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The Bacterial Effector AvrPto Targets the Regulatory Coreceptor SOBIR1 and Suppresses Defense Signaling Mediated by the Receptor-Like Protein Cf-4

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Receptor-like proteins (RLPs) and receptor-like kinases (RLKs) are cell-surface receptors that are essential for detecting invading pathogens and subsequent activation of plant defense responses. RLPs lack a cytoplasmic kinase domain to trigger downstream signaling leading to host resistance. The RLK SOBIR1 constitutively interacts with the tomato RLP Cf-4, thereby providing Cf-4 with a kinase domain. SOBIR1 is required for Cf-4–mediated resistance to strains of the fungal tomato pathogen *Cladosporium fulvum* that secrete the effector Avr4. Upon perception of this effector by the Cf-4/SOBIR1 complex, the central regulatory RLK SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3a (SERK3a) is recruited to the complex and defense signaling is triggered. SOBIR1 is also required for RLP-mediated resistance to bacterial, fungal ,and oomycete pathogens, and we hypothesized that SOBIR1 is targeted by effectors of such pathogens to suppress host defense responses. In this study, we show that *Pseudomonas syringae* pv. *tomato* DC3000 effector AvrPto interacts with *Arabidopsis* SOBIR1 and its orthologs of tomato and *Nicotiana benthamiana*, independent of SOBIR1 kinase activity. Interestingly, AvrPto suppresses *Arabidopsis* SOBIR1-induced cell death in *N. benthamiana*. Furthermore, AvrPto compromises Avr4-triggered cell death in *Cf-4*-transgenic *N. benthamiana*, without affecting Cf-4/SOBIR1/SERK3a complex formation. Our study shows that the RLP coreceptor SOBIR1 is targeted by a bacterial effector, which results in compromised defense responses.

The innate immune system of plants against invading pathogens consists of two layers, which are termed microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI) (Couto and Zipfel 2016; Janeway 1989; Jones and Dangl 2006; Macho and Zipfel 2015). MAMPs are conserved structural components of pathogens, whereas effectors are typically in planta–induced proteins of microbial pathogens. Effectors suppress plant defense responses, thereby causing effector-triggered susceptibility (ETS) (Dodds and Rathjen 2010; Feng and Zhou 2012; Jones and Dangl 2006). Essentially, there are two subcellular locations of pathogen perception, the apoplast and the cytoplasm (Cui et al. 2009; Dodds and Rathjen 2010; Zipfel 2014). MAMPs and secreted effectors that end up in the apoplast are generally recognized by plasma membrane–associated pattern recognition receptors (PRRs) (Dodds and Rathjen 2010; Zipfel 2014). Effectors that are translocated from fungal or oomycete haustoria, which are specialized feeding structures that are formed in host cells, or are injected into the cell by the type three secretion system (T3SS) of bacteria are perceived by cytoplasmic immune receptors (Dodds and Rathjen 2010). These cytoplasmic receptors mostly carry a nucleotide binding site and leucine-rich repeats (NB-LRRs) (Dodds and Rathjen 2010).

PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Couto and Zipfel 2016; Zipfel 2014). Compared with RLKs, RLPs lack a kinase domain to trigger downstream signaling. Recently it was observed that RLPs constitutively interact with the RLK SUPPRESSOR OF BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (SOBIR1) and require SOBIR1 for their function (Gust and Felix 2014; Liebrand et al. 2013, 2014). *Arabidopsis thaliana* SOBIR1 was originally identified as a suppressor of the *bir1-1* phenotype, partially rescuing *bir1* knockout plants that mount a constitutive defense response (Gao et al. 2009). AtSOBIR1 was found to function as a positive regulator of cell death, as overexpression of *AtSOBIR1* triggered enhanced basal defense and reduced colonization by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Gao et al. 2009). Recently, the RLK SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3)/BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1), further referred to as SERK3, has been reported as another suppressor of *bir1-1* (Liu et al. 2016). Both SOBIR1 and SERK3 are required for *bir1-1*–triggered autoimmunity, and interaction was found between SOBIR1 and SERK3 in *BIR1*-silenced *Nicotiana benthamiana* (Liu et al. 2016).

The tomato (*Solanum lycopersicum*) RLP Cf-4 confers resistance to the pathogenic biotrophic fungus *Cladosporium fulvum*, by recognition of the secreted effector Avr4. It was

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recently shown that SERK3 is also required for Cf-4 signaling and is recruited to the Cf-4/SOBIR1 complex upon recognition of Avr4 (Postma et al. 2016). Increasing evidence on signaling by RLP/SOBIR1/SERK3-containing complexes indicates that SOBIR1 constitutively forms a complex with RLPs, whereas SERK3 is specifically recruited to the RLP/SOBIR1 bipartite RLK upon ligand recognition by the RLP (Albert et al. 2015; Zhang et al. 2013a). SOBIR1 and SERK3 are also required for tomato Ve1-, I-, and *Brassica napus* LepR3-mediated resistance to *Verticillium dahliae* expressing Ave1 (Liebrand et al. 2013), *Fusarium oxysporum* f. sp. *lycopersici* expressing Avr1 (Catanzariti et al. 2017), and *Leptosphaeria maculans* expressing AvrLm1 (Ma and Borhan 2015), respectively, although for these particular RLPs the association of SERK3 with the RLP/SOBIR1 complex remains to be shown. SOBIR1 is also required for the functionality of various additional RLPs playing a role in immunity (Hegenauer et al. 2016; Jehle et al. 2013; Zhang et al. 2014), though the requirement of SERK3 for the functionality of these RLPs remains currently unknown. Furthermore, SOBIR1 is involved in resistance to the oomycete pathogen *Phytophthora parasitica* (Peng et al. 2015) and the fungus *Magnaporthe oryzae* (Takahashi et al. 2016).

The *Arabidopsis* RLK FLAGELLIN-SENSING 2 (FLS2) confers resistance to *P. syringae* pv. *tomato* DC3000 (Couto and Zipfel 2016). Flg22, a 22–amino acid peptide derived from a conserved domain of bacterial flagellin, is perceived by FLS2 (Gómez-Gómez and Boller 2000) together with SERK3 (Chinchilla et al. 2007; Sun et al. 2013). Recognition of flg22 triggers a rapid defense response, including calcium spiking (Boudsocq et al. 2010), reactive oxygen species synthesis (Kadota et al. 2015; Ma 2014) and MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) activation (Asai et al. 2002). The receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE1 (BIK1) constitutively interacts with FLS2 and SERK3, and association with FLS2 or SERK3 is not affected in single *serk3*- or *fls2*-knockout mutants, respectively (Lu et al. 2010). However, flg22-induced BIK1 phosphorylation is both FLS2- and SERK3-dependent, as BIK1 phosphorylation does not take place in *fls2* or *serk3* mutants (Lu et al. 2010). BIK1 is a substrate of SERK3, which, in turn, phosphorylates SERK3 and FLS2 (Lu et al. 2010). After flg22 perception, BIK1 is released from FLS2 and triggers early defense responses (Ma 2014; Zhang et al. 2010).

Pathogenic bacteria inject their effectors into the host cells via the T3SS to suppress MTI by directly interfering with PRR function or with downstream signaling pathways (Bi and Zhou 2017; Couto and Zipfel 2016; Lee et al. 2013; Tang et al. 2017). For example, the effector AvrPto, also referred to as AvrPto1, is injected into host cells by *P. syringae* pv. *tomato* DC3000 and localizes to the plasma membrane (Shan et al. 2000). AvrPto targets FLS2 and suppresses the phosphorylation of its kinase domain (Xiang et al. 2008). AvrPto suppresses the flg22-activated MTI (He et al. 2006), although it is not clear if SERK3 is also targeted by AvrPto (Shan et al. 2008; Xiang et al. 2008, 2011). Eventually, also BIK1 phosphorylation is suppressed, and the MAPK signaling pathway is inhibited (He et al. 2006; Lu et al. 2010; Xiang et al. 2011).

The resistance gene *Pto* (for resistance against *P. syringae* pv. *tomato* race 0) encodes a protein kinase with serine-threonine specificity (Loh and Martin 1995), which competes with FLS2 for AvrPto to trigger ETI (Xiang et al. 2008). In tomato, Pto confers resistance to *P. syringae* pv. *tomato* DC3000 carrying AvrPto (Martin et al. 1993) in a *Pseudomonas* resistance and fenthion (Prf)–dependent manner. Transient overexpression of *AvrPto* in *Pto/Prf*-transgenic *N. benthamiana* causes a hypersensitive response (HR) (Balmuth and Rathjen 2007; Salmeron et al. 1994; Scofield et al. 1996). However, AvrPto does not affect association between Pto and Prf (Mucyn et al. 2006).

SOBIR1 is generally required for RLP-mediated resistance to bacterial, fungal, and oomycete pathogens (Albert et al. 2015; Catanzariti et al. 2017; Hegenauer et al. 2016; Jehle et al. 2013; Liebrand et al. 2013; Ma and Borhan 2015; Peng et al. 2015; Takahashi et al. 2016; Zhang et al. 2013a, 2014). Therefore, we hypothesized that this regulatory coreceptor is targeted by cytoplasmic effectors of such pathogens in order to suppress host defense responses. Here, we show that tomato SOBIR1, its homolog SlSOBIR1-like, and its orthologs from *Arabidopsis* and *N. benthamiana* interact with AvrPto, independent of SOBIR1 kinase activity. In addition, AvrPto suppresses AtSOBIR1-induced constitutive immunity in *N. benthamiana*. Furthermore, AvrPto was found to suppress the Cf-4/Avr4-triggered HR, without affecting Cf-4/SlSOBIR1/SlSERK3a complex formation.

RESULTS

AvrPto interacts with SOBIR1 in planta.

To investigate whether AvrPto and SOBIR1 interact, a binary construct containing C-terminally hemagglutinin (HA) epitope–tagged AvrPto was generated. AvrPto-HA was coinfiltrated with C-terminally tagged SlSOBIR1-eGFP (enhanced green fluorescent protein) (Liebrand et al. 2013) in *N. benthamiana* by *Agrobacterium*-mediated transient expression (agroinfiltration). AvrPto-HA was also coinfiltrated with C-terminally tagged SlSERK3a-eGFP as a positive control, because AtSERK3 was earlier reported to be targeted by AvrPto (Shan et al. 2008). Coimmunoprecipitation (Co-IP) experiments reveal that AvrPto-HA copurifies with SlSOBIR1-GFP upon pull-down of the latter using GFP beads (Fig. 1A). Interaction between SlSERK3a and AvrPto was also observed (Fig. 1A). In a reciprocal experiment in which SlSOBIR1, SlSERK3a, and β-glucuronidase (GUS) were fused to HA and coinfiltrated with AvrPto-eGFP, a pull-down of SlSOBIR1 and SlSERK3a using HA beads also resulted in copurification of AvrPto-eGFP (Fig. 1B).

In tomato, the SlSOBIR1 homolog SlSOBIR1-like shares a high similarity in amino acid sequence with SlSOBIR1, and, in *N. benthamiana*, two clear SOBIR1 orthologs are present (Liebrand et al. 2013). In addition, the function of SOBIR1 appears to be highly conserved, as both AtSOBIR1 and SlSOBIR1 interact with Cf-4 (Liebrand et al. 2013), and AtSOBIR1 can complement the loss of Avr4-triggered cell death in *NbSOBIR1*(*-like)*-silenced *Cf-4*-transgenic *N. benthamiana* plants (Bi et al. 2016; Liebrand et al. 2013). To study if the SOBIR1 homolog and its orthologs are also targeted by AvrPto, C-terminally eGFP-tagged SlSOBIR1-like and NbSOBIR1 were generated and, together with SlSOBIR1-eGFP and AtSOBIR1-eGFP (Bi et al. 2016; Liebrand et al. 2013), were coinfiltrated with AvrPto-HA in *N. benthamiana*. Co-IP experiments reveal that, in all cases, AvrPto copurifies with the SOBIR1 variants (Fig. 2).

Kinase activity of SOBIR1 is not required for its interaction with AvrPto.

SOBIR1 is a so-called "RD" kinase and by its constitutive interaction with RLPs, bipartite RLKs are formed. In this bipartite RLK, SOBIR1 is thought to provide the interacting RLP with a kinase domain to initiate defense signaling upon ligand recognition by the RLP (Gust and Felix 2014; Liebrand et al. 2014). It has been reported that AvrPto targets Pto and FLS2, while the interaction is dependent on kinase activity of these two targets (Xiang et al. 2008; Xing et al. 2007). To determine if kinase activity of SOBIR1 is required for its interaction with AvrPto, eGFP-tagged wild type and kinase-dead SOBIR1 variants (Liebrand et al. 2013) (mutated in the catalytic aspartate [D] of the kinase domain) of *Arabidopsis* (AtSOBIR1^{D489N}) and tomato SOBIR1 (SlSOBIR1^{D473N}) were coinfiltrated with AvrPto-HA. Co-IP experiments reveal that AvrPto copurifies with all SOBIR1 variants upon their purification using GFP beads, indicating that kinase activity of SOBIR1 is not required for its interaction with AvrPto (Fig. 3).

Overexpression of *AtSOBIR1* induces constitutive immunity, which requires a functional kinase domain.

SOBIR1 is a positive regulator of immunity (Gao et al. 2009), and we anticipated that the protein would constitutively induce an immune response, visualized as cell death, when it accumulates at relatively high levels. To investigate this, eGFP-tagged AtSOBIR1, SlSOBIR1, SlSOBIR1-like, and NbSOBIR1 were transiently overexpressed in leaves of *N. tabacum* by agroinfiltration. Interestingly, only overexpression of *AtSOBIR1* induced cell death, which was visible within 2 to 3 days after agroinfiltration (Fig. 4A). The other SOBIR1 variants that were tested did not exhibit this constitutive immunity symptom. Although protein accumulation was not tested in *N. tabacum*, the same constructs were expressed in *N. benthamiana* and resulted in clear protein accumulation for all constructs (discussed below) (Supplementary Fig. S1A). To determine if kinase activity of AtSOBIR1 is specifically required to induce constitutive immunity, eGFP-tagged $AtSOBIR1^{D489N}$ was transiently overexpressed in leaves of *N. tabacum* (Fig. 4B). This kinase-dead SOBIR1 mutant did not induce cell death upon its transient overexpression, indicating that a functional kinase domain is required for AtSOBIR1 to induce constitutive immunity in *N. tabacum*. This suggests that the constitutive immune response is triggered as a result of perturbation of the immune system of the plant, through constitutive activation of downstream immune signaling components.

The same constructs were tested for constitutive immune activation in *N. benthamiana*. No cell death was observed for any of the *SOBIR1* variants when expressed alone (data not shown), although all proteins do accumulate. However, when coexpressed with the silencing suppressor *P19* (Voinnet et al. 2015), cell death could be observed for AtSOBIR1 at 2 to 3 days after infiltration and, again, not for any of the other tested SOBIR1 variants (Fig. 4A). *N. tabacum* is generally more sensitive to overexpression of immunity-related proteins and, probably, therefore does not require coexpression of a silencing suppressor to provoke AtSOBIR1 constitutive immunity (Van der Hoorn et al. 2000; Zhang et al. 2013b).

To obtain additional support that this cell-death phenotype indeed reflects an immune response, we analyzed MAPK activation, which is a key downstream step in defense activation (Stulemeijer et al. 2007; Xu et al. 2014). Upon Avr4 recognition by Cf-4 in stable transgenic *N. benthamiana* expressing tomato *Cf-4*, immune blots showed a typical MAPK activation pattern

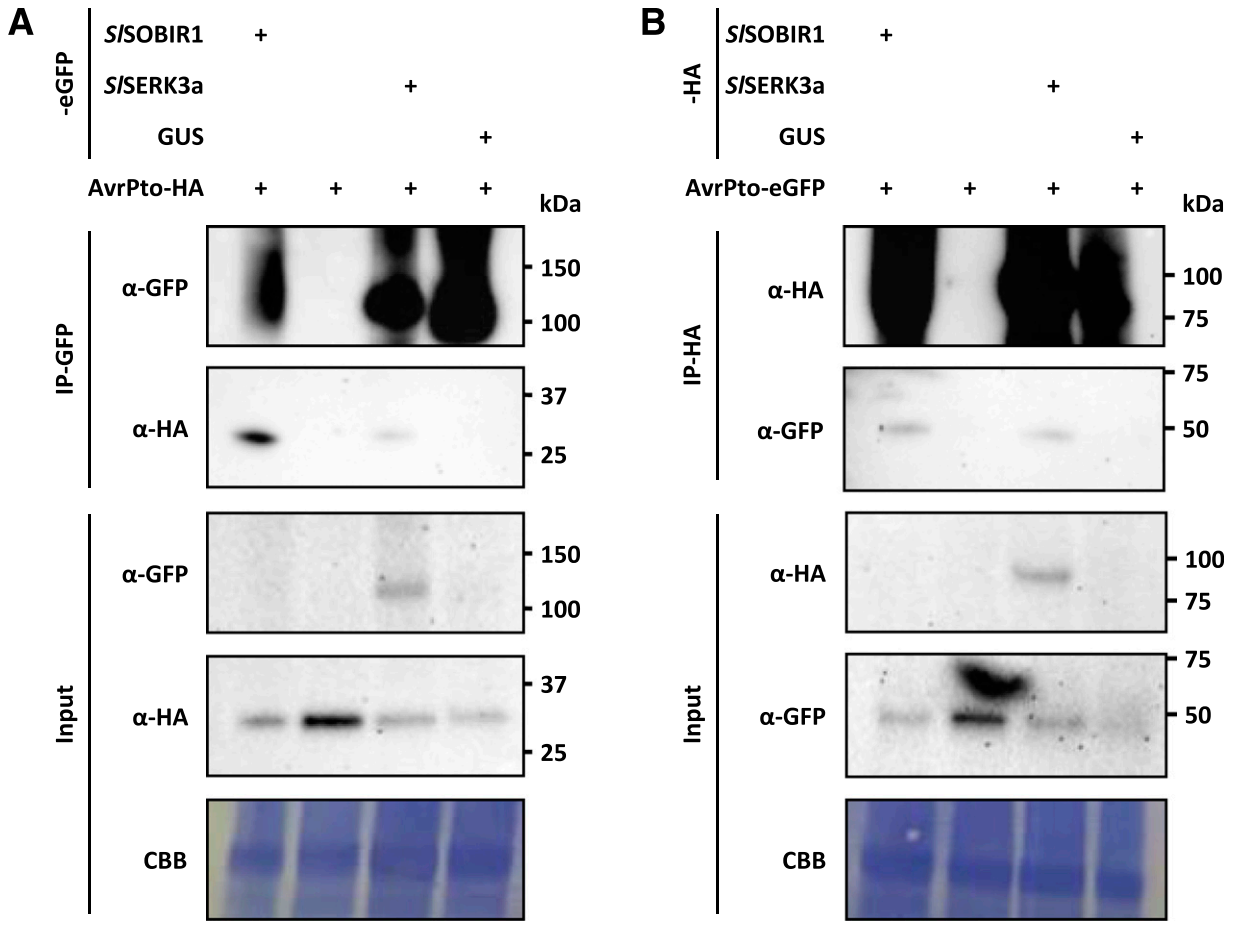

Fig. 1. AvrPto interacts with SlSOBIR1 in planta. **A,** AvrPto-hemagglutinin (HA) interacts with SlSOBIR1-eGFP (enhanced green fluorescent protein). C-terminally eGFP-tagged *SlSOBIR1*, *SlSERK3a*, and *GUS* (β-glucuronidase) were coexpressed with *AvrPto-HA* by agroinfiltration in *Nicotiana benthamiana*. Two days later, total proteins were extracted and were subjected to immunoprecipitation using GFP_TrapA affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were blotted to polyvinylidene diflouride (PVDF) membrane. Blots were probed with α-GFP antibodies, to detect the immunoprecipitated eGFP-tagged proteins, and with α-HA antibodies, to detect coprecipitated AvrPto-HA. CBB = Coomassie brilliant blue. Experiments were performed at least three times and representative images are shown. **B,** AvrPto-eGFP interacts with SlSOBIR1-HA and SlSERK3a-HA. C-terminally HA-tagged *SlSOBIR1*, *SlSERK3a*, and *GUS* were coexpressed with *AvrPto-eGFP* in *N. benthamiana*. Two days later, total proteins were extracted and subjected to immunoprecipitation using HA magnetic beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to SDS-PAGE and were blotted to PVDF membrane. Blots were probed with α-HA antibodies to detect the immunoprecipitated HA-tagged proteins and with α-GFP antibodies to detect coprecipitated AvrPto-eGFP. Experiments were performed at least three times and representative images are shown.

(Fig. 4C). Notably, induction of cell death upon *AtSOBIR1* overexpression in *N. benthamiana* also coincided with MAPK activation (Fig. 4C and D). In conclusion, these data show that the cell death caused by AtSOBIR1 is dependent on its kinase activity and represents a constitutive immune response.

AvrPto suppresses AtSOBIR1-induced constitutive immunity.

To determine whether AvrPto can suppress the cell death response induced by overexpression of *AtSOBIR1*, AvrPto-eGFP and GUS-eGFP were coinfiltrated with AtSOBIR1-eGFP and P19 in *N. benthamiana*. Coinfiltration of GUS-eGFP does not affect AtSOBIR1-induced cell death, whereas the cell-death response is strongly suppressed by AvrPto-eGFP, indicating that AtSOBIR1-induced constitutive immunity is suppressed by AvrPto (Fig. 5).

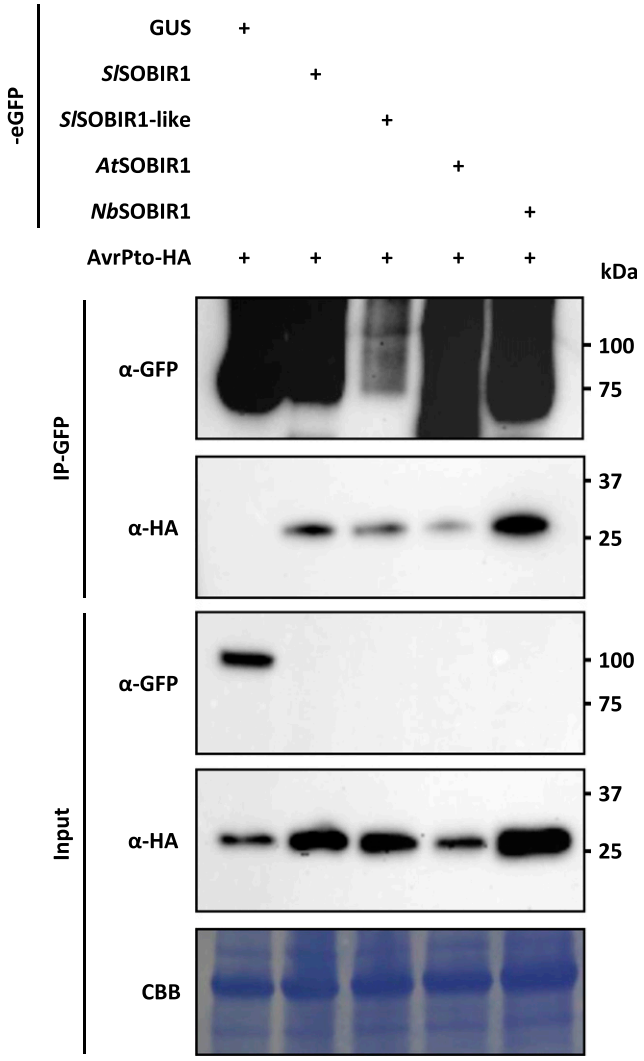

Fig. 2. AvrPto interacts with SlSOBIR1, SlSOBIR1-like, AtSOBIR1, and NbSOBIR1. C-terminally enhanced green fluorescent protein (eGFP)-tagged *GUS* (β-glucuronidase), *SlSOBIR1*, *SlSOBIR1-like*, *AtSOBIR1*, and *NbSOBIR1* were coexpressed with *AvrPto-HA* (hemagglutinin) by agroinfiltration in *Nicotiana benthamiana*. Two days later, total proteins were extracted and subjected to immunoprecipitation using GFP_TrapA affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted to polyvinylidene diflouride membrane. Blots were probed with α-GFP antibodies to detect the immunoprecipitated eGFP-tagged proteins and with α-HA antibodies to detect coprecipitated AvrPto-HA. CBB = Coomassie brilliant blue. Experiments were performed at least three times and representative images are shown.

AvrPto suppresses the Avr4-triggered cell death in *Cf-4*-transgenic *N. benthamiana* plants.

SOBIR1 is required for the Cf-4–mediated cell death upon recognition of Avr4 and for resistance to *C. fulvum* (Liebrand et al. 2013). Furthermore, kinase activity of SOBIR1 is necessary for Cf-4 function, as a kinase-inactive variant of AtSOBIR1 fails to complement the loss of Avr4-triggered cell death in *NbSOBIR1 (-like)*-silenced *Cf-4*-transgenic *N. benthamiana* plants (Bi et al. 2016; Liebrand et al. 2013).

To study if AvrPto is able to suppress the Avr4-triggered cell death through suppression of SOBIR1-mediated defense signaling, *AvrPto-eGFP* and *GUS-eGFP* were transiently overexpressed in *Cf-4*-transgenic *N. benthamiana* plants, 2 days before agroinfiltration of Avr4. Figure 6 shows that cell death triggered by Avr4 is strongly suppressed upon infiltration of AvrPto-eGFP when compared with infiltration of GUS-eGFP. Together with the observation that AvrPto suppresses AtSOBIR1-induced constitutive immunity (Fig. 5), the suppression of Avr4-triggered cell death by AvrPto indicates that AvrPto compromises Avr4-triggered

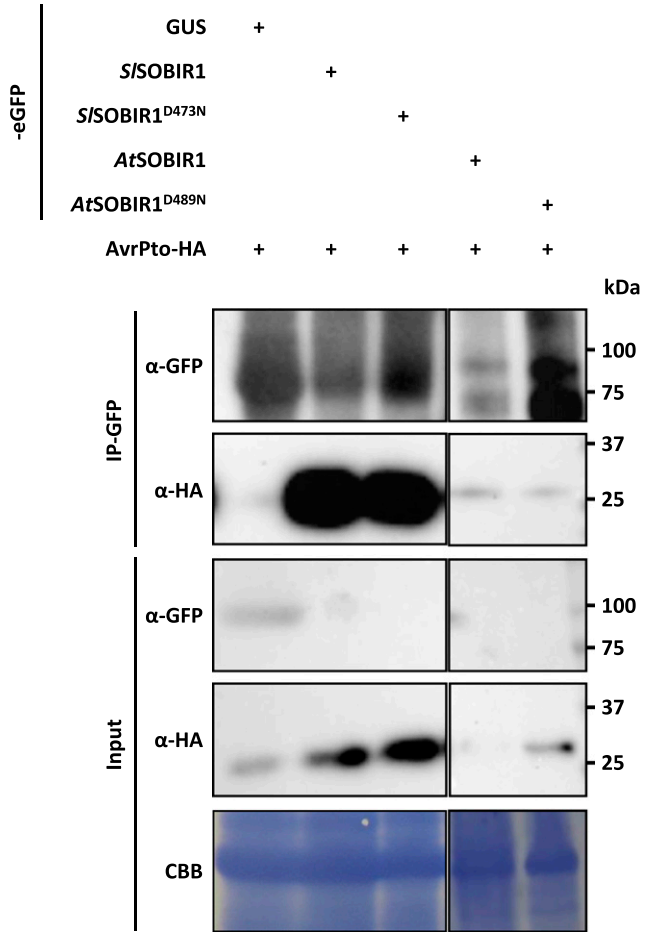

Fig. 3. Kinase activity of SlSOBIR1 and AtSOBIR1 is not required for their interaction with AvrPto. C-terminally enhanced green fluorescent protein (eGFP)-tagged *GUS* (β-glucuronidase), *SlSOBIR1*, kinase-dead *SlSOBIR1*^{D473N}, *AtSOBIR1*, and kinase-dead *AtSOBIR1*^{D489N} were coexpressed with *AvrPto-HA* (hemagglutinin) by agroinfiltration in *Nicotiana benthamiana*. Two days later, total proteins were extracted and subjected to immunoprecipitation, using GFP_TrapA affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted to polyvinylidene diflouride membrane. Blots were probed with α-GFP antibodies to detect the immunoprecipitated eGFP-tagged proteins and with α-HA antibodies to detect coprecipitated AvrPto-HA. CBB = Coomassie brilliant blue. Experiments were performed at least three times and representative images are shown.

cell death through suppression of SOBIR1-mediated defense signaling.

AvrPto does not affect Cf-4/SlSOBIR1/SlSERK3a complex formation.

Recently it was shown that SlSERK3a is involved in the Cf-4–triggered signaling pathway (Postma et al. 2016). SOBIR1 constitutively interacts with Cf-4 (Liebrand et al. 2013), whereas SlSERK3a interacts with Cf-4 in an Avr4-dependent manner, as SlSERK3a is specifically recruited to the Cf-4/SlSOBIR1 complex in the presence of Avr4 (Postma et al. 2016). Silencing of either *NbSOBIR1(-like)* or *NbSERK3a/b* compromises the Avr4-triggered cell death (Liebrand et al. 2013; Postma et al. 2016).

To study the effect of AvrPto on Cf-4/SlSOBIR1/SlSERK3a complex formation, AvrPto-HA was coinfiltrated with Cf-4-eGFP, SlSOBIR1-HA, and SlSERK3a-Myc, followed by infiltration of Avr4 protein. Cf-4-eGFP and SlSOBIR1-HA are invisible in the input but are readily detectable in the immunoprecipitate (Fig. 7), which is consistent with our earlier published studies (Liebrand et al. 2013; Postma et al. 2016). Co-IP experiments reveal that AvrPto does not affect the interaction between Cf-4 and SlSOBIR1. Moreover, the recruitment of SlSERK3a to the Cf-4/SlSOBIR1 complex upon infiltration of Avr4 protein is not affected (Fig. 7). These results indicate that AvrPto does not suppress the Avr4-triggered cell death by hampering Cf-4/SlSOBIR1/SlSERK3a complex formation upon Avr4 recognition by Cf-4.

DISCUSSION

In this study, we provide evidence that AvrPto interacts with AtSOBIR1 and its orthologs of tomato and *N. benthamiana*, independent of the kinase activity of SOBIR1. Overexpression of *AtSOBIR1* induces constitutive immunity, which is suppressed by AvrPto. In addition, AvrPto also suppresses Cf-4/Avr4-triggered cell death without affecting Cf-4/SlSOBIR1/SlSERK3a complex formation.

AtSOBIR1 induces a constitutive immune response.

AtSOBIR1 is a functional ortholog of NbSOBIR1 (Liebrand et al. 2013) and, in contrast to NbSOBIR1 or SlSOBIR1,

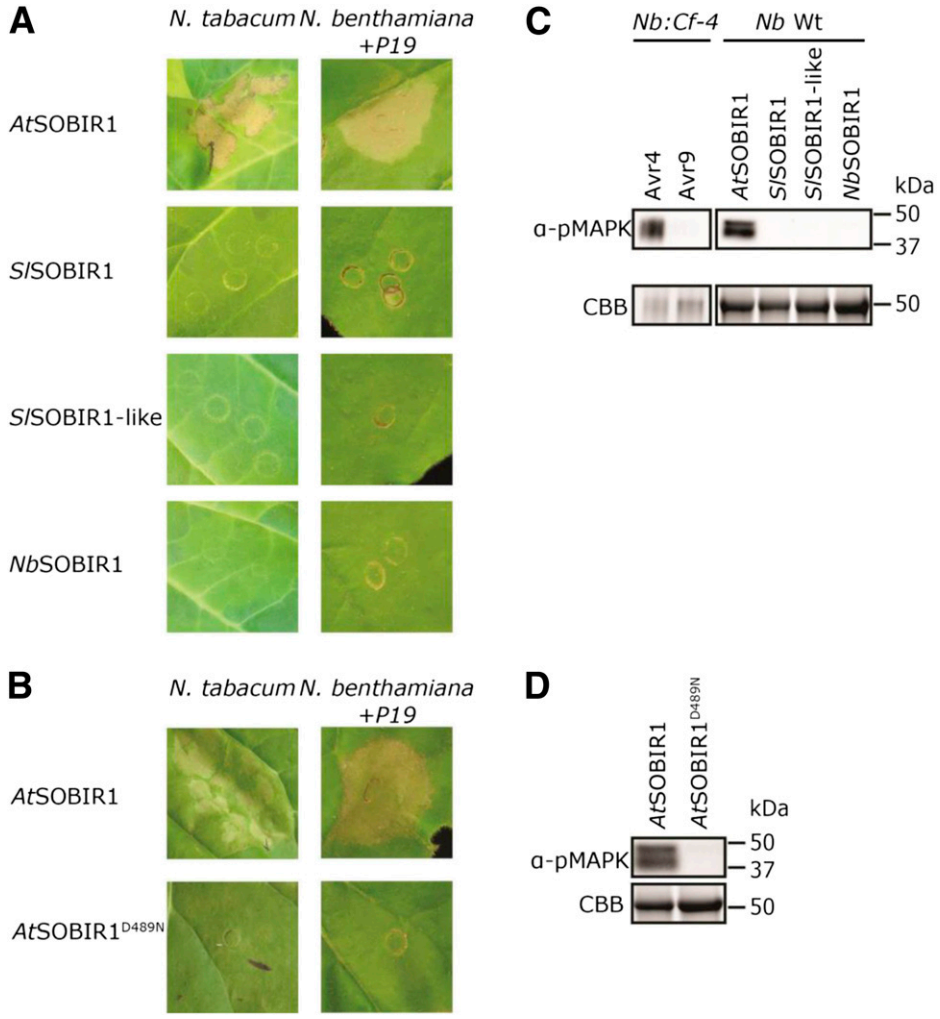

Fig. 4. Constitutive immunity induced by overexpression of AtSOBIR1 is dependent on a functional kinase domain. **A,** Transient expression of *AtSOBIR1* induces cell death in *Nicotiana tabacum* and *N. benthamiana.* C-terminally enhanced green fluorescent protein (eGFP)-tagged *AtSOBIR1*, *SlSOBIR1*, *SlSOBIR1-like*, and *NbSOBIR1* were transiently expressed by agroinfiltration at an optical density (OD) of 1; in *N. benthamiana*, all constructs were coexpressed with the silencing suppressor *P19* at an OD of 1. Pictures were taken at 2 days postinfiltration (dpi) for *N. tabacum* and at 3 dpi for *N. benthamiana.* **B,** Transient expression of C-terminally eGFP-tagged kinase-dead $AtSOBIR1^{D489N}$ does not induce cell death in *N. tabacum* or *N. benthamiana.* The experiment was performed as described in A. **C,** Mitogen-activated protein kinases (MAPKs) are activated upon Avr4 recognition by Cf-4 and upon overexpression of *AtSOBIR1*. Left, 2 μM of Avr4 or Avr9 protein was infiltrated in *Cf-4*-transgenic *N. benthamiana* plants, and after 15 min, total protein was extracted and analyzed for MAPK activation, using α-p42/p44-erk antibody. Right, C-terminally eGFP-tagged *AtSOBIR1*, *SlSOBIR1, SlSOBIR1-like*, and *NbSOBIR1* were transiently coexpressed with *P19* in *N. benthamiana*. At 2 dpi, total protein was extracted and analyzed for MAPK activation, using α-p42/p44-ERK antibody. **D,** MAPKs are not activated upon overexpression of $AtSOBIR1^{D489N}$. The experiment was performed as described in C. Experiments were performed at least three times and representative images are shown.

overexpression of *AtSOBIR1* in *N. tabacum* or *N. benthamiana* induces constitutive immunity, which is observed as MAPK activation and cell death (Fig. 4). This observation is in agreement with the cell death and defense gene activation observed upon overexpression of *AtSOBIR1* in *Arabidopsis* (Gao et al. 2009). The lack of MAPK activation and cell death upon transient expression of NbSOBIR1 and SlSOBIR1 could be an effect of proper SOBIR1 signaling regulation in *N. tabacum* and *N. benthamiana*. The signaling capacity of immune receptors is tightly regulated, as immune homeostasis must be maintained and its deregulation may be lethal (Couto et al. 2016).

A way of regulating immune signaling activity is at the level of phosphorylation of the kinase domain of the signaling components. For instance, the *Arabidopsis* phosphatases PP2A and PP2C38, associating with SERK3 and BIK1, respectively, were found to negatively regulate the activity of their target kinases (Couto et al. 2016; Segonzac et al. 2014). Potential intrinsic differences between phosphatases, from *Arabidopsis* and solanaceous plants, involved in negatively regulating SOBIR1 signaling could explain the lack of MAPK activation and absence of cell death upon expression of SlSOBIR1 or NbSOBIR1 in contrast to AtSOBIR1 in solanaceous plants. It is likely that solanaceous plants can fine-tune the activity of endogenous versions of SOBIR1 in a more controlled manner than the activity of heterologously expressed AtSOBIR1. Thus, it could be that endogenous phosphatases of *N. tabacum* and *N. benthamiana* can properly negatively regulate solanaceous SOBIR1 immune signaling, whereas these phosphatases might, for example, have lower affinity for the more distantly related ortholog AtSOBIR1. This could result in a higher basal level of phosphorylation of the kinase domain of AtSOBIR1. This higher level of phosphorylation might perturb the immune system of the plant and trigger a constitutive immune response in *N. tabacum* and *N. benthamiana*, which is also reflected by constitutive activation of MAPK signaling by AtSOBIR1 (Fig. 4C).

Immunoblot analysis demonstrated that the accumulation levels of the AtSOBIR1 and AtSOBIR1^{D489N} proteins inversely correlates with their capacity to activate constitutive immunity. Kinase-dead AtSOBIR1^{D489N} accumulates to relatively high levels when compared with wild-type AtSOBIR1 (Fig. 3). Transient expression of wild-type AtSOBIR1 causes constitutive MAPK activation and cell death (Fig. 4), eventually resulting in impaired protein production. AtSOBIR1^{D489N} lacks constitutive immune activity (Fig. 4) and does not affect the vitality of the cells when transiently expressed, allowing this kinase-dead mutant to accumulate to higher levels than AtSOBIR1 wild type. Although transient expression of SlSOBIR1 does not cause MAPK activation and cell death (Fig. 4), its lower accumulation as compared with kinase-dead SlSOBIR1^{D473N} (Fig. 3) suggests that SlSOBIR1 also has some residual constitutive immunity activity. This residual activity affects the vitality of the expressing cells and hampers SlSOBIR1 accumulation, albeit not as strong as for AtSOBIR1.

AvrPto suppresses Cf-4/SlSOBIR1/SlSERK3a-triggered cell death without affecting complex formation.

AvrPto has been reported to suppress cell death triggered by *P. syringae* pv. *tomato* T1 in *N. benthamiana* (Kang et al. 2004). In addition, AvrPto has been shown to suppress the flg22/elf26-triggered immune response, the latter which is a fragment of the bacterial MAMP ELONGATION FACTOR-TEMPERATURE UNSTABLE (EF-Tu) that is perceived by the RLK EF-Tu RECEPTOR (EFR) (He et al. 2006; Xiang et al. 2008; Zipfel et al. 2006). Here, we show that AvrPto is able to suppress AtSOBIR1-induced constitutive immunity (Fig. 5) as well as the Cf-4–mediated cell-death response triggered by Avr4

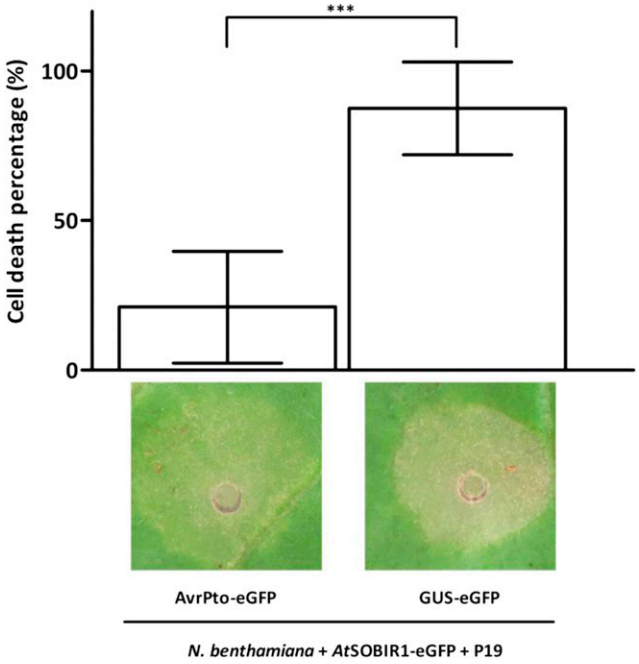

Fig. 5. Constitutive immunity induced by overexpression of *AtSOBIR1* is suppressed by AvrPto. *AtSOBIR1-eGFP* (enhanced green fluorescent protein) and *P19* were coexpressed with β-glucuronidase (*GUS*)-*eGFP* or *AvrPto-eGFP* by agroinfiltration in *Nicotiana benthamiana* at an optical density of 1. Pictures were taken at 4 days postinfiltration (dpi). Cell death at 4 dpi was scored by its intensity. The means of the cell death percentages are shown. Asterisks indicate a statistically significant difference according to a Student's *t* test (*** $P < 0.0001$), with $n = 20$. Experiments were performed at least three times and representative images are shown.

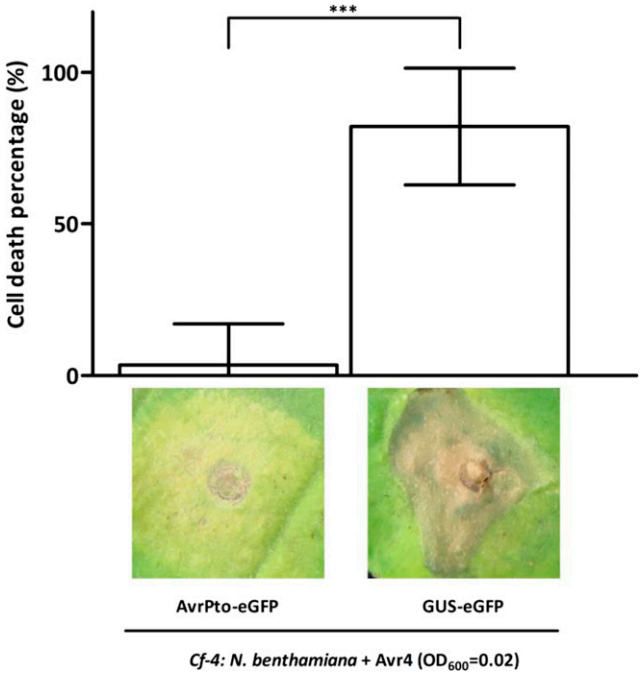

Fig. 6. The Avr4-triggered cell death in *Cf-4*-transgenic *Nicotiana benthamiana* plants is suppressed by AvrPto. *AvrPto-eGFP* (enhanced green fluorescent protein) and β-glucuronidase (*GUS*)-*eGFP* were expressed at an optical density (OD) of 1 in *Cf-4*-transgenic *N. benthamiana* plants at 2 days before agroinfiltration of Avr4 at an OD of 0.02. Pictures were taken at 5 days postinfiltration (dpi) of Avr4. Cell death at 5 dpi was scored by the intensity. The means of the cell death percentages are shown. Asterisks indicate a statistically significant difference according to a Student's *t* test (*** $P < 0.0001$), with $n = 15$. Experiments were performed at least three times and representative images are shown.

(Fig. 6). Previously, it was reported that AvrPto fails to suppress the cell death triggered by Cf-9/Avr9 (Kang et al. 2004). Cf-9 also interacts with SOBIR1 (Liebrand et al. 2013), and we anticipate that Cf-9 requires SOBIR1 for its function. The absence of AvrPto-mediated suppression of Cf-9/Avr9-induced cell death might be caused by using a lower amount of *Agrobacterium* expressing AvrPto (optical density at 600 nm [OD_{600}] = 0.4, as compared with OD = 1.0 in our studies). Furthermore, Kang et al. (2004) coinfiltrated AvrPto with Cf-9/Avr9, whereas, in our study, AvrPto was infiltrated 2 days before infiltration of Avr4 in *Cf-4*-transgenic *N. benthamiana*.

It has been shown that AvrPtoB, which is another T3SS effector of *P. syringae* pv. *tomato* DC3000 (Pedley and Martin 2003) and AvrPto both interact with FLS2 and SERK3, whereas only the association of AvrPtoB and FLS2 gets stronger upon flg22 treatment (Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008). In addition, the flg22-induced FLS2-SERK3 interaction is suppressed by both AvrPtoB and AvrPto (Shan et al. 2008). Furthermore, the dissociation of BIK1 from FLS2 and SERK3 in the presence of flg22 is also suppressed by AvrPto (Lu et al. 2010; Zhang et al. 2010). In view of developmental regulation, SERK3 is involved in the BRASSINOLIDE (BR)-triggered signaling pathway, since *serk3* mutants show reduced sensitivity to BR (Li et al. 2002). SERK3 constitutively interacts with the BR receptor BRI1 (Li et al. 2002) and this interaction is also suppressed by AvrPto (Shan et al. 2008).

Altogether, these observations indicate that AvrPto suppresses flg22-triggered plant immunity by interrupting PRR complex formation with the essential coreceptor SERK3 and suppresses BRI1 and SERK3 constitutive complex formation involved in BR-mediated developmental regulation (Shan et al. 2008). In this study, we show that AvrPto is able to suppress Cf-4/Avr4-triggered cell death without affecting the constitutive interaction between Cf-4/SlSOBIR1 and the Avr4-dependent recruitment of SlSERK3a to the Cf-4/SlSOBIR1 complex (Figs. 6 and 7). As we found that AvrPto interacts with both SlSOBIR1 and SlSERK3 (Fig. 1), in this case, AvrPto might interfere in the phosphorylation status of one or both SOBIR1 and SERK3 to suppress Cf-4/Avr4-triggered cell death (Fig. 6).

T3SS effectors interfere in host protein phosphorylation.

Effector-mediated modifications of defense-related proteins have been shown to be an effective way to suppress the immune response (Lee et al. 2013). Interestingly, it has been shown that effectors interfere with the phosphorylation status of important host kinase proteins, including PRRs, RLCKs and MAPKs (Bi and Zhou 2017; Couto and Zipfel 2016; Lee et al. 2013; Macho and Zipfel 2015; Tang et al. 2017). For example, RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV *MACULICOLA 1* (RPM1)-INTERACTING PROTEIN 4 (RIN4) associates with and is required for RPM1-mediated resistance (Mackey et al. 2002). The *P. syringae* effector AvrB interacts with RIN4 and induces its phosphorylation, thereby activating the RPM1 signaling pathway leading to resistance to the bacterium (Lee et al. 2015; Mackey et al. 2002). Similar to AvrB, AvrRpm1 also induces RIN4 phosphorylation (Mackey et al. 2002).

Pseudomonas HopAO family members all have tyrosine phosphatase activity, which is required for pathogen virulence (Bretz et al. 2003; Castañeda-Ojeda et al. 2017). For instance,

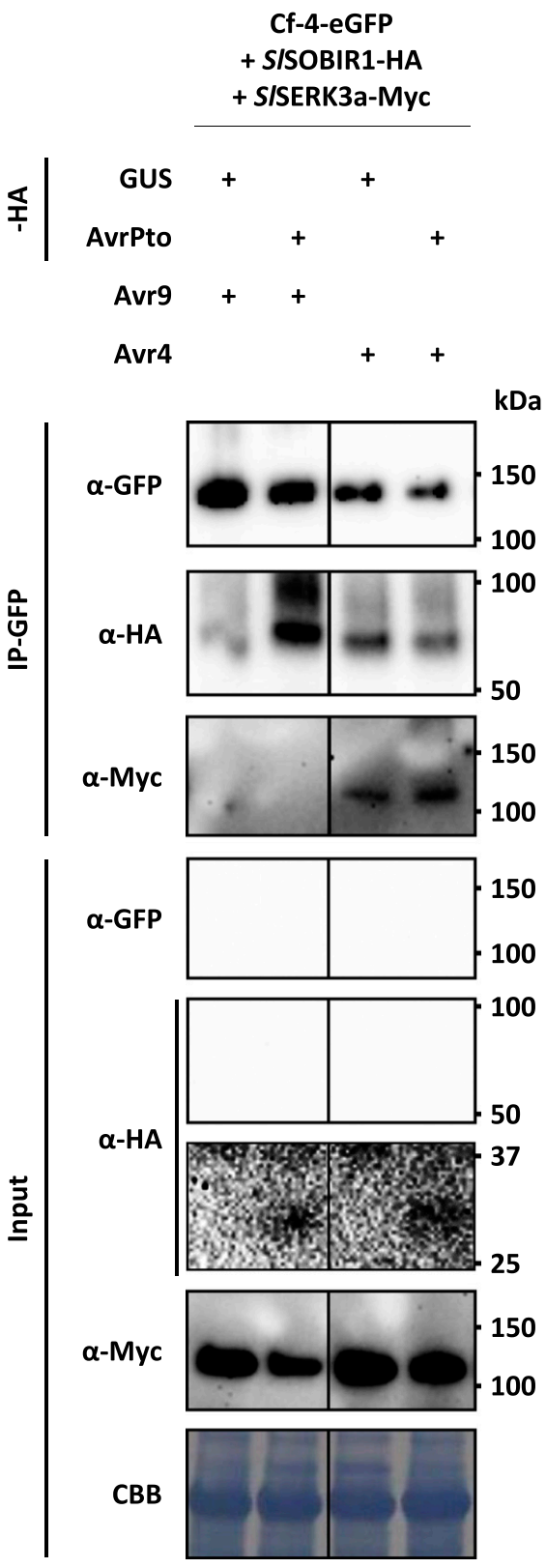

Fig. 7. Avr4-mediated SlSERK3a recruitment to the Cf-4/SlSOBIR1 complex is not affected by AvrPto. *Cf-4-eGFP* (enhanced green fluorescent protein), *SlSOBIR1-HA* (hemagglutinin), and *SlSERK3a-Myc* were coexpressed with either β-glucuronidase (*GUS*)-*HA* or *AvrPto-HA* by agroinfiltration in *Nicotiana benthamiana*. Two days later, Avr4 or Avr9 protein (10 nM) was infiltrated in the same area, and leaves were harvested 30 min later. Total proteins were extracted and subjected to immunoprecipitation, using GFP_TrapA affinity beads. Total proteins (Input) and immunoprecipitated proteins (IP) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted to polyvinylidene diflouride membrane. Blots were probed with α-GFP antibodies to detect immunoprecipitated Cf-4-eGFP, with α-HA antibodies to detect coprecipitated SlSOBIR1-HA, and with α-Myc antibodies to detect coprecipitated SlSERK3a-Myc. CBB = Coomassie brilliant blue. Experiments were performed at least three times and representative images are shown. The lower α-HA panel from the input shows an overexposed blot to reveal accumulation of AvrPto-HA.

the phosphatase catalytic activity of HopAO1 is required for suppressing callose deposition and MAPK activation upon bacterial colonization of *Arabidopsis* (Castañeda-Ojeda et al. 2017; Macho et al. 2014; Underwood et al. 2007). Recently, HopAO1 was reported to dephosphorylate EFR on an essential tyrosine residue, a modification occurring upon elf18, another EF-Tu-derived peptide sharing equal activity to elf26 (Zipfel et al. 2006), treatment without interfering in the EFR-SERK3 interaction (Macho et al. 2014). Dephosphorylation of this particular tyrosine residue was found to result in suppression of MTI (Macho et al. 2014).

In another study, it was found that SERK3, which interacts with FLS2 and EFR upon defense activation, is targeted by HopF2, AvrPtoB, and AvrPto (Yasuda et al. 2017). HopF2 targets SERK3 to suppress BIK1 phosphorylation, thereby compromising MAPK activation (Wu et al. 2011; Zhou et al. 2014).

AvrPtoB targets *Arabidopsis* LysM-RLK CHITIN RECEPTOR KINASE1 (CERK1) to degrade the protein, and this effector also seems to suppress CERK1 kinase activity (Gimenez-Ibanez et al. 2009). In addition, kinase activity of the AvrPtoB tomato-interacting 9 (Bti9) protein, the closest tomato homolog of CERK1, is also suppressed by AvrPtoB (Zeng et al. 2012). AvrPtoB triggers degradation of FLS2/EFR but does not directly affect their kinase activity (Göhre et al. 2008), which contrasts with SERK3, which is not degraded but inhibited in its kinase activity (Cheng et al. 2011).

Cytoplasmic RLCKs, which play a role downstream of PRRs, are phosphorylated by ligand-activated PRRs and, in their turn, transphosphorylate the kinase domain of these PRRs (Couto and Zipfel 2016). Phosphorylation of the RLCK AvrPphB-SUSCEPTIBLE 1 (PBS1) (Swiderski and Innes 2001) is required for its interaction with RESISTANCE TO *PSEUDOMONAS SYRINGAE* 5 (RPS5) (Warren et al. 1998; Ade et al. 2007). PBS1 is cleaved by HopAR1 (AvrPphB) from *P. syringae* pv. *tomato* DC 3000 to activate RPS5-mediated auto immunity (Ade et al. 2007; Shao et al. 2003). HopAR1 also dephosphorylates BIK1 to suppress the flg22-induced signaling pathway (Zhang et al. 2010). In addition, BIK1 is also dephosphorylated by the *Xanthomonas* T3SS effector AvrAC (Feng et al. 2012).

Activation of the MAPK cascade is a common downstream event in plant resistance to pathogens, and the kinase activity of these MAPKs is modified by several bacterial effectors (Lee et al. 2013). HopF2 interacts with MKK5 to prevent its autophosphorylation (Wang et al. 2010), and HopAI1 targets MPK3, MPK6, and MPK4 to dephosphorylate them (Zhang et al. 2007, 2012).

AvrPto has many targets, including SERK3, FLS2, EFR, CERK1, and Pto. Recently, it was reported that both *AvrPto* overexpression and a double mutation in both *SERK3* and *BAK1-LIKE1* (*BKK1*), also referred to as SERK4, similarly suppress oligogalacturonide-induced defense signaling (Gravino et al. 2017). However, it is not clear if AvrPto is able to directly suppress SERK3 kinase activity. AvrPto binds to the kinase domain of FLS2 and EFR, thereby acting as a kinase inhibitor and blocking FLS2 and EFR autophosphorylation to compromise flg22/elf26-triggered immunity (Xiang et al. 2008). Here, we show that AvrPto interacts with SOBIR1; however, it is not known whether AvrPto directly binds to the kinase domain of SOBIR1 or whether this interaction occurs indirectly through another protein that interacts with the SOBIR1 kinase domain. Furthermore, AvrPto targets Pto, thereby suppressing Pto kinase activity (Xing et al. 2007). Surprisingly, the association between BRI1 and SERK3 is interrupted by AvrPto, but the phosphorylation of BRI1 and SERK3 upon BR treatment remains unaffected (Shan et al. 2008).

The overview provided above shows that, except for the BR-triggered phosphorylation of BRI1 and SERK3 that is not affected by AvrPto, the level of phosphorylation of the AvrPto-targeted kinases is lowered by AvrPto. Although there is a debate on the interaction between AvrPto and SERK3 (Xiang et al. 2011), we found that AvrPto targets both SOBIR1 and SERK3 (Fig. 1). Therefore, we anticipate that AvrPto might play a role in altering the phosphorylation status of either SOBIR1, SERK3, or both to suppress Cf-4/Avr4-triggered cell death (Fig. 6), without affecting Cf-4/SlSOBIR1/SlSERK3a complex formation (Fig. 7). Changing the phosphorylation status of the kinase domain of one or both SOBIR1 and SERK3 could be the result of inhibition of the kinase activity of these RLKs through their targeting by AvrPto.

The requirement of kinase activity of targeted proteins for their interaction with effectors.

Targeting of host kinases by pathogen effectors is a common strategy to suppress MTI. In most cases, the kinase domain itself is targeted and kinase activity of the targeted proteins plays a role in the interaction. For example, AvrAC interacts with BIK1 to uridylylate the BIK1 phosphorylation site (Feng et al. 2012). Kinase activity of BIK1 is not required for the interaction, but it affects the uridylylation by AvrAC (Feng et al. 2012).

As mentioned above, AvrPtoB targets the kinase domain of SERK3 (Shan et al. 2008), Pto (Kim et al. 2002), FLS2 (Göhre et al. 2008), CERK1 (Gimenez-Ibanez et al. 2009), and Bti9 (Zeng et al. 2012). Kinase activity of SERK3 (Cheng et al. 2011) and Pto (Dong et al. 2009) is required for the interaction with AvrPtoB. However, whether kinase activity is required for interaction with FLS2, CERK1 and Bti9 is unknown.

Kinase activity of Pto is required for its interaction with AvrPto, as it has been shown that the threonine 204 mutant Pto^{T204N}, having low kinase activity, only weakly interacts with AvrPto, whereas the kinase-inactive variant Pto^{S226D} completely loses its interaction with the effector (Xing et al. 2007). Threonine 204 of Pto is highly conserved among the kinase domains of SERK3, SERK4, FLS2, and EFR (Shan et al. 2008). In SERK3, threonine 455 is equivalent to threonine 204 of Pto and a $SERK3^{T455N}$ mutant has reduced kinase activity (Lin et al. 2014). However, this substitution in SERK3 does not affect its interaction with AvrPto (Shan et al. 2008). It has been shown that Pto has evolved to compete for AvrPto interaction with FLS2 through its ATP-binding site (Xing et al. 2007) and has higher affinity for AvrPto than FLS2 (Xiang et al. 2008). AvrPto targets the ATP-binding site in the kinase domain of FLS2, and a mutation (K898H) in the ATP-binding site of FLS2, which is anticipated to have a lower kinase activity, abolishes its interaction with AvrPto (Xiang et al. 2008; Xing et al. 2007). Although it is unknown whether the kinase domain of SOBIR1 by itself is sufficient for interaction with AvrPto, kinase activity of SOBIR1 appears not to be required (Fig. 3). This observation reveals different requirements for the interaction of AvrPto with its targets, indicating that AvrPto utilizes different mechanisms to target and affect plant kinases involved in defense signaling.

MATERIALS AND METHODS

Plant materials and growth conditions.

Nicotiana benthamiana, *Cf-4*-transgenic *N. benthamiana* (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 agroinfiltrations.

A fragment consisting of the AvrPto coding sequence in an entry vector was transferred to the destination vector pBIN-KS (SOL 2095; for C-terminally tagging with eGFP), to generate

AvrPto-eGFP, and pGWB14 (SOL 2749; for C-terminally tagging with the HA epitope), to generate AvrPto-HA. SlSERK3a-HA, SlSOBIR1-HA, SlSOBIR1-like-eGFP, SlSOBIR1^{D473N}-eGFP, NbSOBIR1-eGFP, GUS-eGFP, and GUS-HA were generated from entry vectors that have been described previously (Bi et al. 2016; Liebrand et al. 2013). SlSERK3a-eGFP, SlSOBIR1-eGFP, SlSOBIR1-Myc, Cf-4-eGFP, AtSOBIR1-eGFP, AtSOBIR1^{D489N}-eGFP, and SlSERK3a-Myc have been described previously (Bi et al. 2016; Liebrand et al. 2013; Postma et al. 2016). Silencing suppressor P19, which was included in all Co-IP experiments, has been described previously (Voinnet et al. 2015).

Binary vectors were transformed to *Agrobacterium tumefaciens* C58C1 carrying helper plasmid pCH32. Infiltration of *Agrobacterium* sp. into plant leaves was performed as described at OD_{600} = 1, unless indicated otherwise (Van der Hoorn et al. 2000).

Co-IPs and immunoblotting.

Co-IPs were performed as described previously (Liebrand et al. 2012). The following antibodies were used: α-p42/p44-ERK (New England Biolabs), in combination with goat α-rabbit (Sigma) as a secondary antibody, α-GFP (Miltenyi Biotec GmbH), α-HA (clone 3F10; Roche Applied Science), α-cMyc (9E10; Santa Cruz Biotechnology), with sheep α-mouse (Amersham) as a secondary antibody.

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