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ORIGINAL RESEARCH

Alerted Defense System Attenuates Hypersensitive Response-Associated Cell Death in *Arabidopsis siz1* Mutant

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Abstract Plants defend themselves by inducing sophisticated multilevel defense responses against pathogenic attack. The first line of defense against microbial pathogens is the process of nonself-recognition, which mediates the activation of the necessary defense repertoire. The hypersensitive response (HR), a macroscopic collapse of plant leaves in primary infection site, is one of such plant resistance responses. Subsequently, the HR triggers a general resistance mechanism called systemic acquired resistance (SAR), rendering uninfected parts of the plants less sensitive to further pathogenic attacks. Here, we show that *SIZ1* mutation-mediated preexisting SAR attenuates HR-associated cell death in *Arabidopsis thaliana*. In *siz1* mutant, the amount of PR1 and PR5 stayed high level, and the growth of pathogenic bacteria *Pseudomonas syringae* pv. *maculicola* (Pma) strain M6CΔE was reduced. Early callose deposition, spontaneous formation of microscopic cell death, and reactive oxygen species (ROS) were also observed in *siz1* mutant.

Keywords Plant defense response · SUMO E3 ligase · Hypersensitive response · Cell death · Systemic acquired resistance

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Introduction

Plants are continuously exposed to a variety of potentially pathogenic microbes including bacteria, fungi, and viruses. To defend themselves, they utilize a combination of chemical and physical barriers, which are either preformed or induced after infection (Yang et al. 1997; Jones and Dangl 2006). One response of an attacked plant is formation of papillae, which include structural components, such as callose, and minor amounts of other substances, such as phenolics, reactive oxygen intermediates, and proteins (Jacobs et al. 2003; Nishimura et al. 2003). Callose is an amorphous, high molecular weight (1→3)-β-D-glucan that can be visualized by UV light-induced fluorescence of the aniline blue fluorochrome (Stone et al. 1985). Callose is widely distributed in higher plants during normal plant growth and development (Stone and Clarke 1992). In addition, plant cells respond to wounding and microbial attacks by synthesizing and depositing callose in close proximity to the invading pathogen (Vance et al. 1980; Donofrio and Delaney 2001). It has been postulated that the papillae act as a physical barrier to impede microbial penetration (Brown et al. 1998). The induction of pathogenesis-related (PR) proteins on microbial invasion is also an example of a defense mechanism (Kloek et al. 2001). PR proteins are involved in the basal resistance as well as in systemic acquired resistance (SAR), which enhances a plant's resistance in uninfected tissue (Lee and Hwang 2005). Another examples of chemical responses is the synthesis of antimicrobial compounds like phytoalexins and defensins that can affect the outcome of an interaction between a plant and a pathogen (Pegadaraju et al. 2005; Veronese et al. 2006).

Gram-negative bacteria including *Pseudomonas syringae* utilize a type III secretion system (TTSS) to deliver

numerous effector proteins into cells of the host. These effectors are powerful weapons that contribute to bacterial virulence (Gálan and Collmer 1999). Although the biological function of individual type III effector proteins are mostly unknown, one of their virulence functions is to manipulate the host in a way that helps the pathogen avoid or overcome induced plant defense responses (Chang et al. 2004; Kim et al. 2005a). For example, overexpression of AvrPto, a type III effector from Pto DC3000 in *Arabidopsis*, abrogated cell wall-based basal defense in response to the TTSS defective *hrcC* mutant of Pst DC3000 (Hauck et al. 2003). It also has shown that two other TTSS effector proteins, AvrRpm1 and AvrRpt2, suppress plant defense responses induced by Pto DC3000(*hrcC*) or flg22 (a synthetic peptide derived from conserved N-terminal region of flagellin (Felix et al. 1999)) and thus improve condition for bacterial growth (Kim et al. 2005b).

Infection of plants with pathogens usually results in a distinct host response depending on the genetic constitution of the plant and the pathogen. In an incompatible interaction, the pathogen remains localized at the primary infection sites that often are visible as necrotic local lesions on the leaves. This local defense reaction is referred to as the hypersensitive response (HR). The HR cell death is often preceded by changes in ion fluxes, oxidative burst, and cross-linking of cell wall proteins (Holt et al. 2000; Shirasu and Schulze-Lefert 2003). SAR is usually correlated with the "gene-for-gene" disease resistance. Subsequently, the HR triggers a general resistance mechanism rendering uninfected parts of the plant less sensitive to further attack by pathogens, a phenomenon called SAR. The elicitation of the HR and SAR reactions is accompanied by the coordinated induction of a heterogeneous group of proteins in the infected and uninfected leaves, commonly referred to as PR proteins (Hammond-Kosack and Jones 1996; Ryals et al. 1996).

Now it is well-known concept that all organisms including plants use a variety of covalent modifications for the posttranslational control of proteins that affect growth, development, defense, and homeostasis. Using a diverse set of polypeptide tags for the reversibly altering protein function is rapid and energetically inexpensive mechanism. The reversible conjugation of the small ubiquitin-related modifier (SUMO) peptide to protein substrates (sumoylation) is emerging as a major posttranslational regulatory process in animals and other eukaryotes, including plants. Sumoylation shows many common features with ubiquitination, most widely studied covalent post translation control strategy, in 3D structure, and the biochemical steps that catalyze SUMO conjugation and deconjugation of protein substrates (Kerscher et al. 2006). However, SUMO is different with ubiquitin in primary sequence (about 20% similarities), and SUMO contains ~15 additional N-terminal amino acid residues; SUMO and ubiquitin are ~92–103 and 76 a.a, respectively (Stulemeijer and Joosten 2008). SUMO was discovered in 1996 as a peptide that is conjugated to RanGAP1, a small GTPase, which is localized to the nuclear pore complex and promotes nucleocytoplasmic trafficking (Matunis et al. 1996). For the last decade, the functions of SUMO conjugation have been studied widely, and now it is clear that sumoylated proteins are participated in diverse cellular processes including heat and cold stress response, innate immunity, development, abscisic acid (ABA) signaling, phosphate deficiency response, and flowering time control in plant (Yoo et al. 2006; Lee et al. 2007; Miura et al. 2007b; Duan et al. 2008; Jin et al. 2008; Cheong et al. 2009; Ishida et al. 2009; Miura et al. 2009).

Based on a number of reports implicating the involvement of SUMO pathway in plant–pathogen interactions, it is tempting to speculate that SUMO may play an important role in disease response signaling. Thus, we examined the role of *Arabidopsis* SUMO E3 ligase (*SIZ1*) in HR-associated cell death. We show here that mutation in *SIZ1* leads to alert defense response resulting in partially suppressed HR cell death and reduced electrolyte leakage. In addition, *SIZ1* mutation caused reduced pathogen growth, constitutive accumulation of PR proteins, spontaneous formation of microscopic cell death, accumulation of ROS, and early formation of papillae.

Materials and Methods

Plant Material and Growth Conditions

All the plants used in this work were in the wild-type Col-0 background, and the mutant used in this study, *siz1–2*, was given by Dr. Dae-Jin Yun. The plant growth chambers were set at 25/23°C (day/night), 60% to 70% relative humidity, and a photosynthetic photon flux density of 100 to 150 μmol m^{-2} s^{-1} with a 8-h photoperiod to maintain vegetative status.

Bacteria

Bacterial strain was grown at 28°C on KB medium containing the appropriate antibiotics for selection. For PR-1 and PR-5 expressions, 10^8 cfu/ml (OD_{600}=0.2) of *P. syringae* pv. *phaseolicola* (Pph) strain NPS3121 was infiltrated with a needleless 1-cc syringe (Fig. 2). Pph strain NPS3121 harboring empty vector (pVSP61) or derivatives of this plasmid expressing *AvrRpm1* or *AvrRpt2* were used for ion leakage and cell death assay (Fig. 4). A titer of 10^7 cfu/ml was used to score HR phenotype and to measure conductivity. For measurement of ion leakage,

eight leaf disks (8 mM diameter) were removed immediately following infiltration (t=0) and floated in 40 ml of water. After 30 min, the wash water was removed and replaced with 10 ml of freshwater. Conductance of this water was then measured using Fisher brand conductivity meter over time. The virulent bacteria used for Fig. 5 are the *P. syringae* pv. *maculicola* (Pma) strain M6CΔE (Rohmer et al. 2003) harboring empty vector (pVSP61) or derivatives of this plasmid expressing *AvrRpm1* or *AvrRpt2*. Growth experiments were conducted by inoculating bacterial suspensions in 10 mM $MgCl_2$ into leaves of 5-week-old plants with a needleless 1-ml syringe. After the indicated periods of time, three leaf disks for each sample were ground in 10 mM $MgCl_2$ and serially diluted and plated to count bacterial number. For Fig. 6, Pma M6CΔE was infiltrated at 10^4 cfu/ml and was co-infiltrated with water, 10 μM flg22 or 10^8 cfu/ml Pto(*hrcC*). The sequence of flg22 is QRLSTGSRINSAKDDAAGLQIA, and it was synthesized by EZBiolab Company in Westfield, IN, USA.

Protein

Soluble protein preparation and Western blot analysis were performed following previously described method (Kim et al. 2005b). Briefly, about 3 cm^2 of leaf tissue from three leaves was ground in 100 μl of grinding buffer (20 mM Tris-HCl (ph=7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT, and plant protease inhibitor cocktail (Sigma-Aldrich)). Followed by centrifugation at 20,000×g for 10 min at 4°C, the soluble supernatant was recovered and protein concentration was determined by the Bio-Rad protein assay (Bio-Rad). Samples were resolved on 12% SDS-PAGE gels (Mini-PROTEAN, Bio-Rad) and transferred to PVDF membrane (Bio-Rad). Western blots were done by standard methods using anti-PR-1 sera (Kliebenstein et al. 1999) at a dilution of 1:5,000.

Aniline Blue, DAB, and Trypan Blue Staining

For Fig. 3, 4-week-old leaves were syringe-infiltrated with 100 μM flg22 and collected after 8 h. Whole leaves were collected, stained with aniline blue (Hauck et al. 2003), mounted in 50% glycerol, and examined with epifluorescent illumination from a Nikon microscope. Four leaves were prepared for each treatment. Representative views of these pictures were randomized, and the number of callose deposits was counted blind. To visualize H_2O_2 in situ, 3,3′-diaminobenzidine (DAB) staining was performed on 4-week-old *Arabidopsis* leaves. Leaves were placed in 1 mg DAB/ml H_2O at pH 3.2 and incubated under vacuum for 3 h. DAB deposits were revealed after washing leaves in boiled 100% (v/v) ethanol for 15 min to decolorize the leaves except for the deep brown polymerization product from the reaction of DAB with H_2O_2. To observe microscopic cell death, leaves were immersed in the trypan blue staining solution (about 0.083 mg trypan blue in a mixture of 1 volume of lactophenol alcohol (a 1:1:1:1 volume mix of glycerol, saturated phenol, lactophenol, and deionized water) and 2 volumes of 95% ethanol (EtOH)) and incubated for 5 min at 95°C and then for an additional 4 h at room temperature. After the removal of the trypan blue staining solution, the leaf samples were incubated for 30 min at 65°C and subsequently washed with fresh lactophenol alcohol for 4 h and with 50% EtOH for 24 h at room temperature. Then the leaf samples were destained with the chloral hydrate solution, which was made by dissolving 250 g of chloral hydrate in 100 ml of distilled water.

Results

Mutation in *SIZ1* Gene Confers Alerted Defense System

Previous studies reported that several constitutive resistant mutants show stunted growth, probably due to the fitness cost. They also display high salicylic acid (SA) level, accumulation of ROS, and spontaneous formation of lesions (Bowling et al. 1997; Kirik et al. 2001; Lu et al. 2005; Jing and Dijkwel 2008; Lu et al. 2009). We thus tried to answer whether *siz1–2* mutant carries the characteristics of constitutive resistant plant. When grown under 8-h photoperiod to maintain vegetative status, young *siz1–2* mutants were indistinguishable from the wild type. We realized, however, that 3- to 4-week-old *siz1–2* mutants displayed mild growth reduction, and newly formed leaves were crumpled and serrated compared with the same aged Col-0 plants. It was more distinct when plants reach 5 to 6 weeks old (Fig. 1a). To check spontaneous cell death triggered by *SIZ1* mutation, we performed trypan blue staining and could not find any macroscopic cell death lesions with naked eyes. Although free of macroscopic lesions, *siz1–2* mutants exhibited a small number (30–50/leaf) of microscopic lesions. These individual or small groups of cells accumulated frequently in the vicinity of the vascular bundles. Col-0 wild-type plants did not display these symptoms (Fig. 1b). HR associated cell death followed by SAR induces number of biochemical changes, and the accumulation of ROS is one of the biochemical characteristics of SAR. As shown in Fig. 1c, constitutive accumulation of ROS was detected in *siz1–2* mutant. H_2O_2 or avirulent pathogen treatment significantly increased ROS accumulation both in Col-0 and *siz1–2* mutant indistinguishably (data not shown).

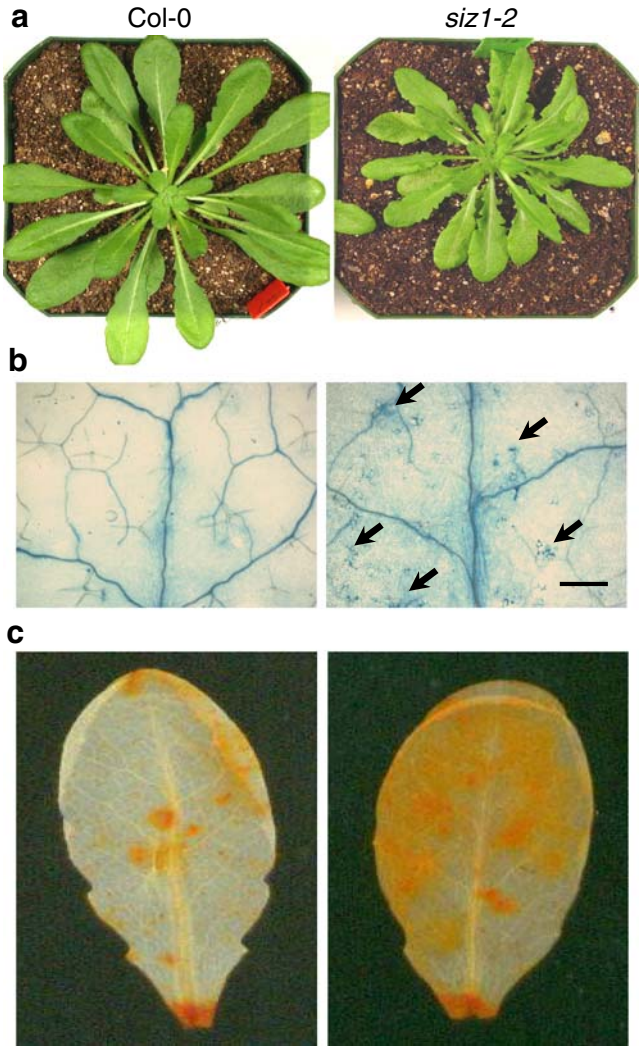

Fig. 1 The loss-of-function mutant *siz1–2* exhibits reduced growth, microscopic cell death, and H_2O_2 accumulation. **a** Photo was taken 7 weeks after seed germination and is the representative from five independent sets of experiment. **b** Distribution of microscopic cell death stained with trypan blue. *Arrowheads* indicate microscopic cell death areas. *Scale bar* is 0.2 mm. **c** H_2O_2 accumulation in the *Arabidopsis* leaves detected by diaminobenzidine (DAB) staining. Brown precipitates correlated with the presence of H_2O_2

SIZ1 Mutation Causes Early Defense Responses

Active host resistance in plants is often accompanied by the induction of *PR-1* gene which is a classic marker of SA-dependent plant defense (Ward et al. 1991). We have reported that Pph strongly induced PR-1 accumulation, while Pto did not (Ham et al. 2007). In addition, we also showed that TTSS defective Pph induced significantly less PR-1 than wild-type Pph, but did induce a low level similar to that induced by TTSS defective Pto or a purified microbe-associated molecular patterns (MAMPs). Meaning TTSS is required for the majority of PR-1 accumulation by Pph while recognition of MAMPs apparently makes a relatively minor contribution to Pph-induced PR-1 expression. Based on these observations, we monitored the accumulation of PR-1 protein over a 3-day period to answer whether *SIZ1* mutation affects the Pph-induced PR-1 accumulation. As shown in Fig. 2, Pph induced relatively high level PR-1 2 days after Pph inoculation in Col-0 wild-type plants. The *siz1–2* plants constitutively accumulated PR-1 protein at detectable levels, and Pph induced PR-1 expression more swiftly and strongly compared to wild-type. Moreover, *siz1–2* plants constitutively produced PR-5 protein in extremely high level. PR-5 protein level was weaker than in *siz1–2* and fluctuated over a time monitored in wild-type plants.

The known outputs of MAMP-induced defenses include the localized callose deposition in the cell wall, a putative physical barrier at the site of infection (Gomez-Gomez et al. 1999; Zipfel et al. 2004). We wondered whether *SIZ1* mutation affect callose deposition induced by the inoculation of 10 μM flg22, 22 amino acid long synthetic peptide derived from conserved N-terminal region of bacterial flagella. Figure 3 shows that flg22 induced callose deposition more rapidly and clearly in *siz1–2* plants compared to wild type. As shown in graphical representation, the number of callose deposits were considerably increased in 4 h and reached on maximum level in 8 h after treatment in *siz1–2* plants (Fig. 3b). The maximal level maintained thereafter. Callose deposition was relatively delayed and attenuated in wild-type plants compared to *siz1–2* plants even though the number of callose in wild-type plants caught up with that of *siz1–2* plants in the long run.

Delayed and Attenuated HR in *siz1–2* Mutant

Gram-negative plant pathogenic bacteria secrete complex sets of type III effectors directly into host cells via TTSS. For example, wild-type Pto delivers at least 33 and perhaps as many as 50 type III effectors (Collmer et al. 2002; Chang

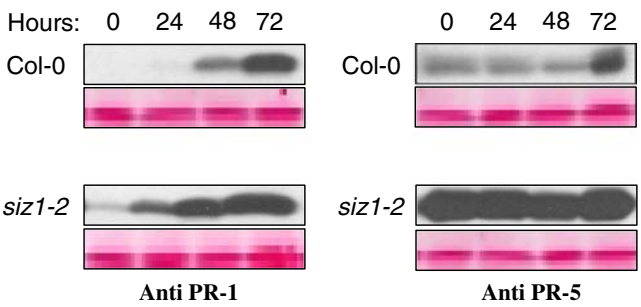

Fig. 2 Western blot analysis to check accumulation of PR-1 and PR-5 in Col-0 and *siz1–2* plants. Leaves were collected at the indicated times following infiltration of 10^8 cfu/ml Pph NPS3121. Ponceau S staining was used as a loading control. This experiment is one of five independent replicates

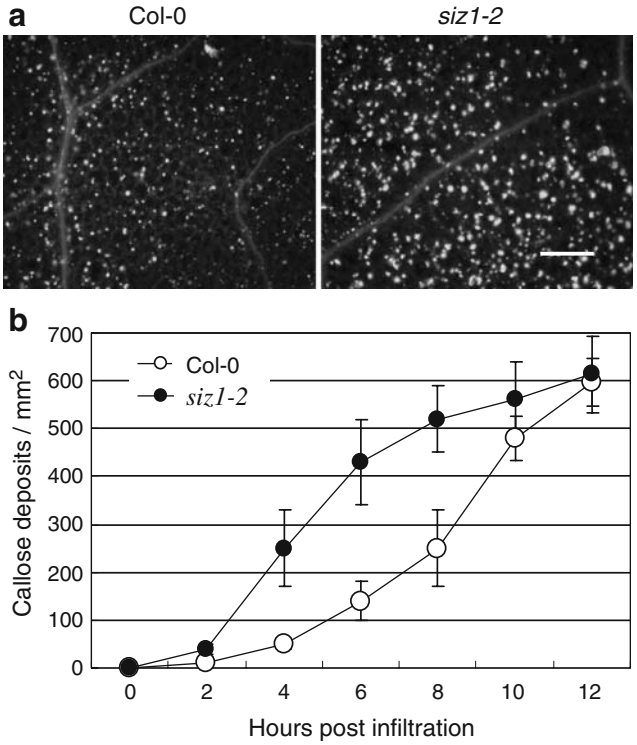

Fig. 3 Mutation in *SIZ1* causes early deposition of flg22-induced callose. **a** Aniline blue staining to detect callose deposits. The representative pictures were taken 8 h after 10 μM flg22 inoculation. *Scale bar* is 0.2 mm. **b** Graphical representation of average and standard deviation of the number of callose deposits per 1.1 mm^2 from four independent leaves. This experiment is one of five independent replicates

et al. 2005). It is cumulative effect of multiple effector proteins if we observe HR using commonly used bacterial strain Pto. Thus, it is almost impossible to detect the HR induced solely by single effector protein. To rule out that possibility, we sought and determined to utilize Gram-negative phytopathogenic bacteria *P. syringae* pv. *phaseolicola* (Pph) strain NPS3121 carrying empty vector or plasmid expressing either *AvrRpm1*or *AvrRpt2*. Pph does not trigger HR, which can obscure other defense responses. Pph is a model pathogen that causes halo blight in bean but not in *Arabidopsis* (Lindgren et al. 1986). Thus, it made it possible to measure the effect of either AvrRpm1 or AvrRpt2 on HR and electrolyte leakage. To characterize the HR of *siz1–2* against avirulent bacterial pathogens, we infiltrated the leaves of 6-week-old *siz1–2* plants with Pph expressing *AvrRpm1* (Pph (AvrRpm1)) at a dosage of 10^7 cfu/ml (see "Materials and Methods" section). Within 5 to 6 h, Col-0 plants showed confluent collapse of tissue at the site of pathogen infiltration, a characteristic feature of HR-associated cell death. However, *siz1–2* plants did not show any serious HR, and a small percentage developed a very weak HR until 6 h. The weak HR in the mutant plants was restricted to a small area surrounding the point of infiltration and was not confluent. Confluent tissue collapse was observed in several inoculated leaves of *siz1–2* plants 9 h after inoculation. AvrRpt2 which induces late HR compared to AvrRpm1 also triggered confluent collapse of tissue in wild-type plants, but a small percentage of infiltrated leaves developed confluent collapse of tissue (Fig. 4a).

Electrolyte leakage due to membrane damage on plant–pathogen interaction is a characteristic feature and a quantitative measure of HR-associated cell death (Mackey et al. 2003). To test the relationship between attenuated HR and membrane damage, we measured the electrolyte leakage in Col-0 and in *siz1–2* after Pph (AvrRpm1) or Pph (AvrRpt2) infection. The Col-0 plants infiltrated with 10^7 cfu/ml Pph (AvrRpm1) reached close maximal conductivity between 8 and 12 h (see "Materials and Methods" section). The *siz1–2* mutant displayed similar pattern but the magnitude was significantly lower than in wild-type plants (Fig. 4b). Pph (AvrRpt2) inoculation gradually increased electrolyte leakage until 24 h in both Col-0 and in *siz1–2*. But the numbers of Col-0 were significantly higher than those of *siz1–2*. Taken together, these results indicate that, unlike the wild-type parent Col-0, the *siz1–2* mutant is significantly impaired in its ability to elicit HR cell death against avirulent bacterial pathogens.

Effects of *SIZ1* Mutation on Growth of Bacterial Pathogen

Consistent with the increased expression of defense-related genes (Fig. 2), spontaneous formation of microscopic cell death (Fig. 1b), accumulation of ROS (Fig. 1c), and early callose depositions (Fig. 3), the *SIZ1* mutation conferred increased resistance to the virulent and avirulent bacterial pathogen *P. syringae* pv. *maculicola* (Pma) strain M6CΔE (Fig. 5). About 7- to 8-fold lower number of Pma (AvrRpm1) or Pma (AvrRpt2) resulted from infection of *siz1–2* compared to wild-type plants. Growth reduction by *SIZ1* mutation was more distinct against virulent pathogen Pma carrying empty vector Pma (EV). Pma (EV) proliferated about 70–80-fold lower in *siz1–2* mutant than in Col-0 plants.

When co-infiltrated into the leaves of *Arabidopsis*, flg22 can inhibit the growth of virulent Gram-negative bacteria and AvrRpt2 overcomes MAMP-induced growth repression (Zipfel et al. 2004; Kim et al. 2005a). In addition, growth repression by MAMP co-infiltration was more distinct when MAMP was infiltrated 24 h ahead (Zipfel et al. 2004). As shown in Fig. 6, co-infiltration of flg22 or TTSS defective mutant Pto(*hrc*C) repressed the growth of Pma (EV) as a magnitude of 70–80-fold compared with water co-infiltrated plants (denoted by asterisk). In *siz1–2* plant, Pma (EV) multiplied into comparable level which is suppressed by MAMP co-infiltration in Col-0 plants, and further growth repression was not observed.

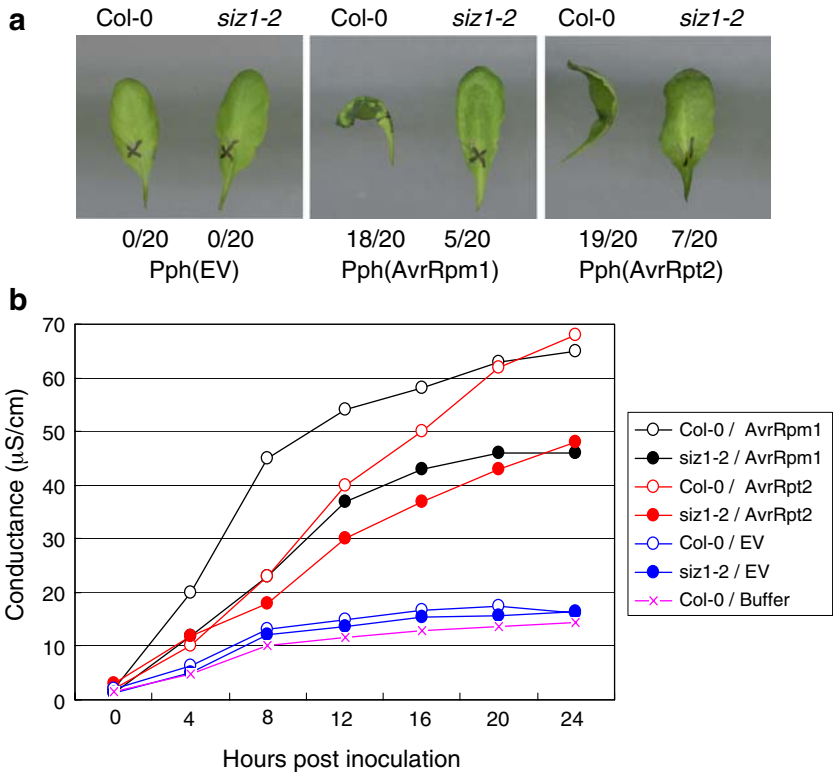

Fig. 4 Mutation in *SIZ1* represses HR cell death and electrolyte leakage. **a** Suppressed HR of *siz1–2* in response to Pph expressing *AvrRpm1* or *AvrRpt2*. Leaves of 6-week-old Col-0 and *siz1–2* plants were infiltrated with 10^7 cfu/ml and photographed 9 h after Pph (AvrRpm1) and 20 h after Pph (AvrRPt2) infiltrations. Beneath each pictured leaf is the number of the leaves that showed macroscopic HR over the total number of leaves infiltrated. **b** Electrolyte leakage in Col-O and *siz1–2* plants. Plants were infiltrated with Pph carrying empty vector or expressing plasmid either *AvrRpm1* or *AvrRpt2* at a dosage of 10^7 cfu/ml, and leaf disks were removed for conductivity measurement over time

Discussion

To study the role of sumoylation in pathogen perception and defense signaling in plants, we obtained and analyzed *Arabidopsis* SUMO E3 ligase mutant *siz1–2*. It has been proven that the impairment of sumoylation process somehow induces SAR and desensitizes pathogen perception. We also showed that the preexisting defense responses antagonize HR-associated cell death. The HR was partially compromised when the *AvrRpm1* and/or *AvrRpt2* was expressed within the *siz1–2* plants, suggesting that the repressed HR cell death in *siz1–2* might be universal symptom. Along with elevated SA level (Lee et al. 2007), accumulation of ROS, spontaneous formation of microscopic cell death, constitutively expressed PR proteins might provide favorable circumstance for these symptoms.

Single genes can give rise to a diversity of RNA transcripts because of gene silencing and each of these

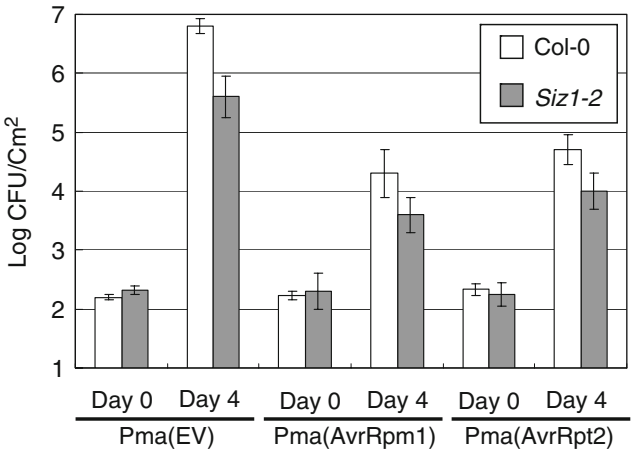

Fig. 5 Growth of Pma M6CΔE carrying empty vector or expressing a plasmid either *AvrRpm1* or *AvrRpt2* in Col-O and *siz1–2* plants. Plants were infiltrated with Pma M6CΔE carrying empty vector or expressing a plasmid either AvrRpm1 or AvrRpt2 at a dosage of 10^4 cfu/ml. The bacterial titer in leaves was determined at the indicated time points. Values are represented as colony-forming unit per square centimeter with standard deviation. This experiment is one of four independent replicates

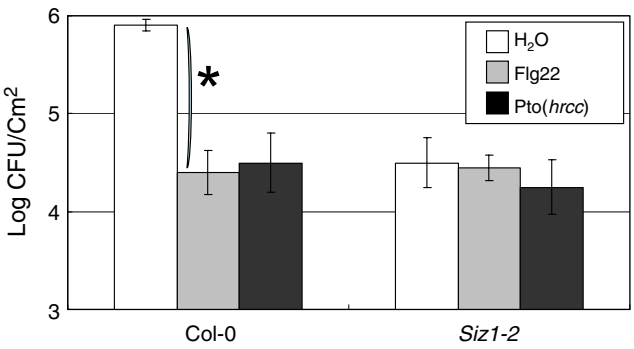

Fig. 6 Mutation in *SIZ1* suppresses MAMP-induced bacterial growth repression. Growth analysis of Pma M6CΔE co-infiltrated at 10^4 cfu/ml with either water, 10 μM flg22, or 10^8 cfu/ml Pto(*hrcC*). Bacterial growth was measured 4 days after inoculation. This experiment is one of three independent replicates

transcripts is translated into a protein that subsequently can be proteolytically processed and/or posttranslationally modified. Posttranslational modifications (PTMs) are responsible for a major increase in complexity from genome to proteome, and the reports suggesting the role of PTM on a variety of cellular processes in plants are accumulating. Currently, more than 300 types of PTMs have been described (Jensen 2004; Stulemeijer and Joosten 2008). To activate a rapid response, receptor-mediated pathogen perception and subsequent downstream signaling depend on PTM of components essential for defense signaling (Stulemeijer and Joosten 2008). Initial plant defense responses occur extremely fast on recognition of invader, which implies the involvement of PTM of pre-existing proteins in signal transduction cascades (Nurnberger and Scheel 2001; Laxalt and Munnik 2002; Laxalt et al. 2007). An obvious role of PTM in defense signaling became apparent with the discovery of protein phosphorylation in parsley cells on elicitor treatment and with the observation that mitogen-activated protein kinases (MAPKs), which require phosphorylation for activation, are involved in the primary resistance response of parsley to *Phytophphyhora sojae* (Bogre et al. 1997; Ligterink et al. 1997). In recent years, the general importance of PTMs in signal transduction cascades has become clear, and its relevance for successful plant defense signaling was further confirmed by reports describing direct manipulation of PTMs by effector protein secreted into plant cell from pathogens in order to suppress plant immune responses (Kim et al. 2005b; Mudgett 2005; Shan et al. 2007).

Sumoylation is one of the most predominant covalent modifications of proteins and implies the reversible attachment of a small ubiquitin-related modifier (SUMO) moiety to an amino acid residue (Novatchkova et al. 2004; Miura et al. 2007a). Like ubiquitination, sumoylation occurs in a series of biochemical steps referred to as SUMO E1 activation, E2 conjugation, and E3 ligation. Deconjugation of SUMO substrates is catalyzed by ubiquitin-like SUMO-specific proteases (ULP). The sumoylation and desumoylation effector proteins are conserved in plants, though there is variation in gene complexity for each in different species (Miura et al. 2007a). Sumoylation is known to play a role in defense signaling, although evidence for this remains scarce. Thus far, there are only two reports that show an increase in protein sumoylation upon exposure to abiotic stress conditions such as heat shock, H_2O_2, ethanol, and the defensive compound against herbivores, canavanine (Kurepa et al. 2003; Saracco et al. 2007). However, overexpression of SUMO in tobacco appears to block HR cell death upon xylanase infiltration (Hanania et al. 1999), and a SUMO E3 ligase, SIZ1, was reported to regulate SA-dependent innate immunity in *Arabidopsis* (Lee et al. 2007). *Xanthomonas campestris* effector XopD provides the best example for the importance of sumoylation in defense signaling from the observation that pathogen effector proteins interfere with the host sumoylation cascade. XopD from *X. campestris* effector is injected into the host cell upon infection and encodes an active cysteine protease with plant-specific SUMO substrate specificity. XopD specifically desumoylates host proteins, thereby most likely interfering with the host defense signaling cascade upon infection (Hotson et al. 2003).

Expeditious recognition and activation of the necessary defense repertoire are required for successful host resistance against pathogen invasion. One such robust response in plants involves resistance *R* gene-dependent recognition of effector proteins and initiation of localized cell necrosis at the site of pathogen infection (Ryals et al. 1996). The induction of HR cell death in resistant plants upon pathogen attack is probably the most well-recognized active resistance response. Although the exact role of cell death during HR is unclear, the controlled initiation and execution of HR cell death are thought to limit the spread of pathogens and other unwanted toxic products into healthy cells (Morel and Dangl 1997). Host cellular machinery thus needs to precisely control the untoward spread of HR cell death to protect themselves. Host R-protein RPM1 undergoes rapid degradation soon after HR initiation strongly suggests the existence of a negative feedback loop modulating the extent of cell death at the site of infection (Boyes et al. 1998). The best line of evidence for the genetic control of HR-like cell death stems from the analyses of several lesion mimic mutants that may be perturbed in regulating certain aspects of pathogen-induced cell death. The *Arabidopsis dnd1* mutant, originally identified in a screen for reduced HR against *P. syringae* pv. *glycinea* (AvrRpt2), was later found to be a rare/conditional lesion mimic mutant (Clough et al. 2000). However, why such a mutation that suppresses HR leads to systemic resistance is not clear. Alternatively, the systemic resistance itself may be responsible for the reduced HR in the *dnd1* mutant. If so, what are the possible mechanisms that suppress HR in *siz1–2* plants? There are numerous signaling steps in the HR cascade, which, when affected, can influence HR. For example, downregulation of K^+ and Cl^- efflux channel activities in *siz1–2* plants could lead to a severe reduction in HR. Ca^{2+} channel blockers have been shown to inhibit HR in tobacco and soybean (*Glycine max*) cells (Atkinson et al. 1990, 1996). Continuous generation of ROS and other antimicrobial compounds such as phytoalexins may render *siz1–2* mutant refractory to changes in membrane permeability that is crucial for HR cell death. Rapid turnover of various *R* gene products might prevent HR induction (Boyes et al. 1998). Although we cannot accurately predict which step is perturbed in *siz1–2* that leads to suppression of HR, our

results indicate that an induced SAR response down-regulates further HR cell death.

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