Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection

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The plant immune system is activated by microbial patterns that are detected as nonself molecules. Such patterns are recognized by immune receptors that are cytoplasmic or localized at the plasma membrane. Cell surface receptors are represented by receptor-like kinases (RLKs) that frequently contain extracellular leucine-rich repeats and an intracellular kinase domain for activation of downstream signaling, as well as receptor-like proteins (RLPs) that lack this signaling domain. It is therefore hypothesized that RLKs are required for RLPs to activate downstream signaling. The RLPs Cf-4 and Ve1 of tomato (*Solanum lycopersicum*) mediate resistance to the fungal pathogens *Cladosporium fulvum* and *Verticillium dahliae*, respectively. Despite their importance, the mechanism by which these immune receptors mediate downstream signaling upon recognition of their matching ligand, Avr4 and Ave1, remained enigmatic. Here we show that the tomato ortholog of the *Arabidopsis thaliana* RLK Suppressor Of BIR1-1/Evershed (SOBIR1/EVR) and its close homolog *S. lycopersicum* (*Sl*) SOBIR1-like interact *in planta* with both Cf-4 and Ve1 and are required for the Cf-4– and Ve1-mediated hypersensitive response and immunity. Tomato SOBIR1/EVR interacts with most of the tested RLPs, but not with the RLKs FLS2, SERK1, SERK3a, BAK1, and CLV1. SOBIR1/EVR is required for stability of the Cf-4 and Ve1 receptors, supporting our observation that these RLPs are present in a complex with SOBIR1/EVR *in planta*. We show that SOBIR1/EVR is essential for RLP-mediated immunity and propose that the protein functions as a regulatory RLK of this type of cell-surface receptors.

plant innate immunity | receptor complex | development | defense signaling | plant–microbe interaction

Plants rely on an innate immune system, which is activated upon recognition of pathogen-derived nonself molecules, or host-derived damage products (1, 2). Conserved microbe-associated molecular patterns (MAMPs) are perceived by pattern recognition receptors (PRRs) that activate MAMP-triggered immunity (MTI). Pathogenic microbes promote virulence by secretion of effector proteins, many of which suppress MTI (3, 4). In resistant plants, these effector proteins are detected by resistance proteins that activate effector-triggered immunity (ETI), frequently resulting in the hypersensitive response (HR), a localized programmed host cell death response (1). Conceptually, MTI and ETI function in a similar fashion by using immune receptors that mount a suitable defense response to halt pathogen ingress upon recognition of appropriate ligands that betray pathogen presence (5).

Most PRRs have been identified as transmembrane receptor-like kinases (RLKs) that frequently contain an extracellular leucine-rich repeat (LRR) domain or lysin-motif (LysM) for ligand recognition and an intracellular kinase domain for activation of downstream signaling (6). The LysM–RLK Chitin Elicitor Receptor Kinase 1 mediates immunity against fungi by recognizing fungal chitin (7, 8), whereas the LRR–RLKs Flagellin Sensing 2 (FLS2) and EF-Tu Receptor (EFR) are involved in recognition

of bacterial flagellin and the elongation-factor Tu protein, respectively (9, 10). Upon ligand recognition, both FLS2 and EFR form a receptor complex with the LRR–RLK Somatic Embryogenesis Receptor Kinase 3/Brassinosteroid Insensitive 1 (BRI1)-Associated receptor Kinase 1 (SERK3/BAK1) and its close paralog BAK1-like 1 (BKK1) (11–13). The transphosphorylation events that follow, together with the dissociation of the cytoplasmic kinase Botrytis-Induced Kinase 1 from the receptor complex, subsequently activate downstream defense signaling (14, 15). SERK3/BAK1 is not involved in ligand binding to FLS2 and EFR but, rather, plays a role in downstream signaling upon its recruitment by FLS2 and EFR after ligand binding (2). Hence, SERK3/BAK1 and BKK1 likely function as signal enhancers and can be regarded as coregulatory RLKs in FLS2- and EFR-mediated immunity (6, 16).

Receptor-like proteins (RLPs) form a second major class of cell-surface receptors in plants. RLPs are structurally similar to RLKs but lack a cytoplasmic kinase domain (17, 18). RLPs function in defense, such as the Cf proteins and Ve1, as well as in development (18). Examples of the latter are Clavata2 (CLV2), which plays a role in meristem maintenance, and Too Many Mouths (TMM), which regulates stomatal patterning (18). Because RLPs lack a cytoplasmic kinase domain, it is anticipated that proteins containing such a domain are recruited to activate downstream signaling (19, 20). Indeed, *Arabidopsis thaliana* CLV2 forms a complex with the transmembrane kinase Coryne and the LRR–RLK CLV1 (21–23), whereas TMM requires the LRR–RLK Erecta to activate downstream signaling (24).

In tomato (*Solanum lycopersicum*), resistance to specific races of the fungal pathogens *Cladosporium fulvum* (causing leaf mold disease) and *Verticillium dahliae* (causing vascular wilt disease) is mediated by LRR-containing RLPs (25, 26). Cf proteins confer immunity upon recognition of *C. fulvum* race-specific secreted effectors [also referred to as avirulence (Avr) proteins] (27), whereas Ve1 recognizes the Ave1 effector protein secreted by race 1 *V. dahliae* strains (28). Cf-9 was the first identified RLP (19), and since its discovery several attempts have been made to understand Cf-mediated defense signaling by identifying Cf-interacting proteins. Yeast two-hybrid analyses resulted in the isolation of several potential interactors of the cytoplasmic C terminus of Cf-9 (29–31). However, an RLK or Coryne-like protein,

recruited by Cf proteins and providing a cytoplasmic kinase domain through which Cf-mediated signaling would occur, remained to be identified (20). Recently, by immunopurification of a functional Cf-4–enhanced green fluorescent protein (eGFP) fusion protein from plants, we identified endoplasmic reticulum (ER)-resident chaperones as *in planta* interactors of Cf proteins that are required for Cf protein biogenesis (32). Here, following a similar approach, we describe the identification of the tomato ortholog of the *Arabidopsis* RLK Suppressor Of BIR1-1/Evershed (SOBIR1/EVR; hereafter referred to as SOBIR1) (33, 34) and its close homolog SOBIR1-like as Cf interactors. Interestingly, both tomato homologs and *Arabidopsis* SOBIR1 interact with Ve1, in addition to Cf-4, and we show that SOBIR1 is required for Cf-2–, Cf-4–, and Ve1-mediated immunity. Our work reveals an essential role for SOBIR1 in the plant immune response activated by two distinct RLPs involved in resistance to fungal pathogens and suggests that SOBIR1 functions as an essential regulatory RLK of this type of cell-surface receptors.

Results

Identification of Tomato SOBIR1 and SOBIR1-like as Interactors of Cf-4 and Ve1. To identify Cf-interacting proteins, we stably transformed Money Maker (MM)–Cf-0 tomato, lacking *Cf* resistance genes to *C. fulvum*, with a construct driving constitutive expression of a Cf-4–eGFP fusion protein (32). Transgenic line (TL) TL3 showed recognition resulting in a specific HR upon infiltration with the *C. fulvum* Avr4 effector, whereas TL21 did not show a response to Avr4 (Fig. S1*A*). Cf-4–eGFP was successfully immunopurified from TL3, whereas the fusion protein could not be detected in TL21 (Fig. S1*B*). To identify proteins copurifying with Cf-4, tryptic on-bead digestion of the purified proteins was performed, and the generated peptides were analyzed by mass spectrometry. Interestingly, in the sample originating from TL3, but not in the one from TL21, in addition to peptides originating from Cf-4–eGFP itself, peptides matching to two tomato RLKs encoded by Solyc06g071810.1.1 and Solyc03g111800.2.1 were identified (Table S1). The alignments presented in Fig. S2*A* show that the amino acid sequences of these tomato RLKs are highly homologous to each other (~74% identical) and are closely related to the *Arabidopsis* RLK SOBIR1 (~60% identity). Both tomato RLKs are more distantly related to *S. lycopersicum* (*Sl*)SERK3a/BAK1 (~25% identical) (33, 34). Fig. S2*B* also shows that the nucleotide sequences of both tomato RLKs and *A. thaliana* (*At*)SOBIR1 are very similar throughout their coding regions. Hence, we named the genes encoding the two tomato RLKs *SlSOBIR1* and *SlSOBIR1-like*. Similar to *At*SOBIR1, *Sl*SOBIR1 and *Sl*SOBIR1-like have five predicted LRRs, in contrast to *Sl*SERK3a/BAK1, which has only four LRRs. The SOBIR1 sequences of tomato and *Arabidopsis* are highly similar, both in their extracellular LRR and cytoplasmic kinase domains, whereas the homology of SOBIR1 to *Sl*SERK3a/BAK1 is mostly restricted to their kinase domains (Fig. S2*A*). No peptides originating from any other RLKs were identified in the peptide sample originating from TL3.

Cf-4–eGFP is also functional in *Nicotiana benthamiana* (32), and immunopurification of transiently expressed Cf-4–eGFP from this plant also yielded peptides from copurifying RLKs potentially matching SOBIR1 and SOBIR1-like (Table S2). The presence of *SlSOBIR1* orthologs in *N. benthamiana* and *Nicotiana tabacum* was assessed by searching public databases, indeed revealing two candidate *N. benthamiana* homologs, referred to as *NbSOBIR1* and *NbSOBIR1-like*, and one *N. tabacum* homolog (*NtSOBIR1*) (Fig. S2*C*). To also identify proteins interacting with Ve1, eGFP-tagged Ve1 (35) was immunopurified upon its transient expression in *N. benthamiana*. Also for this RLP, peptides matching *Nb*SOBIR1 and *Nb*SOBIR1-like were identified, whereas again no peptides from other RLKs were detected (Table S3).

Tomato SOBIR1 and SOBIR1-like and *Arabidopsis* SOBIR1 Interact with Cf-4 and Ve1. C-terminally Myc epitope-tagged versions of the tomato and *Arabidopsis* SOBIR1 RLKs (*Sl*SOBIR1–Myc, *Sl*SOBIR1-like–Myc, and *At*SOBIR1–Myc) were generated to perform coimmunopurification experiments with Cf and Ve1. Transient coexpression in *N. benthamiana* revealed that all three SOBIR1 proteins interact with Cf-4 and Ve1 (Fig. 1 and Fig. S1*C*). Coexpression of constructs encoding *Sl*SOBIR1–eGFP and Cf-4–Myc similarly revealed interaction of Cf-4–Myc with *Sl*SOBIR1–eGFP (Fig. S3*A*). We then examined whether the SOBIR1 proteins also interact with RLKs known to be involved in defense and/or development. Interestingly, C-terminally (e)GFP-tagged *Sl*SERK1, *Sl*SERK3a/BAK1 (36), *Sl*FLS2 (37), or *At*CLV1 (38), did not copurify with SOBIR1 (Fig. 1 and Fig. S1*C*).

To determine whether SOBIR1 requires a functional kinase domain for interaction with Cf-4, the core catalytic aspartate (D) of its conserved RD kinase motif was substituted to an asparagine (N) residue. For all tested RLKs containing the catalytic D, among which is SERK3a/BAK1, this mutation causes a loss of kinase activity (39). Interestingly, C-terminally Myc-tagged SlSOBIR1^{D473N}, SlSOBIR1-like^{D486N}, and AtSOBIR1^{D489N} all still interact with Cf-4–eGFP, showing that kinase activity of SOBIR1 is not required for interaction with the RLP (Fig. S3*B*). It was subsequently tested whether the presence of the Cf-4 ligand, Avr4, would lead to loss of the interaction between SOBIR1 and Cf-4. Cf-4–eGFP and *Sl*SOBIR1–Myc were transiently coexpressed with Avr4 or the nonrecognized effector Avr9 infiltrated at two different optical densities. Interaction between Cf-4 and *Sl*SOBIR1 was still observed in the presence of Avr4 and Avr9, indicating that the Cf-4/*Sl*SOBIR1 complex does not dissociate upon recognition of Avr4 by Cf-4 (Fig. S3*C*). We further studied whether *Sl*SOBIR1 forms homodimers and/or heterodimerizes with *Sl*SOBIR1-like or *At*SOBIR1. For this experiment, *Sl*SOBIR1–eGFP was coexpressed with *Sl*SOBIR1–Myc, *Sl*SOBIR1-like–Myc, or *At*SOBIR1–Myc, whereas coexpression with Cf-4–Myc was used as a control. Upon pull-down of *Sl*SOBIR1–eGFP, Cf-4–Myc strongly copurified with the RLK. However, we did not observe copurification of *Sl*SOBIR1–Myc, *Sl*SOBIR1-like–Myc, or *At*SOBIR1–Myc,
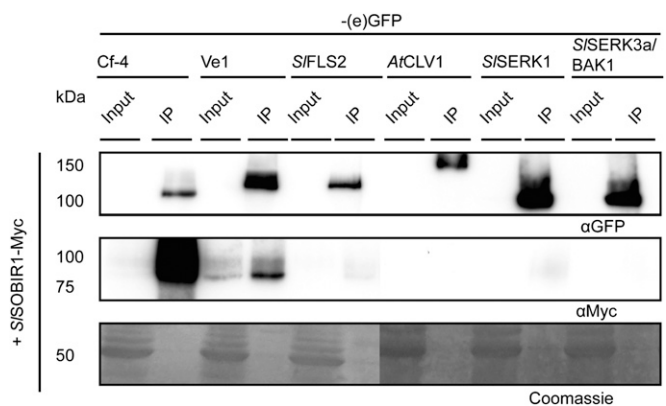
Fig. 1. Tomato *Sl*SOBIR1 interacts with Cf-4 and Ve1, but not with various RLKs. Tagged versions of Cf-4, Ve1, *At*CLV1, *Sl*SERK1, *Sl*SERK3a/BAK1, and *Sl*FLS2 (all fused to eGFP, except for *Sl*FLS2, which was fused to GFP) were coexpressed with *Sl*SOBIR1–Myc in *N. benthamiana*. Total protein extracts of transiently transformed leaf tissue were subjected to immunopurification by using GFP-affinity beads. Total proteins (Input) and immunopurified proteins (IP) were subjected to SDS/PAGE and blotted. Blots were incubated with α-GFP antibody to detect the immunopurified (e)GFP fusion proteins and incubated with α-Myc antibody to detect coimmunopurifying SOBIR1–Myc proteins. Coomassie-stained blots showing the 50-kDa Rubisco band present in the input samples confirm equal loading. Representative results for three independent experiments are shown.

indicating that SOBIR1 does not form homo- or heterodimers with SlSOBIR1-like or AtSOBIR1 (Fig. S3D).

SlSOBIR1 Localizes to the Plasma Membrane and Cytoplasmic Vesicles.
It has been reported that AtSOBIR1–YFP, when expressed under control of its own promoter in Arabidopsis, localizes to the plasma membrane and internal membrane compartments of epidermal leaf petiole cells and epidermal root cells (33). Confocal-laser scanning microscopy performed on N. benthamiana epidermal leaf cells transiently expressing SlSOBIR1–eGFP under control of the 35S promoter revealed that SlSOBIR1 mainly localizes to the plasma membrane (Fig. S4A). In addition, fluorescence signals were observed in mobile cytoplasmic vesicles (Fig. S4A). As previously shown, the GFP–HA control protein localizes to the cytoplasm and nucleus, whereas SlFLS2–GFP localizes to the plasma membrane (37) (Fig. S4 B–D).

Targeting SOBIR1 Compromises the Cf-4/Avr4–Induced and Ve1/Ave1-Induced HR. The observation that the two SOBIR1 homologs from tomato and N. benthamiana interact with Cf-4 and Ve1 (Fig. 1, Fig. S1C, and Tables S1–S3) suggests that both proteins play a role in Cf-4– and Ve1-mediated defense signaling in Solanaceous plants. Therefore, recombinant tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) constructs were generated to target expression of the NbSOBIR1 homologs, either individually or simultaneously (Fig. S2C), and transgenic N. benthamiana expressing Cf-4 was inoculated with the different TRV constructs. Three weeks after viral inoculations, plants were transiently transformed to express Avr4 (40). Inoculation with TRV:NbSOBIR1/NbSOBIR1-like resulted in a severely compromised Avr4-triggered HR, similar to inoculation with a TRV construct targeting Cf-4 itself (TRV:Cf-4) (Fig. 2). The Avr4-triggered HR was also strongly compromised when NbSOBIR1 was targeted. When NbSOBIR1-like was targeted, the HR was affected to a much lesser extent (Fig. 2). Quantitative RT-PCRs (qRT-PCRs) revealed that expression of NbSOBIR1 was strongly reduced upon inoculation with TRV:NbSOBIR1/NbSOBIR1-like or TRV:NbSOBIR1, compared with inoculation with TRV:β-glucuronidase (GUS) (Fig. S5 A and B). Interestingly, we did not detect transcripts of NbSOBIR1-like in TRV:GUS-inoculated or TRV:NbSOBIR1/NbSOBIR1-like-inoculated plants, suggesting that NbSOBIR1-like is not expressed or is at a very low level. We therefore reasoned that the slight reduction of the Avr4-triggered HR upon inoculation of N. benthamiana:Cf-4 with TRV:NbSOBIR1-like (Fig. 2) could be attributed to cross-silencing of NbSOBIR1 by the TRV:NbSOBIR1-like construct. Indeed, qRT-PCR confirmed that NbSOBIR1 expression levels were ~30% reduced upon inoculation with TRV:NbSOBIR1-like (Fig. S5B). Together these results indicate that NbSOBIR1 is the RLK that is required for the Cf-4–

mediated HR in N. benthamiana. The Cf homolog Peru2 from Solanum peruvianum is autoactive in N. benthamiana, causing an effector-independent HR when transiently expressed (41). Interestingly, the Peru2–eGFP-triggered HR was also strongly compromised upon expression in TRV:NbSOBIR1/NbSOBIR1-like–inoculated N. benthamiana plants (Fig. S6A). To check whether the silenced plants were still able to mount programmed cell death, fully expanded leaves were also transiently transformed to express an autoactive variant of the Nucleotide Binding (NB)–LRR immune receptor Rx (RxD460V) (42) and the proapoptotic factor Bcl2-Associated protein X (BAX) (43). Because RxD460V and BAX still triggered a strong cell death, we concluded that the ability of the plants to mount programmed cell death was not compromised (Fig. 2).

Unlike in N. benthamiana, coexpression of Ve1 with Ave1 triggers an HR in N. tabacum, a plant for which TRV-based VIGS was recently established (28, 35). N. tabacum plants (cultivar Samsun) were inoculated with TRV:NbSOBIR1/NbSOBIR1-like, which also targets the NtSOBIR1 homolog (Fig. S2C), and TRV:Enhanced Disease Susceptibility 1 (EDS1) as a positive control, because EDS1 is required for Ve1-mediated immunity (26). Inoculation with TRV:GFP was included as a negative control. We used the TRV:NbSOBIR1/NbSOBIR1-like construct because we anticipated that N. tabacum, of which the currently available genome sequence is very similar to that of N. benthamiana, may contain an NtSOBIR1-like homolog in addition to NtSOBIR1, although we did not identify an NtSOBIR1-like candidate in public databases. Three weeks after inoculation with the different recombinant TRV constructs, Ve1 and Ave1 were coexpressed, revealing that plants inoculated with the VIGS constructs targeting NtSOBIR1/NtSOBIR1-like and EDS1 did not mount an HR, in contrast to the TRV:GFP-inoculated plants (Fig. S6B). Together, these results show that SOBIR1 is required for Cf-4– and Peru2-mediated HR in N. benthamiana and Ve1-mediated HR in N. tabacum.

Kinase Activity of SOBIR1 Is Required for Cf-4–Mediated HR. To determine whether SOBIR1 requires a functional kinase domain for the Cf-4–mediated HR, we inoculated N. benthamiana:Cf-4 with TRV:NbSOBIR1/NbSOBIR1-like. These plants were then spot-infiltrated to transiently express the combinations Avr4 and AtSOBIR1–Myc or Avr4 and AtSOBIR1^{D489N}–Myc. As a control, GUS was expressed in combination with Avr4. We reasoned that AtSOBIR1 would not be targeted by this RNA silencing because there is not sufficient sequence homology between the NbSOBIR1 genes and AtSOBIR1, and therefore AtSOBIR1, being a functional homolog of NbSOBIR1, would complement the loss of NbSOBIR1 and reconstitute the Avr4-triggered HR. However, if SOBIR1 kinase activity is required for Cf-4–mediated HR, AtSOBIR1^{D489N}–Myc would not be able to complement.

Coexpression of GUS with Avr4 in the NbSOBIR1-silenced plants did not restore the Cf-4–mediated HR (Fig. S5C). When AtSOBIR1–Myc was coexpressed with Avr4, an HR was observed. However, when the kinase-dead mutant AtSOBIR1^{D489N}–Myc was coexpressed with Avr4, the Avr4-triggered HR was strongly compromised, indicating that SOBIR1 kinase activity is required for Cf-4–mediated HR (Fig. S5C). RT-PCR analysis showed that full-length AtSOBIR1–Myc and AtSOBIR1^{D489N}–Myc transcripts were present in the plants inoculated with TRV:NbSOBIR1/NbSOBIR1-like (Fig. S5D), confirming that Arabidopsis SOBIR1 is indeed not targeted by the VIGS construct. These results show that AtSOBIR1 complements NbSOBIR1 and the C-terminal Myc epitope tag does not appear to affect AtSOBIR1 function with respect to its role in Cf-4–mediated HR. Importantly, these results show that SOBIR1 kinase activity is required for the Cf-4–mediated HR.

SOBIR1 Is Required for Cf- and Ve1-Mediated Resistance to C. fulvum and V. dahliae, Respectively. To determine whether SOBIR1 is required for Cf-4–mediated resistance of tomato to C. fulvum,
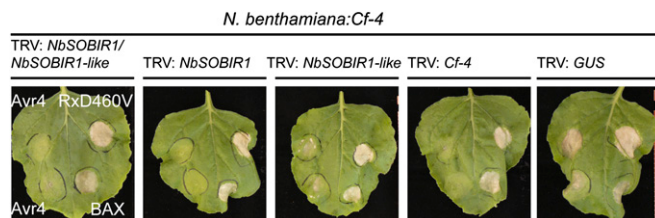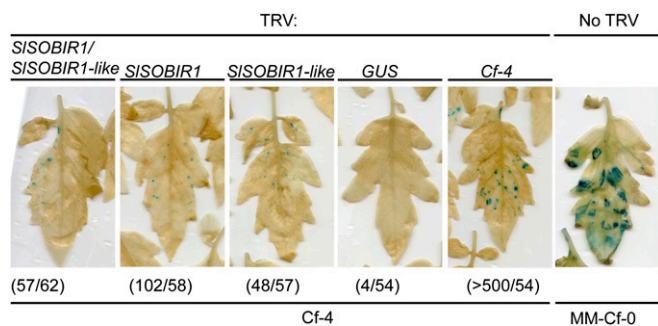

Fig. 2. SOBIR1 is required for Cf-4–mediated HR. Transgenic N. benthamiana:Cf-4 plants were subjected to VIGS by inoculation with the TRV constructs indicated above each image. TRV:NbSOBIR1/NbSOBIR1-like targets NbSOBIR1 and NbSOBIR1-like simultaneously. TRV:Cf-4 and TRV:GUS served as controls. Three weeks after TRV inoculation, Avr4 (in duplicate), autoactive Rx (RxD460V), and BAX were transiently expressed in the order indicated in the left image, and leaves were photographed 3 d later. The experiment was performed three times with three plants for each TRV construct, and representative pictures for the experiment are shown.
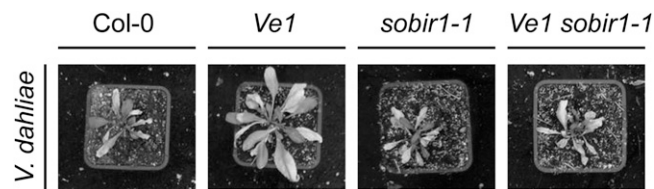
TRV constructs targeting tomato *SlSOBIR1* and *SlSOBIR1-like* individually or both genes simultaneously were generated (Fig. S2B). As a positive control, plants were inoculated with TRV: *Cf-4*, whereas TRV:*GUS*-inoculation served as a negative control. *Cf-4*–expressing tomato was inoculated with the different TRV constructs, and 3 wk later, plants were inoculated with a race 5 strain of *C. fulvum*, expressing *Avr4* and the *GUS* reporter gene. To detect fungal colonization, leaflets were GUS-stained after 2 wk. Inoculation with constructs targeting the two *SlSOBIR1* homologs either individually or simultaneously, resulted in increased fungal colonization as indicated by the much higher number of successful colonization attempts compared with the TRV:*GUS*-inoculated plants. This result shows that both tomato SOBIR1 homologs contribute to Cf-4–mediated resistance (Fig. 3). We also targeted both *SlSOBIR1* homologs in tomato expressing *Cf-2.2* and inoculated these plants with the same *C. fulvum* strain as used above, because this race 5 strain also expresses *Avr2*. Also in this case, increased fungal colonization was observed compared with the TRV:*GUS* control (Fig. S6C).

To test the role of the *SlSOBIR1* homologs in resistance to *V. dahliae*, tomato cultivar Motelle that carries the *Ve1* gene was also inoculated with TRV:*SlSOBIR1*, TRV:*SlSOBIR1-like*, and TRV:*SlSOBIR1*/*SlSOBIR1-like*. As controls, plants were inoculated with TRV:*Ve1* and TRV:*GFP*. Three weeks after TRV inoculation, plants were either inoculated with a race 1 strain of *V. dahliae* expressing Ave1 or mock-treated and subsequently monitored for development of disease symptoms (e.g., stunted growth and reduced canopy area). Targeting of the two *SlSOBIR1* homologs either individually or simultaneously, as well as *Ve1* itself, resulted in clear stunting and a strongly reduced canopy area compared with the mock-treated plants. These disease symptoms were not observed in plants inoculated with TRV:*GFP* (Fig. S6D).

SOBIR1 Is Required for Ve1-Mediated Resistance to *V. dahliae* in *Arabidopsis*. Ve1 provides resistance to *V. dahliae* when introduced in *Arabidopsis* (44). To study the requirement of *At*SOBIR1 for Ve1-mediated resistance in this plant, we tested whether Ve1 still mediates resistance to *V. dahliae* in an *Arabidopsis sobir1-1* mutant (34). Similar to the Columbia 0 (Col-0) wild-type, the *sobir1-1* mutant is susceptible to *V. dahliae* race 1, as shown by the stunted appearance and chlorosis upon fungal infection (Fig. 4). When transformed with the *Ve1* gene, the Col-0 wild-type gains resistance to *V. dahliae* race 1 (Fig. 4). Strikingly, when the *Ve1* gene was introduced into the *sobir1-1* mutant background, the

Fig. 4. Ve1 is not functional in an *Arabidopsis sobir1-1* mutant background. *Arabidopsis* ecotype Col-0 is susceptible to *V. dahliae* race 1 expressing Ave1 (Col-0). When transformed with the *Ve1* gene, expressed under control of the 35S promoter, Col-0 gains resistance to the fungus (Ve1). Similar to the Col-0 wild-type, *sobir1* mutants are susceptible (*sobir1-1*), whereas *sobir1-1* mutant plants transformed with *Ve1* remain susceptible to the fungus (*Ve1 sobir1-1*). The inoculation experiments and qRT-PCR quantifications (Fig. S7A) were performed three times, with similar results. A representative picture is shown.
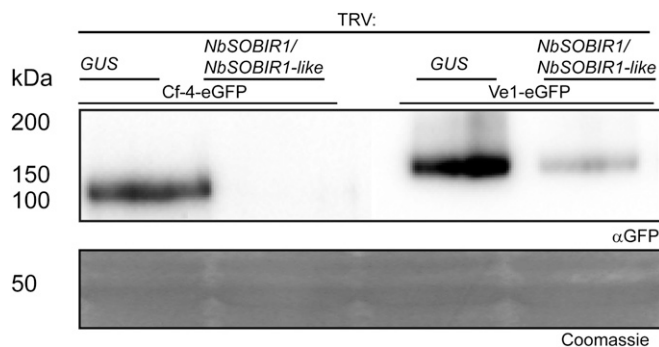
plants did not gain resistance to the pathogen, because stunting and chlorosis were still observed after inoculation with the fungus (Fig. 4). Quantitative measurement of fungal biomass confirmed these results, because only in the Col-0 wild-type plants transformed with Ve1 fungal colonization was very limited (Fig. S7A). This result indicates that in addition to its requirement in tomato, SOBIR1 is required for Ve1-mediated resistance to *V. dahliae* in *Arabidopsis*.

Targeting *SOBIR1* in *N. benthamiana* Leads to Reduced Cf-4 and Ve1 Protein Levels. To investigate whether targeting *SOBIR1* affects Cf-4 and Ve1 protein levels, we inoculated *N. benthamiana* with TRV:*NbSOBIR1*/*NbSOBIR1-like* or the control TRV:*GUS*, and after 3 wk fully expanded leaves were transiently transformed to individually express eGFP-tagged Cf-4 or Ve1. Subsequently, the steady-state levels of the RLPs were determined by their immunopurification and detection by immunoblotting. Both Cf-4 and Ve1 protein levels were strongly reduced upon targeting *SOBIR1*, compared with the TRV:*GUS*-inoculated plants, indicating that SOBIR1 is required for the accumulation of Cf-4 and Ve1, and thus stabilizes these RLPs (Fig. 5). RT-PCRs revealed that *Cf-4* and *Ve1* are normally expressed in plants inoculated with TRV:*NbSOBIR1*/*NbSOBIR1-like* and TRV:*GUS*, indicating that reduced accumulation of the Cf-4 and Ve1 proteins is not due to reduced expression levels (Fig. S7B).

Tomato SOBIR1 Homologs Interact with a Broad Range of RLPs. To test whether the tomato SOBIR1 homologs interact with additional RLPs, Cf-2.2, Cf-4E, Cf-9, and the Cf-like protein Peru2 from *S. peruvianum* were coexpressed as eGFP fusions with *Sl*SOBIR1–Myc or *Sl*SOBIR1-like–Myc in *N. benthamiana*. This experiment revealed that both SOBIR1 homologs copurify with the various Cf proteins (Fig. S8A). We expanded our study and examined whether more distantly related tomato RLPs also interact with the tomato SOBIR1 homologs. We fused *Sl*EIX2 (45), mediating perception of the ethylene-inducing xylanase from *Trichoderma viride*, and the closest tomato orthologs of *Arabidopsis* CLV2 (Solyc04g056640.1), TMM (Solyc12g042760.1), and the Suppressor of Non-expressor of pathogenesis-related genes 1-1 (Npr1-1), Constitutive 2 (SNC2; Solyc02g072250.1) (46) to eGFP and coexpressed them with the Myc-tagged SOBIR1 homologs in *N. benthamiana*. Immunopurification of the RLPs revealed that *Sl*EIX2, *Sl*CLV2, and *Sl*TMM, but not *Sl*SNC2, interact with *Sl*SOBIR1 and *Sl*SOBIR1-like (Fig. S8B).

Discussion

For signal initiation by Cf proteins, a mechanistic model was proposed based on the early model of the Clavata1 (CLV1) signaling pathway, in which the RLP CLV2 interacts with the RLK CLV1. This RLK acts as a coreceptor that allows binding of the extracellular endogenous ligand CLV3 and subsequently mediates

Fig. 3. Targeting *SOBIR1* and *SOBIR1-like* suppresses Cf-4–mediated resistance of tomato. Cf-4 tomato was inoculated with the indicated TRV constructs, and 3 wk later plants were inoculated with an Avr4-secreting, *GUS*-transgenic strain of *C. fulvum*. A non TRV-inoculated susceptible MM-Cf-0 plant was included as control. Two weeks later, leaflets were stained for GUS activity to detect *C. fulvum* colonization. For the Cf-4 tomato plants, the amount of successful colonization attempts (blue spots) vs. the total amount of leaflets analyzed for that particular experiment is indicated between parentheses. The experiment was performed three times, and representative pictures are shown.

Fig. 5. SOBIR1 is required for the accumulation of Cf-4 and Ve1 proteins. Cf-4 and Ve1, fused to eGFP, were expressed in leaves of *N. benthamiana* subjected to VIGS by inoculation with the indicated TRV constructs. Transiently expressed fusion proteins were immunopurified and subjected to SDS/PAGE, and blots were incubated with αGFP antibody for detection of the expressed proteins. The Coomassie-stained blot shows the 50-kDa Rubisco band present in the input samples to confirm equal loading. The experiment was repeated three times with similar results, and a representative picture is shown.

downstream signaling through its kinase domain (20, 47). Here, we report that the RLK SOBIR1 interacts with various RLPs of tomato, including the Cf proteins, Ve1 and *Sl*EIX2, which are all involved in immunity, as well as the tomato homologs of *Arabidopsis Sl*TMM and *Sl*CLV2, which are involved in development (Fig. 1 and Figs. S1C and S8). However, not all RLPs interact with SOBIR1, as is exemplified by *Sl*SNC2 (Fig. S8B). In addition, no interaction of SOBIR1 with any of the tested RLKs was found (Fig. 1 and Fig. S1C). We show that SOBIR1 is required for Cf-2.2–, Cf-4–, and Ve1-mediated immune responses (Figs. 2–4 and Figs. S5 and S6).

SOBIR1 was initially identified in a suppressor screen of the *Arabidopsis bak1-interacting receptor kinase 1-1* (*bir1-1*) mutant and was referred to as Suppressor Of BIR1-1, 1 (34). BIR1 encodes another RLK, which interacts with SERK3/BAK1, and the *bir1-1* mutant shows a constitutive defense phenotype, indicating that BIR1 is a negative regulator of defense responses. The *bir1-1* phenotype is suppressed by the *sobir1-1* mutation, suggesting that SOBIR1 is a positive regulator of defense signaling (34). In line with this finding, overexpression of *SOBIR1* in *Arabidopsis* leads to constitutive defense activation (34). Although no direct interaction between SOBIR1 and BIR1 was observed, it was hypothesized that BIR1 functions in a signal transduction pathway that is dependent on SOBIR1 and which promotes pathogen resistance and cell death (34). As mentioned above, a mutation in *AtSOBIR1* suppresses the *bir1-1* phenotype, whereas an additional mutation in *At Phytoalexin Deficient* 4 (*PAD4*) fully reverts the *bir1-1 sobir1-1* mutant phenotype back to that of wild-type plants. It was suggested that BIR1 regulates two parallel pathways—one involving resistance proteins that are dependent on PAD4, such as the Toll-Interleukin 1 Receptor (TIR)–NB–LRRs, and one involving another class of resistance proteins requiring SOBIR1 (34). We propose that the RLPs are members of this latter class of resistance proteins.

We also observed *in planta* interaction of SOBIR1 with RLPs involved in development. Indeed, a role of SOBIR1 in development has been described. *Arabidopsis* mutants in the gene encoding the ADP ribosylation factor GTPase-activating protein Nevershed (NEV) show impaired floral organ shedding after flowering (48). A screen for mutations in *nev* plants that restore organ shedding identified a mutation in *SOBIR1* resulting in premature floral organ shedding. Hence, the name Evershed (EVR) was coined as a synonym for this RLK, which in this case functions as an inhibitor of abscission (33). Because SOBIR1/EVR was found to localize to the plasma membrane and cytoplasmic vesicles, it was proposed that the RLK regulates the signaling and

internalization of other ligand-binding RLKs involved in floral organ shedding (33). Interestingly, when transiently expressed in *N. benthamiana*, we likewise found *Sl*SOBIR1–eGFP to localize to the plasma membrane and mobile, cytoplasmic vesicles (Fig. S4). Similar to SOBIR1, SERK3/BAK1 also plays a role both in development and defense, and this RLK was initially identified as an interactor of the RLK BRI1, which is involved in brassinosteroid (BR) perception and signaling (49, 50). SERK3/BAK1 was also identified to act as a regulator of the RLK-type PRRs FLS2 (11, 13), EFR (12), and PEP1 Receptor protein-1, an RLK involved in perceiving endogenous peptides (51). Because Cf and Ve1 interact with SOBIR1 *in planta* and require SOBIR1 for mediating HR and resistance, it is tempting to speculate that SOBIR1 is involved in signaling and possible internalization of RLP-containing immune receptor complexes, similar to the function of SERK3/BAK1 in relation to RLKs involved in defense (52).

The current paradigm for several LRR–RLK-type PRRs is their rapid heterodimerization with SERK3/BAK1 upon ligand perception (11–13). By contrast, interaction between SOBIR1 and the various RLPs studied here is ligand-independent, because we did not coexpress the corresponding ligands in most of our coimmunopurification experiments and still detected copurification of SOBIR1 with the RLPs (Fig. 1 and Figs. S1C and S8). In addition, the presence of Avr4 did not affect the interaction of Cf-4 with SOBIR1 (Fig. S3C). Through mutation of its highly conserved RD motif, we showed that a functional SOBIR1 kinase domain is required for Cf-4–dependent HR (Fig. S5C), but not for interaction with Cf-4 (Fig. S3B). Possibly, the phosphorylation status of SOBIR1 changes upon ligand perception by Cf proteins, thereby allowing additional proteins to associate with the complex. Such proteins could be the previously identified Cf interactors Cf-9–Interacting Thioredoxin (CITRX) (31), the protein kinase Avr9/Cf-9–Induced Kinase 1 (ACIK1) (29), the Soluble *N*-ethyl-maleimide-sensitive factor Adaptor protein Receptor (SNARE) protein Vesicle-Associated Protein 27 (VAP27) (30), and RLKs that reside in the active Cf-containing receptor complex. For example, recently it was shown that SERK1 is also required for Cf-4–mediated resistance of tomato. Furthermore, SERK1 and SERK3/BAK1 are both required for full Ve1-mediated resistance (26, 44). Because SOBIR1 constitutively interacts with a broad range of RLPs, either involved in defense or in development, it may be that SOBIR1 functions as a scaffold protein stabilizing receptor complexes in which RLPs take part. Alternatively, SOBIR1 could play a role as an integral part of the signaling pathway triggered by RLPs involved in different processes. In that case, downstream signaling specificity might be determined by the particular phosphorylation status of the cytoplasmic kinase domain of this regulatory RLK. For example, recent characterization of the *bak1-5* mutation in *Arabidopsis* revealed that the function of SERK3/BAK1 in MTI, the BR response and cell death control can be mechanistically uncoupled (39). The *bak1-5* mutation is in the kinase domain of SERK3/BAK1 and results in strongly impaired FLS2- and EFR-mediated immune signaling but does not affect BR signaling and the control of the cell death response (39). Such a situation might also hold for SOBIR1 in relation to signaling triggered by the different RLPs.

Together, our studies support the existence of a SOBIR1/RLP complex *in planta*, in which SOBIR1 is required for RLP-mediated immunity against two fungal pathogens that exhibit a different lifestyle. SOBIR1 appears to function as a regulatory RLK for RLP-containing immune receptor complexes in plants. Future experiments focusing on the cell biology of SOBIR1 and determination of its phosphorylation status and downstream interactors, in the presence and absence of the ligand that is perceived by the interacting RLP, should specify the precise role of SOBIR1 in RLP-containing signaling complexes.

Materials and Methods

Plant Materials and Growth. Growth conditions for *N. benthamiana*, *A. thaliana*, and *S. lycopersicum* (tomato) are described in *SI Materials and Methods*.

Primers and Vector Construction. Sequences of primers and corresponding targets can be found in Table S4. Construction of plasmids containing Cf-2.2, -4, -4E, -9, Peru2, and Ve1, C-terminally fused to either eGFP or the Myc epitope-tag, has been described (32, 35). The construction of additional vectors for *A. tumefaciens*-mediated transient transformation and VIGS is described in *SI Materials and Methods*.

Plant Transformations. Plasmid pBIN-KS-35S::Cf-4–eGFP (Sol 2701) (32) was used for transformation of tomato MM–Cf-0, which does not carry a functional *Cf-4* gene. Transformations and plant selections were performed as described in *SI Materials and Methods*.

Protein Immunopurification and Identification. Immunopurifications were essentially performed following the protocol described with minor modifications (32). Immunopurifications from stable transgenic tomato expressing Cf-4–eGFP were performed as described in *SI Materials and Methods*.

VIGS and Disease Assays. VIGS experiments in *N. benthamiana*, tobacco, and tomato were performed as described (32, 35). *C. fulvum* disease assays were performed as described (32), and *V. dahliae* disease assays were performed as described in *SI Materials and Methods*.

Further experimental details can be found in *SI Materials and Methods*.

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