K^+ channel K2PI (Rajan *et al.*, 2005). *EDS1* and *PAD4* are partitioned between the nucleus and cytoplasm, and, when localized in the nucleus, form complexes with *SAG101* (Feys *et al.*, 2005). *EDS1* and *PAD4* proteins exhibit a high degree of similarity with TGA lipase, and may convert TGA to fatty acids and glycerol. In addition,

SAG101 exhibits acyl hydrolase activity, hydrolysing TAG to fatty acids and di- or mono-acylglycerol or glycerol (He and Gan, 2002). Given that glycerol induces the accumulation of SA, leading to SAR activation that is dependent upon *EDS1/PAD4*, but independent of *NPR1* (Kachroo *et al.*, 2005), *EDS1/PAD4* proteins may positively control SA levels through regulation of glycerol metabolism. As sumoylation usually causes suppression of the activity of target proteins (Gill, 2005; Hay, 2005), it is possible that *EDS1*, *PAD4* and/or *SAG101* are sumoylated by *SIZ1* to repress their activities in glycerol metabolism and subsequent SA biosynthesis and signalling. It is also quite possible that *SIZ1* facilitates sumoylation of an unknown cellular target protein(s) that then represses the expression of *EDS1* and *PAD4*. By either of these mechanisms, desumoylation of target protein(s) could trigger activation of SA biosynthesis in *siz1* plants. High levels of SA enhance formation of the *EDS1/PAD4* complex that activates TIR-NBS-type *R* gene pathways as well as basal resistance. Consistent with this scenario, we observed up-regulation of the TIR-NBS class of disease resistance *R* genes and genes regulating SA biosynthesis in *siz1* plants (Table 1, Figure 5 and Table S4). In addition, the *EDS1/PAD4*-mediated specific *R* gene pathway was hyperactivated in *siz1* plants (Figure 7). These results may explain how the gene-for-gene resistance to various pathogens that is conferred by specific *R* genes also depends upon the level of SA and not only upon *NPR1* function (Rairdan and Delaney, 2002; Van der Biezen *et al.*, 2002).

SIZ1 is the only Arabidopsis protein that appears to contain all of the prototypical domains of PIAS/*SIZ*-type proteins such as PINIT, SXS, NLS, SAP and SP-RING (Kotaja *et al.*, 2002). Interestingly, three proteins (At5g41580, At1g08910 and At3g15150) contain the SP-RING domain that is necessary for SUMO E3 ligase activity (Novatchkova *et al.*, 2004; Takahashi and Kikuchi, 2005) even though these proteins do not have the other PIAS/*SIZ*-type domains that are conserved in *SIZ1*. At3g15150 has high similarity to human MMS21 and *Schizosaccharomyces pombe* NSE2, which also have SUMO E3 ligase activity (Andrews *et al.*, 2005; Potts and Yu, 2005). Because the *siz1* mutation is not lethal, it is possible that these or other proteins have redundant SUMO E3 ligase activity.

Regulation of SA signalling by SIZ1 in a sumoylation-independent manner

Because SUMO1/2 conjugation was not induced during pathogen infection (data not shown), it is possible that defence signalling is regulated by *SIZ1* in a sumoylation-independent manner. Indeed, mammalian PIAS proteins can affect the activity of transcriptional regulators through SUMO E3 ligase-mediated sumoylation and/or by SUMO-independent effects (Sharrocks, 2006). For instance, PIASy acts as a transcriptional co-repressor of STAT1 and the

androgen receptor (Gross *et al.*, 2001; Liu *et al.*, 2001), and this trans-repression activity remains even after mutation of PIASy has removed its sumoylation ability (Gross *et al.*, 2004). PIAS1 acts on Msx1, controlling its DNA binding specificity by recruiting Msx1 to target genes (Lee *et al.*, 2006).

PIASy and PIASx α control the localization into nuclear bodies of transcription factors LEF1 and FLI-1, respectively (Sachdev *et al.*, 2001; Van den Akker *et al.*, 2005). It is thought that relocalization to nuclear bodies is required for repression of LEF1/FLI1 activities. SIZ1 is preferentially localized to nuclear speckles (Miura *et al.*, 2005). Nuclear localization of EDS1, PAD4 and/or SAG101 may be controlled by interaction with SIZ1, with or without the involvement of SUMO E3 ligase activity. Thus, by either a sumoylation-dependent or -independent mechanism, the *siz1* mutation may release EDS1, PAD4 and/or SAG101 into the nucleoplasm, resulting in the accumulation of glycerol and subsequent biosynthesis of increased amounts of SA (Figure 3).

NPR1 is a transcription factor that is shuttled from the cytosol to the nucleus (Kinkema *et al.*, 2000) under the control of SA. It has been observed that the *snc1 npr1* double mutant accumulates more SA than the *snc1* single mutant, and this has been interpreted to mean that SA biosynthesis is under negative feedback regulation by NPR1 (Palma *et al.*, 2005). The *npr1 siz1* double mutant accumulates similar levels of SA as the *snc1 npr1* double mutant (Figure 3). Therefore, it appears that SA biosynthesis in *siz1* mutant plants may also be negatively controlled by NPR1 (Figure 9). As the negative control of SA biosynthesis by NPR1 occurs in the *siz1* mutant background, NPR1 does not seem to be a critical target for SIZ1-mediated regulation of innate immunity in plants.

In summary, SIZ1 controls SA-mediated plant defence signalling. However, SIZ1 does not appear to regulate the *B. cinerea*-induced JA-mediated defence signalling pathway (Figure 9). Although much remains to be discovered about the link between SUMO modification and SA-mediated innate immunity, our results demonstrate that the plant SIZ1 is very likely to be an important factor in both processes.

Experimental procedures

Growth conditions

Arabidopsis plants were grown on soil (Metro-Mix200; Grace-Sierra, Malpitas, CA, USA) in a growth room with a 16-h photoperiod and a light intensity of 100–120 $\mu\text{E m}^{-2} \text{sec}^{-1}$ at 22°C. To aid uniform germination, all seeds were incubated at 4°C for at least 2 days prior to placing in the growth room.

Genetic analysis

The mutants *siz1-2*, *siz1-3*, *nahG*, *npr1-1*, *pad4-1* and *ndr1-1* were in the *Arabidopsis thaliana* Columbia (Col-0) background. The T-DNA

insertion mutants *siz1-2* and *siz1-3* were identified by PCR (Miura *et al.*, 2005) using the following primers: SIZ1-F (5'-CTGATGG-TAGCCTTGCCCT-3') and SIZ1-R (5'-CAACTAACTCCT-GAAACGTCAG-3'). The *nahG siz1-2* mutant was isolated by screening F₂ plants for *nahG* morphology and using diagnostic PCR. The *siz1-2* mutation in *pad4 siz1-2*, *ndr1 siz1-2* or *npr1 siz1-2* was identified by diagnostic PCR using primers SIZ1-F and SIZ1-R. The mutations *pad4*, *ndr1* and *npr1* were confirmed as described previously (Cao *et al.*, 1997; Century *et al.*, 1997; Feys *et al.*, 2005).

Pathogen infections

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000) was grown with empty vector (pVSP61) or vector containing *avrRpm1* or *avrRps4* at 28°C on King's agar plates or in liquid medium (King *et al.*, 1954) supplemented with 50 $\mu\text{g ml}^{-1}$ rifampicin and 50 $\mu\text{g ml}^{-1}$ kanamycin. In brief, bacteria were resuspended in 10 mM MgCl₂, adjusted to 1×10^5 cfu ml⁻¹, and pressure-infiltrated into leaves using a needleless syringe. Leaf discs were combined from leaves of three independent plants, then ground in 10 mM MgCl₂, serial-diluted by 1:10, and plated onto King's B medium containing the appropriate antibiotics. Plates were incubated at 28°C for 2 days, after which the colonies were counted. Statistical analyses were performed using Student's *t*-test (Sokal and Rohlf, 1981).

For protein expression analyses, the *vir* (*Pst* DC3000) and *avr* (*Pst* DC3000 *avrRpm1*) pathogens were resuspended into 10 mM MgCl₂, and the concentrations were adjusted to OD₆₀₀ = 0.1 (5×10^7 cfu ml⁻¹). Bacteria were infiltrated into leaves of 4-week-old plants. Infected leaves were harvested from each line at the indicated time points.

Culture of *B. cinerea*, and disease assays were performed as described previously (Mengists *et al.*, 2003). In order to determine the susceptibility to *Botrytis* infection, the spore suspension (2.5×10^5 spores ml⁻¹) was sprayed onto soil-grown 4-week-old plants.

RNA analysis

Tissue samples for RNA gel blot analysis were harvested from soil-grown 4-week-old plants. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (10 μg) was separated on formaldehyde agarose gels and transferred to a hybridization membrane (Bio-Rad, Hercules, CA, USA) as described previously (Ausubel *et al.*, 1994). Genomic *PR1* (At2g14610), *PR2* (At3g57260), *PR5* (At1g75040) and *PDF1.2* (At5g44420) were amplified by PCR as described previously (Rogers and Ausubel, 1997), and ³²P-radiolabelled probes were generated from genomic DNA using an oligolabeling kit (Stratagene, La Jolla, CA, USA). Northern hybridization was performed as described previously (Ausubel *et al.*, 1994).

Semi-quantitative RT-PCR analyses, using the primers outlined in Table S3, were performed using ThermoScript reverse transcriptase (Invitrogen) and *Taq* polymerase (Promega Corp., Madison, WI, USA) as described previously (Shirano *et al.*, 2002).

Oligonucleotide microarray analysis

Total RNA (70 μg) was isolated with Trizol reagent (Invitrogen) from 1-week-old WT and *siz1-2* seedlings grown in MS liquid medium (Miura *et al.*, 2005). RNA samples were reverse-transcribed (SuperScript III; Invitrogen), and cDNAs were labelled with Cy3 or

Cy5 by indirect labelling (Gong *et al.*, 2005). The microarray slides used in the study include 25 425 probes spotted as 70-mer oligonucleotides (<http://www.ag.arizona.edu/microarray>; Gong *et al.*, 2005). To avoid bias in microarrays as a consequence of dye-related differences in labelling efficiency, dye labelling for each paired sample (mutant/WT) was swapped in one of three independent hybridizations. Two biological repeats were carried out. Signal intensities for each array element were collected (GENEPIX 4000B; Axon Instruments, Union City, CA, USA) and images analysed (GENEPIX PRO 4.0). Spots with intensities lower than background or with an aberrant spot shape were flagged by the GENEPIX software and checked manually. The resulting GPR files were analysed in Microsoft EXCEL.

Protein expression analyses

Four-week-old leaves were ground in liquid nitrogen and resuspended in lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA and one tablet of protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) per 50 ml extraction buffer. Samples were centrifuged at 14 000 *g* for 20 min, and the protein concentration was determined using Bradford reagent (Bio-Rad). Total protein (2 µg) was separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). For immuno-detection, after overnight incubation with 1:5000-diluted anti-PR1 antibody (Wang *et al.*, 2005), membranes were incubated with 1:5000-diluted horseradish peroxidase-conjugated antirabbit secondary antibody (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) for 1 h. Specific protein bands were visualized using the ECL Plus kit (Amersham Bioscience).

Salicylic acid and MeJA treatments

Four-week-old plants that had been grown in the greenhouse were treated with 50 or 100 µM MeJA (Sigma-Aldrich, Milwaukee, WI, USA). Treatments were applied using foliar sprays and plants were harvested at the times indicated. For determination of PR1 expression levels, 2 mM SA with 0.005% Silwet L-77 (Sigma-Aldrich, Milwaukee, WI, USA) were sprayed onto plants, and plants were kept under humid conditions using a shade cloth or plastic cover for 4 h. Plants were harvested 24 h after treatment.

Salicylic acid measurement

Leaf tissues were collected from 4-week-old soil-grown plants, weighed and frozen in liquid nitrogen. For each sample, 0.3 g of the frozen tissue was used for measurement of free SA and SA β-glucoside (SAG). In brief, each tissue was extracted in 6 ml of ice-cold methanol at 4°C for 24 h, then 3.6 ml of ice-cold water and 3 ml of chloroform with 10 mM of a 3,4,5-trimethoxy-*trans*-cinamic acid internal standard were added. After vortexing, the samples were kept at 4°C for 12 h. The combined supernatants were dried in a speed vacuum. The residue was resuspended in 0.6 ml of cold-ice water/methanol (1:1 v/v), and analysed as described previously (Freeman *et al.*, 2005).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Complementation of *siz1-2* by full length *At5g60410* cDNA.

Figure S2. (a) HR phenotypes in WT and *siz1* plants following inoculation with *pst* DC3000, *pst* DC3000 *avrRpm1*, and *pst* DC3000 *avrRps4*. (b) Electrolyte leakage in WT and *siz1* plants.

Table S1 Genes > 3.0 fold up-regulated in *siz1-2* plants

Table S2 Genes > 3.0 fold down-regulated in *siz1-2* plants

Table S3 Primers used for semi-quantitative RT-PCR analysis

Table S4 *R* gene expression profiles in *siz1-2* plants

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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