SOBIR1 requires the GxxxG dimerization motif in its transmembrane domain to form constitutive complexes with receptor-like proteins

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SUMMARY

Receptor-like proteins (RLPs), forming an important group of transmembrane receptors in plants, play roles in development and immunity. RLPs contain extracellular leucine-rich repeats (LRRs) and, in contrast with receptor-like kinases (RLKs), lack a cytoplasmic kinase required for the initiation of downstream signalling. Recent studies have revealed that the RLK SOBIR1/EVR (SUPPRESSOR OF BIR1-1/EVERSHED) specifically interacts with RLPs. SOBIR1 stabilizes RLPs and is required for their function. However, the mechanism by which SOBIR1 associates with RLPs and regulates RLP function remains unknown. The Cf immune receptors of tomato (*Solanum lycopersicum*), mediating resistance to the fungus *Cladosporium fulvum*, are RLPs that also interact with SOBIR1. Here, we show that both the LRR and kinase domain of SOBIR1 are dispensable for association with the RLP Cf-4, whereas the highly conserved GxxxGxxxG motif present in the transmembrane domain of SOBIR1 is essential for its interaction with Cf-4 and additional RLPs. Complementation assays in *Nicotiana benthamiana*, in which endogenous *SOBIR1* levels were knocked down by virus-induced gene silencing, showed that the LRR domain as well as the kinase activity of SOBIR1 are required for the Cf-4/Avr4-triggered hypersensitive response (HR). In contrast, the LRRs and kinase activity of SOBIR1 are not required for facilitation of Cf-4 accumulation. Together, these results suggest that, in addition to being a stabilizing scaffold for RLPs, SOBIR1 is also required for the initiation of downstream signalling through its kinase domain.

Keywords: defence signalling, development, plant immunity, receptor complex, receptor-like kinase (RLK), receptor-like protein (RLP), SOBIR1.

INTRODUCTION

Plants employ pattern recognition receptors (PRRs) triggering a first layer of innate immunity to defend themselves against pathogens (Dodds and Rathjen, 2010; Macho and Zipfel, 2014). PRRs are either cell surface-localized receptor-like kinases (RLKs), which contain an extracellular domain for pathogen perception and an intracellular kinase domain involved in downstream cytoplasmic defence signalling, or receptor-like proteins (RLPs), which lack such a cytoplasmic signalling domain (Böhm *et al.*, 2014).

Well-characterized PRRs are FLAGELLIN SENSING 2 (FLS2) and the ELONGATION FACTOR-TU RECEPTOR (EFR), which are RLKs involved in the recognition of bacterial flagellin and the elongation-factor Tu protein, respectively (Gómez-Gómez and Boller, 2000; Macho and Zipfel, 2014; Zipfel *et al.*, 2006). On ligand recognition, both FLS2 and EFR form a complex with the regulatory LRR-RLK SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3/BRI-ASSOCIATED RECEPTOR KINASE 1 (SERK3/BAK1), and intracellular signalling is subsequently initiated by the transphosphorylation events that take place between the cytoplasmic kinase domains of recruited BAK1 and FLS2 or EFR, which come into close proximity to each other (Oh *et al.*, 2010; Schwessinger *et al.*, 2011).

Tomato (*Solanum lycopersicum*, Sl) Cf resistance genes, encoding leucine-rich repeat (LRR)-RLPs, confer resistance to specific races of the extracellular biotrophic fungal pathogen *Cladosporium fulvum*, causing leaf mould disease of tomato (Rivas and Thomas, 2005; Stergiopoulos and de Wit, 2009). Cf proteins trigger immunity on recognition of their matching ligands, which are so-called avirulence (Avr) proteins that are secreted by *C. fulvum* and function as effectors in the apoplastic space of tomato leaves (Rivas and Thomas, 2005; Stergiopoulos and de Wit, 2009). Resistance of tomato to *C. fulvum* is associated with the hypersensitive response (HR), which is a form of localized programmed cell death (De Jong *et al.*, 2004; Jones and Dangl, 2006; Rivas and Thomas, 2005).

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Because RLPs lack a kinase domain, it has been hypothesized that these cell surface receptors recruit one or more RLKs to initiate cytoplasmic signalling. Indeed, in *Arabidopsis thaliana* (At), the RLP TOO MANY MOUTHS (TMM), which regulates stomatal patterning, interacts with the LRR-RLK ERECTA (Lee *et al.*, 2012), whereas CLAVATA 2 (CLV2), which is involved in meristem maintenance, forms a complex with the transmembrane (TM) kinase CORYNE, as well as with the LRR-RLK CLV1, to activate downstream signalling (Bleckmann *et al.*, 2010; Müller *et al.*, 2008; Zhu *et al.*, 2010). Recently, it has been reported that the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, hereafter referred to as SOBIR1) (Gao *et al.*, 2009; Leslie *et al.*, 2010) constitutively interacts with Cf proteins and is required for their function (Liebrand *et al.*, 2013, 2014b). SOBIR1 was originally identified as a suppressor of the *Arabidopsis* *bir1-1* mutant (Gao *et al.*, 2009). This mutant shows a constitutive defence response, which is suppressed by an additional mutation in *AtSOBIR1* (Gao *et al.*, 2009). This suggests that *AtSOBIR1* is required for defence signalling. In agreement with this, overexpression of *AtSOBIR1* resulted in a constitutive defence phenotype in *Arabidopsis* (Gao *et al.*, 2009). It was observed that tomato SOBIR1 (*S/SOBIR1*) interacts with a number of additional RLPs, such as Ve1, *LeEix2* and the closest tomato homologues of *AtCLV2* and *AtTMM* (Liebrand *et al.*, 2013). Ve1 mediates recognition of the effector Ave1 of the fungal pathogen *Verticillium dahliae* (de Jonge *et al.*, 2012; Zhang *et al.*, 2013b), whereas *LeEix2* recognizes fungal xylanase (Ron and Avni, 2004). Interestingly, SOBIR1 does not appear to interact with RLKs (Liebrand *et al.*, 2013), suggesting that SOBIR1 is specifically required for RLP function. Additional studies in *Arabidopsis* revealed that *AtSOBIR1* also interacts with *AtRLP23*, whose function is unknown, and with RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES 1 (RBPG1)/*AtRLP42*, recognizing fungal endopolygalacturonases (Bi *et al.*, 2014; Zhang *et al.*, 2014). Recently, *AtRLP30* was identified as the receptor recognizing a novel proteinaceous elicitor, referred to as SCLEROTINIA CULTURE FILTRATE ELICITOR 1 (SCFE1), of the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Zhang *et al.*, 2013a). RLP30 function was also found to depend on SOBIR1, because *Arabidopsis* *sobir1* knock-outs were strongly compromised in RLP30-mediated immunity (Zhang *et al.*, 2013a). In the same study, it was shown that these knock-out mutants are not compromised in immune responses mediated by FLS2, supporting the hypothesis that SOBIR1 specifically plays a role in RLP function (Liebrand *et al.*, 2014b; Zhang *et al.*, 2013a). *Nicotiana benthamiana* (Nb) contains two closely related SOBIR1 homologues: *NbSOBIR1* and *NbSOBIR1-like*. Remarkably, silencing of endogenous SOBIR1(-like) in *N. benthamiana* reduces Cf-4 and Ve1 protein levels on transient expression of the encoding genes (Liebrand *et al.*, 2013), whereas, in *Arabidopsis* *bak1* knock-out mutants, FLS2 accumulates to the same levels as

seen in wild-type plants (Chinchilla *et al.*, 2007; Heese *et al.*, 2007).

It has been reported that Ve1 interacts with SOBIR1 through its C-terminal part (Fradin *et al.*, 2014). The C-terminal part of the various LRR-RLPs of *Arabidopsis* shows conserved features, such as the presence of two cysteines between the LRR domain and the extracellular juxtamembrane (eJM) domain, the latter containing a relatively large amount of acidic amino acid residues (Gust and Felix, 2014). Furthermore, the TM domain of most RLPs contains typical GxxxG motifs (in which G is glycine and x can be any amino acid), either single or in tandem (Gust and Felix, 2014). A GxxxGxxxG motif, referred to as a 'glycine zipper', is also present in the TM of SOBIR1 (Gust and Felix, 2014), and consists of a double GxxxG motif that has been shown to be important in the dimerization of two TM proteins through helix-helix interactions (Cymer *et al.*, 2012; Fink *et al.*, 2012; Li *et al.*, 2012). In addition, structural modelling of the TM domain of the human TM receptor neuropilin-1 (NRP1), complemented with biochemical analyses, showed that the TM domain drives the homodimerization of NRP1 via its GxxxGxxxG motif (Roth *et al.*, 2008).

It has been shown that SOBIR1 associates with a large number of LRR-RLPs which act as immune receptors, and that this RLK is required for RLP-mediated signalling (Böhm *et al.*, 2014; Gust and Felix, 2014; Jehle *et al.*, 2013; Liebrand *et al.*, 2013; Zhang *et al.*, 2013a). However, it is not known which domains of SOBIR1 are required for the interaction with RLPs and which domains are required for functionality of the RLP/SOBIR1 complex. Here, we show that the GxxxGxxxG motif present in the TM of SOBIR1 is necessary for its interaction with a number of RLPs. The LRR and kinase domains of SOBIR1 are dispensable for interaction with Cf-4; however, both the LRR domain and kinase activity of SOBIR1 are required for Cf-4-mediated HR.

RESULTS

Analysis of SOBIR1 homologues and construction of a phylogenetic tree

To understand which domains of SOBIR1 are required for its interaction with RLPs and to determine how conserved these domains are among the members of the SOBIR1 family, we performed a multiple sequence alignment of SOBIR1 homologues using Mafft (Stamatakis *et al.*, 2008), including *S/SOBIR1*, *AtSOBIR1* and two SOBIR1 homologues of the primitive moss *Physcomitrella patens* (*Pp*) (Fig. S1, see Supporting Information). From this alignment, an unrooted maximum likelihood tree was made using RAXML. The tree clearly shows the relation between the various SOBIR1 homologues, with *AtBAK1* as an outgroup. *PpSOBIR1* and banana (*Musa* spp.) SOBIR1 are grouped relatively distant from all other SOBIR1 homologues (Fig. 1A).

Fig. 1 Phylogenetic analysis of SOBIR1 homologues. (A) Phylogenetic tree of SOBIR1 homologues originating from 20 plant species, compared with AtBAK1. This unrooted maximum likelihood tree, including bootstrap values, was made from a multiple sequence alignment of SOBIR1 homologues, using RAxML. (B) Sequence alignments of the transmembrane and juxtamembrane regions of the various SOBIR1 homologues and receptor-like proteins (RLPs). Acidic amino acid residues are

*S*SOBIR1 and *At*SOBIR1, both of which interact with Cf-4, with the latter complementing the loss of endogenous *Nb*SOBIR1(-like) in *Nb*SOBIR1(-like)-silenced *N. benthamiana* (Liebrand *et al.*, 2013), have five predicted LRRs, a single TM domain and a cytoplasmic kinase domain (Gao *et al.*, 2009; Leslie *et al.*, 2010; Liebrand *et al.*, 2013). All SOBIR1 homologues included in the multiple sequence alignment share these features (Fig. S1) and, when excluding the relatively distantly related *Pp*SOBIR1 homologues, the intracellular kinase domains of all aligned SOBIR1 homologues share a minimal identity of 73% and a minimal similarity of 81%. This is much higher than the minimal identity of 60% and minimal similarity of 67% for the extracellular SOBIR1 LRR domains. Clearly, the intracellular kinase domain of the various SOBIR1 homologues is highly conserved (Fig. S1). We further zoomed in on the TM domain and observed the presence of a conserved GxxxGxxxG motif in all aligned SOBIR1 homologues (Fig. 1B), a motif that has been suggested to play a role in the interaction of SOBIR1 with RLPs (Gust and Felix, 2014). In addition, many positively charged amino acids are located in the eJM of SOBIR1 homologues, in contrast with the mostly negatively charged amino acids located in the eJM of RLPs (Fig. 1B) (Gust and Felix, 2014). This conservation suggests that these features are important for SOBIR1 functioning and its interaction with RLPs.

The LRR and kinase domains of *S*SOBIR1 are dispensable for its interaction with Cf-4

To identify which domains of *S*SOBIR1 are required for its interaction with Cf-4, we initially designed deletion constructs encoding *S*SOBIR1 lacking the first two N-terminal LRRs (*S*SOBIR1 Δ 2LRRs), *S*SOBIR1 lacking the majority of its extracellular region (*S*SOBIR1 Δ Ncaps Δ 5LRRs) and a construct encoding only the kinase domain of *S*SOBIR1 (*S*SOBIR1-kinase) (Fig. 2A). C-terminally enhanced green fluorescent protein (eGFP) epitope-tagged versions of these truncated variants of tomato SOBIR1 were generated and transiently co-expressed with Myc-tagged Cf-4 (Liebrand *et al.*, 2013) in *N. benthamiana*. As a control, we also transiently co-expressed full-length *S*SOBIR1-eGFP and Cf-4-Myc. Co-immunoprecipitation (co-IP) experiments revealed that Cf-4 co-purifies with *S*SOBIR1 Δ 2LRRs and *S*SOBIR1 Δ Ncaps Δ 5LRRs, but not with *S*SOBIR1-kinase (Fig. 2B).

To further confirm that the LRR and kinase domains of *S*SOBIR1 are not required for its interaction with Cf-4, we designed a deletion construct coding only for the eJM, TM and intracellular juxtamembrane (iJM) of *S*SOBIR1 (*S*SOBIR1-eJM-TM-iJM). Considering that *S*SOBIR1-eJM-TM-iJM is smaller in size than the eGFP tag itself, we fused this truncated protein to the much smaller haemagglutinin (HA) tag and performed co-IP experiments with Cf-4-eGFP. As a negative control for interaction, *S*/FLS2-GFP was included. co-IP experiments revealed that *S*SOBIR1-eJM-TM-

iJM co-purifies with Cf-4-eGFP, but not with the negative control *S*/FLS2-GFP (Fig. 2C). These results indicate that the eJM-TM-iJM domain of *S*SOBIR1 is sufficient and essential for interaction with Cf-4 *in planta*.

The GxxxGxxxG motif present in the TM of *S*SOBIR1 is required for its interaction with RLPs

It has been reported that the GxxxGxxxG motif mediates intermolecular interactions between TM proteins (Cymer *et al.*, 2012; Fink *et al.*, 2012) and, to determine the role of this motif in the interaction with Cf-4, the first, second and third glycine residues (G) of the GxxxGxxxG motif of *S*SOBIR1 were individually replaced by an alanine (A). C-terminally eGFP epitope-tagged versions of the *S*SOBIR1 mutants (*S*SOBIR1^{G274A}, *S*SOBIR1^{G278A} and *S*SOBIR1^{G282A}) were generated and transiently co-expressed with Cf-4-Myc. As a control, we also transiently co-expressed Cf-4-Myc and wild-type *S*SOBIR1-eGFP. co-IP revealed that these single G to A mutants of SOBIR1 still interact with Cf-4 (Fig. 3A). To further elucidate whether the GxxxGxxxG motif is required for interaction with Cf-4, all three glycine residues of the GxxxGxxxG motif were simultaneously replaced by an alanine residue. Again a C-terminally eGFP epitope-tagged version of the mutant (*S*SOBIR1^{3GA}) was generated and transiently co-expressed with Cf-4-Myc. Figure 3B shows that Cf-4-Myc is immunoprecipitated with wild-type *S*SOBIR1-eGFP, but not with *S*SOBIR1^{3GA}-eGFP, showing that an intact GxxxGxxxG motif in the TM of *S*SOBIR1 is essential for its interaction with Cf-4. We regularly observed that pulled down SOBIR1 migrated as multiple, smearing bands (Fig. 3B). A similar observation was made by Liebrand *et al.* (2013) and Zhang *et al.* (2014), and this feature might be the result of post-translational modifications, in addition to partial degradation of SOBIR1.

It has been reported that *S*SOBIR1 interacts with a broad range of RLPs (Gust and Felix, 2014; Liebrand *et al.*, 2013). A C-terminally HA-tagged version of Ve1 has been generated (Fradin *et al.*, 2014; Liebrand *et al.*, 2014a) and, to test whether the GxxxGxxxG motif is also required for the interaction with Ve1, we transiently co-expressed Ve1-HA with *S*SOBIR1^{3GA}-eGFP. As a control, we also transiently co-expressed Ve1-HA and wild-type *S*SOBIR1-eGFP. Ve1-HA co-purified with wild-type *S*SOBIR1-eGFP, but not with *S*SOBIR1^{3GA}-eGFP (Fig. 3C, left panel), indicating that the GxxxGxxxG motif of *S*SOBIR1 is also required for its interaction with Ve1. To test whether the GxxxGxxxG motif is required for interaction with additional RLPs, the C-terminally-tagged HA fusion protein *S*SOBIR1^{3GA}-HA was generated to perform co-IP experiments with C-terminally eGFP-tagged versions of *Le*EIX2 and the closest tomato orthologue of *Arabidopsis* CLV2 (Solyc04g056640.1) (Liebrand *et al.*, 2013). Transient co-expression in *N. benthamiana* revealed that both *Le*EIX2 and *Sl*CLV2 co-purified with wild-type

S/SOBIR1-HA, but not with S/SOBIR1^{3GA}-eGFP (Fig. 3C, right panel). These results indicate that the GxxxGxxxG motif present in the TM of S/SOBIR1 is crucial for its interaction with RLPs.

To check whether the GxxxGxxxG motif is required for S/SOBIR1 localization to the plasma membrane and cytoplasmic vesicles (Leslie et al., 2010; Liebrand et al., 2013), confocal laser scanning microscopy was performed on *N. benthamiana* epidermal leaf cells transiently expressing S/SOBIR1^{3GA}-eGFP under the control of

Fig. 2 Analysis of the requirement of the various S/SOBIR1 domains for interaction with Cf-4. (A) Schematic representation of the S/SOBIR1 protein domain structure and generated truncation variants of S/SOBIR1. Tan, signal peptide for extracellular targeting; yellow, Ncaps domain; light green, leucine-rich repeat (LRR) domain; dark green, extracellular juxtamembrane domain (eJM); black, transmembrane domain (TM); blue, intracellular juxtamembrane domain (iJM); pink, kinase domain. (B) Cf-4 interacts with S/SOBIR1, S/SOBIR1Δ2LRRs and S/SOBIR1ΔNcapsΔ5LRRs, but not with S/SOBIR1-kinase. Full-length and truncation variants of S/SOBIR1, fused to enhanced green fluorescent protein (eGFP), were transiently co-expressed with Cf-4-Myc in *Nicotiana benthamiana*. Total proteins were extracted and subjected to immunoprecipitation using GFP_TrapA affinity beads. Total proteins (Input) and immunopurified proteins (IP) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride (PVDF) membrane. Blots were subsequently incubated with αGFP antibody to detect the immunopurified eGFP fusion proteins and incubated with αMyc antibody to detect co-purifying Cf-4-Myc. Coomassie-stained blots show the 50-kDa Rubisco band present in the input samples to confirm equal loading. (C) Cf-4 interacts with S/SOBIR1-eJM-TM-iJM. Cf-4-eGFP and FLS-2-GFP were transiently co-expressed with S/SOBIR1-eJM-TM-iJM fused to haemagglutinin (HA) in *N. benthamiana*. Immunoprecipitation and detection of proteins were performed as described under (B). All assays were performed three times and a representative photograph for each experiment is shown.

the 35S promoter. This revealed that, similar to S/SOBIR1-eGFP, the triple GA S/SOBIR1 mutant still localizes to the plasma membrane (Fig. 3D). In addition, fluorescence signals were observed in mobile cytoplasmic vesicles (Fig. S2, see Supporting Information). This indicates that the GxxxGxxxG motif present in the TM of SOBIR1 is not required for proper subcellular localization of SOBIR1.

The LRR and kinase domain of SOBIR1 are required for Cf-4-mediated HR

Having identified the requirements of SOBIR1 to interact with RLPs, we set out to determine which domains of SOBIR1 are required for Cf-4 function, i.e. the activation of HR on recognition of the matching avirulence factor, Avr4. For this, we generated deletion constructs encoding AtSOBIR1 lacking Ncaps (AtSOBIR1ΔNcaps), AtSOBIR1 lacking the iJM domain (AtSOBIR1ΔiJM), AtSOBIR1Δ2LRRs, AtSOBIR1ΔNcapsΔ5LRRs, AtSOBIR1-kinase and S/SOBIR1^{3GA} (Fig. 4A). In addition, a kinase-dead mutant (AtSOBIR1^{D489N}) was also included in the experiment (Liebrand et al., 2013). In complementation assays in *N. benthamiana* stably expressing Cf-4, we have shown earlier that a Tobacco rattle virus (TRV)-based silencing construct targeting *NbSOBIR1* (-like) does not target AtSOBIR1, because there is insufficient DNA sequence homology between *NbSOBIR1* (-like) and AtSOBIR1 (Liebrand et al., 2013).

In this way, it was shown that AtSOBIR1 is a functional homologue of *NbSOBIR1* (-like), as it complements a knock-down of

Fig. 3 The GxxxGxxxG motif in the transmembrane domain of *S/SOBIR1* is required for interaction with Cf-4. (A) Cf-4 still interacts with *S/SOBIR1* on replacement of one of the three glycine (G) residues in the GxxxGxxxG motif of *S/SOBIR1* by an alanine (A) residue. *S/SOBIR1* wild-type and versions of *S/SOBIR1* mutated in the GxxxGxxxG motif, *S/SOBIR1*^{G274A}, *S/SOBIR1*^{G278A} and *S/SOBIR1*^{G282A}, fused to enhanced green fluorescent protein (eGFP), were transiently co-expressed with Cf-4-Myc in *Nicotiana benthamiana*. Total proteins were extracted and subjected to immunoprecipitation using GFP_TrapA affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride (PVDF) membrane. Blots were incubated with α GFP antibody to detect the immunoprecipitated eGFP fusion proteins and incubated with α Myc antibody to detect co-purifying Cf-4-Myc. Coomassie-stained blots show the 50-kDa Rubisco band present in the input samples to confirm equal loading. (B) Simultaneous replacement of all three glycine (G) residues present in the GxxxGxxxG motif of *S/SOBIR1* by an alanine (A) residue compromises its interaction with Cf-4. *S/SOBIR1* wild-type and the triple mutant *S/SOBIR1*^{3GA}, both fused to eGFP, were transiently co-expressed with Cf-4-Myc in *N. benthamiana*. Immunoprecipitation and detection of proteins were performed as described under (A). (C) The *S/SOBIR1*^{3GA} mutant also shows compromised interaction with Ve1 (left) and *S/CLV2* and *LeEIX2* (right). (D) *S/SOBIR1*^{3GA}-eGFP localizes to the plasma membrane and cytoplasmic vesicles, similar to *S/SOBIR1*-eGFP. Proteins were transiently expressed in *N. benthamiana* epidermal leaf cells and their subcellular localization is shown. Each panel shows a combination of the eGFP signal (green) and the chloroplast signal (red). Scale bar, 10 μ m. All assays were performed three times and a representative photograph for each experiment is shown.

NbSOBIR1(-like) by virus-induced gene silencing (VIGS) to restore the Cf-4-mediated HR, whereas the *AtSOBIR1*^{D489N} kinase-dead mutant does not (Liebrand *et al.*, 2013). In the same way, 3 weeks after inoculation of *N. benthamiana*:Cf-4 with the TRV:*NbSOBIR1(-like)* VIGS construct (Liebrand *et al.*, 2013), the plants were infiltrated with suspensions of *Agrobacterium tumefaciens* (agroinfiltration) to transiently express *AtSOBIR1*-eGFP and the various truncated versions of *AtSOBIR1*, C-terminally fused to eGFP, in combination with Avr4. As a control, β -glucuronidase (GUS) was expressed in combination with Avr4.

Although co-expression of *AtSOBIR1*-eGFP with Avr4 in the *NbSOBIR1(-like)*-silenced *N. benthamiana*:Cf-4 plants restored the Cf-4-mediated HR, the negative control, consisting of the co-expression of GUS with Avr4, did not (Fig. 4B). Visual inspection of the leaf area agroinfiltrated with Avr4 and *AtSOBIR1*^{D489N}, *AtSOBIR1* Δ Ncaps-eGFP, *AtSOBIR1* Δ 2LRRs-eGFP, *AtSOBIR1* Δ Ncaps Δ 5LRRs-eGFP, *AtSOBIR1*-kinase-eGFP and *AtSOBIR1*^{3GA}, indicated that the Avr4-triggered HR was not fully restored, whereas *AtSOBIR1* Δ iJM complemented the knock-down of endogenous *NbSOBIR1(-like)* (Fig. 4B). In addition, co-IP experiments showed that all eGFP-tagged *AtSOBIR1* truncation and mutation variants were stable and interacted with Cf-4-Myc (Fig. 4C), except for *AtSOBIR1*-kinase-eGFP and *AtSOBIR1*^{3GA}. Furthermore, agroinfiltration of the various *AtSOBIR1* constructs in *N. benthamiana* does not result in constitutive cell death (Fig. S3). This observation indicates that various *AtSOBIR1* mutants interact with Cf-4 and stabilize the protein; however, they are unable to restore the Cf-4-mediated HR.

The LRR domain and kinase activity of *SOBIR1* are not required for Cf-4 accumulation

It has been reported earlier that *SOBIR1* is required for stable accumulation of Cf-4 in *N. benthamiana* (Liebrand *et al.*, 2013). To determine which domains of *SOBIR1* are required for Cf-4 accumulation, stable transgenic *N. benthamiana*:Cf-4-eGFP plants were inoculated with TRV:*NbSOBIR1(-like)* and, 3 weeks later, *AtSOBIR1* Δ Ncaps Δ 5LRRs-Myc or *AtSOBIR1*^{D489N}-Myc was transiently expressed.

AtSOBIR1-Myc served as a positive control, whereas GUS served as a negative control. The amount of Cf-4 protein accumulating on expression of *AtSOBIR1* Δ Ncaps Δ 5LRRs-Myc or *AtSOBIR1*^{D489N}-Myc was similar to the level observed on *AtSOBIR1*-Myc expression, but was higher than when GUS was expressed (Fig. 5). These results show that the LRR domain and kinase activity of *AtSOBIR1* are not required for the accumulation of Cf-4.

DISCUSSION

In previous studies, *SOBIR1* has been found to interact specifically with RLPs and has been shown to be required for RLP accumulation and function. However, how *SOBIR1* forms a complex with RLPs, what domains are required for this and how *SOBIR1* regulates RLP function are poorly understood. Here, we have shown that the highly conserved GxxxGxxxG motif present in the TM domain of *S/SOBIR1* (Fig. 1B) is required for interaction with RLPs (Fig. 3). In complementation assays, we found that the LRR domain of *SOBIR1* and its kinase activity are required for Cf-4-mediated HR (Fig. 4), whereas the LRRs and kinase activity are not required for facilitation of Cf-4 accumulation (Fig. 5).

It has been reported that, in many cases, GxxxG and GxxxGxxxG motifs are responsible for the interaction between two TM proteins (Cymer *et al.*, 2012; Fink *et al.*, 2012; Li *et al.*, 2012). co-IP experiments have shown that the GxxxGxxxG motif present in the TM domain of *S/SOBIR1* is required for its interaction with RLPs (Fig. 3). It may be that the GxxxGxxxG motif is required for the localization of *SOBIR1* in the plasma membrane (Leslie *et al.*, 2010; Liebrand *et al.*, 2013), thereby allowing the RLK to interact with plasma membrane-localized RLPs. Alternatively, the GxxxGxxxG motif may not be essential for plasma membrane localization, but may play a role in mediating the interaction with RLPs by allowing helix-helix interactions in the TM domains of both proteins (Cymer *et al.*, 2012). Indeed, we have shown here that *S/SOBIR1*^{3GA}-eGFP still localizes to the plasma membrane and endosomal vesicles, very similar to wild-type *S/SOBIR1*-eGFP (Figs S3D and S2). Therefore, we conclude that the GxxxGxxxG motif of *SOBIR1* is important for the interaction with RLPs.

Fig. 4 Analysis of the requirement of the various domains of AtSOBIR1 for Cf-4/Avr4-triggered hypersensitive response (HR). (A) Schematic representation of the AtSOBIR1 protein domain structure and variants of AtSOBIR1. Tan, signal peptide; yellow, Ncaps domain; light green, leucine-rich repeat (LRR) domain; dark green, extracellular juxtamembrane domain (eJM); black, transmembrane domain (TM); blue, intracellular juxtamembrane domain (iJM); pink, kinase domain. (B) The LRR domain and kinase activity of AtSOBIR1 are required for the Cf-4/Avr4-triggered HR. Transgenic *Nicotiana benthamiana*:Cf-4 plants were subjected to virus-induced gene silencing (VIGS) of endogenous *NbSOBIR1* by inoculation with *TRV:NbSOBIR1(-like)*. Three weeks later, mature leaves were agroinfiltrated to express Avr4 in combination with the different AtSOBIR1 variants fused to enhanced green fluorescent protein (eGFP), wild-type AtSOBIR1-eGFP and β -glucuronidase (GUS). All assays were performed three times and a representative photograph is shown. Please note that this experiment is performed in *N. benthamiana* expressing Cf-4. As VIGS causes a knock down of the expression of endogenous *NbSOBIR1(-like)*, there will always be some residual functional *NbSOBIR1(-like)* protein present, resulting in background Cf-4/Avr4-triggered HR visible in the infiltrated areas. (C) Stability of truncated and mutated AtSOBIR1 variants and their interaction with Cf-4. Wild-type AtSOBIR1 and the different AtSOBIR1 variants, all fused to eGFP, were transiently expressed in *N. benthamiana* in combination with Cf-4-Myc. Total proteins were extracted and subjected to immunopurification using GFP_TrapA affinity beads. Total proteins (Input) and immunopurified proteins (IP) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride (PVDF) membrane. Blots were subsequently incubated with α GFP antibody to detect the immunopurified eGFP fusion proteins and incubated with α Myc antibody to detect co-purifying Cf-4-Myc (co-immunoprecipitation, co-IP) and Cf-4-Myc in the input. The Coomassie-stained blot shows the 50-kDa Rubisco band present in the input samples to confirm equal loading.

Fig. 5 The leucine-rich repeat (LRR) domain and kinase activity of SOBIR1 are not required for Cf-4 accumulation. Transgenic *Nicotiana benthamiana*:Cf-4-eGFP plants were inoculated with TRV:NbSOBIR1(-like) and, 3 weeks after inoculation, AtSOBIR1^{D489N}-Myc or AtSOBIR1ΔNcapsΔ5LRRs-Myc was transiently expressed in *N. benthamiana*:Cf-4-eGFP. As controls, wild-type AtSOBIR1-Myc and β-glucuronidase (GUS) were transiently expressed in *N. benthamiana*:Cf-4-eGFP. An immunoprecipitation of Cf-4-GFP was performed and blots were incubated with αGFP antibody to detect Cf-4-eGFP and incubated with αMyc antibody to detect co-purifying Myc fusion proteins. Coomassie-stained blots show the 50-kDa Rubisco band present in the input samples to confirm equal loading. All assays were performed three times and a representative photograph for each experiment is shown.

GxxxG and GxxxGxxxG motifs are also highly conserved in the TM domain of most RLPs (Fig. 1B) (Gust and Felix, 2014). Interestingly, a mutation in the second glycine of the GxxxG motif abolishes the function of Cf-9 and it has been hypothesized that this mutation either affects the localization of Cf-9 or impairs its interaction with another protein required for Cf-9-dependent signalling (Wulff et al., 2004). We propose that this interacting protein is SOBIR1. A mutant in the GxxxG motif of SNC2 (AtRLP51), referred to as snc2-1D, and of SNC3 (AtRLP55) leads to a constitutively activated defence response (Zhang et al., 2010). Furthermore, Arabidopsis mesophyll protoplasts trans-

formed with SNC2-GFP and snc2-1D-GFP have revealed that both RLPs localize to the plasma membrane (Zhang et al., 2010). Given that the GxxxGxxxG motif of SOBIR1 mediates the interaction with most RLPs, the GxxxG or GxxxGxxxG motifs present in RLPs may mediate the interaction with SOBIR1. However, it has been observed that SOBIR1 also interacts with some RLPs which do not contain a GxxxG motif, such as LeEIX2 and S/CLV2 (Liebrand et al., 2013). One explanation could be that there are other motifs present in the TM domain of RLPs mediating the interaction with SOBIR1. Indeed, several motifs in TM domains have been reported to mediate the interaction, such as polar residues allowing the formation of hydrogen bonds (Gratkowski et al., 2001; Zhou et al., 2001), serine/threonine-rich sequences (Dawson et al., 2002) and the QxxS motif (Sal-Man et al., 2005).

Although the truncated form of S/SOBIR1, S/SOBIR1-eJM-TM-iJM, still associates with Cf-4, and the TM domain plays an important role in this interaction, we cannot exclude the possibility that the other domains of SOBIR1, i.e. the LRR and kinase domains, and eJM and iJM, also play a role in the interaction with RLPs. Indeed, conserved opposite charges present in the apoplastic eJM domains of RLP and SOBIR1 might allow physical interaction between the two proteins (Fig. 1B) (Gust and Felix, 2014). Here, we found that, when the three glycine residues of the GxxxGxxxG motif of SOBIR1 are simultaneously replaced by alanine residues, its interaction with RLPs is abolished (Fig. 3B,C). We propose that the GxxxGxxxG motifs of the TM domains of both proteins bring SOBIR1 and RLP together, after which the other domains of SOBIR1 and RLP stabilize this interaction.

Overall, our observations indicate that, in addition to a role of SOBIR1 in RLP stabilization, this RLK is also involved in the transduction of defence signalling downstream of the RLP on activation by the matching ligand.

EXPERIMENTAL PROCEDURES

Analysis of SOBIR1 homologues and construction of a phylogenetic tree

Amino acid sequences of S/SOBIR1 and SOBIR1 homologues identified in 19 additional plant species were aligned using the Mafft web-server (Stamatakis et al., 2008). From this alignment, an unrooted maximum likelihood tree was made using RAxML version 8.0.5. The resulting tree was viewed using the iTOL online tree viewing tool (Letunic and Bork, 2007, 2011). Sequence identity and similarity scores between SOBIR1 homologues were calculated using SIAS (<http://imed.med.ucm.es/Tools/sias.html>).

Plant growth conditions

Nicotiana benthamiana, *N. benthamiana*:Cf-4 (Gabriëls et al., 2007), *N. benthamiana*:Cf-4-eGFP [obtained by stably transforming the pBIN-KS-

35S::Cf-4-eGFP (SOL2701) construct (Liebrand *et al.*, 2012) to *N. benthamiana*, according to the method described previously (Gabriëls *et al.*, 2007)] and *N. tabacum* were grown under 16 h of light at 25 °C and 8 h of darkness at 21 °C in climate chambers with a relative humidity of 75%.

Binary vectors for *Agrobacterium* infiltrations (agroinfiltrations) and VIGS

The constructs pBIN-KS-35S::Cf-4-eGFP (Sol 2701), pGWB20-Cf-4-Myc (Sol 2783), pBIN-KS-35S::S/SOBIR1-eGFP (Sol 2774), pGWB20-S/SOBIR1-Myc (Sol 2754), pBIN-KS-35S::AtSOBIR1-eGFP (Sol 2848), pGWB20-AtSOBIR1-Myc (Sol 2849), pBIN-KS-35S::AtSOBIR^{D489N}-eGFP (Sol 2877), pGWB20-AtSOBIR^{D489N}-Myc (Sol 2880), pBIN-KS-35S::LeEIX2-eGFP (Sol 2863), pBIN-KS-35S::S/CLV2-eGFP (Sol 2782) and pTRV::NbSOBIR1(-like) (Sol 2854) have been described previously (Liebrand *et al.*, 2012, 2013). The constructs pB7K40-Ve1-HA (Fradin *et al.*, 2014) and pCambia2300-FLS2p::LeFLS2 (Robatzek *et al.*, 2007) have also been described elsewhere.

To generate truncation constructs S/SOBIR1ΔNcapsΔ5LRRs, S/SOBIR1Δ2LRRs, S/SOBIR1-eJM-TM-iJM, AtSOBIR1Δ2LRRs, AtSOBIR1ΔNcapsΔ5LRRs, AtSOBIR1ΔNcaps, AtSOBIR1ΔiJM and kinase domain only, polymerase chain reactions (PCRs) were performed to amplify the upstream region encoding the predicted signal peptide and downstream region encoding the required C-terminal part of the protein, using pBIN-KS-35S::S/SOBIR1-eGFP (Sol 2774) or pBIN-KS-35S::AtSOBIR1-eGFP (Sol 2848) as a template. The upstream and downstream regions, having an overlap of about 20 base pairs, were fused by subsequent overlap extension PCR. The products were cloned into the pENTR/D-Topo vector (Invitrogen, Life Technologies, Carlsbad, CA, USA). The regions encoding the kinase domain of S/SOBIR1 and AtSOBIR1 were amplified by PCR using pBIN-KS-35S::S/SOBIR1-eGFP (Sol 2774) and pBIN-KS-35S::AtSOBIR1-eGFP (Sol 2848). To generate G to A point mutations in the GxxxGxxxG motif present in the TM domain of S/SOBIR1 and AtSOBIR1, the coding region present in pENTR/D-Topo::S/SOBIR1 (Sol 2746) (Liebrand *et al.*, 2013) was PCR amplified with primers introducing a mismatch nucleotide to generate a G to A codon change. After amplification, the methylated parental plasmid was digested using *DpnI*. Primer sequences used for PCR are listed in Table S1 (see Supporting Information).

All pENTR/D-Topo clones were sequenced and, subsequently, fragments were transferred to the binary transformation vector pBIN-KS-35S::GWY-eGFP (Sol 2095; for C-terminally tagging with eGFP), pGWB20 (Nakagawa *et al.*, 2007) (for C-terminally tagging with the Myc epitope) or pGWB14 (Nakagawa *et al.*, 2007) (for C-terminally tagging with the HA epitope), using Gateway LR Clonase II (Invitrogen). This resulted in plasmids pBIN-KS-35S::S/SOBIR1Δ2LRRs-eGFP (Sol 4092), pBIN-KS-35S::S/SOBIR1ΔNcapsΔ5LRRs-eGFP (Sol 4091), pBIN-KS-35S::S/SOBIR1-kinase-eGFP (Sol 4044), pGWB14-S/SOBIR1-eJM-TM-iJM-HA (Sol 4108), pBIN-KS-35S::S/SOBIR1^{G274A}-eGFP (Sol 4105), pBIN-KS-35S::S/SOBIR1^{G278A}-eGFP (Sol 4106), pBIN-KS-35S::S/SOBIR1^{G282A}-eGFP (Sol 4107), pBIN-KS-35S::S/SOBIR1^{3GA}-eGFP (Sol 4118), pBIN-KS-35S::S/SOBIR1^{3GA}-HA (Sol 4121), pBIN-KS-35S::AtSOBIR1Δ2LRRs-eGFP (Sol 4507), AtSOBIR1ΔNcapsΔ5LRRs-eGFP (Sol 4506), pBIN-KS-35S::AtSOBIR1ΔNcaps-eGFP (Sol 4512), pBIN-KS-35S::AtSOBIR1ΔiJM (Sol 4509), pBIN-KS-35S::AtSOBIR1-kinase-eGFP (Sol 4046), pGWB20-AtSOBIR1ΔNcapsΔ5LRRs-Myc (Sol 4137) and pBIN-KS-35S::AtSOBIR1^{3GA}-

eGFP (Sol 4165). Avr4 was expressed from pMOG800-Avr4. All binary plasmids were transformed to *A. tumefaciens* strain C58C1, carrying helper plasmid pCH32. *Agrobacterium tumefaciens* infiltrations were performed as described previously (Van der Hoorn *et al.*, 2000).

Co-immunopurifications and immunoblotting

Co-immunopurifications were performed as described previously (Liebrand *et al.*, 2012). The following antibodies were used: αGFP-HRP (130-091-833, MACS antibodies, Bergisch Gladbach, Germany), αHA-HRP (clone 3F10; Roche Applied Science, Mannheim, Germany) and αMyc (cMYC9E10, sc-40, Santa Cruz Biotechnology, Heidelberg, Germany), with αMouse-HRP (Amersham, GE Healthcare, Eindhoven, The Netherlands) as a secondary antibody.

Confocal microscopy

Confocal microscopy was performed using the Leica SP5 microscope and software package (Leica Camera AG, Wetzlar, Germany) as described previously (Beck *et al.*, 2012).

VIGS in *N. benthamiana* and functional complementation assays

VIGS experiments in *N. benthamiana*:Cf-4 and wild-type *N. benthamiana* were performed as described previously (Liebrand *et al.*, 2012). For functional complementation analysis with AtSOBIR1 truncated and mutated variants, and GUS and wild-type AtSOBIR1 as negative and positive controls, respectively, 3 weeks after agroinoculation with the TRV VIGS constructs, constructs driving the expression of these proteins were co-expressed with Avr4 [optical density at 600 nm (OD₆₀₀) = 0.03] at an OD₆₀₀ of 0.6.

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REFERENCES

- Beck, M., Zhou, J., Faulkner, C., MacLean, D. and Robatzek, S. (2012) Spatio-temporal cellular dynamics of the *Arabidopsis* flagellin receptor reveal activation status-dependent endosomal sorting. *Plant Cell*, **24**, 4205–4219.
- Bi, G., Liebrand, T., Cordewener, J., America, A., Xu, X. and Joosten, M. (2014) *Arabidopsis thaliana* receptor-like protein AtRLP23 associates with the receptor-like kinase AtSOBIR1. *Plant Signal. Behav.* **9**, e27937.
- Bleckmann, A., Weidtkamp-Peters, S., Seidel, C.A. and Simon, R. (2010) Stem cell signaling in *Arabidopsis* requires CRN to localize CLV2 to the plasma membrane. *Plant Physiol.* **152**, 166–176.
- Böhm, H., Albert, I., Fan, L., Reinhard, A. and Nürnberger, T. (2014) Immune receptor complexes at the plant cell surface. *Curr. Opin. Plant Biol.* **20**, 47–54.

- Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G. and Boller, T. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, **448**, 497–500.
- Cymer, F., Veerappan, A. and Schneider, D. (2012) Transmembrane helix–helix interactions are modulated by the sequence context and by lipid bilayer properties. *Biochim. Biophys. Acta*, **1818**, 963–973.
- Dawson, J.P., Weinger, J.S. and Engelman, D.M. (2002) Motifs of serine and threonine can drive association of transmembrane helices. *J. Mol. Biol.* **316**, 799–805.
- De Jong, C.F., Laxalt, A.M., Bargmann, B.O., De Wit, P.J., Joosten, M.H. and Munnik, T. (2004) Phosphatidic acid accumulation is an early response in the *Cf-4/Avr4* interaction. *Plant J.* **39**, 1–12.
- Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* **11**, 539–548.
- Fink, A., Sal-Man, N., Gerber, D. and Shai, Y. (2012) Transmembrane domains interactions within the membrane milieu: principles, advances and challenges. *Biochim. Biophys. Acta*, **1818**, 974–983.
- Fradin, E.F., Zhang, Z., Rovenich, H., Song, Y., Liebrand, T.W., Masini, L., van den Berg, G.C., Joosten, M.H. and Thomma, B.P. (2014) Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2. *PLoS ONE*, **9**, e88208.
- Gabriëls, S.H., Vossen, J.H., Ekengren, S.K., van Ooijen, G., Abd-El-Halim, A.M., van den Berg, G.C., Rainey, D.Y., Martin, G.B., Takken, F.L. and De Wit, P.J. (2007) An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. *Plant J.* **50**, 14–28.
- Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y.T., Chen, S., Li, X. and Zhang, Y. (2009) Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe*, **6**, 34–44.
- Gómez-Gómez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell*, **5**, 1003–1011.
- Gratkowski, H., Lear, J.D. and DeGrado, W.F. (2001) Polar side chains drive the association of model transmembrane peptides. *Proc. Natl. Acad. Sci. USA*, **98**, 880–885.
- Gust, A.A. and Felix, G. (2014) Receptor like proteins associate with SOBIR1-type of adaptors to form bimolecular receptor kinases. *Curr. Opin. Plant Biol.* **21**, 104–111.
- Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C. and Rathjen, J.P. (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. USA*, **104**, 12 217–12 222.
- Jehle, A., Fürst, U., Lipschis, M., Albert, M. and Felix, G. (2013) Perception of the novel MAMP eMax from different *Xanthomonas* species requires the *Arabidopsis* receptor-like protein ReMAX and the receptor kinase SOBIR. *Plant Signal. Behav.* **8**, e27408.
- Jones, J.D. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.
- de Jonge, R., van Esse, H.P., Maruthachalam, K., Bolton, M.D., Santhanam, P., Saber, M.K., Zhang, Z., Usami, T., Lievens, B. and Subbarao, K.V. (2012) Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proc. Natl. Acad. Sci. USA*, **109**, 5110–5115.
- Lee, J.S., Kuroha, T., Hnilova, M., Khatayevich, D., Kanaoka, M.M., McAbee, J.M., Sarikaya, M., Tamerler, C. and Torii, K.U. (2012) Direct interaction of ligand–receptor pairs specifying stomatal patterning. *Genes Dev.* **26**, 126–136.
- Leslie, M.E., Lewis, M.W., Youn, J.-Y., Daniels, M.J. and Liljegren, S.J. (2010) The EVERSLED receptor-like kinase modulates floral organ shedding in *Arabidopsis*. *Development*, **137**, 467–476.
- Letunic, I. and Bork, P. (2007) Interactive tree of life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, **23**, 127–128.
- Letunic, I. and Bork, P. (2011) Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* **39**, W475–W478.
- Li, E., Wimley, W.C. and Hristova, K. (2012) Transmembrane helix dimerization: beyond the search for sequence motifs. *Biochim. Biophys. Acta*, **1818**, 183–193.
- Liebrand, T.W., Smit, P., Abd-El-Halim, A., de Jonge, R., Cordewener, J.H., America, A.H., Sklenar, J., Jones, A.M., Robatzek, S., Thomma, B.P., Tameling, W.I. and Joosten, M.H. (2012) Endoplasmic reticulum-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. *Plant Physiol.* **159**, 1819–1833.
- Liebrand, T.W., van den Berg, G.C., Zhang, Z., Smit, P., Cordewener, J.H., America, A.H., Sklenar, J., Jones, A.M., Tameling, W.I., Robatzek, S., Thomma, B.P. and Joosten, M.H. (2013) Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proc. Natl. Acad. Sci. USA*, **110**, 10 010–10 015.
- Liebrand, T.W., Kombrink, A., Zhang, Z., Sklenar, J., Jones, A.M., Robatzek, S., Thomma, B.P. and Joosten, M.H. (2014a) Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*. *Mol. Plant Pathol.* **15**, 109–117.
- Liebrand, T.W., van den Burg, H.A. and Joosten, M.H. (2014b) Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends Plant Sci.* **19**, 123–132.
- Macho, A.P. and Zipfel, C. (2014) Plant PRRs and the activation of innate immune signaling. *Mol. Cell*, **54**, 263–272.
- Müller, R., Bleckmann, A. and Simon, R. (2008) The receptor kinase CORYNE of *Arabidopsis* transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell*, **20**, 934–946.
- Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* **104**, 34–41.
- Oh, M.-H., Wang, X., Wu, X., Zhao, Y., Clouse, S.D. and Huber, S.C. (2010) Autophosphorylation of Tyr-610 in the receptor kinase BAK1 plays a role in brassinosteroid signaling and basal defense gene expression. *Proc. Natl. Acad. Sci. USA*, **107**, 17 827–17 832.
- Rivas, S. and Thomas, C.M. (2005) Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*. *Annu. Rev. Phytopathol.* **43**, 395–436.
- Robatzek, S., Bittel, P., Chinchilla, D., Köchner, P., Felix, G., Shiu, S.-H. and Boller, T. (2007) Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of *Arabidopsis* FLS2 exhibiting characteristically different perception specificities. *Plant Mol. Biol.* **64**, 539–547.
- Ron, M. and Avni, A. (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell*, **16**, 1604–1615.
- Roth, L., Nasarre, C., Dirrig-Grosch, S., Aunis, D., Crémel, G., Hubert, P. and Bagnard, D. (2008) Transmembrane domain interactions control biological functions of neuropilin-1. *Mol. Biol. Cell*, **19**, 646–654.
- Sal-Man, N., Gerber, D. and Shai, Y. (2005) The identification of a minimal dimerization motif QXXS that enables homo- and hetero-association of transmembrane helices in vivo. *J. Biol. Chem.* **280**, 27 449–27 457.
- Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A. and Zipfel, C. (2011) Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* **7**, e1002046.
- Stamatakis, A., Hoover, P. and Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web servers. *Syst. Biol.* **57**, 758–771.
- Stergiopoulos, I. and de Wit, P.J. (2009) Fungal effector proteins. *Annu. Rev. Phytopathol.* **47**, 233–263.
- Van der Hoorn, R.A., Laurent, F., Roth, R. and De Wit, P.J. (2000) Agroinfiltration is a versatile tool that facilitates comparative analyses of *Avr9/Cf-9*-induced and *Avr4/Cf-4*-induced necrosis. *Mol. Plant–Microbe Interact.* **13**, 439–446.
- Wulff, B.B., Thomas, C.M., Parniske, M. and Jones, J.D. (2004) Genetic variation at the tomato *Cf-4/Cf-9* locus induced by EMS mutagenesis and intralocus recombination. *Genetics*, **167**, 459–470.
- Zhang, L., Kars, I., Essenstam, B., Liebrand, T.W., Wagemakers, L., Elberse, J., Tagkalaki, P., Tjoitang, D., van den Ackerveken, G. and van Kan, J.A. (2014) Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the *Arabidopsis* receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. *Plant Physiol.* **164**, 352–364.
- Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Boutrot, F.F., Tör, M., Zipfel, C., Gust, A.A. and Brunner, F. (2013a) *Arabidopsis* RECEPTOR-LIKE PROTEIN30 and receptor-like kinase SUPPRESSOR OF BIR1-1/EVERSLED mediate innate immunity to necrotrophic fungi. *Plant Cell*, **25**, 4227–4241.
- Zhang, Y., Yang, Y., Fang, B., Gannon, P., Ding, P., Li, X. and Zhang, Y. (2010) *Arabidopsis* *snc2-1D* activates receptor-like protein-mediated immunity transduced through WRKY70. *Plant Cell*, **22**, 3153–3163.
- Zhang, Z., Fradin, E., de Jonge, R., van Esse, H.P., Smit, P., Liu, C.M. and Thomma, B.P. (2013b) Optimized agroinfiltration and virus-induced gene silencing to study Ve1-mediated *Verticillium* resistance in tobacco. *Mol. Plant–Microbe Interact.* **26**, 182–190.
- Zhou, F.X., Merianos, H.J., Brunger, A.T. and Engelman, D.M. (2001) Polar residues drive association of polyleucine transmembrane helices. *Proc. Natl. Acad. Sci. USA*, **98**, 2250–2255.

- Zhu, Y., Wang, Y., Li, R., Song, X., Wang, Q., Huang, S., Jin, J.B., Liu, C.M. and Lin, J. (2010) Analysis of interactions among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in *Arabidopsis*. *Plant J.* **61**, 223–233.
- Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell*, **125**, 749–760.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Alignment of SOBIR1 amino acid sequences from 20 different plant species. Amino acid residues highlighted in yellow and blue represent 100% identical and 100% similar residues, respectively. The five predicted leucine-rich repeats (LRRs), as identified with <http://www.lrrfinder.com/>, are underlined.

Fig. S2 *S/SOBIR1*^{3GA}-eGFP localizes to the plasma membrane and cytoplasmic vesicles, similar to *S/SOBIR1*-eGFP. Scale bar, 10 µm.

Fig. S3 *AtSOBIR1* variants fused to enhanced green fluorescent protein (eGFP) and wild-type *AtSOBIR1*-eGFP do not induce cell death in *Nicotiana benthamiana*. (A) Transgenic *N. benthamiana*:*Cf-4* plants were subjected to virus-induced gene silencing (VIGS) of endogenous *NbSOBIR1* by inoculation with TRV:*NbSOBIR1*(-like). Three weeks later, mature leaves were agroinfiltrated to express wild-type *AtSOBIR1*-eGFP, the different *AtSOBIR1* variants fused to eGFP and β-glucuronidase (GUS). All assays were performed three times and a representative photograph is shown. (B) Wild-type *N. benthamiana* plants were subjected to VIGS of endogenous *NbSOBIR1* by inoculation with TRV:*NbSOBIR1*(-like). Three weeks later, mature leaves were agroinfiltrated to express wild-type *AtSOBIR1*, *AtSOBIR1*ΔIJM and *AtSOBIR1*^{3GA}, all fused to eGFP. All assays were performed three times and a representative photograph is shown.

Table S1 Overview of the oligonucleotide primers used in this study.