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Letter

Knocking out *SOBIR1* in *Nicotiana benthamiana* abolishes functionality of transgenic receptor-like protein Cf-4

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W.R.H.H., C.S., and S.L.V. performed experiments. R.H. provided the CRISPR/Cas9 constructs. All authors designed the research and analyzed data. W.R.H.H., C.S., and M.H.A.J.J. wrote the letter, with contributions from all the authors.

Dear Editor,

Plants are challenged by a plethora of agents causing biotic stress. To defend themselves, plants have developed a multi-layered immune system (Couto and Zipfel, 2016). The first layer is mediated by pattern recognition receptors (PRRs) that localize on the plasma membrane (PM) and perceive extracellular immunogenic patterns (ExIPs; van der Burgh and Joosten, 2019). So far, all known plant PRRs that carry an extracellular receptor domain consisting of leucine-rich repeats (LRRs) are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs). They share the same overall structure; however, in contrast to RLKs, RLPs lack a cytoplasmic domain for downstream signaling (Couto and Zipfel, 2016). RLPs, such as the tomato (*Solanum lycopersicum*, *Sl*) PRR Cf-4 (Thomas et al., 1997) that mediates resistance against strains of the pathogenic extracellular fungus *Cladosporium fulvum* secreting the matching avirulence factor Avr4 (Joosten et al., 1994), constitutively interact with the RLK SUPPRESSOR OF BIR1-1 (SOBIR1; Gao et al., 2009; Liebrand et al., 2013). SOBIR1 is essential for Cf-4 accumulation and function (Liebrand et al., 2013), and upon recognition of Avr4 by Cf-4, the RLK BRI1-ASSOCIATED KINASE 1 (BAK1), which is a regulatory co-receptor involved in development and defense, is recruited by the activated Cf-4/SOBIR1 complex (Postma et al., 2016). BAK1 recruitment to the activated RLP/SOBIR1 complex appears to be a general process, as this was also shown for the RLP23/SOBIR1 complex (Albert et al., 2015). RLP23 from Arabidopsis (*Arabidopsis thaliana*, *At*) is involved in perception of the ExIP necrosis and ethylene-inducing peptide 1-like protein nlp20, which is produced by several bacterial, fungal, and oomycete species. It has been proposed that subsequent trans-phosphorylation events between the kinase domains of SOBIR1 and BAK1 eventually initiate downstream defense signaling (van der Burgh et al., 2019).

Although important advances have been made in deciphering RLK-mediated downstream immune signaling (Couto and Zipfel, 2016; van der Burgh and Joosten, 2019), little is known about how the RLP/SOBIR1/BAK1 complex functions at the level of complex formation and downstream signal initiation. What is known is that the tomato receptor-like cytoplasmic kinase (RLCK) AVR9/CF-9-INDUCED KINASE 1 (ACIK1) plays an essential role downstream of Cf-4 and the RLP Cf-9, which confers recognition of the secreted *C. fulvum* effector Avr9 (van Kan et al., 1991; Jones et al., 1994; Rowland et al., 2005). In Arabidopsis, the RLCK BOTRYTIS-INDUCED KINASE 1 (BIK1) is swiftly phosphorylated upon the perception of flg22, a peptide derived from bacterial flagellin, by the RLK FLAGELLIN-SENSITIVE 2 (FLS2; Gòmez-Gòmez and Boller, 2000). FLS2 also recruits

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BAK1 upon flg22 binding, and BIK1 phosphorylation is BAK1-dependent (Lu et al., 2010). It was concluded that, similar to ACIK1, BIK1 is a critical component, linking the PM-associated PRR complex to cytoplasmic immune signaling (Lu et al., 2010).

Here, we took advantage of the CRISPR/Cas9 system to knock out *SOBIR1* and its close homolog *SOBIR1-like* in the model plant *Nicotiana benthamiana* (*Nb*), as well as in *N. benthamiana* stably expressing the *Cf-4* transgene. Cf-4 is functional in *N. benthamiana*, and we demonstrate that Cf-4 function is completely abolished in *N. benthamiana:Cf-4 sobir1*(/*sobir1-like*) knock-out mutants. We anticipate that these mutant lines will be important materials for studying the fundamentals of plant immunity mediated by RLPs. SOBIR1 is a positive regulator of plant immunity, as overexpression of *AtSOBIR1* in Arabidopsis, as well as in *N. benthamiana*, leads to constitutive activation of cell death and defense responses (Gao et al., 2009; Wu et al., 2017; van der Burgh et al., 2019). Surprisingly, no symptoms of constitutive immunity were observed when tomato *SlSOBIR1* or *NbSOBIR1* was overexpressed in *N. benthamiana* (Wu et al., 2017). Therefore, over the past years, the *At*SOBIR1-induced constitutive immunity in *N. benthamiana*, visible as a hypersensitive response (HR) at the site of agro-infiltration (transient expression) of *AtSOBIR1*, is commonly employed to decipher the mechanism behind RLP/SOBIR1-mediated plant immunity. For this, endogenous *NbSOBIR1*(*-like*) genes are silenced in *N. benthamiana:Cf-4* by virus-induced gene silencing (VIGS), and subsequent complementation studies are performed by transiently expressing *AtSOBIR1* and various mutants of this RLK (Liebrand et al., 2013; van der Burgh, 2018). However, as VIGS only generates a gene knock-down, such complementation experiments require high amounts of repetition due to variation caused by the presence of varying background levels that remain of the endogenous *Nb*SOBIR1(-like) protein. With the advent of the CRISPR/Cas9 gene-editing system, it is now possible to generate stable gene knock-outs in various plant species (Belhaj et al., 2015), and this development prompted us to knock out functional *SOBIR1* in *N. benthamiana* and *N. benthamiana:Cf-4*, thereby generating a perfect system for complementation studies with mutants of SOBIR1.

To knock out both *SOBIR1* and *SOBIR1-like* in *N. benthamiana*, six single-guide RNAs (sgRNAs) targeting the open reading frames (ORFs) of both genes (Supplemental Figure S1 and Table S1) were designed using CRISPR-P 2.0 (Liu et al., 2017). Together with Cas9 and the selection marker BIALAPHOS RESISTANCE (BAR), sgRNA1, 2, 5, and 6 were assembled into the acceptor backbone (pAGM4723), referred to as Construct 1. In addition, sgRNA3, 4, 5, and 6 were cloned into the same acceptor backbone, referred to as Construct 2. The effectiveness and efficiency of the generated constructs were confirmed by studies based on their transient expression (Supplemental Figure S2), before stable transformation to explants of *N. benthamiana*. CRISPR/Cas9-induced mutations were detected by amplifying and sequencing the targeted gene regions, using isolated genomic DNA of the generated transformants as a template (Supplemental Table S3). Two homozygous *sobir1*/*sobir1*-like double knock-out lines (Supplemental Figure S3), and also one single *sobir1* knock-out line, generated by Construct 2, were obtained. *N. benthamiana sobir1*/*sobir1-like* line #1 contains a 1 bp deletion in the sgRNA3-target region and a 6 bp deletion in the sgRNA4-target region in the ORF of *SOBIR1*, whereas a 1 bp insertion is present in the ORF of *SOBIR1-like* (Figure 1A). Similarly, there is a 1 bp insertion and a 1 bp deletion in *SOBIR1*, and a 4 bp deletion in *SOBIR1-like* in the *N. benthamiana sobir1*/*sobir1-like* line #2 (Figure 1A). The *N. benthamiana* single *sobir1* mutant only contains a 1 bp insertion in *SOBIR1* (Figure 1A).

Transient co-expression of Cf proteins with their matching Avr ligands in *N. benthamiana* triggers a typical HR (Figure 1B; van der Hoorn et al., 2000). Compared to the wild-type plant, none of the double knock-out lines was responsive to the Avr4/Cf-4 or Avr9/Cf-9 combination (Figure 1B), indicating that SOBIR1 and SOBIR1-like are indeed non-functional in the two mutant lines, due to disruption of their ORFs. Additionally, the *N. benthamiana sobir1* single knock-out mutant was non-responsive to the *Avr4*/*Cf-4* or *Avr9*/*Cf-9* combination (Figure 1B), similar to the two double knock-out mutants. This observation is in accordance with our earlier finding that *NbSOBIR1-like* is not expressed or expressed only at a very low level, indicating that this gene is not functional (Liebrand et al., 2013). Interestingly, complementation of the *SOBIR1(-like)* knock-outs through transient expression of *NbSOBIR1*, *SlSOBIR1*, or *SlSOBIR1*-like, together with the *Avr4*/*Cf-4* combination, restored the HR (Figure 1C). Complementation did not take place upon co-expression of the corresponding kinase-dead mutants of *SOBIR1*, as in this case, the leaf tissue remained non-responsive to the *Avr4*/*Cf-4* combination (Figure 1C). These results reinforce the conclusion that SOBIR1/SOBIR1-like plays a pivotal role in RLP-mediated immunity and that the *N. benthamiana sobir1*/*sobir1-like* mutant plants form a robust basis for complementation studies.

We use *N. benthamiana*:*Cf-4* for the elucidation of the molecular mechanisms of signal transduction events triggered by Cf-4 upon Avr4 recognition (Liebrand et al., 2013; Wu et al., 2017; van der Burgh et al., 2019). Therefore, *SOBIR1* and *SOBIR1-like* were also knocked out in *N. benthamiana*:*Cf-4* (Supplemental Table S3). Two homozygous double knock-out mutant lines were obtained with disruptions in both the ORF of *SOBIR1* and *SOBIR1-like*, which were introduced by Construct 1 (Figures 2A and Supplemental Figure S3). In addition, one single *sobir1* knock-out line, generated by Construct 2, was also obtained (Figure 2B). In agreement with our previous finding, transient expression of *Avr4* triggered an HR in *N. benthamiana*:*Cf-4* plants; however, the knock-out lines all were non-responsive to Avr4 (Figure 2C), again confirming that Cf-4 functionality requires functional SOBIR1(-like). Complementation with *Nb*SOBIR1, *Sl*SOBIR1, or *Sl*SOBIR1-

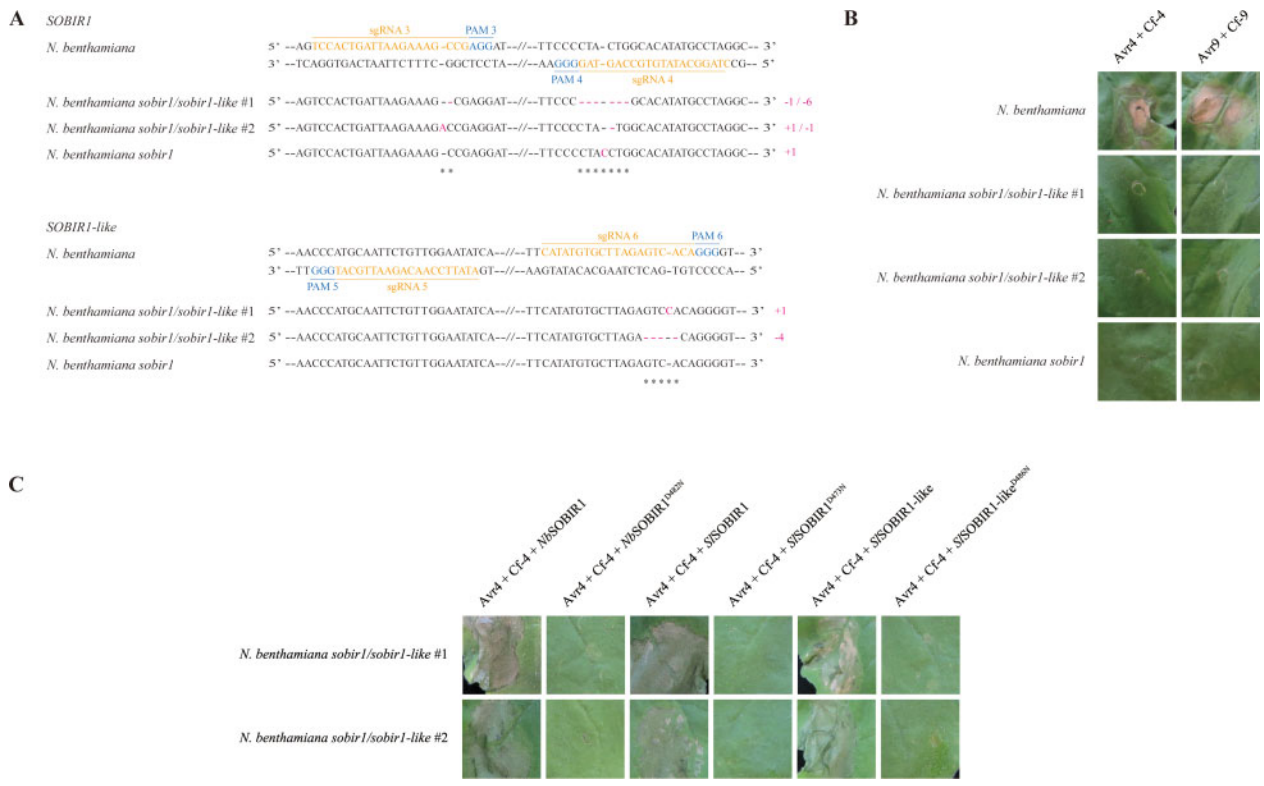

Figure 1 CRISPR/Cas9-induced targeted knockout of *SOBIR1* in *N. benthamiana* abolishes the responsiveness to matching Avr/Cf combinations. A, Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel), targeted by single-guide RNAs (sgRNAs) in the two *N. benthamiana* double *sobir1/sobir1-like* knock-out lines and the *N. benthamiana* single *sobir1* knock-out line, with wild-type *SOBIR1* and *SOBIR1-like* sequences, respectively. The sgRNA sequences are indicated in orange, and the protospacer-adjacent motifs (PAMs) are indicated in blue. The deleted nucleotides in the generated transformants are indicated with carmine dashes, and the inserted nucleotides are denoted with carmine letters. The type of mutations and the numbers of deleted/inserted nucleotides are shown on the right. B, Transient co-expression of *Cf-4* with the matching *C. fulvum* effector *Avr4*, or of *Cf-9* with its matching *C. fulvum* effector *Avr9*, by *Agrobacterium*-mediated transient expression, triggers a rapid HR in the leaves of wild-type *N. benthamiana* plants (upper two panels), whereas neither Avr4/Cf-4-, nor Avr9/Cf-9-induced cell death was observed in the two double *sobir1/sobir1-like* knock-out lines (middle four panels) and in the single *sobir1* knock-out line (lower two panels). C, Complementation by transient expression of *NbSOBIR1*, *SlSOBIR1*, or *SlSOBIR1-like*, restores the Avr4/Cf-4-specific HR in the *N. benthamiana sobir1/sobir1-like* mutants, whereas this complementation does not take place upon transient expression of the corresponding kinase-dead mutants. *NbSOBIR1*, *SlSOBIR1*, or *SlSOBIR1-like*, as well as their corresponding kinase-dead mutants (negative controls), was transiently co-expressed with *Avr4*/*Cf-4*, in the leaves of the *N. benthamiana sobir1/sobir1-like* knock-out lines. Each construct was agro-infiltrated at an optical density at 600 nm (OD_{600}) of 0.5, and all leaves were photographed at 5 d post infiltration (dpi). Experiments were repeated at least three times, and similar results were obtained. Representative pictures are shown.

like in the double knock-out *N. benthamiana*:*Cf-4* plants, in combination with transient expression of *Avr4*, again resulted in a Cf-4-mediated HR, whereas complementation did not take place upon co-expression of the corresponding kinase-dead mutants of *SOBIR1* (Figure 2D).

These mutant lines were further validated by monitoring the production of reactive oxygen species (ROS), which is a very early downstream response upon immune activation, upon treatment with either Avr4 or flg22. Note that FLS2 is also present in *N. benthamiana* and does not interact with SOBIR1 and does not require SOBIR1 for its functionality (Liebrand et al., 2013; Albert et al., 2015). Unlike the rapid and monophasic ROS burst induced by the flg22 peptide, a biphasic ROS accumulation was observed when leaf discs of *N. benthamiana*:*Cf-4* were treated with Avr4 protein. In the latter case, the first transitory response was followed by a second, sustained ROS burst, which was of higher amplitude when compared to the initial ROS burst (Figure 2E). As expected, the biphasic Avr4-triggered ROS burst was completely abolished in all mutant lines (Figure 2E), which further verifies that these mutant lines have become non-responsive to Avr4. Intriguingly, an unexpected second sustained ROS burst, triggered by flg22, was observed in the two *N. benthamiana*:*Cf-4* double *sobir1/sobir1-like* mutants, as well as in the single *N. benthamiana*:*Cf-4 sobir1* mutant (Figure 2E). Our observation suggests that there is potential crosstalk taking place between RLP/SOBIR1- and FLS2-triggered pathways.

We propose that the appearance of a biphasic ROS burst triggered by flg22 in the *SOBIR1(-like)* knock-out mutants uncovers the presence of inhibitory activity of the RLP/SOBIR1 signal transduction pathway on the signaling route employed by FLS2. In addition to the work presented here, recently, a large number of immune-related genes, including

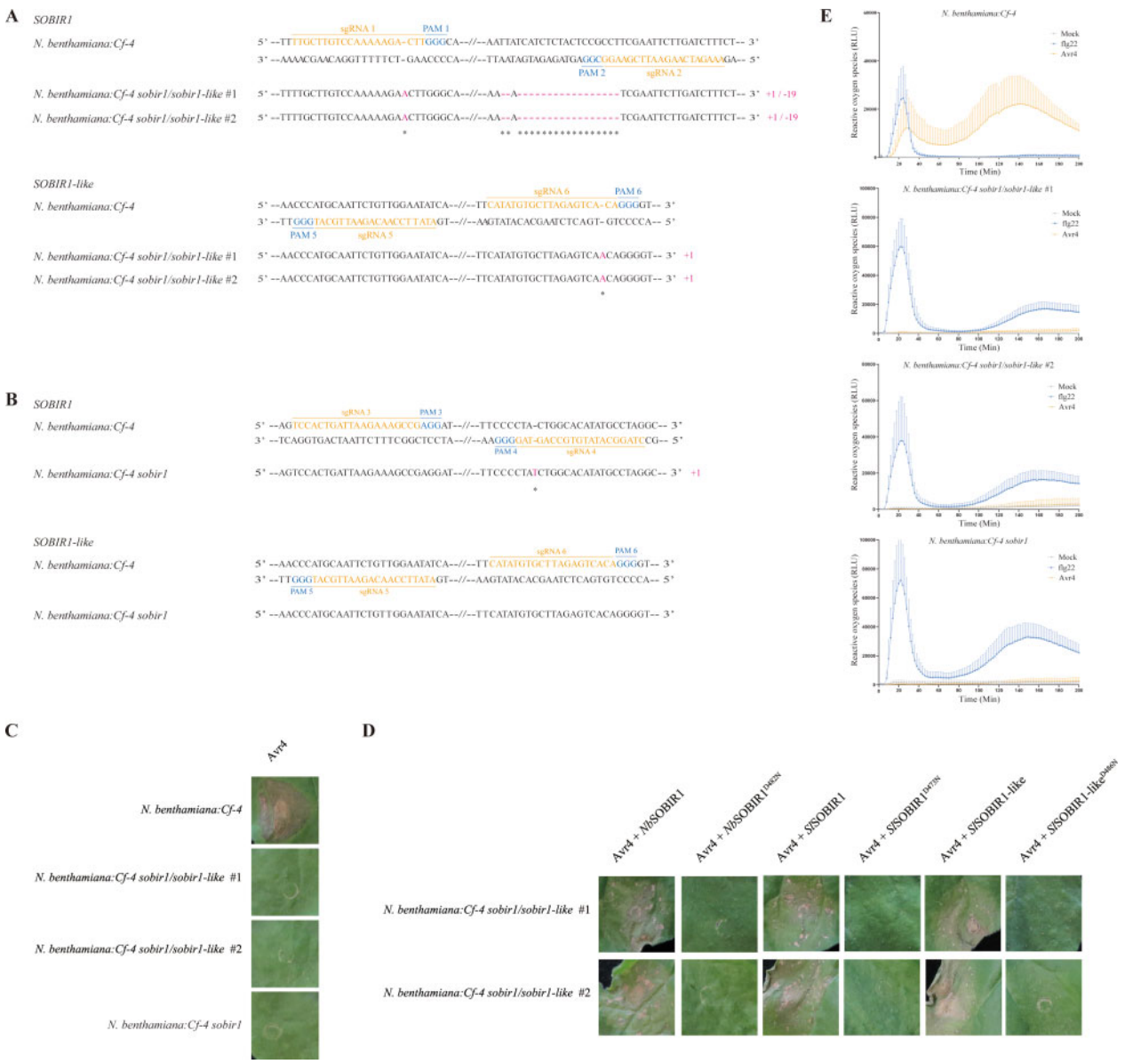

Figure 2 CRISPR/Cas9-induced targeted knockout of *SOBIR1* in transgenic *N. benthamiana*:*Cf-4* abolishes the functionality of the *Cf-4* transgene. A, Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel) targeted by sgRNAs in the two *N. benthamiana*:*Cf-4* double *sobir1*/*sobir1-like* knock-out lines, with wild-type *SOBIR1* and *SOBIR1-like* sequences, respectively. The sgRNA sequences are shown in orange, and the PAM sites are indicated in blue. The deleted nucleotides in the generated transformant are indicated with carmine dashes, and the inserted nucleotides are denoted with carmine letters. The type of mutations and the numbers of deleted/inserted nucleotides are shown on the right. B, Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel) targeted by sgRNAs in the *N. benthamiana*:*Cf-4* single *sobir1* knock-out line, with wild-type *SOBIR1* and *SOBIR1-like* sequences, respectively. The sgRNA sequences are shown in orange, and the PAM sites are indicated in blue. The deleted nucleotides in the generated transformant are indicated with carmine dashes, and the inserted nucleotides are denoted with carmine letters. The type of mutations and the numbers of deleted/inserted nucleotides are shown on the right. C, *Agrobacterium*-mediated expression of *Avr4* in *N. benthamiana*:*Cf-4* plants results in a rapid HR at the site of infiltration (upper panel), whereas agro-infiltration of *Avr4* failed to induce cell death in the two *N. benthamiana*:*Cf-4* double *sobir1*/*sobir1-like* knock-out lines (middle two panels) and in the *N. benthamiana*:*Cf-4* single *sobir1* knock-out line (lower panel). D, Complementation by transient expression of *NbSOBIR1*, *Sl*SOBIR1, or *SlSOBIR1-like*, restores the *Avr4*/*Cf-4*-specific HR in the two *N. benthamiana*:*Cf-4* *sobir1*/*sobir1-like* mutants, whereas this complementation does not take place upon transient expression of the corresponding kinase-dead mutants. *NbSOBIR1*, *SlSOBIR1*, or *SlSOBIR1-like*, as well as their corresponding kinase-dead mutants (negative controls), was transiently co-expressed with *Avr4*, in the leaves of the two *N. benthamiana*:*Cf-4* *sobir1*/*sobir1-like* knock-out lines. Each construct was agroinfiltrated at an OD_{600} of 0.5, and the leaves were photographed at 5 dpi. E, Avr4 fails to induce a ROS burst in the two *N. benthamiana*:*Cf-4* *sobir1*/*sobir1-like* knock-out lines and in the *N. benthamiana*:*Cf-4* single *sobir1* knock-out line. Leaf discs of *N. benthamiana*:*Cf-4* (upper panel), the two *N. benthamiana*:*Cf-4* double *sobir1*/*sobir1-like* knock-out lines (middle two panels), and the *N. benthamiana*:*Cf-4* single *sobir1* knock-out line (lower panel), were treated with 0.1 μM Avr4 or 0.1 μM flg22 (positive control), or with water (mock) (negative control). ROS production is expressed as relative light units (RLUs), and the data are represented as mean + SD. Experiments were repeated at least three times, and similar results were obtained. Representative pictures are shown. Note that in the *N. benthamiana*:*Cf-4* *sobir1*/*sobir1-like* knock-out lines, as well as in the *N. benthamiana*:*Cf-4* single *sobir1* knock-out line, the response to flg22 manifests itself as a biphasic ROS burst, whereas in *N. benthamiana*:*Cf-4* the flg22-triggered ROS burst is monophasic.

SOBIR1 and *SOBIR1-like*, were targeted by CRISPR/Cas9 technology in tomato (Zhang et al., 2020). These resources will allow the study of the role of these genes in resistance of tomato to *C. fulvum*, by crossing these mutants to tomato carrying the *Cf-4* resistance gene.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Methods

Supplemental Figure S1 Nucleotide sequence of *NbSOBIR1* and *NbSOBIR1-like*.

Supplemental Figure S2 Determination of the effectiveness and efficiency of the generated CRISPR/Cas9 constructs.

Supplemental Figure S3 Phenotypes of wild-type *N. benthamiana* and the various mutant lines. The plants were extracted from different photos and placed on a black background.

Supplemental Table S1 Nucleotide sequences of the six single-guide RNAs.

Supplemental Table S2 Nucleotide sequences of the primers used in this study.

Supplemental Table S3 Number of transgenic *N. benthamiana* lines screened and the types of mutations that were obtained.

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Conflict of interest statement. The authors declare that there is no conflict of interest.

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