Tomato *SOBIR1/EVR* Homologs Are Involved in Elicitin Perception and Plant Defense Against the Oomycete Pathogen *Phytophthora parasitica*

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Submitted 19 December 2014. Accepted 17 February 2015.

During host-pathogen interactions, pattern recognition receptors form complexes with proteins, such as receptor-like kinases, to elicit pathogen-associated molecular pattern-triggered immunity (PTI), an evolutionarily conserved plant defense program. However, little is known about the components of the receptor complex, as are the molecular events leading to PTI induced by the oomycete *Phytophthora* pathogen. Here, we demonstrate that tomato (*Solanum lycopersicum*) *SISOBIR1* and *SISOBIR1*-like genes are involved in defense responses to *Phytophthora parasitica*. Silencing of *SISOBIR1* and *SISOBIR1*-like enhanced susceptibility to *P. parasitica* in tomato. Callose deposition, reactive oxygen species production, and PTI marker gene expression were compromised in *SISOBIR1*- and *SISOBIR1*-like-silenced plants. Interestingly, *P. parasitica* infection and elicitin (ParA1) treatment induced the relocalization of *SISOBIR1* from the plasma membrane to endosomal compartments and silencing of *NbSOBIR1* compromised ParA1-mediated cell death on *Nicotiana benthamiana*. Moreover, the *SISOBIR1* kinase domain is indispensable for ParA1 to trigger *SISOBIR1* internalization and plant cell death. Taken together, these results support the idea of participation of solanaceous *SOBIR1/EVR* homologs in the perception of elicitins and indicate their important roles in plant basal defense against oomycete pathogens.

To prevent infection by pathogens, plants have developed two distinct types of immunity: i) pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and ii) effector-triggered immunity (ETI) (Dodds and Rathjen 2010; Jones and Dangl 2006). PAMPs (also known as microbe-associated molecular patterns [MAMPs]), derived from microbial pathogens, are conserved molecular structures that are recognized by pattern recognition receptors (PRR) and elicit the first layer of plant immunity, i.e., PTI. On the other hand, some adapted plant pathogens can produce effectors to suppress PTI and thus promote disease development. Through the arms race with

pathogens, plants have evolved resistance (R) proteins to recognize cognate effectors or effector-mediated alteration of host targets and, thereby, activate ETI (Dodds and Rathjen 2010; Jones and Dangl 2006).

Most plant PRR are members of the receptor-like kinase (RLK) or receptor-like protein (RLP) family, implicated in multiple physiological programs, including plant development and immunity to microbial infection (Morillo and Tax 2006; Shiu and Bleecker 2003). In *Arabidopsis thaliana*, the most studied PRR are FLAGELLIN SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR), both leucine-rich repeat (LRR)-RLK responsible for perceiving bacterial PAMPs flg22 (derived from flagellin) and elf18/elf26 (derived from elongation factor Tu), respectively (Gómez-Gómez and Boller 2000; Zipfel et al. 2004, 2006). As well, CHITIN ELICITOR RECEPTOR KINASE 1, a Lysine motif-RLK, recognizes fungal chitin and mediates resistance to fungal pathogens (Miya et al. 2007). When PRR perceive their cognate PAMPs, downstream defense signaling pathways are activated that involve ion fluxes, activation of calcium-dependent protein kinases and mitogen-activated protein kinases, reactive oxygen species (ROS) production, callose deposition, and defense gene induction (Altenbach and Robatzek 2007; Boller and Felix 2009; Zipfel 2009). As a consequence, plants display basal resistance to a broad range of pathogens.

Recent studies to elucidate molecular events immediately after flg22 perception indicated that FLS2 forms a heterodimer with BRASSINOSTEROID (BR) INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1; also known as SOMATIC EMBRYOGENESIS RLK3 [SERK3]), which was first identified due to its interaction with BRI1, a receptor of the plant hormone BR (Chinchilla et al. 2007; Heese et al. 2007). Upon perception of BR, BAK1 forms a heterodimer with BRI1, which is followed by a rapid reciprocal transphosphorylation of cytoplasmic kinase domains and activation of a downstream signaling pathway (Wang et al. 2008). Null *bak1* mutant plants are hyposensitive to BR, which indicates that BAK1 acts as a positive regulator of the BRI1 pathway (Chinchilla et al. 2007; Heese et al. 2007). In addition to binding with BRI1, BAK1 forms receptor complexes with FLS2 and EFR in a ligand-dependent manner and is also required for responses triggered by other PAMPs, such as lipopolysaccharides, peptidoglycans, and HrpZ, as well as INF1, an elicitor identified in *Phytophthora infestans* (Chinchilla et al. 2007; Heese et al. 2007; Shan et al. 2008). Therefore, BAK1 is considered a central regulator of plant basal immunity (Chinchilla et al. 2009). Two additional RLK function as regulators of defense responses in *A. thaliana*, *BAK1-interacting RLK (BIR1)* and *Suppressor of*

Nucleotide sequence data is available in the GenBank database under accession numbers KF268115 (*SISOBIR1*), KF977196 (*SISOBIR1*-like), and KF998581 (*NbSOBIR1*).

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*The e-Xtra logo stands for “electronic extra” and indicates that nine supplementary figures and one supplementary table are published online.

BIR1-1/Evershed (*SOBIR1/EVR*; hereafter called *AtSOBIR1*) (Gao et al. 2009). Moreover, SERK1 and BAK1-LIKE 1 (BKK1; also known as SERK4), both involved in regulating cell death and senescence (He et al. 2007), were found to be components of the ligand-induced FLS2 and EFR receptor complexes and required for FLS2- and EFR-dependent responses, which reinforces the idea that PRR functions as part of multiprotein complexes at the plasma membrane with the triggering of plant basal immunity (Monaghan and Zipfel 2012; Roux et al. 2011). Of note, FLS2 undergoes ligand-induced endocytosis and subsequent degradation upon elicitation by flg22 (Geldner and Robatzek 2008; Robatzek et al. 2006). Similarly, LeEix2 receptor is internalized in response to treatment with the ethylene-inducing xylanase (EIX) from *Tichoderma viride*, a potent elicitor of plant defense responses (Bar and Avni 2009). These results highlight the important role of receptor-mediated endocytosis in the regulation of plant defense signaling (Bar and Avni 2014; Leborgne-Castel et al. 2010).

Oomycetes belong to a phylogenetically distinct group of eukaryotic microorganisms that resemble true fungi in morphologic features and pathogenic life style (Baldauf et al. 2000). Members of the genus *Phytophthora* are of special interest because many of them cause devastating diseases in economically important crops and ornamental plants (Erwin and Ribeiro 1996; Kamoun et al. 2014). PAMPs identified in genus *Phytophthora* thus far include CBD (derived from cellulose binding elicitor lectin [CBEL]) of *Phytophthora parasitica* (Gaulin et al. 2006; Mateos et al. 1997), Pep-13 (derived from calcium-dependent transglutaminase GP42) of *Phytophthora sojae* (Brunner et al. 2002; Nürnberger et al. 1994), and elicitors such as INF1 (Kamoun et al. 1997). Elicitors are a family of structurally related extracellular proteins that are secreted by all tested *Phytophthora* and *Pythium* species (Ponchet et al. 1999). These proteins induce the hypersensitive response (HR) and other defense responses in a limited range of dicot plants, including *Nicotiana* spp. (Kamoun et al. 1993a and b, 1997; Ricci et al. 1989). For example, ParA1, a close homolog of INF1 identified in *P. parasitica*, induces a necrotic HR on tobacco leaves (Kamoun et al. 1993a). INF1 elicits HR cell death and ROS production in *Nicotiana benthamiana*. Knocking down the expression of *SERK3-like* abrogated INF1-induced cell death (Heese et al. 2007). On the other hand, silencing of the *N. benthamiana* homologs of *SERK3/BAK1*, including both *NbSERK3A* and *NbSERK3B*, enhanced plant susceptibility to *P. infestans* and reduced the cell death responses triggered by INF1 in *N. benthamiana* (Chaparro-Garcia et al. 2011). INF1 also interacted with a lectin-like receptor kinase, NbLRK1, and downregulation of this gene delayed INF1-mediated HR cell death (Kanzaki et al. 2008). Nonetheless, our current knowledge about the nature of elicitor receptors and components of the receptor complex remains very limited, not to mention other LRR-RLK that might be involved in elicitor-mediated cell death and plant basal defense against *Phytophthora* infection.

In attempts to identify tomato genes that are involved in plant immunity against *P. parasitica*, we performed microarray analyses and found a variety of genes that are upregulated in response to pathogen infection, including one that encodes *SISOBIR1*, a tomato (*Solanum lycopersicum*) homolog of *AtSOBIR1*. Just recently, *SISOBIR1/EVR* and its close homolog *SISOBIR1-like* were found to interact with both Cf-4 and Ve1 in planta and were required for HR and for plant resistance to *Cladosporium fulvum* and *Verticillium dahliae* mediated by these two RLP (Liebrand et al. 2013). Here, we demonstrate that *SISOBIR1* and *SISOBIR1-like* are involved in tomato basal defense against *P. parasitica* infection. Notably, both pathogen

infection and ParA1 treatment induced the endocytosis of *SISOBIR1*. Furthermore, a *SOBIR1/EVR* homolog of *N. benthamiana*, *NbSOBIR1*, is required for full induction of ParA1-mediated cell death. These results imply the participation of *SISOBIR1* in the formation of the receptor complex, which mediates elicitor perception and defense signaling against oomycete pathogen infection.

RESULTS

Molecular cloning and sequence analysis of *SISOBIR1* and *SISOBIR1-like*.

To identify tomato genes involved in the plant defense against *P. parasitica*, we performed microarray analyses and found various genes upregulated in response to pathogen infection (R. F. Liou unpublished data), including one that encodes a putative RLK, tomato gene model Solyc06g071810.1.1 (unigene SGN-U565512; corresponding to probe ID Les.5205.1. S1_at [Affymetrix]). By searching the Sol Genomics Network (SGN), we found a close homolog with about 80% nucleotide identity with this gene, Solyc03g111800.1.1 (SGN-U568730). These two genes were reported recently by Liebrand et al. (2013) and were named *SISOBIR1* (Solyc06g071810) and *SISOBIR1-like* (Solyc03g111800), respectively.

The cDNA fragments for both genes were cloned with mRNA from tomato (cv. Summer Sweet) as the template (GenBank accession numbers: KF268115 [*SISOBIR1*] and KF977196 [*SISOBIR1-like*]). When compared with the sequences in the SGN database, the amino acid sequence of KF268115 is identical to the *SISOBIR1* sequence, whereas KF977196 possesses an extra segment of 50 amino acid residues when compared with *SISOBIR1-like* (Supplementary Fig. S1A). BLASTP analysis with the National Center for Biotechnology Information and SGN websites revealed the presence of respective homologs for *SISOBIR1* and *SISOBIR1-like* in *Solanum tuberosum*, with identity of 92.7% between Sotub06g029250.1.1 and *SISOBIR1* and 95.6% between Sotub03g023250.1.1 and *SISOBIR1-like*. *N. benthamiana* also contains two homologs, namely, *NbSOBIR1* (NbS00037616 g0014.1) and *NbSOBIR1-like* (NbS00006092g0015.1). We have determined the cDNA sequence of *NbSOBIR1* (KF998581) and found that it is identical to that shown in the database. However, we failed to detect any expression of *NbSOBIR1-like*. There is only one *SOBIR1* homolog of *N. tabacum* in GenBank, AB073628, which is close to *NbSOBIR1*, as revealed by phylogenetic analysis.

Transcription of *SISOBIR1* and *SISOBIR1-like* is induced in response to *P. parasitica* infection and salicylic acid (SA) treatment.

To confirm the extent to which *SISOBIR1* and *SISOBIR1-like* are induced by *P. parasitica* infection, we analyzed their transcriptional expression in response to pathogen inoculation. Compared with mock-inoculated controls, the expression of *SISOBIR1* was induced highly (24-fold) in tomato leaves at 1 h postinoculation (hpi) and to a lesser extent at 3 and 6 hpi but upregulated again at 12 hpi (Fig. 1A). In contrast, the expression of *SISOBIR1-like* was significantly upregulated only at 12 hpi.

To test whether plant-hormone signaling cascades regulate the transcription of *SISOBIR1* and *SISOBIR1-like*, we treated plants with different plant hormones and analyzed their expression. The expression of both *SISOBIR1* and *SISOBIR1-like* was induced at 3 h posttreatment (hpt) with SA and was downregulated to a background level at 6 hpt (Fig. 1B). With ethylene (ET) treatment, *SISOBIR1* was significantly induced at 3 hpt (Fig. 1C), whereas *SISOBIR1-like* was downregulated

at 6 hpt. In contrast, treatment with methyl jasmonate (MeJA) downregulated the expression of both *SISOBIR1* and *SISOBIR1-like* (Fig. 1D). Thus, the expression of *SISOBIR1* and *SISOBIR1-like* was induced by SA but downregulated by JA. With ET treatment, only *SISOBIR1* was induced.

Silencing of *SISOBIR1* and *SISOBIR1-like* enhances plant susceptibility to *P. parasitica*.

To determine whether *SISOBIR1* and *SISOBIR1-like* are involved in disease resistance, we silenced the expression of *SISOBIR1* or *SISOBIR1-like*, alone or together, in tomato plants by *Tobacco rattle virus* (TRV)-mediated gene silencing and, then, challenged plants with *P. parasitica*. Four weeks after virus inoculation, plants were inoculated with zoospores of *P. parasitica* and were examined for the development of disease symptoms as well as the expression levels of *SISOBIR1* and *SISOBIR1-like*. In TRV-*SISOBIR1*-infected tomato plants, the expression of *SISOBIR1* but not *SISOBIR1-like* was downregulated in systemic leaves as compared with control plants (TRV) (Supplementary Fig. S2A). Similarly, in plants infected with TRV-*SISOBIR1-like*, only *SISOBIR1-like* was downregulated. In contrast, in plants infected with TRV-*SISOBIR1*&*SISOBIR1-like*, containing a conserved sequence of *SISOBIR1* and *SISOBIR1-like*, both genes were downregulated. With silencing of *SISOBIR1* or *SISOBIR1-like* individually, plants became more susceptible to infection by *P. parasitica*, as revealed by the appearance of disease symptoms (Fig. 2A) and disease severity scores (Fig. 2B). To further confirm the effect of *SISOBIR1* silencing on *P. parasitica* colonization, we employed quantitative polymerase

chain reaction (qPCR) to analyze the accumulation of *P. parasitica* WS21 on the inoculated plants. WS21 encodes a ribosomal protein (Yan and Liou 2006) and here serves as an indicator of pathogen biomass. The biomass of *P. parasitica* on *SISOBIR1*-silenced plants was more abundant than that of the control plants with the empty vector (TRV) at both 48 and 72 hpi, whereas significant difference was detected for the *SISOBIR1-like*-silenced plants only at 72 hpi (Fig. 2C). When both genes were silenced simultaneously, the plants became even more susceptible to *P. parasitica* infection, as revealed by the enhanced disease symptoms (Fig. 2A), disease severity scores (Fig. 2B), and the highly increased level of pathogen biomass at both 48 and 72 hpi (Fig. 2C). Thus, both *SISOBIR1* and *SISOBIR1-like* are required for the basal disease resistance of tomato to *P. parasitica*.

Downregulation of *SISOBIR1* and *SISOBIR1-like* causes reduced plant defense response against *P. parasitica*.

To further determine whether *SISOBIR1* and *SISOBIR1-like* regulate plant defense responses during pathogen infection, we examined tomato leaves for ROS accumulation, callose deposition, and expression of PTI marker genes after TRV-mediated gene silencing and pathogen inoculation. Control plants infected by *P. parasitica* showed prominent production of H₂O₂ at 3 hpi (TRV) (Fig. 3A and C), accompanied by callose deposition at 12 hpi (TRV) (Fig. 3B and D). The expression of PTI marker genes, including *Pti-5*, *WRKY28*, *Gras2*, and *Lrr22*, was significantly induced at 6 hpi (Supplementary

Fig. 1. Expression of *SISOBIR1* and *SISOBIR1-like* genes is induced in response to infection by *Phytophthora parasitica* and treatment with plant hormones. After pathogen inoculation or hormone treatment of 4-week-old tomato (*Solanum lycopersicum* cv. Summer Sweet), total RNA isolated from the leaves were analyzed by quantitative reverse transcription-polymerase chain reaction. *eIF5A-4* was used as an internal control. Data from three independent experiments are presented as mean ($\pm$ standard deviation) fold change relative to time 0. An asterisk (*) indicates $P < 0.05$, two (**) indicate $P < 0.01$ by Student *t* test. hpi = hours postinoculation with pathogen; hpt = hours posttreatment with plant hormone. **A**, Inoculation with zoospores of *P. parasitica*; **B**, Treatment with salicylic acid (SA), **C**, ethylene (ET), and **D**, methyl jasmonate (MeJA).

Fig. S3). In contrast, plants with silencing of one or both *SISOBIR1* and *SISOBIR1-like* showed significantly reduced H_2O_2 production (Fig. 3A and C) and callose deposition (Fig. 3B and D). Moreover, silencing of *SISOBIR1* reduced expression of *Pti-5*, *WRKY28*, and *Gras2*, and simultaneous silencing of both genes reduced expression of *Pti-5* and *WRKY28* (Fig. 3E to G), with no change in expression of *Lrr22* (Fig. 3H). At this time point of infection, the expression of one or both *SISOBIR1* and *SISOBIR1-like* was still suppressed by the silencing constructs. Therefore, both *SISOBIR1* and *SISOBIR1-like* play essential roles in triggering plant basal defense responses to *P. parasitica* infection,

although their roles for the regulation of PTI marker genes may differ.

P. parasitica inoculation and ParA1 treatment induce the endocytosis of *SISOBIR1*.

Receptor-mediated endocytic trafficking pathways play important roles for PAMP perception and cellular responses during pathogen attack (Geldner and Robatzek 2008; Leborgne-Castel et al. 2010). A recent report has established that *SISOBIR1* and *SISOBIR1-like* encode transmembrane domain-containing, receptor-like protein kinases that localize to the plasma membrane (Liebrand et al. 2013). To study functions of these proteins during

Fig. 2. Silencing of *SISOBIR1* and *SISOBIR1-like* genes enhances tomato susceptibility to *Phytophthora parasitica*. **A**, Downregulation of one or both *SISOBIR1* and *SISOBIR1-like* enhances tomato susceptibility to *P. parasitica*. At 4 weeks post-agroinfection for *Tobacco rattle virus* (TRV)-mediated gene silencing of one or both *SISOBIR1* and *SISOBIR1-like*, tomato plants were inoculated with zoospores of *P. parasitica* and were examined for disease development. Plants infiltrated with the empty vector (TRV) served as a control. Images were obtained at 36 h postinoculation (hpi). Experiments were repeated three times, each with 10 plants. **B**, Disease index of tomato plants. Disease severity was scored on a scale from 0 to 5, according to symptoms developed on tomato plants: 0, healthy, no water-soaking lesions; 1, <25% lesions; 2, 25 to 50% lesions; 3, 50 to 75% lesions; 4, 75 to 100% lesions; and 5, complete wilting. One asterisk (*) indicates $P < 0.05$, two (**) indicate $P < 0.01$ by Wilcoxon rank-sum test. **C**, Quantification of *P. parasitica* biomass by quantitative polymerase chain reaction (qPCR). After pathogen inoculation of tomato plants silenced in one or both *SISOBIR1* and *SISOBIR1-like*, DNA was isolated from systemic leaves at 24, 48, and 72 hpi, respectively. The quantity of *P. parasitica* WS21 and tomato *elf5A-4* was determined by qPCR. Data of *P. parasitica* WS21 were normalized with tomato *elf5A-4* and are presented as mean ($\pm$ standard deviation) fold change relative to plants infiltrated with the empty vector (TRV) at 24 hpi from three independent experiments. One asterisk (*) indicates $P < 0.05$, two (**) indicate $P < 0.01$ by Student *t* test.

Fig. 3. Downregulation of *SISOIR1* and *SISOIR1-like* genes attenuates tomato defense responses to *Phytophthora parasitica*. At 4 weeks post-agroinfection for Tobacco rattle virus (TRV)-mediated gene silencing of one or both *SISOIR1* and *SISOIR1-like*, tomato plants were inoculated with zoospores of *P. parasitica* and were then subjected to 3,3'-diaminobenzidine (DAB) staining, aniline blue staining, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Plants infiltrated with the empty vector (TRV) served as a control. **A**, Staining with DAB at 3 h postinoculation (hpi) to detect H₂O₂ accumulation. Bar = 1 cm. **B**, Staining with aniline blue at 12 hpi to detect callose deposition. The images were captured by fluorescence microscopy. Bar = 25 μ m. **C**, Quantification of DAB staining. Values are mean ($\pm$ standard deviation [SD]) from three independent experiments (each with 10 leaves). Asterisks (*) indicate $P < 0.05$ by Student *t* test compared with TRV. **D**, Quantification of aniline blue staining. Values are mean ($\pm$ SD) from three independent experiments. Asterisks (*) indicate $P < 0.05$ by Student *t* test. **E** to **H**, qRT-PCR analysis of defense genes. Total RNAs isolated from inoculated plants at 6 hpi were analyzed for the expression of *Pti-5* (E), *WRKY28* (F), *Gras2* (G), and *Lrr22* (H), with *eIF5A-4* as the internal control. Values are mean ($\pm$ SD) fold change relative to empty vector (TRV) inoculation from three independent experiments. Asterisks (*) indicate $P < 0.05$ by Student *t* test.

P. parasitica infection, we expressed green fluorescent protein (GFP)-fusion proteins of *SISOBIR1*- and *SISOBIR1-like* on *N. benthamiana* by agroinfiltration. Confocal microscopy results revealed that *SISOBIR1*-GFP localized to the plasma membrane, as visualized by colocalization with the staining of the lipophilic

Fig. 4. *SISOBIR1*-GFP moved to vesicle-like structures in response to *Phytophthora parasitica* infection. **A**, Subcellular localization of *SISOBIR1*-GFP and *SISOBIR1-like*-GFP. At 23 h post-agroinfiltration, *Nicotiana benthamiana* leaves were examined under a confocal microscope. Bar = 25 μ m. **B**, Subcellular localization of *SISOBIR1*-GFP in response to *Phytophthora parasitica* infection. At 23 h post-agroinfiltration for expressing *SISOBIR1*-GFP, *N. benthamiana* leaves were inoculated with zoospores of *P. parasitica* (or double-distilled H_2O as a control) and were examined under a confocal microscope at 3 h postinoculation. The first and second rows show the green fluorescent protein (GFP) images, and the bottom row shows an overlay of GFP with images recorded under red light and differential interference contrast microscopy, respectively. Images to the right are enlargements of the outlined regions in the left panel. Arrowheads point to *SISOBIR1*-GFP-labeled vesicle-like structures (second row) and germinating cysts (bottom row). Bars in the left and right panels represent 25 and 10 μ m, respectively.

styryl dye FM4-64 (Fig. 4A; Supplementary Fig. S4A), consistent with findings by Liebrand et al. (2013). However, in addition to plasma membrane, *SISOBIR1*-like-GFP also accumulated on some internal structures (Fig. 4A), indicative of localization to multiple membrane compartments or vesicles. Interestingly, when we challenged tobacco leaves expressing *SISOBIR1*-GFP with *P. parasitica*, we observed increasing amounts of internal puncta labeled with *SISOBIR1*-GFP in a substantial population of cells (Fig. 4B). As germinating cysts are often found adjacent to these cells (Fig. 4B), we suggest that the accumulation of *SISOBIR1*-GFP internal puncta may correlate with *P. parasitica* infection.

To test whether the perception of PAMPs from *Phytophthora* spp. cause similar effects, we coexpressed *SISOBIR1*-GFP with various PAMPs on leaves of *N. benthamiana*. Interestingly, we found that coexpression with the *P. parasitica* elicitor ParA1 dramatically relocalized *SISOBIR1*-GFP from plasma membrane to multiple internal puncta (Fig. 5A). Further staining with FM4-64 confirmed the intactness of the plasma membrane and internalization of the fluorescent dye after ParA1 treatment. These results suggest that the *P. parasitica* elicitor ParA1 is sufficient to trigger *SISOBIR1* internalization. Given that, in nature, ParA1 is secreted from *P. parasitica* to the apoplastic plant-pathogen interface, we next tested whether the ParA1-induced *SISOBIR1* internalization requires secretion of ParA1 to extracellular space. We found that *SISOBIR1*-GFP remains on the plasma membrane when it is coexpressed with ParA1- Δ SP, a ParA1 variant lacking the N-terminal signal peptide (Fig. 5A). Thus, ParA1 is likely secreted to the apoplastic space and perceived by the plant cell at the cell surface and, then, causes *SISOBIR1* internalization.

To directly address whether treatment of ParA1 triggers *SISOBIR1* internalization, we set up a simple system by which we expressed *SISOBIR1*-GFP in *N. benthamiana* protoplasts and treated the cells with purified recombinant ParA1 protein. To avoid observing *SISOBIR1*-GFP accumulation on transport vesicles or compartments along the secretory pathways, we incubated the protoplasts with 50 μ M cycloheximide (CHX) to block protein synthesis 30 min before and during ParA1 addition. When compared with the control, *SISOBIR1*-GFP, indeed, trafficked from the plasma membrane to highly mobile internal structures upon ParA1 treatment (Fig. 5B). Quantification results further indicated that the number of these internal structures in ParA1-treated protoplasts was significantly higher than that of buffer-treated, control protoplasts (Fig. 5C). We next tested the extent to which treatment of latrunculin B (LatB), a drug that blocks endocytosis by disrupting the assembly of actin cytoskeleton, could block the endocytosis of *SISOBIR1*. Addition of LatB effectively impaired ParA1-induced *SISOBIR1*-GFP internalization (Fig. 6A), as the number of vesicle-like structures formed in ParA1-treated protoplasts in the presence of LatB was significantly reduced to a level similar to a mock control (Fig. 6B).

To confirm that the punctate structures observed are endocytic vesicles, we coexpressed *SISOBIR1*-GFP with the early/late endosome marker AtARA6-mRFP (monomeric red fluorescence protein) and the early endosome marker mRFP-AtARA7 (Lu et al. 2012). As shown in Figure 6C, AtARA6-mRFP (70.8% $\pm$ 10.9%) and mRFP-AtARA7 (89.9% $\pm$ 12.4%) largely colocalized with ParA1-induced *SISOBIR1*-GFP vesicle, supporting the idea that these structures are, indeed, endosomal compartments. Taken together, we conclude that perception of *P. parasitica* elicitor ParA1 at the plant cell surface triggers the endocytosis of *SISOBIR1* from plasma membrane to various endosomal compartments in the cell.

Interestingly, we found that flg22 treatment did not trigger the endocytosis of *SISOBIR1*-GFP, although the same treatment

relocalized the well-studied RLK FLS2 from the plasma membrane to punctate structures in the cytoplasm (Supplementary Fig. S5A). Similarly, treatment with CBEL in an agroinfiltration experiment failed to cause SISOBIR1-GFP internalization. Thus, our data clearly showed that SISOBIR1 responds specifically to ParA1 and, perhaps, other PAMPs but not all PAMPs. As SISOBIR1 is a RLK, we further asked whether the kinase domain of SISOBIR1 is essential for its internalization triggered by ParA1. Similar to SISOBIR1-GFP, the kinase-defective mutant SISOBIR1^{D473N}-GFP (Liebrand et al. 2013) localized mainly to the plasma membrane, although the signal was much weaker (Supplementary Fig. S6). Treatment with ParA1 resulted in the formation of a very small number of SISOBIR1^{D473N}-GFP-labeled puncta nearby the plasma membrane. Thus, the kinase activity is very likely required for ParA1-triggered endocytosis of SISOBIR1-GFP.

***NbSOBIR1* and *NbSERK3/BAK1* are required for ParA1-mediated cell death.**

The observation of ParA1 triggering SISOBIR1 endocytosis suggests that *SOBIR1* homologs are involved in elicitor perception in solanaceous plants. Hence, we silenced the expression of *NbSOBIR1* by TRV to determine whether it affects ParA1-mediated cell death. We confirmed the silencing of *NbSOBIR1* by quantitative reverse transcription (qRT)-PCR at 3 weeks after

virus inoculation (Supplementary Fig. S7A) and, then, expressed ParA1 in *N. benthamiana* leaves by agroinfiltration. Three days after agroinfiltration, necrosis appeared on control *N. benthamiana* leaves with the TRV empty vector (Fig. 7A, TRV). ParA1 treatment also induced H₂O₂ production and callose deposition (Fig. 7A, TRV). In contrast, the ParA1-induced necrosis, H₂O₂ production, and callose deposition were significantly compromised in *NbSOBIR1*-silenced leaves (Fig. 7A, TRV-*NbSOBIR1*). To know whether these effects were, indeed, caused by *NbSOBIR1* silencing, we coexpressed ParA1 with *NbSOBIR1*-GFP in the silenced tobacco plants. Moreover, P19 of *Tomato bush stunt virus* was coexpressed to attenuate the effect of TRV-mediated gene silencing. In contrast to coexpression of GFP as a control, coexpression of *NbSOBIR1* and P19 rescued ParA1-induced necrosis, H₂O₂ production, and callose deposition on *NbSOBIR1*-silenced tobacco leaves (Fig. 7B to E). Similarly, coexpression of *SISOBIR1*-GFP, individually or in combination with *SISOBIR1*-like-GFP, rescued the effects caused by ParA1 on *NbSOBIR1*-silenced tobacco leaves (Fig. 7B to E). However, coexpression with *SISOBIR1*-like-GFP only recovered callose deposition but not ParA1-induced necrosis and H₂O₂ accumulation (Fig. 7B to E). In *A. thaliana*, overexpression of *SOBIR1* is known to cause cell death (Gao et al. 2009). To exclude the possibility that the necrosis symptom observed in the complementation experiment

Fig. 5. SISOBIR1-GFP moved to vesicle-like structures in response to ParA1 treatment. **A**, Subcellular localization of SISOBIR1-GFP coexpressed with ParA1 or ParA1-ΔSP on *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* harboring ParA1, ParA1-ΔSP, or an empty vector (EV) expression construct was coinfiltrated with that carrying SISOBIR1-GFP expression construct, and confocal images were captured at 23 h post-agroinfiltration. Images to the right are enlargements of the outlined regions in the left panel. Arrowheads indicate SISOBIR1-GFP-labeled vesicle-like structures. Bars in the left and right panels represent 25 and 10 μm, respectively. **B**, Confocal images of SISOBIR1-GFP in tobacco protoplasts in the presence of ParA1. After transformation with the SISOBIR1-GFP expression construct, tobacco protoplasts were treated with 50 μM cycloheximide (CHX) for 30 min, and then, 300 μM ParA1 recombinant protein (or buffer as a control) for another 30 min. Arrowheads point to SISOBIR1-GFP-labeled vesicle-like structures. Images in the upper (Z1) and lower panels (Z2) were captured with different z-stacks. Bar = 10 μm. **C**, The number of SISOBIR1-GFP-labeled vesicle-like structures in tobacco protoplasts. Values are the numbers of vesicle-like structures in each protoplast ($n = 50$) from one of the three biological repeats, with the mean marked by a horizontal line. The asterisk (*) indicates $P < 0.05$ by Student t test.

was caused by overexpression of *SISOBIR1* and *SISOBIR1-like*, we expressed either of these two genes in *N. benthamiana*. By 72 h post-agroinfiltration, no symptom of necrosis developed on the leaves expressing *SISOBIR1* or *SISOBIR1-like* nor were H₂O₂ accumulation and callose deposition detectable, which is similar to results obtained with the control leaves expressing

GFP (Supplementary Fig. S8). In contrast, expression of *ParA1* caused necrosis, H₂O₂ accumulation, and callose deposition, as seen in the *ParA1*-expressing plants infected with the TRV empty vector (Fig. 7A). To test if the kinase activity of *SISOBIR1* is essential for plant response to *ParA1*, we coexpressed *ParA1* with kinase-defective mutant, *SISOBIR1*^{D473N} or *SISOBIR1-like*^{D486N}

Fig. 6. Endocytosis of *SISOBIR1*-GFP in response to *ParA1* treatment. **A**, Latrunculin B (LatB) impaired the movement of *SISOBIR1*-GFP in response to *ParA1* treatment. *Nicotiana benthamiana* protoplasts expressing *SISOBIR1*-GFP were treated with 50 μ M cycloheximide (CHX) for 30 min, and then, 300 μ M *ParA1* recombinant proteins along with 20 nM LatB (or buffer as a control) for another 30 min, followed by capture of the confocal images with different *z*-stacks (Z1 and Z2). Bar = 10 μ m. **B**, LatB reduced the number of *ParA1*-induced, *SISOBIR1*-GFP-labeled vesicle-like structure in the cytoplasm. Values are the numbers of vesicle-like structures in each protoplast ($n = 50$) from one of the three biological repeats, with the mean marked by a horizontal line. Asterisks (*) indicate $P < 0.05$ by Student *t* test. **C**, *SISOBIR1*-GFP colocalized with endocytic markers in *Nicotiana benthamiana* leaves in response to *ParA1* treatment. At 23 h post-agroinfiltration for coexpression of *SISOBIR1*-GFP, *ParA1*, and the marker (AtARA6-mRFP or mRFP-AtARA7), images were captured under a confocal microscope. Values to the right of the figure represent mean ($\pm$ standard deviation) of the percentage of vesicles colabeled by *SISOBIR1*-GFP and the marker to those labeled only by *SISOBIR1*-GFP from three biological repeats. Bar = 10 μ m.

(Liebrand et al. 2013), and found neither of them rescued plant response to ParA1 (Fig. 7). Therefore, solanaceous *SOBIR1* homologs are involved in ParA1-induced necrosis and the kinase activity is essential for the response. It has been shown that *NbSERK3/BAK1* regulates immune responses triggered by INF1 (Chaparro-Garcia et al. 2011). To test if *NbSERK3/BAK1* is also required for ParA1-induced responses, we performed an experiment to silence the expression of *NbSERK3A* and *NbSERK3B* simultaneously by using a silencing construct that contains a conserved region of both genes. ParA1-induced necrosis was compromised in *N. benthamiana* leaves with *NbSERK3/BAK1*-silencing, as evaluated by the symptoms and necrosis index (Supplementary Fig. S9A and B, respectively). Therefore, the immune responses mediated by oomycete elicitor in solanaceous plants appear to require both *SERK3/BAK1* and *SOBIR1/EVR* homologs.

DISCUSSION

In this article, we demonstrate that *SISOBIR1* and its close homolog *SISOBIR1-like* are involved in plant basal immunity to *P. parasitica* infection as well as plant responses triggered by elicitors. *SOBIR1* of *A. thaliana* was originally identified as a suppressor of *BIR1* mutation and its overexpression led to enhanced plant resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (Gao et al. 2009). In contrast to *A. thaliana*, tomato contains two genes homologous to *AtSOBIR1* (i.e., *SISOBIR1* and *SISOBIR1-like*), as do other plants of family Solanaceae, such as potato and *N. benthamiana*. When either of these two genes was silenced, plants became more susceptible to pathogen infection, as revealed by enhanced disease severity and pathogen biomass present in the inoculated plants. Notably, with both genes silenced simultaneously, plants became even more susceptible to pathogen infection, which indicated the important role of both *SISOBIR1* and *SISOBIR1-like* in plant basal defense. Hallmark events of PTI signaling encompass an enhancement of callose deposition and ROS production as well as induction of PTI marker genes (Boller and Felix 2009; Nguyen et al. 2010). Both callose deposition and ROS production were compromised in *SISOBIR1* or/and *SISOBIR1-like*-silenced plants (Fig. 3). Moreover, the expression of *Pti-5*, *WRKY28*, and *Gras2* was compromised in *SISOBIR1*-silenced plants. Thus, *SISOBIR1* and *SISOBIR1-like* play important roles in plant basal immunity toward *P. parasitica*, especially regarding the regulation of callose deposition and ROS production, but their roles in the control of defense gene expression are likely different.

In accordance with our results, the role of *AtSOBIR1* in basal defense response of *A. thaliana* was demonstrated in two recent independent studies (Zhang et al. 2013, 2014). *SCLEROTINIA CULTURE FILTRATE ELICITOR1* (SCFE1), an elicitor identified in *Sclerotinia sclerotiorum*, induced typical MAMP-triggered immune responses that engaged a signaling pathway dependent on RECEPTOR-LIKE PROTEIN30, BAK1, and *AtSOBIR1* (Zhang et al. 2013). Similar to mutants of *RLP30* and *BAK1*, *AtSOBIR1* mutants were more susceptible to infection by necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Zhang et al. 2013). On the other hand, *AtSOBIR1* and *REPONSIVENESS TO BOTRYTIS POLYGALACTURONASE 1* (RBPGL1), an LRR-RLP that acts as a receptor to recognize endopolygalacturonases (PG) of *Botrytis cinerea*, are involved in the formation of necrosis caused by PG (Zhang et al. 2014). When *A. thaliana* plants carrying a *sobir1* mutation were treated with PG, no symptom of necrosis developed and PG-induced resistance to *Hyaloperonospora arabidopsidis* was compromised (Zhang et al. 2014). It is thus obvious that *SOBIR1* homologs play essential roles in plant basal defense against a broad range of pathogens,

including bacteria, fungi, and oomycetes. Furthermore, both *SISOBIR1* and *SISOBIR1-like* are required for HR induced by Cf-4 and Ve1, as well as for Cf-4- and Ve1-mediated tomato resistance to *Cladosporium fulvum* and *Verticillium dahliae*, respectively (Liebrand et al. 2013). In addition to their roles in plant innate immunity, *SOBIR1/EVR* is involved in floral abscission and functions downstream of NEVERSHED, which encodes an ADP-ribosylation factor GTPase-activating protein (Leslie et al. 2010). In this regard, *SOBIR1/EVR* is similar to BAK1, which is involved not only in plant development but also in plant immunity.

Upon pathogen infection, PRR interact with PAMPs, which is usually accompanied by the formation of receptor complexes and, then, receptor-mediated endocytosis (Geldner and Robatzek 2008; Leborgne-Castel et al. 2010; Monaghan and Zipfel 2012; Roux et al. 2011). When *SISOBIR1*-GFP and *SISOBIR1-like*-GFP were expressed by agroinfiltration in *N. benthamiana*, *SISOBIR1*-GFP localized predominantly to the plasma membrane, similar to results reported by Liebrand et al. (2013). In contrast, *SISOBIR1-like*-GFP also localized in numerous cytoplasmic puncta in the *N. benthamiana* epidermal cells, likely due to constitutive endocytosis driven by endogenous signals, as reported for other RLK (Beck et al. 2012; Geldner et al. 2007). Intriguingly, *SISOBIR1*-GFP translocated from the plasma membrane to cytoplasmic puncta in response to *P. parasitica* infection. When various PAMPs were tested, we found that only ParA1 carrying an intact signal peptide could trigger internalization of *SISOBIR1*-GFP (Fig. 5A) and this process required an active kinase domain. Although the *SISOBIR1*-GFP-labeled cytoplasmic puncta may represent endocytic or secretory vesicles, our data clearly ruled out the latter case, because pretreatment with CHX, a protein synthesis inhibitor, did not impair *SISOBIR1*-GFP internalization (Figs. 5 and 6). In yeast and mammals, actin cytoskeletons are known to play an important role during receptor-mediated endocytosis (Mooren et al. 2012). Similarly, an intact actin cytoskeleton is indispensable for endocytosis of plant RLK, such as the flagellin receptor FLS2 (Robatzek et al. 2006). To further characterize the phenomena of ParA1-induced *SISOBIR1*-GFP internalization, we treated tobacco protoplasts with the actin polymerization inhibitor (Spector et al. 1989) LatB, which has been shown to impair the internalization of FLS2 induced by flg22 (Robatzek et al. 2006). When tobacco protoplasts were treated with ParA1 and LatB, the number of *SISOBIR1*-GFP-labeled puncta was significantly reduced compared with the level of the control experiment treated only with ParA1 (Fig. 6). We also found that *SISOBIR1*-GFP colocalized with AtARA6 or AtARA7. In *A. thaliana*, both ARA6 and ARA7 play important roles in endocytic trafficking, with ARA6 mainly in late endosomes and ARA7 at both early and late endosomes (Ebine et al. 2011; Ueda et al. 2001). Thus, our findings indicate that ParA1 treatment triggers endocytosis of *SISOBIR1*-GFP, which traffics through ARA6- and ARA7-positive endosomal compartments in the cytoplasm. The fact that neither marker exhibits perfect colocalization with *SISOBIR1*-GFP suggests that, once being endocytosed, *SISOBIR1*-GFP is sorted in and out by multiple trafficking pathways among the complex endosomal compartments (Mooren et al. 2012). Further analyses are required to elucidate how exactly these endosomal trafficking pathways contribute to either recycling, turnover, or both of *SISOBIR1*-GFP and how the kinase activity of *SISOBIR1* coordinates its interaction with other proteins upon PAMP perception.

Elicitins can induce necrosis in tobacco plants and some radish and turnip cultivars (Kamoun et al. 1993b). NbLRK1, a lectin-like receptor kinase, is known to interact with INF1 and mediate INF1-induced cell death in *N. benthamiana* (Kanzaki et al. 2008).

Moreover, *NbSERK3/BAK1*-silencing abrogated HR induced by INF1 (Chaparro-Garcia et al. 2011). In our study, ParA1-induced necrosis on *N. benthamiana* leaves was compromised when the expression of *NbSOBIR1* was downregulated (Fig. 7), similar to the effects caused by silencing of *NbSERK3/BAK1* (including both *NbSERK3A* and *NbSERK3B*). The importance

of *SOBIR1/EVR* homologs in plant response to elicitors were further verified by complementation experiments showing that both *NbSOBIR1* and *SISOBIR1* but not *SISOBIR1-like* could revert ParA1-induced responses in the silenced plants and, most importantly, the kinase activity is essential for complementation. Therefore, in addition to *NbSERK3/BAK1*,

NbSOBIR1 is required for ParA1-induced plant responses, and the function of *NbSOBIR1* is well conserved in *SISOBIR1* but not *SISOBIR1-like*, despite of the high identity between these two proteins.

The fact that only ParA1 with an intact signal peptide can trigger endocytosis of SOBIR1-GFP indicated that the whole event involves an interaction between ParA1 and its receptor from the cell surface. Currently, no elicitor receptor was identified, although high-affinity binding sites specific to cryptogein, an elicitor from *Phytophthora cryptogea*, have been reported (Wendehenne et al. 1995) and treatment with cryptogein rapidly stimulated both FM4-64 internalization and clathrin-mediated endocytosis in tobacco bright yellow-2 (BY-2) cells (Leborgne-Castel et al. 2008). It is likely that, following perception of ParA1, the ParA1 receptor interacts with other proteins in the plasma membrane, such as *SISOBIR1*, thereby resulting in the formation and subsequent endocytosis of receptor complexes for downstream signaling. In *A. thaliana*, *AtSOBIR1* was shown to interact with RBPG1 (Zhang et al. 2014). As well, tomato *SISOBIR1/EVR* homologs can interact with several RLP, including Cf proteins, Ve1, and LeEix2 (Liebrand et al. 2013). Accordingly, it has been proposed that *SOBIR1/EVR* homologs may function as a regulatory LRR-RLK that is involved in the formation of a plethora of plasma membrane-associated receptor complexes (Liebrand et al. 2014). Identification of proteins that interact with *NbSOBIR1* may help to identify other components of the ParA1-induced receptor complexes and to uncover the early events that occur after recognition of elicitors.

MATERIALS AND METHODS

Growth of *P. parasitica* and plant materials.

P. parasitica (isolate 94069) was routinely grown on 5% V8 juice agar (5% Campbell's V8 juice, 0.02% CaCO₃, and 2% agar) at 25°C in the dark. Tomato plants (*S. lycopersicum* cv. Summer Sweet) were grown at 22°C under a 16-h light and 8-h dark cycle, and *N. benthamiana* was grown at 25°C under a 12-h light and dark cycle, both in a mixture of peat moss, perlite, and vermiculite.

Cloning and sequence analysis of *SOBIR1/EVR* homologs.

Total RNA samples were prepared from tomato and tobacco tissues by using the Plant Total RNA extraction kit (Viogene-Biotek, Taipei, Taiwan). The Turbo-DNA free kit (Ambion, Huntingdon, U.K.) was used to remove residual DNA in RNA samples. First-strand cDNA was synthesized by use of SuperScript III reverse transcription (Life Technologies-Invitrogen, Carlsbad, CA, U.S.A.) and was used for amplification of *SISOBIR1*, *SISOBIR1-like*, and *NbSOBIR1* with the primers listed in Supplementary Table S1. The amplified DNA fragments

were cloned into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) for sequencing and further subcloning. Sequences have been deposited in GenBank [accession numbers KF268115 (*SISOBIR1*), KF977196 (*SISOBIR1-like*), and KF998581 (*NbSOBIR1*)]. Multiple sequence alignment involved use of ClustalX. A phylogenetic tree was generated by the neighbor-joining algorithm, implemented in MEGA 5 with the default parameters. Nodal support of the tree was estimated by bootstrapping with 3,000 pseudoreplicate data sets.

Inoculation of *P. parasitica*, scoring of disease severity, and quantification of pathogen biomass by qPCR.

Zoospores of *P. parasitica* were prepared as described (Yan and Liou 2006). For inoculation, zoospore suspensions (5×10^5 zoospores/ml) were sprayed evenly on the aerial parts of tomato plants until run-off, and inoculated plants were kept in a moisture chamber to facilitate disease development. Disease severity was scored on a scale from 0 to 5, according to symptoms developed on tomato plants: 0, healthy, no water-soaking lesions; 1, <25% lesions; 2, 25 to 50% lesions; 3, 50 to 75% lesions; 4, 75 to 100% lesions; and 5, complete wilting. To quantify pathogen biomass by qPCR, genomic DNA samples were prepared from inoculated plants by using EasyPure genomic DNA spin kit (Bioman Scientific, New Taipei, Taiwan). qPCR involved use of StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) and Power SYBR green PCR master mix (Applied Biosystems), using primers targeting *P. parasitica* WS21 (CF891675) and tomato *elf5A-4* (NM_001247786), respectively. The experiments were repeated three times. Raw data from WS21 were normalized with those from tomato *elf5A-4* and are presented as fold change relative to plants infiltrated with the empty vector (TRV) at 24 hpi.

Treatment with plant hormones.

Four-week-old tomato plants were treated with 2 mM SA (Sigma-Aldrich, St. Louis) or 1.25 mM MeJA (Sigma-Aldrich). For ET treatment, 4-week-old tomato plants were exposed to 10 ppm ET gas in a 9.4-liter air-tight container for 24 h in the dark. Final concentration of the ET gas in the container was measured by gas chromatography at the end of each treatment, to confirm the treatment. Leaf samples were collected at the indicated times for gene expression analysis.

qRT-PCR.

Plant total RNA and cDNA were prepared as described in the previous section. qPCR involved use of StepOnePlus real-time PCR system and Power SYBR green PCR master mix with primers. The transcript of *elf5A-4* was used as an internal control for analysis in tomato and *EF1α* (AF120093) in *N. benthamiana*.

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Fig. 7. Silencing of *NbSOBIR1* compromises ParA1-induced necrosis. **A**, ParA1-induced necrosis, reactive oxygen species accumulation, and callose deposition were compromised in *NbSOBIR1*-silenced *Nicotiana benthamiana* leaves. At 21 days after virus inoculation for *Tobacco rattle virus* (TRV)-mediated gene silencing of *NbSOBIR1*, ParA1 was expressed in systemic leaves by agroinfiltration. 3,3'-Diaminobenzidine (DAB) and aniline blue staining were performed at 48 h, whereas images of necrosis were obtained at 72 h post-agroinfiltration. TRV is the empty vector serving as a control. Bars in the left and middle panels represent 5 mm and, in the right panel, 1 μm. **B**, *NbSOBIR1* and *SISOBIR1* can rescue the effect caused by ParA1 on *NbSOBIR1*-silenced *Nicotiana benthamiana* leaves. At 21 days after virus inoculation for *Tobacco rattle virus* (TRV)-mediated gene silencing of *NbSOBIR1*, ParA1 (or the empty vector [EV] as a control) and the indicated proteins were coexpressed in systemic leaves by agroinfiltration. DAB and aniline blue staining were performed at 48 h, whereas images of necrosis were obtained at 72 h post-agroinfiltration. Bars in the left and middle panels represent 5 mm and, in the right panel, 1 μm. **C**, Necrosis index. ParA1-induced necrosis on *N. benthamiana* leaves were scored for necrosis severity as follows: 0, no symptoms; 1, yellowing at the infiltrated area; 2, necrosis, <50% of the infiltrated area; 3, necrosis, ≥50% of the infiltrated area; 4, necrosis, 100% of the infiltrated area. Values are mean (± standard deviation [SD]) from three independent experiments. Different letters indicate significant difference among treatments by one-way analysis of variance (ANOVA) at $P < 0.01$. **D**, Quantification of DAB staining. Values are mean (± SD) from three independent experiments. Different letters indicate significant difference among treatments by one-way ANOVA at $P < 0.01$. **E**, Quantification of aniline blue staining. Values are means (± SD) from three independent experiments. Different letters indicate significant difference among treatments revealed by one-way ANOVA at $P < 0.01$.

Virus-induced gene silencing.

Virus-induced gene silencing was performed as described (Liu et al. 2002), with some modifications. Briefly, partial sequences of target genes were amplified by PCR with respective primers and were cloned into pTRV2 (pYL279) by Gateway cloning (Life Technologies-Invitrogen). Plasmids were then transformed into *Agrobacterium tumefaciens* GV3101. For virus inoculation, bacteria were grown in Luria-Bertani broth with rifampicin and kanamycin at 28°C for 18 h. The culture was diluted 200× with induction medium (Luria-Bertani broth with 20 µM acetosyringone and 50 ppm kanamycin) and was incubated at 28°C for 16 h. After centrifugation, bacteria were resuspended with MMA (10 mM MES, pH 5.6, 10 mM MgCl₂, 200 µM acetosyringone) and were adjusted to an optical density at 600 nm (OD₆₀₀) = 2. Bacteria harboring TRV1 (pYL192) were mixed with an equal volume of bacteria harboring TRV2 and were infiltrated onto cotyledons of 7-day-old tomato seedlings or leaves of 18-day-old *N. benthamiana* seedlings by use of 1-ml syringes.

3,3'-Diaminobenzidine (DAB) staining.

DAB staining was performed as described (Bindschedler et al. 2006). Tomato leaves were immersed in DAB-HCl solution (1 mg of DAB per milliliter, 0.05% [vol/vol] Tween 20, and 10 mM Na₂HPO₄, pH 3.0) for at least 8 h and were then decolorized in bleaching solution (95% ethanol/acetic acid/glycerol [3:1:1]) for 10 min. Leaves were fixed and preserved in 95% ethanol. Images were captured by use of a Nikon digital camera D50. Intensity of DAB staining was quantified using ImageJ and is presented as the ratio of DAB dark-brown to total pixels. For each treatment, ten leaves from five plants were analyzed, with all the experiments repeated three times.

Staining and quantification for callose deposition.

Callose staining was performed as described (Luna et al. 2011), with some modifications. Tomato leaf disks were incubated in 95% ethanol at 37°C until transparent, were then stained with 0.1% aniline blue (Sigma-Aldrich) in 150 mM K₂HPO₄ (pH 9.5), and were incubated in the dark overnight. Callose deposition at the adaxial surface was examined by use of a Leica DM LB microscope (Buffalo Grove, IL, U.S.A.) equipped with filter cube A (BP 340 to 380 nm, LP 425 nm) and Canon digital camera EOS 550D. Callose deposition was quantified by the number of depositions per square millimeter microscopic field by use of ImageJ. For each treatment, ten leaf discs and three images for each leaf disc were analyzed, with all the experiments repeated three times.

Transient protein expression by agroinfiltration.

Coding sequences of *SISOBIR1* and *SISOBIR1-like* were subcloned into pK7FWG2 by use of the pENTR/D-TOPO cloning kit and Gateway LR Clonase II enzyme mix (Life Technologies-Invitrogen). Kinase-defective mutants of *SISOBIR1* and *SISOBIR1-like* were generated according to Liebrand et al. (2013). Plasmid constructs for AtARA6-mRFP and mRFP-AtARA7 were gifts from S. Robatzek and K. Schumacher. Coding sequences of AtARA6-mRFP and mRFP-AtARA7 were subcloned into pK7WG2 by use of the pENTR/D-TOPO cloning kit and Gateway LR Clonase II enzyme mix. The young expanding leaves of four-week-old *N. benthamiana* were used for transient protein expression. To express protein, *Agrobacterium tumefaciens* C58C1 strains carrying the expression constructs or *Agrobacterium tumefaciens* GV3101 strains carrying pK7FWG2::FLS2 (Schwessinger et al. 2011) were prepared as described in the previous section and were adjusted to OD₆₀₀ = 0.6 with MMA. Staining of the plasma

membrane by 5 µg of FM4-64 per milliliter (Life Technologies-Invitrogen) was performed as described (Bolte et al. 2004). Fluorescence signals from GFP and mRFP were visualized by use of a Zeiss LSM 510 Meta confocal microscope (GFP: excitation 488 nm and emission 500 to 550 nm; mRFP: excitation 543 nm and emission 565 to 615 nm). Colocalization of fluorescent proteins was evaluated by use of the MetaMorph NX microscopy automation and Image analysis software (Molecular Devices, Sunnyvale, CA, U.S.A.). For each treatment, 50 photographs from five different leaf discs were used for statistical analysis, with all the experiments repeated three times.

Transient protein expression in *N. benthamiana* protoplasts.

Protoplasts of *N. benthamiana* were prepared as described by Wu et al. (2011) with some modifications. Leaves of 6-week-old tobacco were cut in pieces that were incubated in the protoplast enzyme digestion buffer (per milliliter: 1 mg of bovine serum albumin, 12 mg of cellulose [Yakult, Tokyo], and 0.6 mg of macerozyme [Yakult]) for 16 h at room temperature. The lysate was then filtered through a 600-mesh cloth into sterile glass tubes and was centrifuged at 300 rpm (Eppendorf 5810) for 5 min, followed by resuspension of pellets in mannitol buffer (0.55 M mannitol, 0.1% MES, pH 5.7). After addition of an equal volume of sucrose solution (0.55 M), the protoplasts were harvested by centrifugation at 300 rpm for 5 min and were then resuspended with MMg buffer (0.55 M mannitol, 15 mM MgCl₂·H₂O, and 4 mM MES, pH 5.7) in a clean glass tube to a final concentration of 10⁶ protoplasts/ml. For polyethylene glycol (PEG)-mediated transformation, the protoplast suspension was mixed with 15 µg of plasmid DNA per microliter in a PEG solution (0.4 g of PEG-4000 per milliliter [Sigma-Aldrich], 0.55 M mannitol, and 0.1 M CaCl₂) and was incubated at room temperature for 45 min. Afterward, the protoplasts were rinsed twice with W5 buffer (154 mM NaCl, 125 mM CaCl₂·H₂O, 5 mM KCl, and 2 mM MES, pH 5.7) to get rid of PEG and were then incubated at room temperature for 8 h under light. Of the expression constructs, p35S-SISOBIR1-GFP was obtained by subcloning the coding sequence of *SISOBIR1* into p35S-MCS-GFP. To block protein synthesis and endocytosis, the protoplasts were treated with 50 µM CHX (Sigma-Aldrich) and 20 µM latrunculin B (Sigma-Aldrich), respectively, for 30 min as described by Gibbon et al. (1999). Fluorescence signals were visualized by use of a Zeiss LSM 510 Meta confocal microscope (GFP: excitation 488 nm and emission 500 to 550 nm), and the number of fluorescent protein-labeled vesicles per square millimeter microscopic field was quantified for 50 protoplasts, with all the experiments repeated three times.

Transient expression of CBEL and ParA1.

CBEL, *ParA1*, and *ParA1* devoid of the signal peptide (*ParA1*-ΔSP) were amplified by using cDNA of *P. parasitica* and primers. The cDNA fragments were cloned into pBI-V-MCS (courtesy of Y. C. Chang, National Taiwan University), a modified pBI121 binary vector (Chen et al. 2003) with the multiple cloning sites from pBluescript SK+ replacing the β-glucuronidase sequence, under the control of the 35S promoter. For transient expression, *Agrobacterium tumefaciens* C58C1 strains harboring the expression plasmids were grown as described previously and were adjusted to a final concentration of OD₆₀₀ = 0.1 with MMA. The severity of cell death induced by *ParA1* on *N. benthamiana* leaves was scored at 48 h post-agroinfiltration as follows: 0, no symptoms; 1, yellowing at the infiltrated area; 2, necrosis, <50% of the infiltrated area; 3, necrosis, ≥50% of the infiltrated area; 4, necrosis, 100% of the infiltrated area.

Expression and purification of ParA1 recombinant protein.

The coding sequence of *ParA1* devoid of the signal peptide was subcloned into the expression vector pET20b (+), and the recombinant plasmid was transformed into *Escherichia coli* C43 (DE3). After growth of the bacteria at 37°C to reach OD₆₀₀ = 1, the expression of recombinant protein was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactoside, accompanied by further incubation at 30°C for 24 h. The recombinant proteins were purified by use of Ni-NTA agarose (Qiagen, Valencia, CA, U.S.A.) as described by Hofzumahaus and Schallmeyer (2013).

Treatment with flg22.

Flg22 was synthesized by Genomics (New Taipei, Taiwan). After resuspension in 0.1 M MES to a final concentration of 1 μM, flg22 was infiltrated into *N. benthamiana* leaves by use of 1-ml syringes.

Statistical analysis.

Statistically significant differences were analyzed by Student *t* test, one-way analysis of variance (ANOVA) or Wilcoxon rank-sum test. Significance was set at $P < 0.05$ or $P < 0.01$ and was determined by PASW Statistics 18 (SPSS Inc., Chicago).

ACKNOWLEDGMENTS

We thank C. P. Cheng (National Taiwan University) for technical assistance on the TRV-mediated gene silencing experiments, T. T. Wang (National Taiwan University) for the ethylene treatment experiments, C. Zipfel (The Sainsbury Laboratory, U.K.) for FLS2-GFP construct, and S. Robatzek (The Sainsbury Laboratory) and K. Schumacher (Centre for Organismal Studies, Heidelberg University, Germany) for endosome markers. We are grateful to have M.-J. Fang and J.-Y. Huang of the Live-Cell Imaging Core Laboratory (IPMB, Academia Sinica) for advice on imaging. This work was supported by Ministry of Science and Technology, Taiwan (98-2313-B-002-035-MY3 and 102-2628-B-002-021-MY3).

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