

Activation and signal transduction of SOBIR1/BAK1-containing immune complexes present at the plant cell surface



Wen R.H. Huang

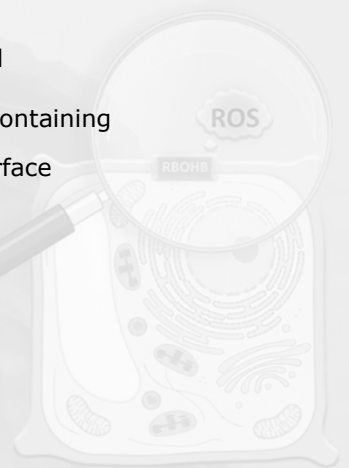
Propositions

1. Auto-phosphorylation of SOBIR1 is a prerequisite for the activation of SOBIR1-containing immune complexes.
(this thesis)
2. Receptor-like cytoplasmic kinases are the initial cytoplasmic transducers of the extracellular signal perceived by cell-surface receptors.
(this thesis)
3. Genome editing in humans by CRISPR/Cas9 should be tightly regulated.
4. The biological material should be made accessible for sharing when a resource paper is published.
5. Human activities have promoted the evolution of the COVID-19 virus.
6. The last leg of a journey marks the halfway point.

Propositions belonging to the thesis, entitled
Activation and signal transduction of SOBIR1/BAK1-containing
immune complexes present at the plant cell surface

Wen R.H. Huang

Wageningen, 28 June 2022



**Activation and signal transduction of
SOBIR1/BAK1-containing immune
complexes present at the plant cell surface**

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Activation and signal transduction of SOBIR1/BAK1-containing immune complexes present at the plant cell surface

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Thesis

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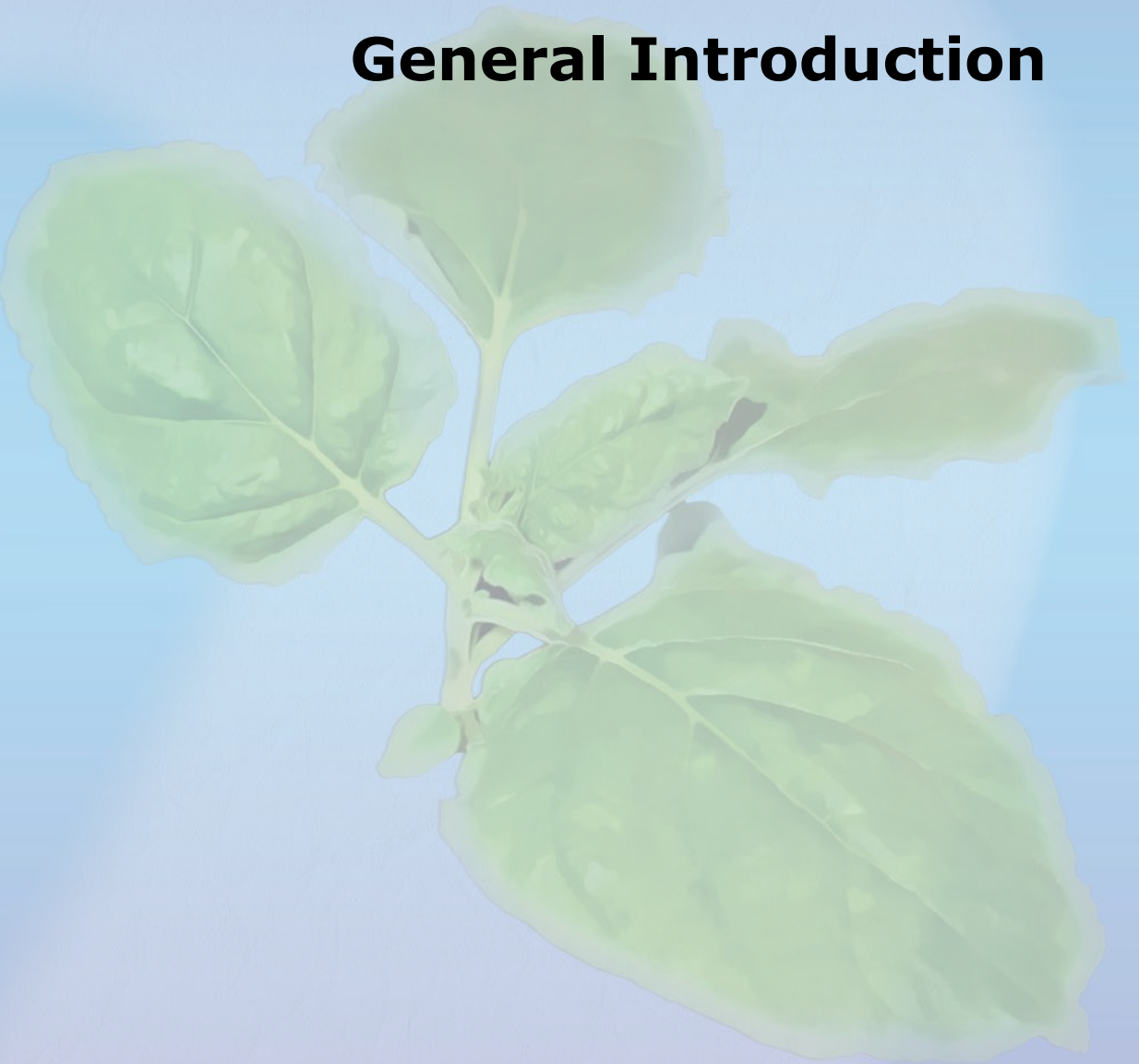
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Chapter 1

General Introduction



The plant immune system

Plants, which live in an environment surrounded by various microbial pathogens, rely on a two-layered innate immune system to monitor the presence of a variety of immunogenic signals (van der Burgh & Joosten, 2019). Cell-surface receptors, consisting of receptor-like kinases (RLKs) and receptor-like proteins (RLPs), perceive extracellular immunogenic patterns (ExIPs) (Dangl & Jones, 2001; Jones and Dangl, 2006; Couto and Zipfel, 2016; van der Burgh and Joosten, 2019). ExIPs comprise conserved microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and extracellular effector proteins (Jones and Dangl, 2006; Zipfel, 2008; Boller and Felix, 2009). The recognition of ExIPs by cell-surface receptors triggers a series of plant immune responses, eventually resulting in resistance to the attacking microbe (Bigeard et al., 2015; Yu et al., 2017). This so-called extracellularly-triggered immunity (ExTI), collectively sets up the frontline of plant immunity, which plays a prominent role in fending off the invasion by a broad spectrum of pathogens and pests (Dodds and Rathjen, 2010; Gust et al., 2017; van der Burgh and Joosten, 2019).

To facilitate invasion and colonization of the host tissues, adapted pathogens secrete intracellular immunogenic patterns (InIPs), which are mainly effector proteins, into the plant cells to interfere with host immunity (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Xin et al., 2018; van der Burgh and Joosten, 2019). Therefore, as a result of co-evolution, plants have evolved a second layer of surveillance machinery, consisting of intracellular immune receptors formed by nucleotide-binding, leucine-rich repeat proteins (NLRs), to specifically recognize accumulating InIPs within plant cells (Dangl and Jones, 2001; Jones and Dangl, 2006; Cui et al., 2015). Either direct or indirect recognition of InIPs activates intracellularly-triggered immunity (InTI) (Cui et al., 2015; van der Burgh and Joosten, 2019). In their turn, pathogens can evade the activation of InTI and further manipulate the host immune system, by discarding or mutating the recognized InIPs, or by gaining new InIPs. Thus, ExTI and InTI are partially in line with the two branches of the plant immune system as proposed in the classical Zigzag model (Jones and Dangl, 2006).

Upon perception of ExIPs by cell-surface receptors, or InIPs by NLRs, a suite of subsequent immune responses is triggered, including the generation of reactive oxygen species (ROS), the activation of mitogen-activated protein kinases (MAPKs), a transient increase of cytosolic calcium, the activation of calcium-dependent protein kinases (CDPKs), an increased biosynthesis of defense hormones, a global transcriptional reprogramming of defense-related genes, and in some cases the activation of a hypersensitive response (HR) at the infection site (Torres et al., 2006; Qi et al., 2017; Yu et al., 2017; Zhang et al., 2018). Collectively, these immune responses result in disease resistance.

Cell-surface receptors

Our research focuses on cell-surface receptors. Such receptors are either RLKs or RLPs, and are responsible for the activation of the first layer of the plant immune system (Macho and Zipfel, 2014; Ben Khaled et al., 2015). RLKs comprise an N-terminal ectodomain (ECD) that is involved in ligand recognition, a single transmembrane domain, and an intracellular kinase domain that is essential for the initiation of downstream defense signaling. RLPs share the same overall structure but lack an obvious intracellular domain (Monaghan and Zipfel, 2012; Böhm et al., 2014). The ECDs of cell-surface receptors are highly variable, which contain for example leucine-rich repeats (LRRs) to bind to proteins and peptides, lysin motif (LysM) to perceive N-acetylglucosamine-containing ligands, lectin motifs to recognize extracellular ATP or bacterial lipopolysaccharides (LPS), or an epidermal growth factor (EGF)-like domain to detect plant cell wall-derived oligogalacturonides (Macho and Zipfel, 2014; Couto and Zipfel, 2016).

LRR-RLKs and LRR-RLPs form the largest family of cell-surface receptors and harbor the most extensively studied members. Well known examples of LRR-RLKs are *Arabidopsis* (*Arabidopsis thaliana*, *At*) FLAGELLIN-SENSING 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR), which perceive bacterial flagellin by recognizing its highly conserved N-terminal 22-amino acid epitope (flg22) and bacterial elongation factor Tu by recognizing its derived N-acetylated 18-amino acid peptide (elf18), respectively (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001; Zipfel et al., 2006). Upon recognition of their matching ligands, both FLS2 and EFR recruit another *Arabidopsis* LRR-RLK BRI-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1), after which immunity against bacterial infection is triggered (Figure 1A) (Heese et al., 2007). Moreover, flg22 directly binds to the ectodomains of FLS2 and BAK1, acting as “molecular glue” to stabilize the FLS2-BAK1 dimerization (Sun et al., 2013).

Concerning LRR-RLPs, RLP23, RLP30, and RLP42 are well-studied cell-surface receptors from *Arabidopsis*. RLP23 perceives necrosis and ethylene-inducing peptide 1-like proteins (NLPs and the derived peptide, nlp20) from various bacteria, oomycetes, and fungi (Albert et al., 2015). RLP 30 particularly recognizes *Sclerotinia sclerotiorum* filtrate elicitor 1 (SCFE1), while RLP42 could specifically detect fungal polygalacturonases (PGs and the derived peptide, pg13) (Figure 1A) (Zhang et al., 2013; Zhang et al., 2014). Lacking an intracellular kinase domain for downstream signal transduction, RLP23, RLP30, and RLP42 form a ligand-independent complex with the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1), and therefore the LRR-RLP/SOBIR1 heteromeric complex has been proposed to be a structural and functional bimolecular equivalent of LRR-RLK (Gao et al., 2009; Liebrand et al., 2013; Gust and Felix, 2014; Liebrand et al., 2014). Interestingly, in line with ligand-activated LRR-RLKs, in the presence of the matching ligand, the

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RLP23/SOBIR1, RLP30/SOBIR1, and RLP42/SOBIR1 bimolecular complexes associate with the regulator BAK1, thereby initiating subsequent downstream immune signaling (Figure 1A) (Chinchilla et al., 2009; Zhang et al., 2013; Zhang et al., 2014; Liebrand et al., 2014; Albert et al., 2015).

Additionally, the Arabidopsis LysM-RLK LYSIN MOTIF RECEPTOR KINASE 5 (LYK5), which is a pseudokinase with a high affinity to chitin, forms a chitin-induced complex with the related LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) to facilitate chitin perception (Figure 1B), which leads to the auto-phosphorylation of CERK1 and activation of an immune response (Cao et al., 2014; Erwig et al., 2017). Consistently, in rice (*Oryza sativa*, *Os*) CERK1 is also required for chitin perception, however, here the LysM-RLP CHITIN ELICITOR-BINDING PROTEIN (CEBiP), instead of a LysM-RLK, forms a complex with *Os*CERK1 for the activation of downstream signaling (Figure 1C) (Hayafune et al., 2014).

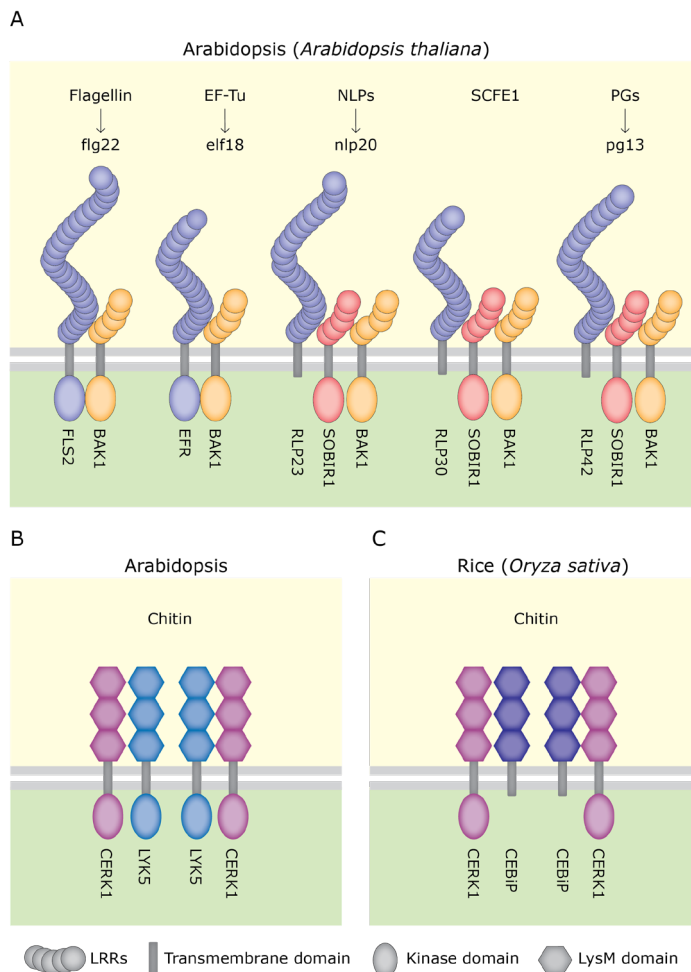


Figure 1. Different types of plant cell-surface receptors and their signaling partners. (A) The Arabidopsis LRR-RLKs FLS2 and EFR, directly detect the conserved epitope flg22 derived from bacterial flagellin and elf18 from bacterial elongation factor Tu (EF-Tu), respectively. Both recognition events result in the recruitment of the regulator BAK1 and the mounting of immunity against bacterial infection. FLS2 and EFR contain 28 and 21 LRRs in their ectodomains, respectively, while BAK1 only comprises 5 LRRs in its ectodomain. The LRR-RLK SOBIR1 constitutively interacts with LRR-RLPs, such as RLP23, RLP30, and RLP42, thereby providing them with an intracellular kinase domain. There are 27, 21, 25, and 5 LRRs in the ectodomains of RLP23, RLP30, RLP42, and SOBIR1, respectively. Upon recognition of the matching ligands, which are respectively NLPs/nlp20, SCFE1, and PGs/pg13, the regulator BAK1 is recruited by the RLP23/SOBIR1, RLP30/SOBIR1, and RLP42/SOBIR1 bimolecular complexes, leading to the activation of subsequent immune responses. **(B)** In Arabidopsis, the LysM-RLK LYK5 is the primary chitin receptor, which exhibits a much higher chitin-binding affinity than that of the LysM-RLK CERK1. Both LYK5 and CERK1 carry 3 extracellular LysM domains. Chitin perception by LYK5 induces the dimerization of LYK5 and CERK1, which is required for chitin-triggered signal transduction. **(C)** In contrast, direct chitin perception in rice is through the LysM-RLP CEBiP, which also possesses 3 extracellular LysM domains but lacks a kinase domain. CEBiP homodimerizes upon chitin perception, followed by the formation of a sandwich-type receptor complex with CERK1.

Receptor-like cytoplasmic kinases

Receptor-like cytoplasmic kinases (RLCKs) are direct substrates of activated receptor complexes, which facilitate the phospho-relay from upstream activated cell-surface receptor complexes to downstream signaling components (Liang and Zhou, 2018). RLCKs lack an extracellular ligand-binding domain, some of them contain a transmembrane helix, whereas others are anchored to the plasma membrane through N-terminal myristoylation or palmitoylation motifs (Lin et al., 2013; Liang and Zhou, 2018). Emerging evidence has shown that RLCKs often functionally and physically associate with RLKs in the resting state and dissociate from cell-surface receptor complexes upon ExIP recognition, which subsequently activates a variety of downstream immune responses, including the generation of a ROS burst and the activation of MAPKs (Macho and Zipfel, 2014; Couto and Zipfel, 2016; Cui et al., 2018; Liang and Zhou, 2018). RLCKs thereby act as key players that fill the gap between cell-surface receptors and intracellular signaling cascades, and have emerged as a major class of kinases that regulate plant innate immunity (Bi et al., 2018).

Accordingly, plants have evolved a large number of RLCKs, with for example 149 members in Arabidopsis and 379 members in rice (Shin-Han Shiu and Bleecker, 2001; Vij et al., 2008). Arabidopsis RLCKs are further divided into 17 classes based on their sequence homology, and among them, the RLCK class VII has been studied intensively (Shiu et al., 2004). This class contains 46 members, and some of them have been reported to play crucial roles in ExTI (Liang and Zhou, 2018; Rao et al., 2018). Arabidopsis BOTRYTIS-INDUCED KINASE 1 (BIK1), a member of RLCK class VII subfamily 8, is the best-studied example. In the resting state, BIK1 associates with both FLS2 and BAK1, whereas upon flg22 perception, BAK1 associates with and phosphorylates FLS2, after which BAK1 phosphorylates BIK1, which in turn phosphorylates both FLS2 and BAK1. Subsequently, activated BIK1 dissociates from

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the FLS2/BAK1 complex, and activates downstream signaling components by phosphorylation (Lu et al., 2010). Similar to FLS2, EFR also requires BIK1 for the transduction of downstream signaling (Zhang et al., 2010). The kinase domain of EFR produced in a bacterial expression system has no or only weak auto-phosphorylation activity; nevertheless, EFR that is produced in insect cells exhibits strong auto-phosphorylation activity and directly phosphorylates BIK1 at serine (Ser/S) 89 and threonine (Thr/T) 90, which are present in a unique extended loop (Lu et al., 2010; Lal et al., 2018). In addition to being present at the plasma membrane, BIK1 has also been reported to localize to the nucleus. Upon mounting the immune response, nuclear BIK1 interacts with and phosphorylates WRKY transcription factors, which are abundant transcription factors having a typical "WRKY" domain and play important roles in the response to biotic and abiotic stress, to regulate the intrinsic level of jasmonic acid (JA) and salicylic acid (SA) (Lal et al., 2018). Moreover, the Arabidopsis PLANT ELICITOR PEPTIDE 1 (Pep1) is a classic DAMP, which is perceived by the LRR-RLKs PEP1 RECEPTOR 1 (PEPR1) and PEP1 RECEPTOR 2 (PEPR2) and that have been reported to specifically associate with BIK1. Upon Pep1 treatment, PEPR1, and likely PEPR2 also, phosphorylate BIK1 directly to mediate DAMP-induced defenses (Liu et al., 2013).

Besides RLCKs belonging to class VII, some members from the RLCK class XII also have been shown to play a role in plant innate immunity. For instance, Arabidopsis BR-SIGNALING KINASE 1 (BSK1), which is involved in BRASSINOSTEROID INSENSITIVE 1 (BRI1)-mediated signaling, physically interacts with FLS2 and is required for the initiation of flg22-triggered responses, including ROS accumulation (Shi et al., 2013).

RLCKs and the ROS burst

Transient production of ROS is one of the very early immune responses that indicate successful recognition of pathogen attacks and subsequent activation of plant defenses. ROS functions not only by being toxic to the invading pathogen but also by acting as intercellular and intracellular signaling molecules to trigger downstream responses. ROS can be produced in various subcellular compartments, including chloroplasts, mitochondria, peroxisomes, and the apoplast (Mittler, 2017; Waszczak et al., 2018). Apoplastic ROS is mainly produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which localize at the plasma membrane and belong to the family of RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) (Kadota et al., 2015; Qi et al., 2017).

In Arabidopsis RBOHs are comprised of 10 members, named AtRBOHA to AtRBOHJ, of which RBOHD and, albeit to a lesser extent, RBOHF, are required for the generation of apoplastic ROS upon recognition of invading pathogens (Torres et al., 2002; Qi et al., 2017; Waszczak et al., 2018). BIK1 plays an essential and central role in multiple ExIP-triggered defenses, as a loss-of-function *bik1* mutant of Arabidopsis shows a reduction in flg22-, elf18- and chitin-induced ROS production (Zhang et al., 2010). Intriguingly,

upon immune activation, BIK1 has been reported to associate with the N-terminus of RBOHD directly, and to phosphorylate RBOHD at both S39 and S343, thereby enhancing ROS generation. Additionally, FLS2 that is present in the stomatal guard cells is involved in controlling the stomatal defense elicited by invading bacteria. Here, the pathway constituted by BIK1 and RBOHD is employed by FLS2 to control stomatal opening in response to bacterial attack, and by which the plant limits the invasion of bacteria into the leaf tissues (Li et al., 2014). Altogether, BIK acts as a long-sought molecular link between cell-surface receptors and early defense outputs.

AvrPphB SUSCEPTIBLE 1 (PBS1), PBS1-LIKE 1 (PBL1), and PBL2, which are all RLCKs from the same class, have also been shown to be required for multiple signaling pathways, including those triggered by FLS2, EFR, and CERK1 (Zhang et al., 2010; Rao et al., 2018). In concert with BIK1, these RLCKs function additively to positively regulate ROS production and consequently plant immunity (Zhang et al., 2010). In contrast, another member from this class, PBL13, has been reported to be a negative regulator of plant innate immunity, as *elf18*- and *flg22*-triggered ROS burst is significantly increased in Arabidopsis *pbl13* knockout mutants. Interestingly, PBL13 is an active kinase and directly interacts with RBOHD *in planta*. Nevertheless, the underlying mechanism through which PBL13 negatively regulates ROS production in response to pathogen stimuli remains to be explored (Lin et al., 2015).

RLCKs and MAPK cascade activation

The rapid activation of MAPK cascades is another hallmark of the immune response required for proper ExTI. A classic MAPK cascade involves a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. Generally, in response to a stimulus MAPKKKs are phosphorylated and thereby activated, after which the phosphorylation signal is transduced linearly from the upstream MAPKKKs to the downstream MAPKs that eventually exert their signaling function (Zhang and Klessig, 2001; Zhang et al., 2018). Strikingly, increasing evidence indicates that the gap in immune signal transduction between cell-surface immune receptors and MAPK cascades is filled by RLCKs.

In rice, the LysM-RLP OsCEBiP constitutes a sandwich-type heterodimer with OsCERK1 upon the perception of chitin, which results in the activation of various defense responses including the activation of various MAPK cascades (Hayafune et al., 2014). Silencing of the RLCK *OsRLCK185* inhibits *OsCEBiP*/*OsCERK1*-triggered MAPK activation and the expression of defense-related genes; conversely, overexpression of *OsRLCK185* increases chitin-mediated activation of *OsMAPK3* and *OsMAPK6* (Yamaguchi et al., 2013). Furthermore, in response to chitin, *OsCERK1* directly phosphorylates *OsRLCK185*, which leads to the dissociation of *OsRLCK185* from the *OsCEBiP*/*OsCERK1* complex. Interestingly, two MAPKKKs, *OsMAPKKK11* and *OsMAPKKK18*, have been reported to function downstream of *OsRLCK185* and upstream of the *OsMAPKK4*-*OsMAPK3*/*OsMAPK6* module (Yamaguchi et al., 2013;

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Wang et al., 2017; Yamada et al., 2017). Therefore, it is concluded that OsRLCK185 acts as a transmitter that connects the OsCEBiP/OsCERK1 complex with a downstream MAPK cascade. Consistently, the homolog of OsRLCK185 in Arabidopsis, PBL27, provides the missing link between the CERK1/LYK5 complex in Arabidopsis and MAPKKK5, which is the Arabidopsis homolog of OsMAPKKK11 and OsMAPKKK18 (Shinya et al., 2014; Yamada et al., 2016; Yamada et al., 2017).

Interestingly, the Arabidopsis RLCK class VII subfamily 4 (consisting of PBL19, PBL20, PBL37, PBL38, PBL39, and PBL40) has been shown to bridge CERK1 and the MAPK cascade, as an *rlck vii-4* mutant, in which all six PBL members are knocked-out, shows a significantly compromised chitin-triggered MAPK activation (Rao et al., 2018). However, flg22- and elf18-induced MAPK activation is not affected in this mutant, as well as in *bik1*, *pbl1* single mutants and in a *bik1 pbl1* double mutant. Therefore, the RLCKs that regulate MAPK activation in response to the perception of flg22/elf18, remain elusive (Ranf et al., 2014; Rao et al., 2018).

Our model system: the interaction between the pathogenic fungus *Cladosporium fulvum* and tomato

The pathosystem consisting of tomato (*Solanum lycopersicum*, *Sl*) and the leaf mold fungal pathogen *Cladosporium fulvum* typically complies with the gene-for-gene model (Person et al., 1962; Flor, 1971). *C. fulvum*, which is an apoplastic, asexual, and non-obligate biotrophic fungus, uses its hyphae to obtain nutrients from the apoplast of tomato leaves, does not form specialized feeding structures such as haustoria, and does not penetrate the host cells (Joosten and de Wit, 1999; Thomma et al., 2005). To promote infection, *C. fulvum* secretes ExIPs, of which some act as avirulence factors (Avrs) in resistant tomato plants carrying the matching resistance (*R*) genes, into the extracellular space of colonized tomato leaves. Tomato, in its turn, has developed various cell-surface receptors to recognize and defend itself against the invading fungus (de Wit, 1995). These cell-surface receptors are LRR-RLPs termed Cf proteins that detect the corresponding Avrs, which results in host resistance to *C. fulvum*. This pathosystem is an excellent model to study the molecular mechanism behind a gene-for-gene interaction between plants and apoplastic pathogens (de Wit, 2016).

Over the years, several tomato Cf genes have been identified, including *Cf-2*, *Cf-4*, *Hcr9-4E*, *Cf-5*, and *Cf-9*, of which the protein products confer recognition of the *C. fulvum* effectors Avr2, Avr4, Avr4E, Avr5, and Avr9, respectively (van Kan et al., 1991; van den Ackerveken et al., 1992; Jones et al., 1994; Joosten et al., 1994; Dixon et al., 1996; Thomas et al., 1997; Dixon et al., 1998; Hammond-Kosack et al., 1998; Takken et al., 1999; Luderer et al., 2002; Westerink et al., 2004; Mesarich et al., 2014). Recently, several additional effectors of *C. fulvum* have been identified that are potentially recognized by several other Cf proteins (Mesarich et al., 2018). Cf receptor proteins are able to, either directly or indirectly, recognize *C. fulvum* Avr proteins. However, defense activation requires at least a third component, as Cf proteins

themselves lack an intracellular kinase domain for downstream signaling (Jones et al., 1994; van der Biezen and Jones, 1998; Joosten and de Wit, 1999; Luderer and Joosten, 2001).

By immuno-purification of stably expressed Cf-4 fused to a GFP affinity tag, followed by mass spectrometry, the tomato LRR-RLK regulator SOBIR1, and its close homolog SOBIR1-like, have been identified as co-purifying Cf interactors (Liebrand et al., 2013). In line with the findings in Arabidopsis, SOBIR1 interacts with various RLPs of tomato, which are involved not only in resistance (like the Cf proteins and Ve1, conferring resistance to the fungal pathogen *Verticillium dahliae* secreting the matching effector Ave1, and the ETHYLENE-INDUCING XYLANASE receptor (LeEix2)), but also in development (like the RLPs CLAVATA and TOO MANY MOUTHS) (Ron and Avni, 2004; Liebrand et al., 2013). In addition to its requirement for resistance to *C. fulvum*, SOBIR1 is also a critical component in LRR-RLP-mediated immunity against bacteria, biotrophic and necrotrophic fungi, and oomycetes (Gao et al., 2009; Liebrand et al., 2013; Zhang et al., 2013; Zhang et al., 2014; Peng et al., 2015; Zhu et al., 2017; Domazakis et al., 2018; Yang et al., 2018). SOBIR1 constitutively interacts with the Cf proteins and additional LRR-RLPs, thereby providing them with a downstream cytoplasmic signaling domain (Figure 2) (Liebrand et al., 2013; Liebrand et al., 2014; Gust and Felix, 2014). The interaction between SOBIR1 and LRR-RLPs is facilitated through the highly conserved GxxxGxxxG dimerization motif present in the transmembrane domain of SOBIR1 (Bi et al., 2016). Interestingly, SOBIR1 also forms constitutive homodimers, which is proposed to be required to keep SOBIR1 kinase activity at a low, steady-state level through auto-phosphorylation (van der Burgh et al., 2019) (Figure 2).

A well-studied example of a matching Avr/Cf couple is the Avr4/Cf-4 gene-for-gene combination. Avr4 is secreted by strains of *C. fulvum* that are avirulent on tomato plants that carry the Cf-4 resistance gene. In response to the detection of Avr4 by Cf-4, the activated Cf-4/SOBIR1 complex recruits BAK1 to initiate downstream signaling, for which the kinase activity of both SOBIR1 and BAK1 is essential (Figure 2) (Liebrand et al., 2013; Postma et al., 2016; van der Burgh et al., 2019). SOBIR1 undergoes homodimerization in the resting state, and it is proposed that, upon Avr4 perception by Cf-4, SOBIR1 undergoes rapid heterodimerization with BAK1, after which SOBIR1 trans-phosphorylates BAK1, activated BAK1 in turn trans-phosphorylates SOBIR1, leading to the full activation of the receptor complex and initiation of downstream signaling (Figure 2) (van der Burgh et al., 2019). It should be further studied what the exact role of the kinase domain of SOBIR1 is in downstream signaling, and what its specific phosphorylation sites are in these phosphorylation events.

Generally, activated cell-surface receptor complexes trigger a series of plant immune signaling steps, including RLCK phosphorylation, a burst of ROS, MAPK activation, CDPK activation, and WRKY transcription factor phosphorylation (Yu et al., 2017; Liang and Zhou, 2018). It has been shown earlier that three tomato MAPKs (*S/MPK1*, *S/MPK2*,

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and *SMPK3*) are simultaneously activated upon the activation of the Cf-4/SOBIR1 complex by Avr4 (Stulemeijer et al., 2007). Although many described ExTI responses confer only a weak basal resistance to a broad spectrum of pathogens, Avr4/Cf-4- and also Avr9/Cf-9-triggered ExTI is associated with a typical HR. This HR is well known to occur upon many InTI responses that are triggered by NLRs (van der Burgh and Joosten, 2019). Intriguingly, this HR is strongly reduced in *NbMAPKKKε*-silenced *N. benthamiana* plants, indicating that *NbMAPKKKε* is required for Avr4/Cf-4- and Avr9/Cf-9-triggered plant immunity (Figure 2) (Melech-Bonfil and Sessa, 2010). Consistently, silencing of its ortholog in tomato, *SIMAPKKKε*, attenuates tomato resistance to strains of *Xanthomonas campestris* and *Pseudomonas syringae* (Melech-Bonfil and Sessa, 2010). In addition, a signaling cascade composed of one MAPKK (MEK2) and two MAPKs, WOUND-INDUCED PROTEIN KINASE (WIPK) and SA-INDUCED PROTEIN KINASE (SIPK), which are the orthologs of Arabidopsis MPK3 and MPK6, respectively, has been identified to function downstream of *MAPKKKε* (Figure 2) (Romeis et al., 1999; Stulemeijer et al., 2007; Melech-Bonfil and Sessa, 2010). It will be interesting to investigate whether knocking down or knocking out *MAPKKKε*, *MEK2*, and/or *MPK1/2/3/6*, compromises resistance of Cf-4 tomato to strains of *C. fulvum* secreting Avr4.

Related to this observation, it is hypothesized that the Cf-4/SOBIR1/BAK1 complex recruits RLCKs to subsequently activate a variety of substrate proteins involved in immune signaling. These substrate proteins include MAPKs and the functional orthologue of Arabidopsis RBOHD in *N. benthamiana* (referred to as *NbRBOHB*), resulting in the activation of a MAPK cascade and, the production of ROS, respectively (Yoshioka et al., 2003; Adachi et al., 2015; Li et al., 2015). The RLCK AVR9-Cf9-INDUCED KINASE-1 (*ACIK1*) has been found to play an essential role in Avr4/Cf-4- and Avr9/Cf-9-mediated HR in *N. benthamiana*, and silencing of *ACIK1* in tomato compromised Cf-9-mediated resistance to *C. fulvum* (Rowland et al., 2005). Nevertheless, it currently remains unknown which RLCKs specifically regulate ROS accumulation through the activation of RBOHB in *N. benthamiana* and tomato, and which particular RLCKs fill the gap between the activated Cf/SOBIR1 cell-surface receptor complex and downstream MAPK cascades. In addition, how these RLCKs eventually activate the resistance of tomato that carries the appropriate Cf gene to *C. fulvum*, requires further studies (Figure 2).

CDPKs, which are unique Ca^{2+} sensor protein kinases, do not only activate RBOHBs to regulate ROS accumulation, but they also play a role in transcriptional reprogramming during the onset of ExTI (Couto and Zipfel, 2016). In potato (*Solanum tuberosum*, *St*), CDPK4 and CDPK5 have been shown to phosphorylate the N-terminal region of RBOHB directly in a calcium-dependent manner (Kobayashi et al., 2007). In line with this finding, Arabidopsis CDPK5, which is activated upon stimulation by ExIPs, also directly phosphorylates RBOHD and mediates plant defense responses (Dubiella et al., 2013). Strikingly, earlier work has revealed that CDPKs are also involved in the Avr/Cf gene-

for-gene interaction. A tobacco (*Nicotiana tabacum*, *Nt*) CDPK, referred to as 67-/70-kD CDPK, has been reported to be activated in *Cf*-9-transgenic tobacco, as its electrophoretic mobility is shifted from 68 to 70 kD after treatment with the Avr9 effector (Romeis et al., 2000). Moreover, silencing of *CDPK2* in *N. benthamiana* compromises both the Avr4/*Cf*-4- and Avr9/*Cf*-9-triggered HR (Figure 2) (Romeis et al., 2001). Further studies are needed to dissect how CDPKs interact with various signaling partners to regulate *Cf*-mediated immune signaling.

Despite locating on the plasma membrane and mediating ExTI, the *Cf*-4/SOBIR1 complex also requires NLRs for downstream signaling. A good example is NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 1 (*NRC1*), which was identified by performing a cDNA-amplified fragment length polymorphism (AFLP) analysis on *Cf*-4- and Avr4-expressing tomato seedlings undergoing a synchronized HR (Gabriëls et al., 2006). Knocking down *NRC1* in tomato dampens the *Cf*-4-dependent HR and resistance to *C. fulvum*, these observations are further substantiated by silencing its ortholog in *N. benthamiana* stably expressing *Cf*-4, by which the Avr4-triggered HR is compromised (Gabriëls et al., 2007). Additionally, the Avr4-induced HR is also reduced in *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*)- and *MEK2*-silenced *N. benthamiana*:*Cf*-4 plants, indicating that, like *NRC1*, *EDS1* also plays a role in *Cf*-4/SOBIR1-mediated signaling. It has been observed that *NRC1* functions downstream of *EDS1* and upstream of a MAPK cascade (Figure 2) (Gabriëls et al., 2007).

Furthermore, to unveil the Avr/*Cf*-specific HR-associated transcriptome in Solanaceae, a transcriptome analysis was performed on the *Cf*-4- and Avr4-expressing tomato seedlings by RNA-sequencing, and subsequent network analysis. The result shows that WRKY transcription factors are the most prominent regulators of the transcriptional reprogramming that is associated with the Avr4/*Cf*-4-mediated HR in tomato (Etalo et al., 2013).

Altogether, over the last two decades, major progress has been made to decipher how plant innate immunity is triggered and regulated. However, the components involved in the Avr4/*Cf*-4 signaling pathway, the way they are interconnected, and the mechanisms by which they regulate tomato resistance to *C. fulvum*, remain largely unknown. Especially when compared to the findings in the model plant *Arabidopsis*, there is still a long way to go. Therefore, in addition to solving the interconnection of known signaling partners, future studies that focus on determining the downstream signaling interactors and their regulatory mechanisms should provide us with a better understanding of how eventually the *Cf*-4/SOBIR1/BAK1 complex triggers a robust resistance against *C. fulvum*.

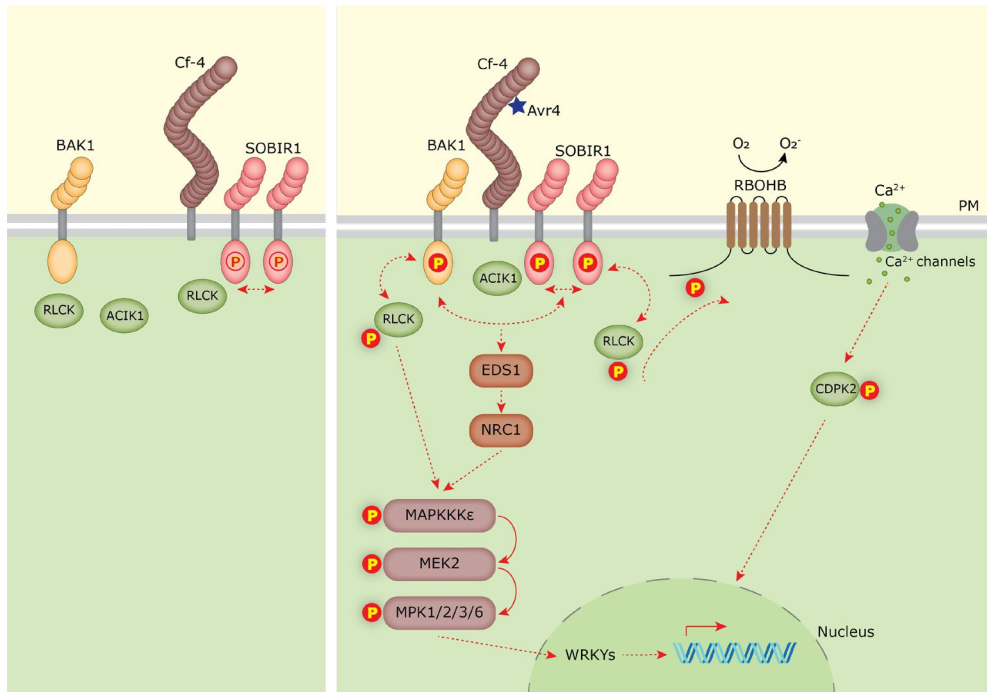


Figure 2. Schematic overview of the proposed signaling events that take place downstream of the Cf-4/SOBIR1/BAK1 complex upon the perception of the matching effector Avr4 from *C. fulvum* in tomato. In the absence of the Avr4 effector (left panel), the resistance protein Cf-4, which is an LRR-RLP localizing at the plasma membrane (PM), constitutively interacts with the LRR-RLK SOBIR1. At the same time, SOBIR1 constitutively forms homodimers that allow basal activation of the SOBIR1 kinase domain through cross-phosphorylation. The common regulator BAK1, which is also an LRR-RLK, is sequestered from the Cf-4/SOBIR1 complex. In addition to the RLCK ACIK1, it is hypothesized that some other RLCKs, which can act either as positive or negative regulators, associate with SOBIR1 and/or BAK1. In response to the perception of Avr4 by Cf-4 (right panel), the Cf-4/SOBIR1 complex recruits BAK1, after which trans-phosphorylation events between SOBIR1 and BAK1 take place and ACIK1 associates with their phosphorylated kinase domains. Likely, the activated Cf-4/SOBIR1/BAK1 complex further phosphorylates additional RLCKs, resulting in the activation of downstream signaling components, such as the RBOHB oxidase and a MAPK cascade. The cytoplasmic lipase-like protein EDS1 and the NLR NRC1 are also required for Avr4/Cf-4-dependent HR and resistance against *C. fulvum*. Most likely, they function upstream of a MAPK cascade consisting of MAPKKKε-MEK2-MPK1/2/3/6. Additionally, the calcium-dependent protein kinase CDPK2 also plays an essential role in the Avr4/Cf-4 signaling pathway. Solid arrows indicate signaling events of which the mechanism is known and which are supported by the literature, whereas dashed arrows indicate proposed events. The red open (left panel) and filled (right panel) circles with 'P' inside represent a low-level and a high-level of phosphorylation, respectively.

Thesis outline

Plants are sessile organisms that lack an adaptive immune system and therefore they largely rely on their innate immune system to cope with the very diverse threats from microbial pathogens. The last two decades have witnessed remarkable progress in our understanding of the initiation and regulation of plant innate immunity, especially in the model plant *Arabidopsis*. However, still relatively little is known concerning Avr4/Cf-4-triggered immunity in the Solanaceous plant tomato. Thus, this thesis was aimed at characterizing how the Cf-4/SOBIR1/BAK1 complex is activated upon Avr4 perception and how the kinase domain of SOBIR1 subsequently transmits specific downstream signals. Furthermore, the work described in this thesis was initiated to identify the cytoplasmic signaling components that are required for Cf-4 functioning.

Cf-4 is a tomato RLP-encoding gene and the Cf-4 resistance protein is also functional in *N. benthamiana*. In **Chapter 2**, we show that we have successfully knocked out *SOBIR1*, as well as its homolog *SOBIR1-like*, in *N. benthamiana* and *N. benthamiana:Cf-4*, by using CRISPR/Cas9 technology. We observed that both the Avr4/Cf-4- and Avr9/Cf-9-triggered HR are abolished in the two *N. benthamiana sobir1/sobir1-like* double knock-out lines and in the *N. benthamiana sobir1* single knock-out line. Consistently, stable transgenic *N. benthamiana* plants expressing *Cf-4*, in which *SOBIR1* is knocked out, are non-responsive to Avr4. As expected, these phenotypes can be complemented by transiently expressing *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like* in leaves of the knock-out plants. Hence, these plants are important materials for studying the fundamentals of plant immunity mediated by LRR-RLPs.

It has been proposed that trans-phosphorylation events take place between the kinase domains of SOBIR1 and BAK1 upon the perception of Avr4 by Cf-4. In **Chapter 3** we provide *in vitro* evidence that supports this model. We, first of all, show that SOBIR1 itself exhibits strong auto-phosphorylation activity. Furthermore, SOBIR1 is able to directly phosphorylate BAK1, whereas BAK1, in its turn, can directly phosphorylate SOBIR1. We propose that these trans-phosphorylation events also occur *in planta* and that these processes eventually result in the full activation of RLP/SOBIR1/BAK1-containing immune complexes. Moreover, the phosphorylatable Thr residue present in the kinase domain of *NbSOBIR1* at position 522, as well as its analogous Thr residue in tomato SOBIR1 and SOBIR1-like, which is actually present in the activation segment of the kinase domain of SOBIR1, was identified to be required for the downstream Avr4/Cf-4-triggered immune responses and for SOBIR1 intrinsic kinase activity.

Chapter 4 investigates the importance of tyrosine (Tyr/Y) residues in the kinase domain of SOBIR1 in regulating plant innate immunity. Similar to the approach followed in Chapter 3, by performing a site-directed mutagenesis screen, combined with complementation studies in the knock-out plants described in Chapter 2, we show that *NbSOBIR1*^{Y469}, as well as its analogous Tyr residues in *SISOBIR1* and *SISOBIR1-like*, plays an essential role in Avr4/Cf-4-triggered MAPK activation and subsequent

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mounting of the HR, and the BcXYG1-triggered HR. Nevertheless, this particular Tyr residue is not required for the intrinsic kinase activity of SOBIR1 and the Avr4/Cf-4-induced ROS production. Furthermore, additional Tyr residues were identified to be crucial for the BcXYG1-triggered HR. Intriguingly, mass spectrometry has not been able to detect phosphorylation of Tyr residues in the kinase domain of SOBIR1, and therefore it is likely that the important Tyr residues in the kinase domain of SOBIR1 regulate the initiation of plant immune responses by binding specific substrates, instead of by their phosphorylation level affecting SOBIR1 signaling activity.

RLCKs are the direct substrates of activated receptor complexes and these cytoplasmic kinases have been recognized to bridge the cell-surface receptors and downstream signaling components. **Chapter 5** aims to identify and characterize the RLCKs that play a role downstream of the Cf-4/SOBIR1/BAK1 complex. By knocking out multiple genes encoding different RLCK-VII subfamily members in *N. benthamiana:Cf-4*, we show that members of the RLCK-VII-6, -7, and -8 subfamilies are required for the Avr4/Cf-4-triggered ROS burst. Subsequent studies focusing on the members from RLCK-VII-6 demonstrate that they are essential for ROS production induced by multiple ExIPs, but not for Avr4/Cf-4-triggered MAPK activation and the HR. Moreover, although the various RLCK-VII-6 members have a different subcellular localization, they all appear to function redundantly to positively regulate the Avr4/Cf-4-triggered ROS burst.

Finally, **Chapter 6** provides recent advances in understanding the mechanisms underlying the activation of plant innate immunity by cell surface receptor complexes. Furthermore, we discuss the most important findings that are described in this thesis in a broader context.

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Chapter 2

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

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Abstract

To cope with diverse threats from microbial pathogens, plants deploy various cell-surface receptor-like kinases (RLKs) and receptor-like proteins (RLPs) to monitor the presence of extracellular immunogenic patterns (ExIPs). Cf-4 and Cf-9, which are well-studied tomato (*Solanum lycopersicum*, *Sl*) leucine-rich repeat (LRR)-RLPs, mediate host resistance against the pathogenic extracellular fungus *Cladosporium fulvum* secreting the matching avirulence factors Avr4 and Avr9, respectively. Cf-4 and Cf-9 lack an intracellular kinase domain for downstream signaling and therefore constitutively interact with the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1). Here, we report that we knock out *SOBIR1*, and its homolog *SOBIR1-like*, in *Nicotiana benthamiana* (*Nb*) using CRISPR/Cas9 gene-editing technology. The *N. benthamiana sobir1/sobir1-like* knock-out plants have become non-responsive to the Avr4/Cf-4 or Avr9/Cf-9 combination, as a hypersensitive response (HR) is abolished in all the knock-out plants. In addition, these plants exhibit increased susceptibility to the hemibiotrophic oomycete pathogens *Phytophthora capsici* and *P. infestans*. Furthermore, knocking out *SOBIR1*(/*SOBIR1-like*) in *N. benthamiana* stably expressing the *Cf-4* transgene eliminates Cf-4 function. We also show that a 22-amino acid epitope from bacterial flagellin, flg22, normally triggers a rapid and monophasic reactive oxygen species (ROS) burst in wild-type *N. benthamiana* plants, whereas this ROS burst becomes biphasic in *N. benthamiana:Cf-4 sobir1*(/*sobir1-like*) mutant plants. This observation reveals potential crosstalk between the signaling pathways downstream of RLP/SOBIR1 and downstream of FLS2. Interestingly, transient expression of *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like*, restores the loss of *NbSOBIR1* in *sobir1/sobir1-like* mutant lines and reconstitutes the Avr4/Cf-4-triggered HR. Therefore, we anticipate that these mutant lines will allow complementation studies that are of use for studying the fundamentals of plant immunity mediated by LRR-RLPs.

Introduction

Plants are challenged by a plethora of agents causing biotic stresses, including pathogenic micro-organisms (Dangl and Jones, 2001; Zipfel, 2014; Zhou and Zhang, 2020). To defend themselves, plants have developed a two-layered innate immune system to recognize non-self and modified-self signals, and subsequently activate immune responses (Dangl and Jones, 2001; Jones and Dangl, 2006; Couto and Zipfel, 2016). The first layer of plant innate immunity is mediated by cell-surface receptors, which localize on the plasma membrane and perceive extracellular immunogenic patterns (ExIPs) (Dodds and Rathjen, 2010; Gust et al., 2017; van der Burgh and Joosten, 2019). The second layer is mediated by the intracellular receptors, which recognize intracellular immunogenic patterns (InIPs) (van der Burgh and Joosten, 2019). InIPs are mainly cytoplasmic effectors, which are secreted by pathogens to suppress immune responses triggered by cell-surface receptors (Dodds and Rathjen, 2010; van der Burgh and Joosten, 2019).

So far, all known plant cell-surface receptors that carry an ectodomain consisting of leucine-rich repeats (LRRs), are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Monaghan and Zipfel, 2012; Böhm et al., 2014; Zipfel, 2014; Ranf, 2017). They share the same overall structure. However, in contrast to RLKs, RLPs lack a cytoplasmic kinase domain for downstream immune signaling (Böhm et al., 2014; Zipfel, 2014; Ranf, 2017). Upon recognition of ExIPs, cell-surface receptors trigger a series of immune events, including the generation of reactive oxygen species (ROS), the activation of mitogen-associated protein kinases (MAPKs), the expression of immune-related genes, and in some cases, a programmed cell death (PCD) at the infection sites, referred to as the hypersensitive response (HR) (Monaghan and Zipfel, 2012; Macho and Zipfel, 2015; van der Burgh and Joosten, 2019; Li et al., 2020).

In the last decades, tremendous advances have been made in deciphering cell-surface receptor-mediated immune signaling. FLAGELLIN-SENSING 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR) are well-characterized RLKs in *Arabidopsis thaliana* (*At*), which perceive bacterial flagellin (or its highly conserved N-terminal 22-amino acid epitope, flg22) and elongation factor TU (or its N-acetylated 18-amino acid peptide, elf18), respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). Upon perception of their matching ligands, FLS2 and EFR recruit another RLK BRI-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1), which is a common regulator involved in plant development and defense. BAK1 recruitment subsequently leads to the rapid trans-phosphorylation of the cytoplasmic kinase domains of FLS2/EFR and BAK1 (Chinchilla et al., 2007; Heese et al., 2007; Lu et al., 2010; Schulze et al., 2010; Roux et al., 2011; Macho and Zipfel, 2014). Hereafter, the receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1) gets phosphorylated, followed by its dissociation from the receptor complex, resulting in the initiation of downstream

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defense signaling (Lu et al., 2010; Li et al., 2014; Macho and Zipfel, 2014; Lal et al., 2018).

RLPs, such as tomato (*Solanum lycopersicum*, *Sl*) Cf-4 that mediates host resistance against the pathogenic extracellular fungus *Cladosporium fulvum* secreting the matching avirulence factor Avr4 (Joosten et al., 1994; Thomas et al., 1997), lack an intracellular kinase domain for immune signaling and have been shown to constitutively interact with the RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1). Thus, the RLP/SOBIR1 complex is postulated to function as a two-component RLK (Gao et al., 2009; Liebrand et al., 2013; Gust and Felix, 2014; Liebrand et al., 2014). Initially, SOBIR1 was identified as a suppressor of the Arabidopsis BAK1-INTERACTING RECEPTOR KINASE 1 (BIR1). BIR1 is a negative regulator of plant defense, as a *bir1-1* knock-out mutant shows constitutive defense responses. Interestingly, these phenotypes are partially suppressed by the *sobir1-1* knock-out mutation, suggesting that SOBIR1 is a positive regulator of immunity (Gao et al., 2009). SOBIR1 has been identified to be essential for Cf-4 accumulation and function, suggesting that this RLK also plays a role as a scaffold protein for RLPs (Liebrand et al., 2013). Consistent with the initiation of FLS2- and EFR-mediated signaling, BAK1 is swiftly recruited by the Cf-4/SOBIR1 complex upon recognition of the matching, secreted *C. fulvum* effector Avr4 by the Cf-4 resistance protein (Postma et al., 2016). BAK1 recruitment to the activated RLP/SOBIR1 complex appears to be a general process, as this is also shown for the activated RLP23/SOBIR1 complex (Albert et al., 2015). RLP23 from Arabidopsis is involved in the perception of the ExIP necrosis and ethylene-inducing peptide 1-like protein nlp20, which is produced by several bacterial, fungal, and oomycete species. Furthermore, the kinase activity of SOBIR1 and BAK1 is required for immune signaling, and it has been proposed that upon BAK1 recruitment, 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 (Liebrand et al., 2014; Couto and Zipfel, 2016; van der Burgh et al., 2019), little is known about how the RLP/SOBIR1/BAK1 complex exactly functions at the level of complex formation and downstream signal initiation. What is known, is that the tomato 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).

As mentioned above, overexpression of *AtSOBIR1* in Arabidopsis, as well as in the model Solanaceous plant *Nicotiana benthamiana* (*Nb*), 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 *SISOBIR1* or *NbSOBIR1* was overexpressed in *N. benthamiana* (Wu et al., 2017). Therefore, over the past years, the *AtSOBIR1*-induced constitutive immunity in *N.*

benthamiana, visible as an HR at the site of agro-infiltration of *AtSOBIR1*, has been commonly employed to decipher the mechanism behind RLP/*SOBIR1*-mediated plant immunity. Similar to tomato, the genome of *N. benthamiana* encodes two homologous *SOBIR1* genes, referred to as *NbSOBIR1* and *NbSOBIR1-like*. Therefore, for performing complementation studies, endogenous *NbSOBIR1(-like)* genes are silenced in *N. benthamiana:Cf-4* by virus-induced gene silencing (VIGS). Subsequent transient expression of *AtSOBIR1* and various mutants of this RLK is possible, as due to large nucleotide sequence differences with the *NbSOBIR1(-like)* genes, these are not targeted by the activated silencing machinery of the plant (Liebrand et al., 2013; Bi et al., 2016; van der Burgh et al., 2019). 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 *NbSOBIR1(-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., 2013; Ran et al., 2013; Belhaj et al., 2015). Therefore, we here took advantage of the CRISPR/Cas9 system to knock out *SOBIR1* and its homolog *SOBIR1-like* in the model plant *N. benthamiana*, as well as in *N. benthamiana* stably expressing the *Cf-4* transgene. *N. benthamiana* is a widely used experimental host, which is highly amenable to transformation and transient expression studies (Goodin et al., 2008). *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. In addition, we show that knocking out *SOBIR1/SOBIR1-like* enhances *N. benthamiana* susceptibility to *Phytophthora capsici* and *P. infestans*, but not to tomato spotted wilt virus (TSWV) and *Botrytis cinerea*. We anticipate that these mutant lines will be very helpful for studying the fundamentals of plant immunity mediated by RLPs.

Results

Generation of *sobir1/sobir1-like* mutants in *N. benthamiana* by CRISPR/Cas9

To knock out both *SOBIR1* and *SOBIR1-like* in *N. benthamiana*, six single-guide RNAs (sgRNAs) targeting the open reading frame (ORFs) of both genes (Figure S1 and Table S1) were designed using CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>) (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. Both constructs were expected to yield a 61 bp deletion in the ORF of *SOBIR1-like*. Construct 1 was designed to generate a 310 bp deletion in the ORF of *SOBIR1*, whereas Construct 2 was made to delete 261 bp in the *SOBIR1* ORF (Figure S1). The effectiveness and efficiency of the generated constructs were confirmed by studies based on their transient expression before their stable transformation to explants of *N. benthamiana* (Figure S2). Compared to wild-

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type (WT) *N. benthamiana*, transient expression of Construct 1 and 2 induced the expected deletions in *SOBIR1*, indicating the high editing efficiency of Construct 1 in sgRNA1- and sgRNA2-target regions, and of Construct 2 in sgRNA3- and sgRNA4-target regions. However, no obvious deletion was observed in *SOBIR1-like* (Figure S2A). As the sgRNA5-target region contains a *HpyCH4V* restriction site and the sgRNA6-target region contains a *HinfI* restriction site (Figure S2B), *SOBIR1-like* amplicons were purified and digested with the two restriction enzymes to determine whether mutations had been introduced in the sgRNA5- or sgRNA6-target regions. Compared to WT, an extra band was observed in Construct 1- and 2-transiently transformed *SOBIR1-like*, indicating that at least one restriction site was missing (Figure S2C). Therefore, it was concluded that both constructs were also able to edit the sequence of *SOBIR1-like*.

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. Two homozygous *sobir1/sobir1-like* double knock-out lines, generated by Construct 2, were obtained (Table S3). *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 sgRNA6-target region ORF of *SOBIR1-like* (Figure 1A). Similarly, there is a 1 bp insertion in the sgRNA3-target region and a 1 bp deletion in the sgRNA4-target region in *SOBIR1*, and a 4 bp deletion in the sgRNA6-target region in *SOBIR1-like* in the *N. benthamiana sobir1/sobir1-like* line #2 (Figure 1A).

Transient co-expression of the 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 plants, 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 the disruption of their ORFs. Interestingly, complementation of the *SOBIR1(-like)* knockouts through transient expression of *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like*, together with agro-infiltration of 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 the RLP-mediated immunity of tomato and that the *N. benthamiana sobir1/sobir1-like* mutant plants form a robust basis for complementation studies.

Knocking out *SOBIR1* in *N. benthamiana*

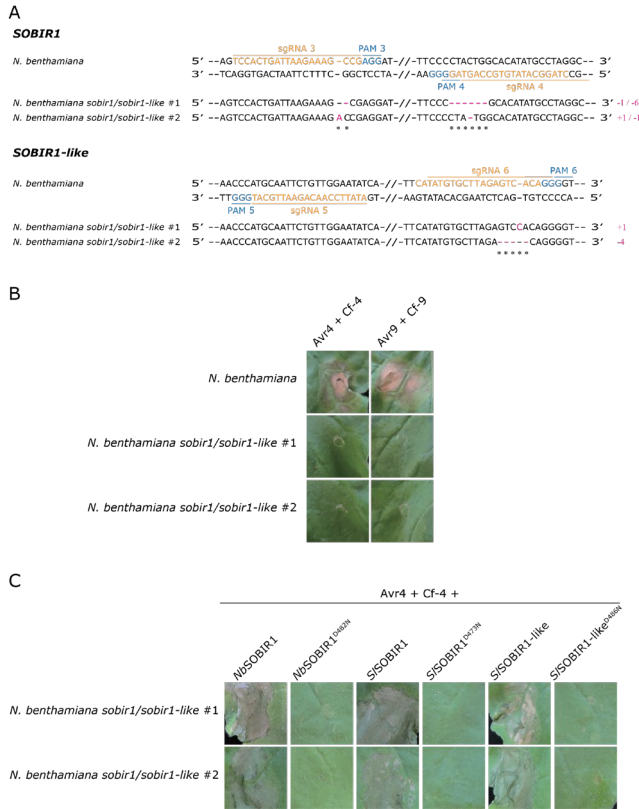


Figure 1. CRISPR/Cas9-induced targeted knockout of *SOBIR1*/*SOBIR1-like* in *N. benthamiana* abolishes the responsiveness to matching Avr/Cf combinations. (A) Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel), targeted by single-guide RNAs (sgRNAs) in the two *N. benthamiana* double *sobir1/sobir1-like* knock-out lines, with wild-type *SOBIR1* and *SOBIR1-like* sequences, respectively. The sgRNA sequences are indicated in orange, and the protospacer associated 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 two panels and lower two panels). **(C)** Complementation by transient expression of *NbSOBIR1*, *SISOBIR1* or *SISOBIR1-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 *SOBIR1* mutants. *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like*, as well as their corresponding kinase-dead mutants (negative controls), were 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 (OD₆₀₀) of 0.5, and all leaves were photographed at 5 days post infiltration (dpi). Experiments were repeated at least three times, and similar results were obtained. Representative pictures are shown.

Generation of *sobir1/sobir1-like* mutants in *N. benthamiana:Cf-4* by CRISPR/Cas9

The transgenic *N. benthamiana* line expressing *Cf-4* is commonly used by us for elucidating the molecular mechanisms of signal transduction events triggered by the *Cf-4* resistance protein 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* by using the CRISPR/Cas9 system. As described before, two CRISPR/Cas9 constructs were transformed to *N. benthamiana:Cf-4*. Two homozygous double knock-out lines were obtained with disruptions in both the ORF of *SOBIR1* and *SOBIR1-like*, which were introduced by Construct 1 (Table S3, Figure 2A and S3). In agreement with our previous finding, transient expression of *Avr4* triggered an HR in *N. benthamiana:Cf-4* plants. However, the knock-out lines were non-responsive to *Avr4* (Figure 2B), again confirming that *Cf-4* functionality requires functional *SOBIR1*(-like). Complementation with *NbSOBIR1*, *S/SOBIR1*, or *S/SOBIR1-like* by their transient expression in leaves of the double knock-out *N. benthamiana:Cf-4* plants, in combination with transient expression of *Avr4*, again restored the *Cf-4*-mediated HR, whereas such restoration did not take place upon co-expression of the corresponding kinase-dead mutants of *SOBIR1* (Figure 2C).

The mutant lines were further validated by monitoring the production of ROS, which is a very early downstream response upon immune activation. FLS2, which is also present in *N. benthamiana*, 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 2D). As expected, the biphasic *Avr4*-triggered ROS burst was completely abolished in the two mutant lines (Figure 2D), 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 sobir1/sobir1-like* double mutants, suggesting that there is potential crosstalk taking place between RLP/*SOBIR1*- and FLS2-triggered pathways.

Knocking out *SOBIR1* in *N. benthamiana*

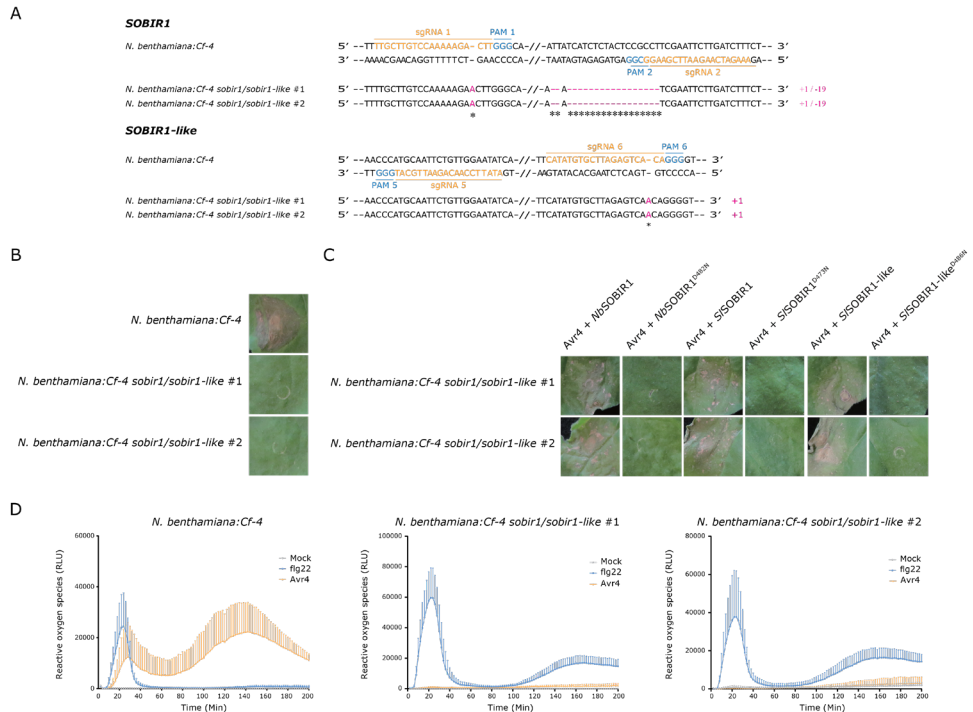


Figure 2. CRISPR/Cas9-induced targeted knockout of *SOBIR1/SOBIR1-like* in transgenic *N. benthamiana*:*Cf-4* abolishes the functionality of the *Cf-4* transgene. (A) Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel) targeted by sgRNAs in the two *N. 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)** *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 and lower panels). **(C)** Complementation by transient expression of *NbSOBIR1*, *SISOBIR1* or *SISOBIR1-like*, restores the *Avr4*/*Cf-4*-specific HR in the two *N. benthamiana*:*Cf-4* *sobir1/sobir1-like* mutants, whereas this restoration does not take place upon transient expression of the corresponding kinase-dead mutants. *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like*, as well as their corresponding kinase-dead mutants (negative controls), were transiently co-expressed with *Avr4*, in leaves of the two *N. benthamiana*:*Cf-4* *sobir1/sobir1-like* knock-out lines. Each construct was agro-infiltrated at an OD₆₀₀ of 0.5, and the leaves were photographed at 5 dpi. **(D)** *Avr4* fails to induce a reactive oxygen species (ROS) burst in the two *N. benthamiana*:*Cf-4* *sobir1/sobir1-like* knock-out lines. Leaf discs of *N. benthamiana*:*Cf-4* (left panel) and the two *N. benthamiana*:*Cf-4* double *sobir1/sobir1-like* knock-out lines (middle and right panels), were treated with 0.1 μ M *Avr4* or 0.1 μ M flg22 (positive control), or with water (mock) (negative control). ROS production is expressed as relative light units (RLU), and the data are represented as mean + standard deviation (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, the response to flg22 manifests itself as a biphasic ROS burst, whereas in *N. benthamiana*:*Cf-4* the flg22-triggered ROS burst is monophasic.

NbSOBIR1-like is not functional

N. benthamiana contains two *SOBIR1* homologs, referred to as *NbSOBIR1* and *NbSOBIR1-like*. It has been reported that the Avr4-triggered HR was severely compromised when *NbSOBIR1* itself was silenced in *N. benthamiana:Cf-4* (Liebrand et al., 2013). In this study, we also obtained two *sobir1* single knock-out lines, for which both the *N. benthamiana sobir1* mutant and the *N. benthamiana:Cf-4 sobir1* mutant contain a 1 bp insertion in the ORF of the *SOBIR1* gene, whereas the sequence of the *SOBIR1-like* gene has remained intact (Table S3 and Figure 3A). To further find out whether *NbSOBIR1-like* plays a role in RLP-mediated immunity, we transiently expressed the Avr4/Cf-4 or Avr9/Cf-9 combination in the *N. benthamiana sobir1* mutant, and Avr4 in the *N. benthamiana:Cf-4 sobir1* mutant. Interestingly, no HR was observed in either the *N. benthamiana sobir1* single knock-out mutant or the *N. benthamiana:Cf-4 sobir1* single knock-out mutant (Figure 3B), similar to their corresponding double knock-out mutants (Figure 1B and 2B). This observation is in agreement with our earlier finding that *NbSOBIR1-like* is not expressed or expressed only at a very low level (Liebrand et al., 2013), indicating that this gene is not functional.

In addition, flg22, but not Avr4, was able to induce a ROS burst in the *N. benthamiana:Cf-4 sobir1* single knock-out mutant, further confirming that this single mutant line is non-responsive to Avr4 and *NbSOBIR1-like* is indeed non-functional. Also, in this case, the flg22-induced ROS burst has become biphasic.

Knocking out *SOBIR1* in *N. benthamiana*

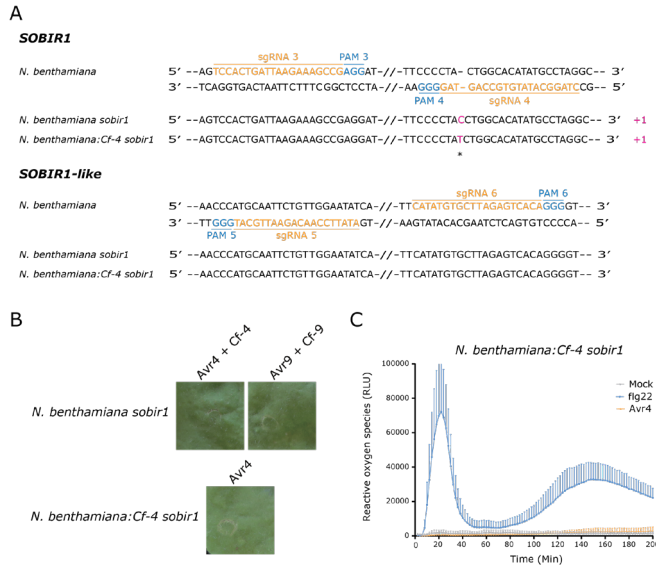


Figure 3. CRISPR/Cas9-induced targeted knockout of *SOBIR1* only in *N. benthamiana* and transgenic *N. benthamiana: Cf-4*, abolishes the responsiveness to matching Avr/Cf combinations and the functionality of the *Cf-4* transgene, respectively. (A) Nucleotide sequence alignment of the regions in *SOBIR1* (upper panel) and *SOBIR1-like* (lower panel) targeted by sgRNAs in the *sobir1* 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. Note that the sequence of *SOBIR1-like* is not altered (B) Transient co-expression of *Cf-4* with *Avr4*, or of *Cf-9* with *Avr9*, failed to induce cell death in the leaves of the *N. benthamiana sobir1* mutant line (upper two panels). Furthermore, *Agrobacterium*-mediated expression of *Avr4* did not trigger cell death in the *N. benthamiana: Cf-4* single *sobir1* knock-out line. Each construct was agro-infiltrated at an OD₆₀₀ of 0.5, and the leaves were photographed at 5 dpi. (C) *Avr4* fails to induce a ROS burst in the *N. benthamiana: Cf-4* single *sobir1* knock-out line. Leaf discs of *N. benthamiana: Cf-4 sobir1* knock-out plants were treated with 0.1 μ M *Avr4* or 0.1 μ M flg22 (positive control), or with water (mock) (negative control). ROS production is expressed as RLU, and the data are represented as mean \pm SD. Experiments were repeated at least three times, and similar results were obtained. Representative pictures are shown. Note that also in this case the response to flg22 becomes biphasic.

Knocking out *SOBIR1/SOBIR1-like* enhances *N. benthamiana* susceptibility to *Phytophthora capsici* and *P. infestans*, but not to tomato spotted wilt virus (TSWV) and *Botrytis cinerea*

Since plants rely on cell-surface receptors to sense ExIPs and activate immunity, and *SOBIR1* is required for RLP function (Liebrand et al., 2014; van der Burgh and Joosten, 2019), we aimed to determine whether *SOBIR1* is involved in disease resistance. It has been reported that *SOBIR1* contributes to defense against the oomycete pathogens *Phytophthora parasitica* in tomato and *P. infestans* in *N. benthamiana* (Peng et al., 2015; Domazakis et al., 2018). However, in both cases, the *SOBIR1/SOBIR1-like* gene

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was only knocked down in plants by VIGS, but not knocked out. Therefore, we here challenged wild-type *N. benthamiana* plants, as well as *sobir1/sobir1-like* double knock-out mutant plants, with the oomycete pathogens *P. capsici* and *P. infestans*. To do this, leaves were taken from the different 5-week-old *N. benthamiana* lines and inoculated with mycelial plugs of *P. capsici* or *P. infestans*. Lesion growth caused by *P. capsici* was quantified at 2 dpi, while the areas of the lesions resulting from infection by *P. infestans* were measured at 5 dpi. In agreement with previous findings, when compared to the wild-type plants, the *N. benthamiana sobir1/sobir1-like* mutant became more susceptible to infection by both *P. capsici* and *P. infestans* (Figure 4A and 4B). It is noteworthy that no disease symptoms appeared on the leaves of wild-type *N. benthamiana* plants after inoculation with *P. infestans* at 5 dpi, whereas by that time lesion development on the mutant was apparent. Taken together, we conclude that SOBIR1 contributes to host resistance against the oomycete pathogens *P. capsici* and *P. infestans*.

Tomato spotted wilt virus (TSWV), one of the most devastating plant viruses, infects over 900 plant species, including the model plants *Arabidopsis* and *N. benthamiana* (Scholthof et al., 2011; Oliver and Whitfield, 2016; Huang et al., 2020). To determine whether SOBIR1 is involved in defense against TSWV, 3-week-old wild-type *N. benthamiana* plants and *sobir1/sobir1-like* knock-out mutant plants were dusted with carborundum powder and rub inoculated with TSWV. Four weeks after inoculation, the systemic presence of TSWV in each plant line was determined by Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA). The result showed that the two *sobir1/sobir1* mutant lines exhibited a similar susceptibility to TSWV as the wild-type *N. benthamiana* plants (Figure 4C), indicating that SOBIR1 is not required for plant basal defense against TSWV.

The grey mold fungus *B. cinerea*, which is an aggressive necrotrophic pathogen, infects more than 1,000 plant species worldwide (Williamson et al., 2007; Veloso and van Kan, 2018). Several ExIPs from *B. cinerea* have been reported to be recognized by RLPs in a SOBIR1/BAK1-dependent manner. For instance, *Arabidopsis* RLP42 recognizes *B. cinerea* ENDOPOLYGALACTURONASE 3 (BcPG3) and activates plant immunity (Zhang et al., 2014). To study whether SOBIR1 contributes to resistance against *B. cinerea*, 4-week-old wild-type *N. benthamiana* plants and *sobir1/sobir1-like* knock-out mutant plants were inoculated with droplets of *B. cinerea* spore suspension, and the lesion sizes were recorded at 2 dpi and 3 dpi. As shown in Figure 4D, no significant differences in lesion size were observed when the tested plant lines were compared with each other, suggesting that knocking out of *SOBIR1/SOBIR1-like* does not change *N. benthamiana* susceptibility to *B. cinerea*.

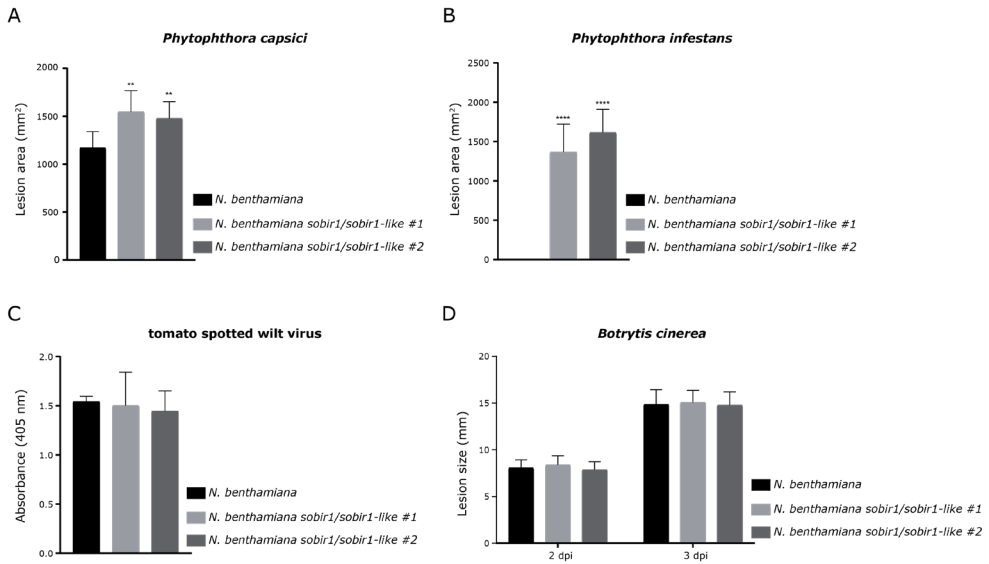


Figure 4. Knocking out *SOBIR1/SOBIR1-like* enhances *N. benthamiana* susceptibility to *Phytophthora capsici* and *P. infestans*, but not to tomato spotted wilt virus (TSWV) and *Botrytis cinerea*. (A) and (B) *N. benthamiana sobir1/sobir1-like* mutants display enhanced susceptibility to *Phytophthora capsici* and *P. infestans*. Lesion areas on leaves of 5-week-old wild-type *N. benthamiana* and two *N. benthamiana sobir1/sobir1-like* mutants were quantified at 2 days after mycelial plug-inoculation with *P. capsici*, or at 5 days after mycelial plug-inoculation with *P. infestans*. Bars represent the mean lesion areas + SD (**, $P < 0.01$, ****, $P < 0.0001$, one-way ANOVA). Experiments were performed in triplicate with similar results. (C) *N. benthamiana sobir1/sobir1-like* mutants do not show altered susceptibility to TSWV. Leaf samples from wild-type *N. benthamiana* and two *N. benthamiana sobir1/sobir1-like* mutants were collected at 4 weeks after TSWV inoculation and the infection levels were determined by Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA). Bars represent the mean absorbance + SD. Experiments were repeated three times and similar results were obtained. (D) *N. benthamiana sobir1/sobir1-like* mutants do not show increased susceptibility to *B. cinerea*. The lesion sizes of wild-type *N. benthamiana* and two *N. benthamiana sobir1/sobir1-like* mutants were measured at 2 and 3 days after inoculation with *B. cinerea*. Bars represent the mean lesion sizes + SD. Experiments were repeated at least three times and similar results were obtained.

Discussion

Since 2012, when the CRISPR/Cas9 system was described as a potential genome editing tool for the first time (Jinek et al., 2012), remarkable advances have been made in employing this powerful technology to edit genes in different plant species, including the model plants *Arabidopsis* and *N. benthamiana* and crop plants, such as tomato, rice, strawberry and potato (Cong et al., 2013; Jiang et al., 2013; Nekrasov et al., 2013; Doudna and Charpentier, 2014; Wang et al., 2015; Ma et al., 2016; Zhou et al., 2018; Zhang et al., 2020). 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*. Construct 1 that we

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generated in this study was designed to knock out 310 bp in the ORF of *SOBIR1* and 61 bp in the ORF of *SOBIR1-like*, whereas Construct 2 was made to generate a 261 bp deletion in the ORF of *SOBIR1* and a 61 bp deletion in the ORF of *SOBIR1-like*. Unlike what we expected, only small deletions or insertions were observed in the homozygous mutant lines (Figure 1A, 2A, and 3A). The possible reason is that both constructs were designed to knock out two genes simultaneously, both *SOBIR1* and *SOBIR1-like*, whereby the targeting efficiency and editing ability of the constructs were reduced, as multiple cleavages in one gene normally lead to larger deletions (Chen et al., 2011; Cong et al., 2013; Ran et al., 2013). Additionally, upon cleavage by Cas9, plants exploit two endogenous DNA repair mechanisms, which are non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Belhaj et al., 2013; Ran et al., 2013; Belhaj et al., 2015). In most cases, DNA cleavage in plants is repaired by NHEJ, as HDR requires the presence of a donor DNA template to initiate this process. Nevertheless, DNA repair by NHEJ easily generates mismatches and gene insertions/deletions, resulting in the loss of gene function (Doudna and Charpentier, 2014; Bortesi and Fisher, 2015). In addition to the work presented here, recently, a large number of immune-related genes, including *SOBIR1* and *SOBIR1-like*, were targeted by CRISPR/Cas9 technology in tomato (Zhang et al., 2020). These resources will allow us to study the role of these genes in the resistance response of tomato to *C. fulvum*, by crossing these mutants to tomato carrying the *Cf-4* resistance gene.

Both *SOBIR1* and *BAK1* were shown to be essential for LRR-RLP-triggered defense responses, which, together with other cell-surface receptor-induced defense responses, form the first line of plant innate immunity (Chinchilla et al., 2009; Liebrand et al., 2014; Albert et al., 2015; Postma et al., 2016; van der Burgh et al., 2019). To promote their pathogenicity, virulent microbial pathogens have evolved sophisticated mechanisms to overcome the first plant immune barrier, with one of their strategies being to target the main plant regulators, such as *BAK1* and *SOBIR1*, by effectors and thereby suppressing plant defense responses (Jones and Dangl, 2006). *Pseudomonas syringae* effectors AvrPto and AvrPtoB were, for example, reported to target *BAK1*, thereby preventing the formation of the receptor-signaling complex (Shan et al., 2008). Interestingly, AvrPto also has been shown to compromise the Avr4/Cf-4-mediated HR by targeting *SOBIR1* (Wu et al., 2017). Plants, in their turn, have evolved strategies to fight back. Knocking out *BAK1*, as well as its functional homolog *BAK1-LIKE 1* (*BKK1*), leads to the constitutive activation of immune responses, resulting in a seeding-lethality phenotype (He et al., 2007). Recent findings show that this cell-death phenotype is caused by the activation of cytoplasmic receptors, unveiling that *BAK1* and *BKK1* are likely guarded by such cytoplasmic receptors (Wu et al., 2020). Unlike what has been observed in *bak1/bkk1* mutants, knocking out *SOBIR1* in Arabidopsis and *N. benthamiana* does not cause an auto-immune phenotype (Figure S3) (Gao et al., 2009; Liu et al., 2016), and therefore *SOBIR1* is unlikely to be guarded.

Strikingly, we observed that flg22 triggers an unexpected second, prolonged ROS burst in the *N. benthamiana sobir1* (*sobir1-like*) mutant plants (Figure 2D and 3B), uncovering the presence of possible crosstalk among different cell-surface receptor-mediated signal transduction pathways. A recent report has shown that bacterial pathogens and/or the bacterial ExIP flg22 induce the phosphorylation of CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), which is the co-receptor of fungal chitin, in a BAK1-dependent manner, leading to CERK1-mediated priming of antifungal immunity (Gong et al., 2019). Furthermore, NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE 1 (NIK1), which is involved in antiviral defense, is phosphorylated by BAK1 upon flg22 perception, resulting in flg22-promoted resistance to viruses in a NIK-dependent manner (Li et al., 2019). Additionally, plant CELL WALL-ASSOCIATED KINASE 1 (WAK1), which recognizes cell-wall-derived oligogalacturonides and initiates plant immune responses, has been reported to form a complex with, and depend on, FLS2 and another RLK (FLS3), for its functionality (Kurt et al., 2020; Zhang et al., 2020). These observations reveal that the crosstalk among cell-surface receptors plays a remarkable role in plant immunity. Different from CERK1, NIK1 and WAK1, SOBIR1 does not interact with FLS2 and is not required for FLS2 functionality (Liebrand et al., 2013; Albert et al., 2015). It has been reported previously that the transcriptional trans-activation of *N. benthamiana* RESPIRATORY BURST OXIDASE HOMOLOGS B (*RBOHB*) through a MAPK-WRKY pathway, is responsible for the sustained second ROS burst (Yoshioka et al., 2003; Adachi et al., 2015). RLCKs are key players to link cell-surface receptors and MAPKs (Cui et al., 2018), therefore, possible crosstalk between RLP/SOBIR1- and FLS2-triggered immune signaling in *N. benthamiana* might take place via an as yet unidentified RLCK. Further studies aimed at identifying the proteins that contribute to the biphasic ROS burst in *N. benthamiana*:*Cf-4* triggered by Avr4 and in the knock-out plants triggered by flg22, should help to understand the mechanism of signal amplification and crosstalk in plant immunity.

To determine the role of SOBIR1 in plant basal defense, in this study we challenged the two *N. benthamiana sobir1/sobir1-like* double knock-out lines with the hemibiotrophic oomycete pathogens *P. capsici* and *P. infestans*, the virus TSWV, and the necrotrophic pathogen *B. cinerea*. Our results showed that *N. benthamiana sobir1/sobir1-like* mutant plants are more susceptible to *P. capsici* and *P. infestans*, but not to TSWV and *B. cinerea* (Figure 4). Previous studies have shown that the ExIPs INF1 and ParA1 from *P. infestans* are recognized by as yet unidentified cell-surface receptors in *N. benthamiana*. Still, they both induce HR in the leaves of *N. benthamiana*, and both SOBIR1 and BAK1 are required for the HR (Peng et al., 2015; Domazakis et al., 2018). These results are in agreement with the previous finding that recognition of INF1 mediates resistance of *N. benthamiana* to *P. infestans* (Kamoun et al., 1998). SOBIR1 does not contribute to the basal defense of *N. benthamiana* against *B. cinerea*, which is in line with what was found in *Arabidopsis* (Zhang et al., 2014). Interestingly, SOBIR1 is essential for the perception of various ExIPs from *B. cinerea*. For instance, the xyloglucanase BcXYG1 and the xylanase BcXyl1 trigger cell death in

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N. benthamiana, and their cell death activity is dependent on both SOBIR1 and BAK1 (Zhu et al., 2017; Yang et al., 2018). Moreover, an nlp20-related peptide from *B. cinerea*, BcNEP2, triggers RLP23/SOBIR1/BAK1-dependent ethylene production in *Arabidopsis* (Oome et al., 2014; Albert et al., 2015). Nevertheless, the RLP/SOBIR1-mediated responses to ExIPs from *B. cinerea* are much slower than the disease development, which explains that knocking out *SOBIR1* does not affect susceptibility to *B. cinerea*. Additionally, being a necrotrophic plant pathogen, *B. cinerea* can utilize the cell death/necrosis of host plants to promote colonization of the host tissues (van Kan, 2006).

Apart from the results shown in this study, the *N. benthamiana* mutant lines we generated allow for more fundamental research on RLP-mediated plant immunity that was difficult or impossible to perform in Solanaceous plants before. For example, upon the perception of Avr4, the activated Cf-4/SOBIR1 complex associates with BAK1, which results in massive phosphorylation events that take place between the two kinase domains of SOBIR1 and BAK1 (van der Burgh et al., 2019). However, which SOBIR1-phosphorylation sites are exactly auto-phosphorylated by SOBIR1 itself, or trans-phosphorylated by BAK1 and possible other signaling partners such as RLCKs, remains unclear. As expected, transient expression of *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1*-like is able to restore the loss of *NbSOBIR1* in *sobir1/sobir1-like* mutant lines and reconstitute the Avr4/Cf-4-mediated HR (Figure 1C and 2C). Therefore, now a site-directed mutagenesis screen of putative phosphorylation sites in the kinase domain of SOBIR1 can be performed. For that, we can transiently co-express various SOBIR1 mutants with Avr4 in the leaves of *N. benthamiana*:Cf-4 *sobir1/sobir1-like* mutant plants. In this way, by checking the effect on Avr4/Cf-4-triggered ROS production, MAPK activation and HR, the importance of each candidate phosphorylation site can be determined. Moreover, as SOBIR1 constitutively interacts with Cf-4, the Cf-4/SOBIR1 complex is considered to function as a two-component RLK (Liebrand et al., 2013; Liebrand et al., 2014). However, it is unclear how the Cf-4/SOBIR1 complex perceives Avr4 and subsequently triggers downstream signal transduction. Emerging evidence has revealed that the signaling specificity is determined by the kinase domain of the primary ligand-receptor (in this case Cf-4/SOBIR1), but not of the co-receptor BAK1 (Hohmann et al., 2020). Therefore, co-expressing Avr4 and a Cf-4-SOBIR1 chimera, comprising the extracellular LRR domain of Cf-4 and the cytoplasmic kinase domain of *NbSOBIR1* or *S/SOBIR1*, in the leaves of *N. benthamiana*:Cf-4 *sobir1/sobir1-like* mutant plants, should help to reveal the mechanism of Avr4 perception and subsequent initiation of immune signaling by the Cf-4/SOBIR1 complex.

Materials and Methods

CRISPR/Cas9 constructs generation and testing

To knock out *SOBIR1* and *SOBIR1-like* in *N. benthamiana*, two constructs were generated using the Golden Gate cloning method (Engler et al., 2014). Both constructs contain four sgRNAs. In total, six different sgRNAs were designed, with sgRNA1/2/3/4 targeting different regions of the ORF of *SOBIR1*, and sgRNA5/6 targeting the ORF of *SOBIR1-like* (Table S1 and Figure S1). Each primer pair for generating the different sgRNAs was annealed into double-stranded DNA (Table S2), followed by assembly into the Level 0 vector pICSL01009:AtU6p (SOL7857). Hereafter, each sgRNA cassette was inserted into the appropriate Level 1 vectors (SOL7861, SOL7862, SOL7863 and SOL7864), whereby pICH47751::AtU6p::sgRNA1 (SOL7867), pICH47761::AtU6p::sgRNA2 (SOL7868), pICH47751::AtU6p::sgRNA3 (SOL7869), pICH47761::AtU6p::sgRNA4 (SOL7870), pICH47772::AtU6p::sgRNA5 (SOL7871) and pICH47781::AtU6p::sgRNA6 (SOL7872), were obtained. Together with pICH47732::NOSp-BAR-NOST (SOL7859), pICH47742::2×35S::hCas9 (SOL7860), pICH41822 end-linker 6 (SOL7865), pICH47772::AtU6p::sgRNA5 and pICH47781::AtU6p::sgRNA6, pICH47751::AtU6p::sgRNA1 and pICH47761::AtU6p::sgRNA2 were assembled into the Level 2 vector pAGM4723 (SOL7866), resulting in CRISPR/Cas9 Construct 1; pAGM4723::BAR::Cas9::sgRNA1::sgRNA2::sgRNA5::sgRNA6 (SOL7880). Next to this, pICH47751::AtU6p::sgRNA3 and pICH47761::AtU6p::sgRNA4 were also assembled into the Level 2 vector pAGM4723, resulting in CRISPR/Cas9 Construct 2; pAGM4723::BAR::Cas9::sgRNA3::sgRNA4::sgRNA5::sgRNA6 (SOL7881).

Each construct was transformed individually into *Agrobacterium tumefaciens* strain GV3101, followed by agro-infiltration at OD₆₀₀ of 1 in leaves of *N. benthamiana* as described before (van der Hoorn et al., 2000). Genomic DNA of the agro-infiltrated leaves was isolated at 5 days post infiltration (dpi) (Fulton et al., 1995), and the targeted fragments of *SOBIR1* and *SOBIR1-like* were amplified, and of which the *SOBIR1-like* fragments were digested with *HpyCH4V* and *HinfI* at 37 °C for 1 h (New England Biolabs) (Table S2 and Figure S2).

Plant transformations and mutation detection

Agrobacterium carrying the different CRISPR/Cas9 constructs was used for transformation into wild-type *N. benthamiana* and *N. benthamiana*:*Cf-4*, respectively (Horsch et al., 1989). To select homozygous transformants and to determine the mutation type, genomic DNA was extracted from each transformant (Fulton et al., 1995) and used as a template to amplify the targeted fragments of *SOBIR1* and *SOBIR1-like* with the specific primers listed in Table S2. Hereafter, the purified fragments were analyzed by Sanger sequencing.

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Plant growth conditions

N. benthamiana, *N. benthamiana*:Cf-4 and all generated transformants of these plants were grown in a climate chamber under 15 h of light at 21 °C and 9 h of darkness at 19 °C, with a relative humidity of ~70%.

Agrobacterium-mediated transient transformation

Agrobacterium-mediated transient transformation of Avr4, the Avr4/Cf-4 combination, or the Avr9/Cf-9 combination, in 5-week-old *N. benthamiana* plants, was performed as described before (OD₆₀₀ = 0.5 for each construct) (van der Hoorn et al., 2000). For complementation studies, eGFP-tagged *NbSOBIR1* (SOL2911), *S/SOBIR1* (SOL2774) or *S/SOBIR1*-like (SOL2773), as well as their corresponding kinase-dead mutants *NbSOBIR1*^{D482N} (SOL7928), *S/SOBIR1*^{D473N} (SOL2875) or *S/SOBIR1*-like^{D486N} (SOL2876) (Liebrand et al., 2013), were mixed with Avr4 (OD₆₀₀ = 0.5 for each construct), and then syringe-infiltrated into fully expanded leaves of *N. benthamiana*:Cf-4 *sobir1/sobir1*-like. The development of an HR was photographed at 5 dpi.

Reactive oxygen species (ROS) assay

ROS production was determined by a luminol-based assay (Keppler et al., 1989). For this, leaf discs from 4- to 5-week-old *N. benthamiana*:Cf-4 and *N. benthamiana*:Cf-4 *sobir1/sobir1*-like plants were collected by using biopsy punches (Ø 5 mm, Robbins Instruments) and incubated in 100 µL of sterile water in a 96-well plate overnight. The water was then replaced carefully by 50 µL of fresh sterile water and the leaf discs were incubated for another hour. 50 µL of a freshly prepared reaction solution, containing 100 µM of luminol (L-012, Fujifilm, Japan), 20 µg/mL horseradish peroxidase (Sigma), and 0.2 µM flg22 (EZBiolab) or 0.2 µM Avr4, was added to each well. For the mock treatment, no flg22 or Avr4 was added. Chemiluminescence was monitored using a CLARIOstar plate reader (BMG Labtech), with a program of 100 cycles and 2 min per cycle.

Pathogen infection assays

The *Phytophthora capsici* isolate BYA5 and the *P. infestans* isolate NL88069 were used for the inoculation assays, and their growth conditions were described before (van West et al., 1998; Wang et al., 2019). Detached leaves, with similar sizes, from 5-week-old wild-type *N. benthamiana* plants and *N. benthamiana* *sobir1/sobir1*-like double knock-out mutant plants, were inoculated on the abaxial side with fresh mycelial plugs (Ø 5 mm) of *P. capsici* or *P. infestans*, after which the leaves were incubated in covered trays at 25 °C with high relative humidity in the dark, as described before (Vleeshouwers et al., 1999). The lesion sizes caused by *P. capsici* were measured at 2 dpi, while the lesion sizes caused by *P. infestans* were quantified at 5 dpi. Hereafter, the lesion areas were calculated.

Knocking out *SOBIR1* in *N. benthamiana*

The first two fully expanded leaves from 3-week-old wild-type *N. benthamiana* plants and *sobir1/sobir1-like* knock-out mutant plants were dusted with carborundum powder after which they were mechanically rub inoculated with the TSWV inoculum. The TSWV inoculum was prepared by collecting 4 leaves of TSWV-infected *Emilia sonchifolia* in a mortar containing 10 mL viral inoculation buffer (10 mM sodium phosphate and 10 mM sodium sulphate) and a dash of carborundum, and then grinding with a pestle. 4 weeks after inoculation, 6 leaf discs (Ø 5 mm) from each plant were collected in a 2 mL microcentrifuge tube containing 200 µL PBS-Tween. Hereafter, the leaf samples were homogenized and tested in the DAS-ELISA, as described (Clark and Adams, 1977).

The *B. cinerea* inoculation assay on *N. benthamiana* was performed as described before (Zhang and van Kan, 2013), and strain B05.10 was used in this study. Each tray contained one plant from each line, and 5 plants in total from each line were used in each experiment. The lesion sizes were measured at 2 and 3 dpi.

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Supplementary Data

A

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B

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TGCTCACTGGATAATGCAAAGGAGAGGCCTAACAGTAAGGATGTTAGATGCATGTTAACTCAGATCAAGC
CTCAAGTAGGTAGAGGATAA

Figure S1. Nucleotide sequence of the open reading frames of *NbSOBIR1* and *NbSOBIR1-like*. The primers used for amplifying and sequencing targeted fragments of *SOBIR1* (A) and *SOBIR1-like* (B) are indicated in yellow. Single-guide RNAs (sgRNAs) are highlighted in orange, followed by protospacer adjacent motifs (PAMs) that are shown in blue.

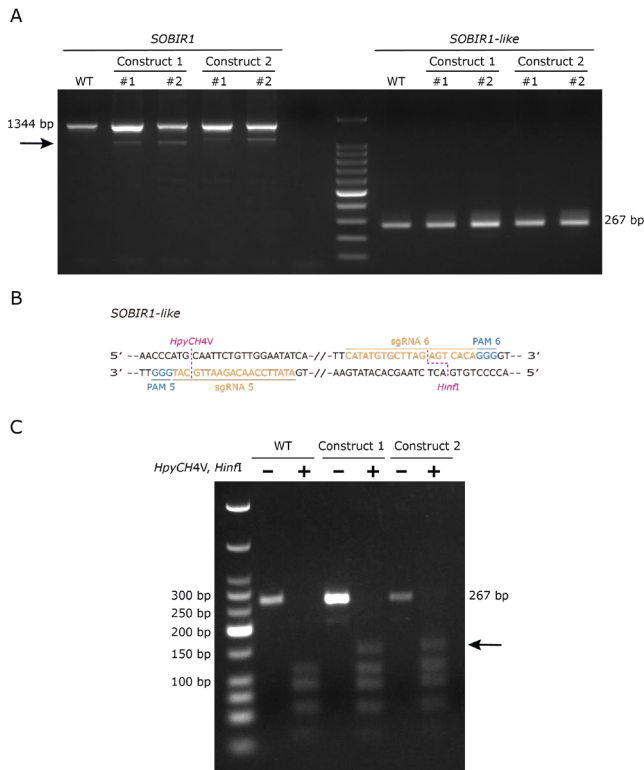


Figure S2. Determination of the effectiveness and efficiency of the generated CRISPR/Cas9 constructs. (A) PCR-based detection of CRISPR/Cas9-induced deletions in *NbSOBIR1* and *NbSOBIR1-like*. The fragments of *NbSOBIR1* (shown on the left) were amplified by PCR with the primer pair Nbsobir1fwd/Nbsobir1rev, while the fragments of *SOBIR1-like* (shown on the right) were amplified with the primer pair Nbsobir1likefwd/Nbsobir1likerev. (B) Nucleotide sequence alignment of the regions in *SOBIR1-like* targeted by sgRNA5 and sgRNA6. The sgRNA sequences are indicated in orange, the PAMs are shown in blue, and the two restriction sites (*HpyCH4V* and *HinfI*) in the sgRNAs are denoted in carmine. (C) Digestion of the *SOBIR1-like* amplicons with *HpyCH4V* and *HinfI*.

Knocking out *SOBIR1* in *N. benthamiana*

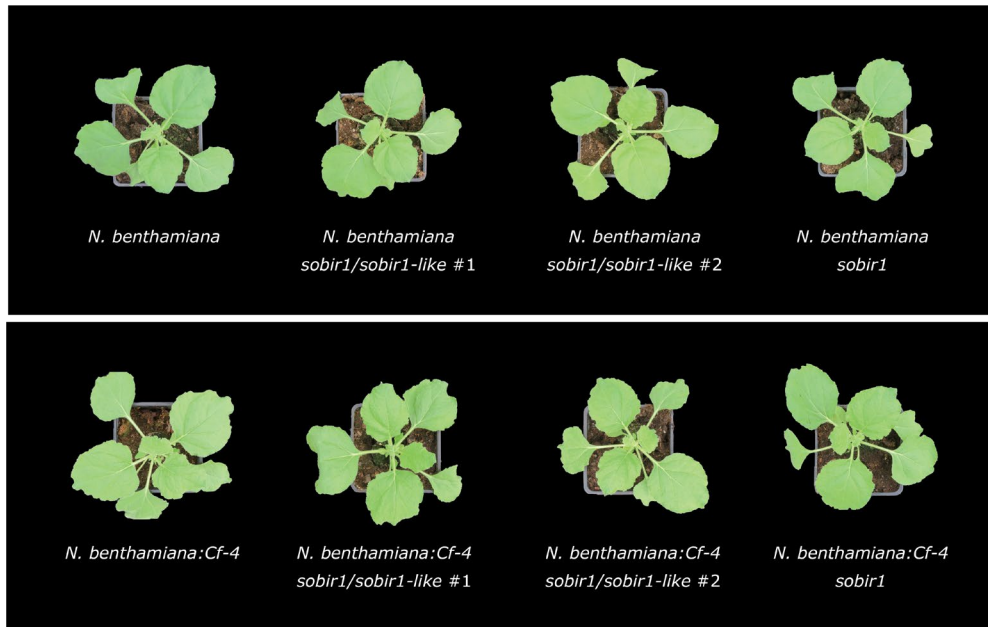


Figure S3. Morphological phenotypes of wild-type *N. benthamiana* and the various mutant lines, as indicated. All plants were grown on soil and photographed when four to five weeks old. Note that the mutant lines do not have an obvious morphological phenotype. The pictures of the plants were extracted from different photos and placed on a black background.

Table S1. Nucleotide sequences of the six single-guide (sg) RNAs.

	Sequence (5'-3')	Target gene
sgRNA1	TTGCTTGCCAAAAAGACTT	<i>NbSOBIR1</i>
sgRNA2	AAAGATCAAGAATTCGAAGG	<i>NbSOBIR1</i>
sgRNA3	TCCACTGATTAAGAAAGCCG	<i>NbSOBIR1</i>
sgRNA4	CTAGGCATATGTGCCAGTAG	<i>NbSOBIR1</i>
sgRNA5	ATATTCCAACAGAATTGCAT	<i>NbSOBIR1-like</i>
sgRNA6	CATATGTGCTTAGAGTCACA	<i>NbSOBIR1-like</i>

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

Code	Name	Sequence (5'-3')	Note
ho51	sgRNA1_fw*	ATTGTTGCTTGTCAAAAAGACTT	sgRNA1
ho52	sgRNA1_rev	AAACAAGTCTTTTGGACAAGCAA	sgRNA1
ho53	sgRNA2_fw	ATTGAAAGATCAAGAATTCGAAGG	sgRNA2
ho54	sgRNA2_rev	AAACCCCTTCGAATTCTTGATCTTT	sgRNA2
ho55	sgRNA3_fw	ATTGTCCACTGATTAAGAAAGCCG	sgRNA3
ho56	sgRNA3_rev	AAACCGGCTTCTTAATCAGTGGA	sgRNA3
ho57	sgRNA4_fw	ATTGCTAGGCATATGTGCCAGTAG	sgRNA4
ho58	sgRNA4_rev	AAACCTACTGGCACAATATGCCTAG	sgRNA4
ho59	sgRNA5_fw	ATTGATATCCAACAGAGTTGCAT	sgRNA5
ho60	sgRNA5_rev	AAACATGCAACTCTGTGGAATAT	sgRNA5
ho61	sgRNA6_fw	ATTGCATATGTGCTTAGAGTCACA	sgRNA6
ho62	sgRNA6_rev	AAACTGTGACTCTAAGCACATATG	sgRNA6
ho11	Nbsobir1_fw	ATGGCCTTCACTGCTTCACAAAT	To amplify <i>NbSOBIR1</i> and test the
ho12	Nbsobir1_rev	CTGGAGGATATCTTGAAGCTCCC	effectiveness of the constructs.
ho13	Nbsobir1like_fw	CCTTTACTCGCCAGATTATAGT	To amplify <i>NbSOBIR1-like</i> and test
ho14	Nbsobir1like_rev	TGAGTTGGGATTCTGTCAAAG	the effectiveness of the constructs.
ho78	NbSOBIR1 1-2 seq1_fw	ACTTTTCGCCTTTTACTTATTGC	To amplify and sequence <i>NbSOBIR1</i> -
ho79	NbSOBIR1 1-2 seq2_rev	TGGAACGAAGTGCTGATCCAAT	containing sgRNA1&2 region.
ho80	NbSOBIR1 2-2 seq2_fw	ATTGGATCAGCACTTCGTCC	To amplify and sequence <i>NbSOBIR1</i> -
ho81	NbSOBIR1 2-2 seq1_rev	AATTCGAGCTCCATATCATCGT	containing sgRNA3&4 region.
ho82	NbSOBIR1-like seq1_fw	CTATCTCTTCTGTCTCTTCCAC	To amplify and sequence <i>NbSOBIR1</i> -
ho83	NbSOBIR1-like seq4_rev	CATAATCTTTTCGATTCAAG	like-containing sgRNA5&6 region.

* fw, forward; rev, reverse.

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

Generation	Number of transgenic plants	Number of edited plants	Mutation rate (%)	Mutation types
<i>N. benthamiana</i>				
T0	24 ¹	14 ¹	58.33	1 <i>sobir1</i> knock-out homozygous line 13 heterozygous lines
	8 ²	7 ²	87.50	1 <i>sobir1</i> knock-out homozygous line 6 heterozygous lines
T1	12 ²	12 ²		2 <i>sobir1/sobir1-like</i> knock-out homozygous lines 10 heterozygous lines
<i>N. benthamiana:Cf-4</i>				
T0	3 ¹	2 ¹	66.67	2 heterozygous lines
	5 ²	4 ²	80.00	4 heterozygous lines
T1	4 ¹	4 ¹		2 <i>sobir1/sobir1-like</i> knock-out homozygous lines 2 heterozygous lines
	5 ²	5 ²		1 <i>sobir1</i> knock-out homozygous line 4 heterozygous lines

¹Plants were transformed with CRISPR/Cas9 Construct 1. ²Plants were transformed with CRISPR/Cas9 Construct 2

² The mutation rate is only calculated for the T0 generation.

Chapter 3

Trans-phosphorylation of SOBIR1 and BAK1 regulates LRR-RLP-triggered plant immunity

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Abstract

The first layer of plant immunity is mediated by receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that perceive extracellular immunogenic patterns (ExIPs) and subsequently initiate the activation of plant immune responses. The leucine-rich repeat (LRR)-RLKs SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1) and BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1) are the common regulatory co-receptors of LRR-RLPs. Phosphorylation of signaling partners is crucial for the activation of receptor complexes, and it is proposed that such phosphorylation events take place between the kinase domains of SOBIR1 and BAK1, which then lead to the initiation of various downstream signaling outputs. These outputs include the accumulation of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase (MAPK) cascades, and in some cases, the hypersensitive response (HR). Here, we perform a site-directed mutagenesis screen of the phosphorylatable threonine (Thr/T) and serine (Ser/S) residues that are present in the activation segment of the kinase domain of SOBIR1, combined with a complementation study in *Nicotiana benthamiana* plants stably expressing the tomato LRR-RLP gene *Cf-4*, and in which the functional *SOBIR1* gene has been knocked out. Thr residue *NbSOBIR1*^{T522}, as well as its analogous residues in tomato SOBIR1 and SOBIR1-like, was found to be essential for the *Cf-4*/SOBIR1/BAK-triggered ROS burst, MAPK activation and the HR, upon recognition of the matching ExIP Avr4 by the *Cf-4* resistance protein. Further *in vitro* phosphorylation assays with the SOBIR1 and BAK1 kinase domains demonstrate that *NbSOBIR1*^{T522}, as well as its analogous residue in tomato SOBIR1 and SOBIR1-like, is required for the intrinsic kinase activity of SOBIR1. Furthermore, we show that SOBIR1 is able to directly phosphorylate BAK1, whereas BAK1, in its turn, can directly phosphorylate SOBIR1. These trans-phosphorylation events are proposed to eventually result in the full activation of SOBIR1/BAK1-containing immune complexes.

Introduction

While constantly being exposed to numerous microbial pathogens, plants have evolved an innate immune system to defend themselves against pathogen attacks (Jones and Dangl, 2006; Dodds and Rathjen, 2010). This innate immune system relies on the recognition of extracellular immunogenic patterns (ExIPs) by cell-surface receptors and the recognition of intracellular immunogenic patterns (InIPs) mainly by nucleotide-binding domain, leucine-rich repeat (NB-LRR) receptors (NLRs) (Dangl and Jones, 2001; Jones and Dangl, 2006; Couto and Zipfel, 2016; van der Burgh and Joosten, 2019). Cell-surface receptors form the front line of plant defense and fall into one of the two receptor classes: receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Boutrot and Zipfel, 2017). RLKs are composed of an extracellular domain that is involved in ligand binding, a single-pass transmembrane domain, and an intracellular kinase domain. In contrast to RLKs, RLPs lack such a cytoplasmic kinase domain for downstream signaling (Zipfel, 2014; Ranf, 2017; Tang et al., 2017).

The extracellular domains of cell-surface receptors are highly different, either containing leucine-rich repeats (LRRs), a lysin motif (LysM), lectin (Lec) motifs, or an epidermal growth factor (EGF)-like domain, thereby providing several means to recognize a wide range of ExIPs (Monaghan and Zipfel, 2012; Böhm et al., 2014; Couto and Zipfel, 2016; Wang and Chai, 2020). To date, the most extensively studied plant cell-surface receptors are the families of the LRR-RLKs and LRR-RLPs. A well-studied example is the Arabidopsis (*Arabidopsis thaliana*, At) LRR-RLK FLAGELLIN-SENSING 2 (FLS2), which recognizes a conserved 22-amino acid epitope (flg22) of bacterial flagellin and subsequently initiates downstream signaling to mount defense (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001; Chinchilla et al., 2007; Sun et al., 2013). Notably, cell-surface receptors do not signal alone; many LRR-RLKs have been shown to require the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family for the receptor complex activation (Heese et al., 2007; Chinchilla et al., 2009; Macho and Zipfel, 2014; Wang and Chai, 2020; Zhou and Zhang, 2020). Even though FLS2 has an intracellular kinase domain with the potential for signaling by itself, it requires ligand-induced dimerization with BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE 1/SERK3 (BAK1/SERK3, further referred to as BAK1), resulting in the rapid trans-phosphorylation of the cytoplasmic kinase domains of BAK1 and FLS2. (Heese et al., 2007; Chinchilla et al., 2009).

LRR-RLPs, which lack an intracellular kinase domain, have been found to constitutively associate with the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1) and to indeed require SOBIR1 for their function in plant immunity (Gao et al., 2009; Liebrand et al., 2013; Liebrand et al., 2014). RLPs are omnipresent in plants, and SOBIR1 is also widely conserved in all sequenced plant genomes for which information is available (Liebrand et al., 2014). For example, the tomato (*Solanum lycopersicum*, Sl) RLPs Cf-4 and Ve1 form a complex with SOBIR1 in

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a ligand-independent manner, which is essential for Cf-4- and Ve1-mediated resistance to the fungal pathogens *Cladosporium fulvum* and *Verticillium dahliae*, respectively (Liebrand et al., 2013; Liebrand et al., 2014). In addition, Arabidopsis RLP23 requires SOBIR1 to mount resistance against various oomycete and fungal plant pathogens that secrete necrosis and ethylene-inducing peptide 1-like proteins (NLPs) (Albert et al., 2015). Furthermore, the RLP ELICITIN RESPONSE PROTEIN (ELR) from the wild potato variety *Solanum microdontum* (Sm) associates with SmSOBIR1 and induces defense responses against *Phytophthora infestans* (Domazakis et al., 2018). Thus, the RLP/SOBIR1 complex is postulated to function as a two-component primary receptor (Gust and Felix, 2014; Liebrand et al., 2014). Intriguingly, reminiscent of FLS2-mediated signaling, upon ligand perception the RLP/SOBIR1 complex also recruits the common regulator BAK1 to trigger downstream signaling (Albert et al., 2015; Postma et al., 2016; Zhang et al., 2021).

Protein phosphorylation, which is a swift and reversible biochemical post-translational modification, plays an important role as a versatile molecular switch in various cellular activities, including the initiation of plant immune responses (Macho and Zipfel, 2014; Mithoe and Menke, 2018; Kong et al., 2021). Phosphorylation by protein kinases in plants takes place on serine (Ser/S), threonine (Thr/T), and less commonly, on tyrosine (Tyr/Y) residues (Afzal et al., 2008; de la Fuente van Bentem and Hirt, 2009). According to whether a conserved arginine (Arg/R) is immediately preceding the highly conserved catalytic aspartate (Asp/D) in their catalytic loop, protein kinases can be subdivided into RD and non-RD kinases (Johnson et al., 1996). Generally, activation of RD kinases requires phosphorylation of one or more residues in their activation segment (Johnson et al., 1996; Nolen et al., 2004; Oliver et al., 2007). A good example is BAK1, which is the common regulator of LRR-RLPs and LRR-RLKs (Chinchilla et al., 2009). BAK1 is a typical RD kinase and exhibits auto-phosphorylation activity, and for example phosphorylation of T455 in the activation segment of Arabidopsis BAK1 is essential for BAK1 to become an active kinase (Wang et al., 2005). In addition, the LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), which is a crucial co-receptor for recognizing fungal chitin oligomers, is also an RD kinase. Phosphorylation of T479, present in the activation segment of Arabidopsis CERK1, is required for the initiation of chitin-triggered defense responses (Miya et al., 2007; Wan et al., 2008; Suzuki et al., 2016). In contrast, non-RD kinases require a regulatory RD kinase, such as BAK1 and CERK1, to promote their phosphorylation and signaling (Dardick et al., 2012).

In tomato, resistance to the biotrophic fungal pathogen *C. fulvum* is mediated by Cf immune proteins that encode LRR-RLPs (Joosten and de Wit, 1999; Luderer and Joosten, 2001; de Wit, 2016). These are plasma membrane-localized receptors that recognize their matching avirulence factors (Avrs) from *C. fulvum* in a gene-for-gene manner (Person et al., 1962; Flor, 1971; Thomma et al., 2005). Similar to other LRR-RLPs, Cf-4 constitutively interacts with SOBIR1 in the resting state, whereas upon

recognition of the effector protein Avr4 from *C. fulvum*, the Cf-4/SOBIR1 complex recruits BAK1, resulting in the activation of numerous downstream signaling events (Liebrand et al., 2013; Liebrand et al., 2014; Postma et al., 2016; Huang et al., 2021). Typically, these events include the swift phosphorylation of downstream receptor-like cytoplasmic kinases (RLCKs), a burst of reactive oxygen species (ROS), and the activation of mitogen-activated protein kinase (MAPK) cascades, eventually resulting in the induction of the hypersensitive response (HR) (van der Hoorn et al., 2000; de Jong, 2002; Stulemeijer et al., 2007; Yu et al., 2017; Huang et al., 2021). Both SOBIR1 and BAK1 are RD kinases, and their kinase activity is required for immune signaling (Liebrand et al., 2013; van der Burgh et al., 2019). It is proposed that there is a series of trans-phosphorylation events occurring between SOBIR1 and BAK1 upon BAK1 recruitment as a result of the recognition of Avr4 by Cf-4 (van der Burgh et al., 2019). However, how these RLKs exactly trans-phosphorylate each other to initiate downstream signaling components, and which phosphorylation sites in the kinase domain of SOBIR1 are essential for its signaling function, remain poorly understood.

In this study, we performed a site-directed mutagenesis screen, combined with a complementation study in *Nicotiana benthamiana* (*Nb*) plants stably expressing tomato *Cf-4* and in which the functional *SOBIR1* gene is knocked out. *NbSOBIR1*^{T522}, *S/SOBIR1*^{T513}, and *S/SOBIR1*-like^{T526} (tomato contains two functional *SOBIR1* genes), which are all Thr residues present in the activation segment of SOBIR1(-like), were found to play an essential role in Avr4/Cf-4-triggered immune signaling, including the initiation of the ROS burst, MAPK activation and the HR. Interestingly, *in vitro* phosphorylation assays demonstrate that the highly conserved Thr residue in the activation segment (*NbSOBIR1*^{T522}, *S/SOBIR1*^{T513}, and *S/SOBIR1*-like^{T526}) is required for SOBIR1(-like) intrinsic kinase activity. In addition, we show that SOBIR1 directly trans-phosphorylates BAK1, whereas, on the other hand, BAK1 is also able to directly trans-phosphorylate SOBIR1. These trans-phosphorylation events are proposed to eventually lead to the full activation of both SOBIR1 and BAK1, and their intrinsic kinase activity is required for these trans-phosphorylation events to take place and the initiation of downstream immune signaling.

Results

***NbSOBIR1*^{T522}, as well as its analogous residues in both tomato SOBIR1s, present in the activation segment of the kinase domain of SOBIR1, is essential for mounting the Avr4/Cf-4-triggered HR**

The regulatory LRR-RLK SOBIR1 appears to be present throughout the plant kingdom, including *N. benthamiana*, which is a versatile experimental host plant (Gao et al., 2009; Liebrand et al., 2013; Liebrand et al., 2014). SOBIR1 is an RD kinase, which suggests that likely SOBIR1 first requires phosphorylation of its activation segment to acquire the kinase-active conformation (Johnson et al., 1996; Oliver et al., 2007). It has been reported that the kinase domain of *AtSOBIR1* auto-phosphorylates at Ser,

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Thr, and Tyr residues *in vitro* (Leslie et al., 2010), and in the activation segment of SOBIR1 of tomato and *N. benthamiana* such residues are present. To investigate which residue(s) in the kinase domain of SOBIR1 is(are) essential for the activation of Cf-4/SOBIR1-triggered signaling pathway, we decided to zoom in on the activation segment of *NbSOBIR1*, *S/SOBIR1* and *S/SOBIR1*-like (the latter is a close homolog of SOBIR1 in tomato) (Liebrand et al., 2013). Indeed, five potential phosphorylation sites are located in this loop of 30 amino acids, including one Ser and four Thr residues. Strikingly, these residues are highly conserved among *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1*-like (Figure 1A).

Cf-4 is functional in *N. benthamiana* and agro-infiltration of Avr4 in stable transgenic *N. benthamiana*:Cf-4 plants triggers a typical HR (Gabriëls et al., 2006). We have described earlier that Cf-4 function is completely abolished in *N. benthamiana*:Cf-4 *sobir1* mutant lines, due to the absence of functional SOBIR1, and complementation through transient expression of functional *NbSOBIR1*, *S/SOBIR1*, or *S/SOBIR1*-like restores the Avr4/Cf-4-mediated HR in such mutant lines (Huang et al., 2021) (Chapter 2). Based on these observations, we carried out site-directed mutagenesis of the activation segment of *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1*-like to substitute individual Ser/Thr residues with alanine (Ala/A) residue, which lacks the phosphorylatable hydroxyl group and thereby cannot be phosphorylated. Subsequently, we performed a complementation study with the 15 different SOBIR1 mutants that we generated, taking along the SOBIR1 wild-types (WTs) and the corresponding D to N (asparagine/Asn) kinase-dead mutants, as positive and negative controls, respectively (van der Burgh et al., 2019). Interestingly, in contrast to *NbSOBIR1* WT, *NbSOBIR1* T522A failed to restore the Avr4/Cf-4-specific HR in the *sobir1* knock-out *N. benthamiana*:Cf-4 plants, similar to the kinase-dead mutant *NbSOBIR1* D482N (Figure 1B). It is worth noting that this phenotype was not caused by a lack of accumulation of the *NbSOBIR1* T522A protein *in planta* (Figure S1). Quantification of the intensity of the HR, which was determined by employing red light imaging (Landeo Villanueva et al., 2021), showed that the intensity of the HR obtained upon transient co-expression of *NbSOBIR1* T522A with Avr4 was much lower than the intensity of the HR obtained when co-expressing *NbSOBIR1* WT with Avr4 (Figure 1C). The four additional mutants of *NbSOBIR1* that we generated showed a complementation capacity that was similar to *NbSOBIR1* WT. Consistently, complementation with *S/SOBIR1* T513A and *S/SOBIR1*-like T526A did not rescue the Avr4/Cf-4-triggered HR in the *sobir1* knock-out plants either, even though they both properly accumulated *in planta*. The differences between the HR intensities obtained upon complementation with the WT and with the other Ser/Thr to Ala mutants were again highly significant (Figure 1D, 1E, 1F, 1G, and S1). Of note, *S/SOBIR1*^{T513} and *S/SOBIR1*-like^{T526} are analogous to *NbSOBIR1*^{T522}, furthermore, these residues are overall highly conserved in SOBIR1 from various plant species (Figure 1A and S2A), which reveals that this specific Thr residue might be crucial for the functionality of SOBIR1 in all plant species. Taken together, these results

Trans-phosphorylation of SOBIR1 and BAK1

indicate that this particular Thr residue, present in the activation segment of SOBIR1, plays a crucial role in mediating the Avr4/Cf-4-specific HR by SOBIR1.

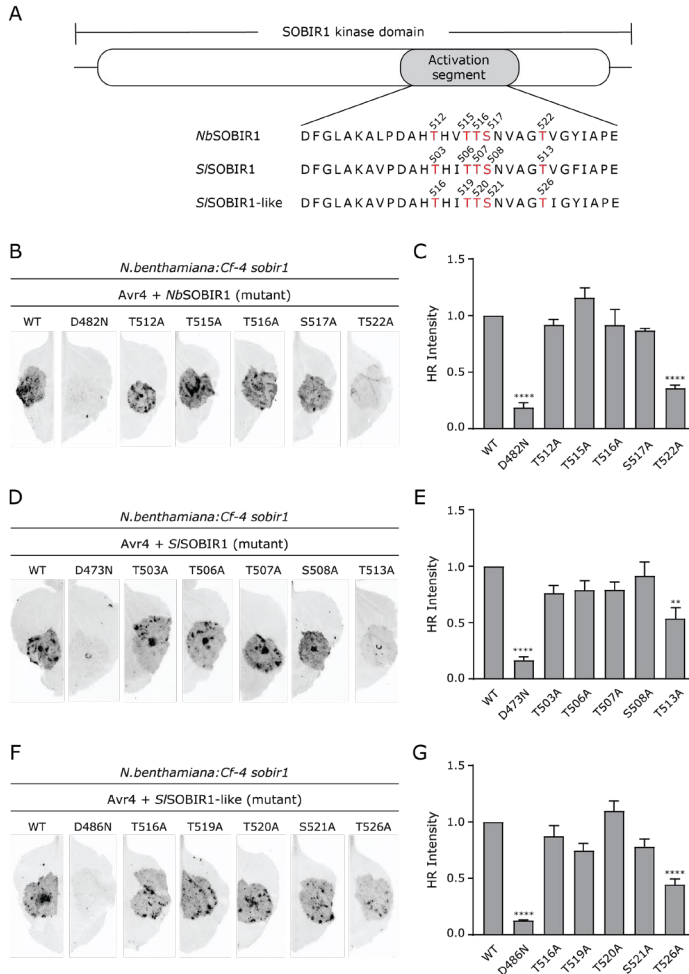


Figure 1. A site-directed mutagenesis screen identifies *NbSOBIR1*^{T522}, *S/SOBIR1*^{T513} and *S/SOBIR1-like*^{T526}, present in the activation segment of the kinase domain, as an essential phosphorylatable Thr residue in Avr4/Cf-4/SOBIR1/BAK1-triggered immune signaling. (A) Schematic diagram of the kinase domain of SOBIR1, with the activation segment indicated. The amino acid sequences of the activation segments of *NbSOBIR1*, *S/SOBIR1* and *S/SOBIR1-like* are aligned and are shown below the diagram. Conserved residues acting as potential phosphorylation sites are denoted in red. **(B to G)** Mutagenesis screen in combination with complementation of all potential phosphorylation sites in the activation segment of *NbSOBIR1* **(B and C)**, *S/SOBIR1* **(D and E)**, and *S/SOBIR1-like* **(F and G)**, to determine their importance in Avr4/Cf-4-triggered immune signaling. All SOBIR1 Thr and Ser mutants were transiently co-expressed with *Avr4* by agro-infiltration (OD₆₀₀ = 0.8) in leaves of *N. benthamiana*:Cf-4 *sobir1* knock-out mutants. Wild-type (WT) *NbSOBIR1*, *S/SOBIR1* and *S/SOBIR1-like* were used as positive controls, whereas kinase-dead versions of *NbSOBIR1* (D482N), *S/SOBIR1* (D473N) and *S/SOBIR1-like* (D486N) were used as negative controls. Leaves were imaged using the ChemiDoc, with the Red Fluorescent Protein (RFP) channel (Filter: 605/50; Light: Green Epi Illumination; Exposure

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time: 2 seconds), at 5 days post infiltration (dpi) (**B**, **D** and **F**). The intensity of the HR was subsequently quantified by Image Lab. Data shown are the average relative intensities of the HR + standard error of the mean (SEM) ($n \geq 6$) that is triggered upon complementation with the various SOBIR1 mutants, when compared to complementation with the respective SOBIR1 WT construct, of which the level of the HR was set to 1 (**C**, **E** and **G**). Statistical significance was determined by a one-way ANOVA test. ** $p < 0.001$; **** $p < 0.0001$. Experiments were repeated at least three times with similar results, and representative results are shown. *Nb*, *Nicotiana benthamiana*; *Sl*, *Solanum lycopersicum*; *S*, serine/Ser; *T*, threonine/Thr; *A*, alanine/Ala.

***Nb*SOBIR1^{T522}, as well as its analogous residues in both tomato SOBIR1s, is essential for Avr4/Cf-4-triggered ROS production and MAPK activation**

The swift production of ROS is a hallmark of the plant immune response and apoplastic ROS are mainly produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, such as the RESPIRATORY BURST OXIDASE HOMOLOGUE B (RBOHB) from *N. benthamiana* and tomato, which localizes at the plasma membrane (Qi et al., 2017; Yu et al., 2017; Waszczak et al., 2018). We have shown that the Avr4 protein triggers a biphasic ROS burst in *N. benthamiana*:*Cf-4*, while this biphasic ROS burst is eliminated in an *N. benthamiana*:*Cf-4 sobir1* knock-out mutant (Huang et al., 2021) (Chapter 2). To examine whether *Nb*SOBIR1^{T522} is crucial for mediating the Avr4-triggered ROS burst, we transiently expressed each *Nb*SOBIR1 mutant in the leaves of *N. benthamiana*:*Cf-4 sobir1* plants, after which the ROS production of discs taken from these leaves was monitored upon adding the Avr4 protein. Intriguingly, in contrast to the other four *Nb*SOBIR1 mutants and the positive control (*Nb*SOBIR1 WT), complementation with neither *Nb*SOBIR1 T522A nor the negative control (*Nb*SOBIR1 D482N) did restore the Avr4-triggered ROS burst in the *Cf-4*-expressing *N. benthamiana sobir1* knock-out line (Figure 2A). Consistently, the analogous residues of *Nb*SOBIR1^{T522} in tomato SOBIR1, which are *S*/SOBIR1^{T513} and *S*/SOBIR1-like^{T526}, gave the same phenotype after complementation with their Thr to Ala mutants (Figure 2B and 2C, respectively). These results suggest that *Nb*SOBIR1^{T522}, and its analogous residues in tomato SOBIR1s, are not only required for the HR, but also for the Avr4/Cf-4-induced ROS production.

Rapid and transient activation of MAPK cascades is another critical downstream event in the resistance of plants to pathogens (Yu et al., 2017; DeFalco and Zipfel, 2021). To determine whether Avr4/Cf-4-triggered MAPK activation in *N. benthamiana* also requires *Nb*SOBIR1^{T522}, *S*/SOBIR1^{T513}, and *S*/SOBIR1-like^{T526}, we transiently co-expressed the mutants *Nb*SOBIR1 T522A, *S*/SOBIR1 T513A or *S*/SOBIR1-like T526A (and their corresponding WTs and kinase-dead D to N mutants as a positive and negative control, respectively) with *Avr4* in the leaves of an *N. benthamiana*:*Cf-4 sobir1* knock-out line, and subsequently detected possible MAPK activation by incubating western blots of a total protein extract with p42/p44-erk antibodies. As expected, similar to the negative kinase-dead controls but in contrast to the positive WT controls, complementation with *Nb*SOBIR1 T522A, *S*/SOBIR1 T513A, or *S*/SOBIR1-

like T526A failed to restore the Avr4/Cf-4-induced MAPK activation in the *SOBIR1* knock-out line (Figure 2D). Notably, these phenotypes were not caused by differences in protein accumulation levels of the tested SOBIR1 variants (Figure S1).

In conclusion, the aforementioned results demonstrate that *NbSOBIR1*^{T522}, and its analogous residues in both tomato SOBIR1s, which are all located in the activation segment of SOBIR1, play a pivotal role in mediating Avr4/Cf-4/SOBIR1-triggered signaling.

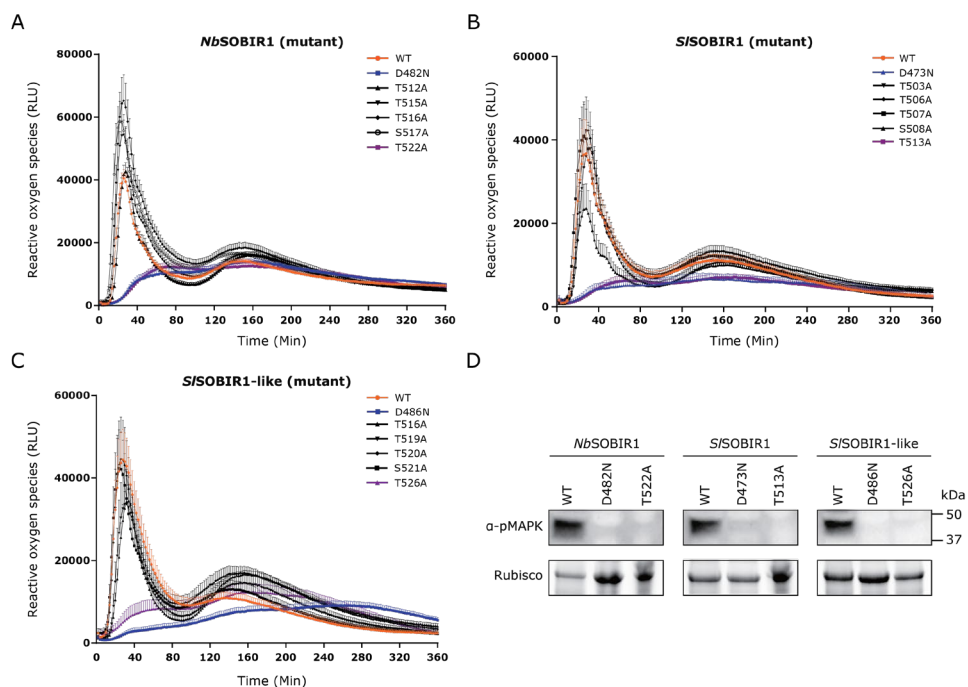


Figure 2. Complementation with *NbSOBIR1* T522A, *S/SOBIR1* T513A or *S/SOBIR1*-like T526A fails to restore the Avr4/Cf-4-triggered ROS burst and MAPK activation in a *N. benthamiana*:Cf-4 *sobir1* knock-out mutant line. Leaf discs of a *N. benthamiana*:Cf-4 *sobir1* mutant line, transiently expressing the five different *NbSOBIR1* mutants (A), the five different *S/SOBIR1* mutants (B), or the five different *S/SOBIR1*-like mutants (C), as well as their corresponding wild types (WT) (positive controls) and kinase-dead (D to N) mutants (negative controls), were treated with 0.1 μ M Avr4 protein, and ROS accumulation was monitored over time. ROS production is expressed as relative light units (RLUs) and the data are represented as mean + SEM. (D) *NbSOBIR1* T522A (left panel), *S/SOBIR1* T513A (middle panel), and *S/SOBIR1*-like T526A (right panel), with their corresponding WT and kinase-dead (D to N) mutants, were agro-infiltrated together with Avr4 in the leaves of an *N. benthamiana*:Cf-4 *sobir1* mutant line. The leaf samples were harvested at 2 dpi and subsequently, total protein extracts were subjected to immunoblotting with a p42/p44-erk antibody to determine the activation of downstream MAPKs by phosphorylation. Experiments were repeated at least three times and similar results were obtained. Representative pictures are shown.

***Nb*SOBIR1^{T522}, as well as its analogous residues in both tomato SOBIR1s, is required for SOBIR1 intrinsic kinase activity**

*Nb*SOBIR1^{T522}, as well as its analogous residues of both tomato SOBIR1s (*S*/SOBIR1^{T513} and *S*/SOBIR1-like^{T526}), is located at the activation segment of the kinase domain of SOBIR1, and this particular phosphorylatable Thr residue plays an important role in Cf-4/SOBIR1-initiated plant immunity. That led us to hypothesize this residue is essential for the intrinsic kinase activity of SOBIR1, and thereby for its auto-phosphorylation. This SOBIR1 auto-phosphorylation represents step 1 in the model that was proposed by van der Burgh et al (2019), in which SOBIR1 and BAK1 act together in immune signaling. To test whether this is the case, we employed *in vitro* phosphorylation assays. The N-terminally GST-tagged cytoplasmic kinase domain from SOBIR1, as well as this domain from various SOBIR1 mutants, were expressed in *Escherichia coli*, which was followed by SDS-PAGE of the *E. coli* lysates, Coomassie brilliant blue (CBB) staining (for determining the accumulation levels of the various mutant kinase domains) and Pro-Q staining (to determine the phosphorylation state of the different kinase domains) (Taylor et al., 2013). Successful production of the various SOBIR1 kinase domains was confirmed by western blotting, using SOBIR1 antibodies (Figure 3). Interestingly, wild-type *Nb*SOBIR1, *S*/SOBIR1, and *S*/SOBIR1-like all exhibited strong auto-phosphorylation activity, similar to their homolog in Arabidopsis (Figure 3) (Leslie et al., 2010). Strikingly, when compared to *Nb*SOBIR1 WT, the auto-phosphorylation activity of *Nb*SOBIR1 T522A was completely abolished, similar to that of the kinase-dead D482N mutant, suggesting that indeed *Nb*SOBIR1^{T522} is essential for the intrinsic kinase activity, and thereby the auto-phosphorylation, of *Nb*SOBIR1 (Figure 3A). In line with this observation for *Nb*SOBIR1 T522A, for *S*/SOBIR1 T513A and *S*/SOBIR1-like T526A also a loss of their intrinsic kinase activity was observed (Figure 3B and 3C). Altogether, these results demonstrate that this specific Thr residue is essential for SOBIR1 intrinsic kinase activity.

In addition to the aforementioned results, we also observed that the bands of SOBIR1 WTs were shifted upwards when compared to their corresponding kinase-dead mutants, which indicates an increase in molecular weight as a result of the auto-phosphorylation of the kinase domains during the expression *in vitro* (Figure 3). Interestingly, similar to the kinase-dead mutants, *Nb*SOBIR1 T522A, *S*/SOBIR1 T513A, and *S*/SOBIR1-like T526A did not display this gel shift, which again suggests that *Nb*SOBIR1^{T522}, *S*/SOBIR1^{T513}, and *S*/SOBIR1-like^{T526} have low phosphorylation levels and thereby this Thr residue is pivotal for their intrinsic kinase activity. More intriguingly, *Nb*SOBIR1 T516A, *S*/SOBIR1 T507A, and *S*/SOBIR1-like T520A exhibited an upward band shift when compared to their corresponding WTs, which reveals that the phosphorylation levels of these mutants might be even higher than those of their corresponding WTs. Therefore, *Nb*SOBIR1^{T516}, *S*/SOBIR1^{T507} and *S*/SOBIR1-like^{T520} might play a negative role in regulating the intrinsic kinase activity of SOBIR1 (Figure 3). Of note, *Nb*SOBIR1^{T516} is analogous to *S*/SOBIR1^{T507} and *S*/SOBIR1-like^{T520}. However, due to its

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low protein accumulation level, the mobility shift of *Nb*SOBIR1 T516A was not that clear when compared to the gel shifts of *S*/SOBIR1 T507A and *S*/SOBIR1-like T520A.

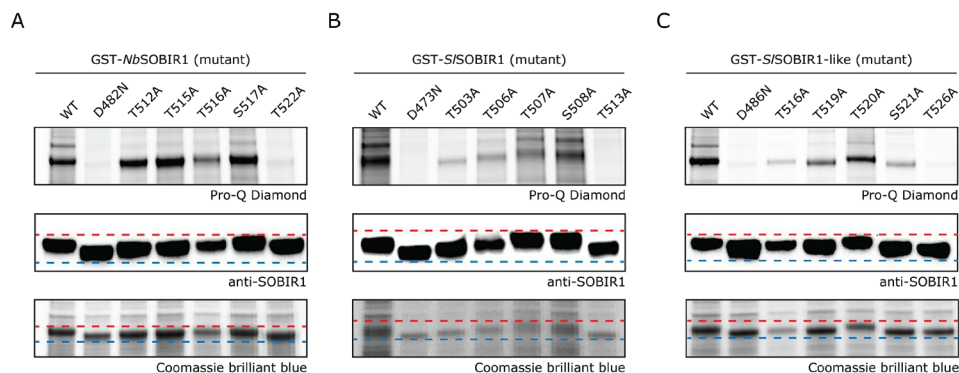


Figure 3. *Nb*SOBIR1^{T522}, *S*/SOBIR1^{T513} and *S*/SOBIR1-like^{T526} are required for the intrinsic kinase activity of SOBIR1. The N-terminally GST-tagged cytoplasmic kinase domains of *Nb*SOBIR1 (**A**), *S*/SOBIR1 (**B**), *S*/SOBIR1-like (**C**), and their Ala substitution mutants were produced in *E. coli*, in addition to their corresponding kinase-dead mutants as negative controls. After SDS-PAGE of the *E. coli* lysates, the recombinant proteins were stained with Coomassie brilliant blue (bottom panels), whereas the phosphorylation status of the kinase domains was determined by performing a Pro-Q Diamond stain (top panels). Production of the various SOBIR1 kinase domains was detected by western blotting, using SOBIR1 antibodies (middle panels). The dotted lines are added in the middle and bottom panels to show the mobility shifts of the different SOBIR1 mutants, with the red dotted lines indicating the top of the highest protein bands, and the blue dotted lines indicating the base of the lowest protein bands. Note that the mobility shifts correlate with the phosphorylation status, with SOBIR1 kinase domain mutants having the lowest phosphorylation levels showing the highest mobility. Experiments were repeated at least three times with similar results, and representative results are shown.

To further examine whether *Nb*SOBIR1^{T522} is actually being phosphorylated in the SOBIR1 kinase domain, we conducted a mass spectrometry (MS)-based analysis of recombinant GST-*Nb*SOBIR1 isolated from SDS gel, with the kinase-dead mutant GST-*Nb*SOBIR1 D482N included as a negative control. MS analyses identified 8 *in vitro* auto-phosphorylation sites (Table 1), of which T317 and T319 are located in the intracellular juxtamembrane (ijM) domain of SOBIR1 that was included in the constructs. Furthermore, S362 and S399, both present in the kinase domain of *Nb*SOBIR1, were also found to be phosphorylated. In addition, T512, T515, T516, and S517, which are all present in the activation segment and are subject of this study, were identified by MS as phosphorylated residues. Unfortunately, T522, which is also located in the activation segment and has been shown in the aforementioned results to be required for the intrinsic kinase activity of SOBIR1 and Avr4/Cf-4-triggered immune signaling, was not detected as being actually phosphorylated. Multiple potential phosphorylatable residues are located in one peptide, which might make the detection of one or more actually phosphorylated sites impossible. In addition, a recent study has shown that *At*SOBIR1^{T529}, which is analogous to *Nb*SOBIR1^{T522}, is a phosphorylated residue (Wei et al., 2022). Therefore, we cannot rule out the possibility that *Nb*SOBIR1^{T522} is also a

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phosphorylated residue in the SOBIR1 kinase domain. Of note, no phosphorylated residues were detected for GST-*Nb*SOBIR1 D482N, confirming that this mutant does not show any auto-phosphorylation activity.

Table 1. *In vitro* phosphorylation sites of *Nb*SOBIR1 identified by mass spectrometry.

Position	Peptide sequence and phosphorylation probabilities*
T317	GKTDGT(1)LTIIYSPLIK
T319	GKTDGT(0.001)LT(0.999)IYSPLIKK
S362	AELPGS(1)NGK
S399	QVKS(1)EIQILGQIR
T512	ALPDAHT(0.493)HVT(0.493)T(0.493)S(0.493)NVAGT(0.022)VG(0.005)IAPEYHQTLLK
T515	ALPDAHT(0.493)HVT(0.493)T(0.493)S(0.493)NVAGT(0.022)VG(0.005)IAPEYHQTLLK
T516	ALPDAHT(0.493)HVT(0.493)T(0.493)S(0.493)NVAGT(0.022)VG(0.005)IAPEYHQTLLK
S517	ALPDAHT(0.493)HVT(0.493)T(0.493)S(0.493)NVAGT(0.022)VG(0.005)IAPEYHQTLLK

* Phosphosite probability indicates the chance of a site being phosphorylated, which is calculated when a peptide only carries one phosphate group but contains more than one phosphorylatable amino acids.

SOBIR1 and BAK1 trans-phosphorylate each other *in vitro*

It was proposed earlier that upon Avr4 perception by Cf-4 there are rapid trans-phosphorylation events taking place between SOBIR1 and recruited BAK1 (van der Burgh et al., 2019). The observed strong auto-phosphorylation of the kinase domains of *Nb*SOBIR1 WT, *S*SOBIR1 WT, and *S*SOBIR1-like WT suggests that they all exhibit substantial intrinsic *in vitro* kinase activity (Figure 3). To elucidate whether SOBIR1 can directly phosphorylate BAK1, corresponding to the proposed SOBIR1 to BAK1 trans-phosphorylation step 2 in the model of van der Burgh et al (2019), we performed an *in vitro* phosphorylation assay. The GST-tagged SOBIR1 WT cytoplasmic kinase domain, or its corresponding kinase-dead mutant, was co-expressed with the His-tagged cytoplasmic kinase domain of the BAK1 kinase-dead mutant. The result showed that, first of all, the kinase-dead variant of *Nb*BAK1, *Nb*BAK1 D418N, of which the conserved "RD" motif in the catalytic loop is changed into "RN", did not have intrinsic auto-phosphorylation activity, as a Pro-Q stain was negative for this mutant when combined with *Nb*SOBIR1 D482N (Figure 4A). However, strikingly, this mutant was properly phosphorylated by kinase-active *Nb*SOBIR1 WT, as visualized by a positive Pro-Q stain for *Nb*BAK1 D418N (Figure 4A). Similarly, the tomato homolog of *Nb*BAK1, *S*BAK1, was also directly phosphorylated by *S*SOBIR1 WT, as well as by *S*SOBIR1-like WT *in vitro*, as in both cases *S*BAK1 D418N was properly phosphorylated. Also here, this trans-phosphorylation fully depended on the intrinsic kinase activity of *S*SOBIR1 WT and *S*SOBIR1-like WT, as their corresponding "RN" kinase-dead mutants did not phosphorylate *S*BAK1 D418N (Figure 4B and 4C).

We next sought to determine whether BAK1 WT can directly phosphorylate SOBIR1, an event that corresponds to the proposed BAK1 to SOBIR1 trans-phosphorylation step 3 in the model of van der Burgh et al (2019). Earlier, we already showed that the strong auto-phosphorylation activity of *E. coli*-produced *Nb*SOBIR1, *S*SOBIR1, and

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S/SOBIR1-like was eliminated in their "RN" kinase-dead variants (Figure 3). Therefore, we performed an additional *in vitro* phosphorylation assay by co-expressing the different SOBIR1 kinase-dead mutants with either BAK1 WT or its kinase-dead mutant. Importantly, *NbSOBIR1* D482N was phosphorylated when co-expressed with *NbBAK1* WT, but not when co-expressed with *NbBAK1* D418N, which demonstrates that indeed *NbSOBIR1* can be trans-phosphorylated by *NbBAK1* (Figure 4D). Consistently, *S/SOBIR1* D473N and *S/SOBIR1*-like D486N were also directly phosphorylated by *S/BAK1* WT (Figure 4E and 4F). Notably, trans-phosphorylation of SOBIR1 by BAK1 also required intrinsic kinase activity of BAK1, as phosphorylation of kinase-dead SOBIR1 did not take place when co-expressed with the BAK1 kinase-dead mutant (Figure 4D, 4E and 4F).

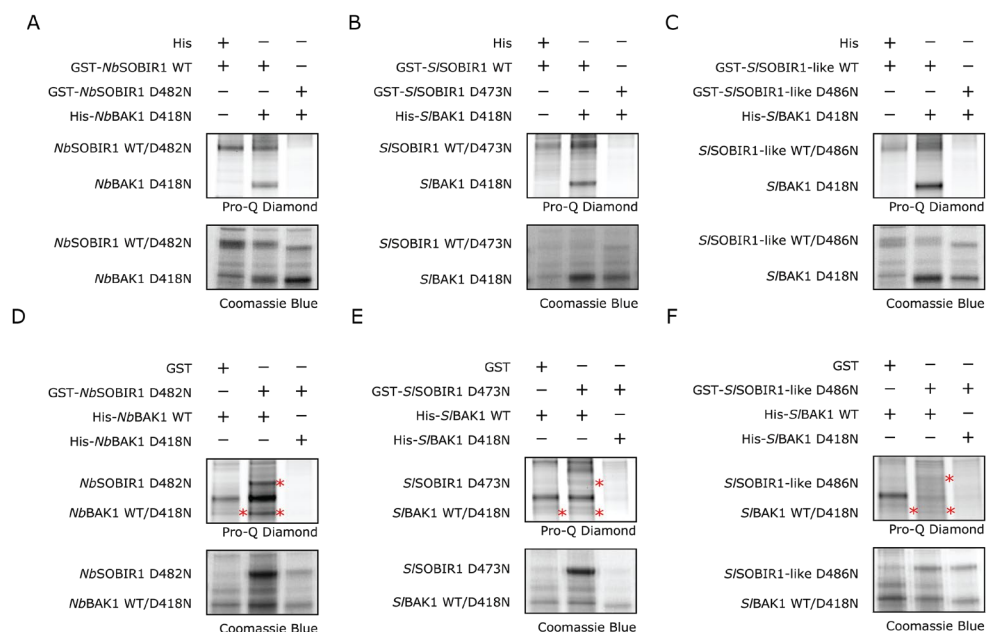


Figure 4. SOBIR1 and BAK1 trans-phosphorylate each other *in vitro*. (A) *NbSOBIR1* WT directly phosphorylates kinase-dead *NbBAK1* D418N, and *S/SOBIR1* WT (B) and *S/SOBIR1*-like WT (C) also directly phosphorylate kinase-dead *S/BAK1* D418N. (D) *NbBAK1* WT directly phosphorylates kinase-dead *NbSOBIR1* D482N and *S/BAK1* WT directly phosphorylates kinase-dead *S/SOBIR1* D473N (E) and *S/SOBIR1*-like D486N (F). The cytoplasmic kinase domains of the different SOBIR1s, as well as their corresponding kinase-dead ("RN") mutants, were fused to a GST tag, whereas the cytoplasmic kinase domain of *NbBAK1* and *S/BAK1*, and their corresponding kinase-dead mutants (*NbBAK1* D418N and *S/BAK1* N418N), were fused to a His tag. GST and His tags only served as negative controls. After co-expressing the indicated recombinant proteins in *E. coli*, the proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue, whereas their phosphorylation status was determined by performing a Pro-Q Diamond stain. Experiments were repeated at least three times with similar results, and representative results are shown. Red stars indicate the expected protein bands.

Trans-phosphorylation between SOBIR1 and BAK1 causes these co-receptors to obtain a unique phosphorylation status

To further show that the trans-phosphorylation events between SOBIR1 and BAK1 might eventually result in the full activation of SOBIR1/BAK1-containing immune complexes, we co-expressed *NbSOBIR1* WT with *NbBAK1* WT in *E. coli*, with the co-expression of *NbSOBIR1* WT and the *NbBAK1* kinase-dead mutant, the *NbSOBIR1* kinase-dead mutant and *NbBAK1* WT, and the *NbSOBIR1* kinase-dead mutant and the *NbBAK1* kinase-dead mutant as controls. We hypothesized that full activation of the SOBIR1/BAK1-containing immune complex would correlate with high phosphorylation levels of both SOBIR1 and BAK1, reflected by a very intense Pro-Q stain. Alternatively, overall phosphorylation levels might be similar to the levels shown in Figure 3, but different residues might be targeted for phosphorylation when WT SOBIR1 and BAK1 kinase domains are co-expressed, eventually requiring MS studies to be confirmed. Although unlike what we anticipated, the staining intensity of co-expressed *NbSOBIR1* WT and *NbBAK1* WT upon Pro-Q staining was not higher when compared to the controls (Figure 5), we did observe that the band representing *NbSOBIR1* WT, when co-expressed with *NbBAK1* WT (lane 1), showed a clear shift upwards when compared to (1) the band of *NbSOBIR1* WT that was co-expressed with the *NbBAK1* kinase-dead mutant D418N (lane 2), (2) the band of the *NbSOBIR1* kinase-dead mutant D482N that was co-expressed with *NbBAK1* D418N (lane 3), or (3) the band of *NbSOBIR1* D482N that was co-expressed with *NbBAK1* WT (lane 4). This observation indicates the possible addition of extra phosphate groups to *NbSOBIR1* WT when co-expressed with *NbBAK1* WT. Similarly, *NbBAK1* WT that was co-expressed with *NbSOBIR1* WT also showed an upward band shift (lane 1), when compared to (1) the band of *NbBAK1* D418N that was co-expressed with *NbSOBIR1* WT (lane 2), (2) the band of *NbBAK1* D418N that was co-expressed with *NbSOBIR1* D482N (lane 3), or (3) the band of *NbBAK1* WT that was co-expressed with *NbSOBIR1* D482N (lane 4), indicating that more phosphate groups were added to *NbBAK1* when co-expressed with *NbSOBIR1* WT (Figure 5). These results support the hypothesis that transphosphorylation events between SOBIR1 and BAK1 result in a unique phosphorylation pattern of both kinase domains, which might lead to the full activation of both co-receptors and thereby the complete Avr4/Cf-4/SOBIR1/BAK1 complex. Further MS studies should reveal whether there are indeed quantitative and/or qualitative differences between the phosphorylation status of the kinase domains of SOBIR1 and BAK1 before and after their co-expression, and when compared with their auto-phosphorylation when expressed alone.

Trans-phosphorylation of SOBIR1 and BAK1

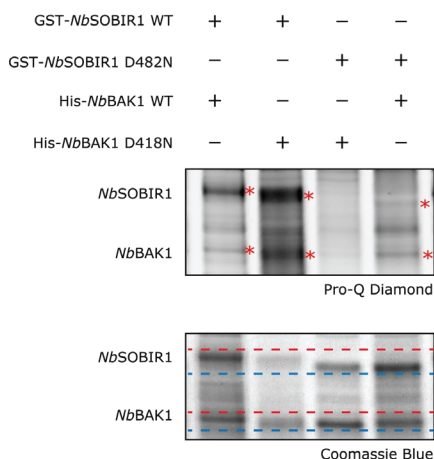


Figure 5. Trans-phosphorylation between SOBIR1 and BAK1 possibly leads to a unique phosphorylation status of both. The cytoplasmic kinase domain of *Nb*SOBIR1 WT or its corresponding kinase-dead mutant D482N, both tagged with GST, was co-expressed with the cytoplasmic kinase domain of either *Nb*BAK1 WT or its corresponding kinase-dead mutant D418N, in *E. coli*. Hereafter, proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (lower panel), whereas their phosphorylation status was determined by performing a Pro-Q Diamond stain (upper panel). The dotted lines are added in the lower panel to show the mobility shifts of *Nb*SOBIR1 or *Nb*BAK1, with the red dotted lines indicating the top of the highest protein bands, and the blue dotted lines indicating the base of lowest protein bands. Experiments were repeated at least three times with similar results, and representative results are shown. Red stars indicate the expected protein bands.

Discussion

Plants have evolved a huge repertoire of cell-surface receptors, including a large number of RLPs, to sense invading pathogens in the extracellular space. SOBIR1 and BAK1 are well-known co-receptors for LRR-RLPs, such as Cf-4, RLP23, and RLP42 (Liebrand et al., 2013; Liebrand et al., 2014; Albert et al., 2015; Postma et al., 2016; Zhang et al., 2021). It has been reported that an RLP/SOBIR1 complex forms heterodimers with BAK1 upon its elicitation and that subsequent trans-phosphorylation events between the kinase domains of SOBIR1 and BAK1 are required for initiating downstream immune signaling (van der Burgh et al., 2019). However, how the kinase domains of SOBIR1 and BAK1 exactly trans-phosphorylate each other, what phosphorylatable amino acid residues are actually important, and how their phosphorylation eventually contributes to RLP/SOBIR1-mediated signaling, remain unclear.

Thanks to the emergence of the CRISPR/Cas9 technique, we have been able to specifically knock out *SOBIR1* in *Cf-4*-expressing *N. benthamiana*, which allowed us to conduct complementation studies with various SOBIR1 mutants in these knock-out plants (Huang et al., 2021) (Chapter 2). In this study, by performing complementation studies in *N. benthamiana*:*Cf-4 sobir1* knock-out plants, we show that *Nb*SOBIR1^{T522}

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and its analogous residues in tomato SOBIR1s (*S/SOBIR1*^{T513} and *S/SOBIR1-like*^{T526}), present in the activation segment of SOBIR1, are essential for the Avr4/Cf-4-mediated HR, the ROS burst and downstream MAPK activation (Figure 1 and 2). Previously, we have reported that overexpression of *AtSOBIR1* in *N. benthamiana* results in constitutive activation of MAPKs and cell death (Liebrand et al., 2013; Bi et al., 2016; Wu et al., 2017). In agreement with the essential role of this Thr residue in the kinase domain of SOBIR1 from *N. benthamiana* and tomato, a mutation of the analogous residue in the kinase domain of *AtSOBIR1*, T529A, (Figure S2) strongly reduces its overall phosphorylation level and also suppresses the *AtSOBIR1*-triggered constitutive immunity in *N. benthamiana* (van der Burgh et al., 2018). These results are further supported by a recent study, which shows that *AtSOBIR1* T529A does not exhibit intrinsic kinase activity, thereby resulting in a complete loss of cell death in *N. benthamiana* upon its overexpression (Wei et al., 2022). In addition, both SOBIR1 and BAK1 are RD kinases, suggesting the possibility of auto-phosphorylation in their activation segment. Further *in vitro* studies demonstrate that, similar to their Arabidopsis homologs, *NbSOBIR1*, *S/SOBIR1*, *S/SOBIR1-like*, *NbBAK1*, and *S/BAK1* exhibit auto-phosphorylation activity (Figure 3 and 4) (Li, 2002; Leslie et al., 2010; Schwessinger, 2011). More interestingly, it has been recently reported that the auto-phosphorylation of *AtSOBIR1* occurs through an intermolecular mechanism, which means that the oligomerization of SOBIR1, as shown by van der Burgh et al. (2019), is important for its auto-phosphorylation (Wei et al., 2022). Notably, *NbSOBIR1*^{T522}, *S/SOBIR1*^{T513}, and *S/SOBIR1-like*^{T526} are crucial for the intrinsic kinase activity of the SOBIR1 kinase domain, as our *in vitro* phosphorylation assay revealed that the *NbSOBIR1* T522A, *S/SOBIR1* T513A, and *S/SOBIR1-like* T526A mutants have completely lost their ability to auto-phosphorylate (Figure 3).

Interestingly, the equivalent Thr residue has been proven essential for many other RD kinases. A good example is Arabidopsis CERK1^{T479} (Figure S2), which is indispensable for the activation of the *AtCERK1* kinase (Suzuki et al., 2016; Suzuki et al., 2019). Furthermore, the Arabidopsis LRR-RLK NUCLEAR SHUTTLE PROTEIN (NSP)-INTERACTING KINASE1 (NIK1) is a virulence target of the begomovirus NSP and is involved in plant antiviral immunity (Mariano et al., 2004; Santos et al., 2010). A mutation at T474, which locates at the activation segment of the kinase domain of NIK1 (Figure S2), attenuates auto-phosphorylation of NIK1 and enhances susceptibility to the Cabbage leaf curl virus (Santos et al., 2009). BRASSINOSTEROID-INSENSITIVE 1 (BRI1) is one of the best-characterized LRR-RLKs, with essential roles in plant growth and development (Chinchilla et al., 2009; Belkhadir et al., 2014). Biochemical and genetic analyses have revealed that T1049, present in the activation segment of the kinase domain of BRI1 (Figure S2), is vital for the intrinsic kinase function of Arabidopsis BRI1, both *in vitro* and *in planta* (Wang et al., 2008). In addition, the LRR-RLK HAESA, which regulates organ abscission in Arabidopsis flowers, requires phosphorylation of S861, present in its activation segment (Figure S2), to activate its kinase activity (Taylor et al., 2016).

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Moreover, another example is *AtBAK1*^{T455}, which is also located in the activation segment (Figure S2), as a single change of this Thr residue to Ala abolishes the kinase activity of *AtBAK1*. Therefore, we speculate that phosphorylation of the analogous residue is also critical for the intrinsic kinase activity of *NbBAK1* and *S/BAK1* (Figure S2). Next to T455, mutating three additional Thr residues in the activation segment of *AtBAK1*, T446, T449, and T450, at the same time while T455 remained unchanged, also results in the loss of *AtBAK1* intrinsic kinase activity. However, their single changes do not affect the kinase activity when compared to *AtBAK1* WT, which demonstrates that phosphorylation of these three Thr residues individually might only play a minor role in regulating the activation of *AtBAK1* (Yun et al., 2009). In this study, we also mutated the analogous residues in *NbSOBIR1* (T512, T515 or S517), *S/SOBIR1* (T503, T506 or S508), and *S/SOBIR1*-like (T516, T519 or S521). Even though all these single mutants still exhibit auto-phosphorylation activity, we did observe that the mobility of some of them was affected, resulting in a band shift when compared to their corresponding wild-type controls (Figure 3). Thus, some of these tested putative phosphorylation sites might also play a minor role in the activation of SOBIR1. Importantly, the substitution of the analogous residues in *AtSOBIR1* does not affect the auto-phosphorylation levels but only slightly decreases the ability to phosphorylate the kinase-dead mutant *AtSOBIR1* K377R (Wei et al., 2022). Future studies involving the generation of double or even triple mutants, followed by determining the effect on the intrinsic kinase activity of these SOBIR1 mutants, should provide more evidence for the relative importance of these residues in auto-activation.

Surprisingly, a mutation at *NIK1*^{T469} does not affect *NIK1* auto-phosphorylation but instead increases its ability to phosphorylate a substrate (Santos et al., 2009). Therefore, phosphorylation of *NIK1*^{T469} plays an inhibitory role in the kinase function. *NIK1*^{T469} corresponds to *NbSOBIR1*^{T516}, *S/SOBIR1*^{T507}, and *S/SOBIR1*-like^{T520}, for which a single mutation of each residue in the SOBIR1 kinase domain displays a pattern of a clear mobility shift to a higher position when compared to the corresponding WT kinase domains, suggesting increased overall phosphorylation levels of these three mutants (Figure 3, S1 and S2). These results indicate that, also in this case, *NbSOBIR1*^{T516}, *S/SOBIR1*^{T507}, and *S/SOBIR1*-like^{T520} might negatively regulate the intrinsic kinase activity of SOBIR1 upon their phosphorylation.

Earlier, we have exploited *AtSOBIR1*-triggered constitutive immunity in *N. benthamiana* to study SOBIR1-mediated immune signaling (Wu et al., 2017). *In planta* evidence has shown that this constitutive immunity requires both kinase-active SOBIR1 and BAK1, and a model was proposed in which *AtSOBIR1* homodimers trigger *AtSOBIR1* auto-phosphorylation and activation, which then phosphorylates and activates BAK1 when being recruited. Subsequently, activated BAK1 phosphorylates, and thereby fully activates, SOBIR1, which leads to *AtSOBIR1*-mediated auto-immunity (Wu et al., 2017; van der Burgh et al., 2019). It was proposed that this model of sequential phosphorylation also applies to *Avr4/Cf-4/SOBIR1/BAK1*-triggered

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signaling, and in this study, we provide *in vitro* support for this model and show that SOBIR1 indeed exhibits strong auto-phosphorylation activity (Figure 3). Furthermore, SOBIR1 indeed can directly phosphorylate BAK1, and the intrinsic kinase activity of SOBIR1 is required for this trans-phosphorylation event (Figure 3 and 4). Accordingly, BAK1 is also able to directly phosphorylate SOBIR1, which again depends on the kinase activity of BAK1 (Figure 3 and 4). Further *in vitro* phosphorylation assays consisting of co-expressing *Nb*SOBIR1 WT and *Nb*BAK1 WT show that *Nb*SOBIR1 WT that is phosphorylated by *Nb*BAK1 WT exhibits an upward band shift, when compared to *Nb*SOBIR1 WT in the absence of *Nb*BAK1 WT, the *Nb*SOBIR1 kinase-dead mutant and the *Nb*SOBIR1 kinase-dead mutant that is phosphorylated by *Nb*BAK1 WT (Figure 5). This observation also holds for *Nb*BAK1 WT (Figure 5). These results collectively demonstrate that the trans-phosphorylation events between SOBIR1 and BAK1 mutually determine the phosphorylation status of each of them. This possibly results in a highly specific phosphorylation pattern of both co-receptors, which subsequently might be responsible for the initiation of further downstream signaling by phosphorylating specific cytoplasmic signaling partners through their kinase domains. Interestingly, our observations are supported by a recent study, which shows that *At*SOBIR1 directly phosphorylates *At*BAK1 *in vitro*, while *At*BAK1 also directly phosphorylates *At*SOBIR1 *in vitro* (Wei et al., 2022). SOBIR1 and BAK1 are common co-receptors for many LRR-RLPs that are involved in plant immunity (Liebrand et al., 2013; Zhang et al., 2013; Bi et al., 2014; Liebrand et al., 2014; Albert et al., 2015; Domazakis et al., 2018; Zhang et al., 2021), and therefore, the model that we proposed earlier (van der Burgh et al., 2019), and is now further supported by this study and by the work of Wei and colleagues (2022), probably also applies to immune signaling triggered by additional two-component RLP/SOBIR1 LRR-RLKs. However, which phosphorylation sites in the kinase domain of SOBIR1 are actually phosphorylated by BAK1 and vice versa, remains to be explored. Further studies involving co-expressing the kinase domains of SOBIR1 and BAK1 in *E. coli* and analyzing the phospho-peptides of SOBIR1 and BAK1 by MS should provide more *in vitro* evidence to answer this question.

Importantly, increasing evidence has indicated that BAK1, which is a common regulator of LRR-RLKs and LRR-RLPs, promotes the activation of the receptor complex upon the perception of an ExIP by the LRRs of the matching primary receptor. However, the signaling specificity is determined by the kinase domain of the primary receptor, which is either an RLK or the constitutive RLP/SOBIR1 complex, whereas BAK1 merely acts as a general complex activator (Hohmann et al., 2020). Therefore, after being trans-phosphorylated by BAK1 (Figure 3 and 4), SOBIR1 is proposed to initiate the trans-phosphorylation events with downstream cytoplasmic signaling components. Such components are for example particular RLCKs that also have kinase activity, thereby triggering a specific type of immune signaling, irrespective of the RLP that is involved in the RLP/SOBIR1 complex. Future studies, for example consisting of transient co-expression of tagged Cf-4 and SOBIR1 in *N. benthamiana sobir1* mutant plants, and a

subsequent pull-down of Cf-4 after treatment with the Avr4 protein, or not, and then determining the exact phosphorylation pattern of the kinase domain of co-purifying SOBIR1, should provide insight into whether the phosphorylation status of SOBIR1 actually changes *in planta* and if so, how SOBIR1 implements its altered phospho-code to trigger plant immunity downstream of any LRR-RLP involved in resistance.

Materials and Methods

Plant growth conditions

N. benthamiana:Cf-4 *sobir1/sobir1-like* knock-out plants were used in this study. As *SOBIR1-like* is not functional in *N. benthamiana*, we further refer to these knock-out plants as *N. benthamiana*:Cf-4 *sobir1* (Huang et al., 2021) (Chapter 2). They were grown under 15 h of light at 21 °C and 9 h of darkness at 19 °C in a climate chamber, with a relative humidity of ~70%.

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

The constructs pBIN-KS-35S::*NbSOBIR1*-eGFP (SOL2911), pBIN-KS-35S::*NbSOBIR1*^{D482N}-eGFP (kinase-dead mutant) (SOL7928), pBIN-KS-35S::*S/SOBIR1*-eGFP (SOL2774), pBIN-KS-35S::*S/SOBIR1*^{D473N}-eGFP (kinase-dead mutant) (SOL2875), pBIN-KS-35S::*S/SOBIR1-like*-eGFP (SOL2773), pBIN-KS-35S::*S/SOBIR1-like*^{D486N}-eGFP (kinase-dead mutant) (SOL2876) and pMOG800-Avr4 have been described previously (Liebrand et al., 2013). The codon change, resulting in a Ser/Thr-to-Ala amino acid change in the activation segment of SOBIR1, was introduced by overlap extension PCR using the plasmids pENTR/D-Topo::*NbSOBIR1* (SOL4064), pENTR/D-Topo::*S/SOBIR1* (SOL2746), and pENTR/D-Topo::*S/SOBIR1-like* (SOL2745) as templates (Liu and Naismith, 2008; Liebrand et al., 2013). Phusion Hot Start II DNA Polymerase (Thermo Scientific) was used for the overlap extension PCR and the primers that were used are listed in Supplemental Table S1. The methylated template plasmids remaining in the PCR products were digested by DpnI (NEB), and after transformation to *E. coli* DH5α, the required SOBIR1 variants carrying individual mutations were selected by Sanger sequencing, and then introduced into pBIN-KS-35S::*GWY*-eGFP (SOL2095; for C-terminally tagging with eGFP), by using Gateway LR Clonase II (Invitrogen). Thereby, the binary vectors pBIN-KS-35S::*NbSOBIR1*^{T512A}-eGFP (SOL7909), pBIN-KS-35S::*NbSOBIR1*^{T515A}-eGFP (SOL7910), pBIN-KS-35S::*NbSOBIR1*^{T516A}-eGFP (SOL7911), pBIN-KS-35S::*NbSOBIR1*^{S517A}-eGFP (SOL7912), pBIN-KS-35S::*NbSOBIR1*^{T522A}-eGFP (SOL7913), pBIN-KS-35S::*S/SOBIR1*^{T503A}-eGFP (SOL7969), pBIN-KS-35S::*S/SOBIR1*^{T506A}-eGFP (SOL7970), pBIN-KS-35S::*S/SOBIR1*^{T507A}-eGFP (SOL7971), pBIN-KS-35S::*S/SOBIR1*^{S508A}-eGFP (SOL7972), pBIN-KS-35S::*S/SOBIR1*^{T513A}-eGFP (SOL7973), pBIN-KS-35S::*S/SOBIR1-like*^{T516A}-eGFP (SOL7950), pBIN-KS-35S::*S/SOBIR1-like*^{T519A}-eGFP (SOL7951), pBIN-KS-35S::*S/SOBIR1-like*^{T520A}-eGFP (SOL7952), pBIN-KS-35S::*S/SOBIR1-like*^{S521A}-eGFP

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(SOL7953) and pBIN-KS-35S::S/SOBIR1-like^{T526A}-eGFP (SOL7954), for *in planta* expression were obtained.

A. *tumefaciens*-mediated transient transformation

All binary plasmids were transformed into *A. tumefaciens* (further referred to as *Agrobacterium*) strain C58C1, carrying the helper plasmid pCH32. Hereafter, each SOBIR1 variant was transiently co-expressed with *Avr4*, at an optical density at 600 nm (OD₆₀₀) of 0.8, in the leaves of 4- to 5-week old *N. benthamiana*:*Cf-4 sobir1* knock-out plants, as previously described (van der Hoorn et al., 2000). The development of cell death was photographed and quantified at 5 days post infiltration (dpi) by red light imaging using the ChemiDoc (Bio-Rad), with an exposure time of 2 seconds (Landeo Villanueva et al., 2021). Statistical analysis was performed using one-way ANOVA by GraphPad Prism 9.

Reactive oxygen species (ROS) assay

The different SOBIR1 variants were transiently expressed by agro-infiltration (OD₆₀₀=0.8) in complete, just fully expanded leaves of *N. benthamiana*:*Cf-4 sobir1* plants, with the corresponding wild-type (WT) SOBIR1 binary construct as a positive control and the kinase-dead SOBIR1 binary construct as a negative control. Leaf discs were collected by using a biopsy puncher (Ø 5 mm, Robbins Instruments), at 24 h after agro-infiltration and were floated on 80 µL of sterile water in a 96-well plate overnight. Hereafter, the water was removed carefully with tissue paper and replaced with 50 µL of fresh sterile water. After another 1 h incubation, 50 µL of the reaction solution, containing 100 µM of luminol (L-012, Fujifilm, Japan), 20 µg/mL horseradish peroxidase (Sigma), and 0.2 µM *Avr4* protein, was added to each well. The level of luminescence was subsequently measured using a CLARIOstar plate reader (BMG Labtech).

Immunoprecipitation (IP) and MAPK assay

eGFP-tagged *NbSOBIR1*, *NbSOBIR1*^{D482N}, *NbSOBIR1*^{T522A}, *S/SOBIR1*, *S/SOBIR1*^{D473N}, *S/SOBIR1*^{T513A}, *S/SOBIR1*-like, *S/SOBIR1*-like^{D486N} or *S/SOBIR1*-like^{T526A}, was transiently co-expressed with *Avr4* (OD₆₀₀=0.8 for each binary vector) in the first fully expanded leaves of *N. benthamiana*:*Cf-4 sobir1* plants. Leaf samples were harvested at 2 dpi, followed by IP using GFP_Trap beads as described previously (Liebrand et al., 2013). Subsequently, the soluble total protein (input) was separated on an SDS-PAGE gel, and activated MAPKs were visualized by immunoblotting with anti-p42/p44-erk antibodies (NEB). Meanwhile, the immunoprecipitated SOBIR1 (variants) were subjected to immunoblot analysis with αGFP-HRP antibodies (Miltenyi Biotec GmbH).

Recombinant protein expression

To produce the recombinant cytoplasmic kinase domain (KD) of SOBIR1 and BAK1 with a GST or 6×His tag in *E. coli*, the vectors pET-GST (Addgene No. 42049) and pET-15b

Trans-phosphorylation of SOBIR1 and BAK1

were employed, respectively. Both vectors were linearized by PCR amplification with the primer pairs pET-GST_fw/rev and pET-15b_fw/rev (Supplemental Table S1). Meanwhile, the coding sequence of *NbSOBIR1* KD, *S/SOBIR1* KD, and *S/SOBIR1*-like KD, as well as all their corresponding mutants, were PCR-amplified from the corresponding pENTR/D-Topo plasmids, using the primers containing the homologous sequence of either pET-GST or pET-15b (Supplemental Table S1). Hereafter, the linearized vector and amplified insert were recombined by using the ClonExpress II One Step Cloning kit (Vazyme, China). After transformation to *E. coli* DH5 α , the correct expression constructs were selected by performing colony PCRs and Sanger sequencing.

For recombinant protein expression, the *E. coli* strain BL21 was used. Bacteria harboring the correct expression construct were cultured at 37 °C overnight in LB liquid medium and subsequently inoculated into fresh LB medium at a ratio of 1:200 (v/v). After culturing at 37 °C for around 3 h, the bacterial population became in an exponential growth phase, with an OD₆₀₀ between 0.6 and 0.8, at which IPTG was added to a final concentration of 0.5 mM, followed by incubating the culture at 22 °C overnight for protein production.

***In vitro* phosphorylation assays**

In vitro phosphorylation assays were performed as previously described (Taylor et al., 2013). In brief, cells from 100 μ L of the *E. coli* cultures that were started for protein expression were collected by centrifugation and then resuspended in 100 μ L of SDS sample buffer (200 mM Tris-HCl, 8% SDS, 40% glycerol, 400 mM DTT, and 0.2% bromophenol blue), followed by boiling for 10 min. Hereafter, the samples were centrifuged for 2 min at maximum speed in an Eppendorf centrifuge, and 8 μ L of the supernatant were loaded onto a precast mini-PROTEIN TGX Polyacrylamide Gel (BIORAD). After running for around 100 min at 160 V, the gel was incubated in fixation solution (50% methanol, 10% acetic acid in H₂O), overnight. Next, the gel was washed in deionized water for 30 min twice, and the phosphorylated proteins were stained using a Pro-Q Diamond solution (Invitrogen). Subsequently, the staining solution was removed by washing the gel in de-staining solution (20% acetonitrile, 50 mM sodium acetate), and proteins were stained with Coomassie brilliant blue (CBB).

MS analysis

To determine the *in vitro* phosphorylation sites in the kinase domain of *NbSOBIR1*, the GST-tagged kinase domains of *NbSOBIR1* and *NbSOBIR1* D482N were produced in *E. coli* BL21 cells and the crude *E. coli* extracts were loaded onto a precast mini-PROTEIN TGX Polyacrylamide Gel as described above. After running the gel, in-gel protein treatment was performed as described before (Karlova, 2009). Note that this experiment has only been done once.

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Supplementary Data

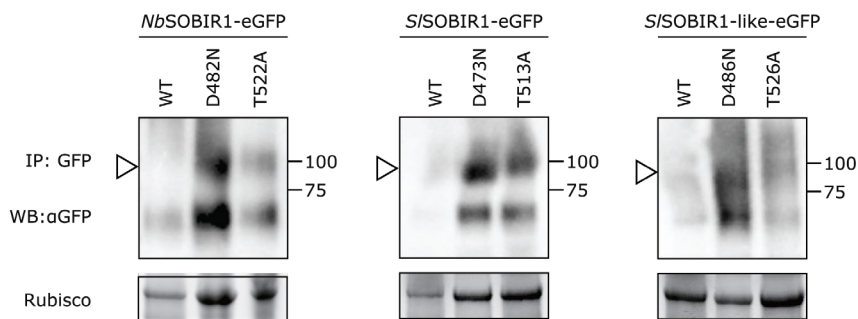


Figure S1. Accumulation levels of *NbSOBIR1* T522A, *S/SOBIR1* T513A and *S/SOBIR1*-like T526A *in planta*, in comparison to their wild-type (WT) and kinase-dead ("RN") versions. *NbSOBIR1* T522A, *S/SOBIR1* T513A and *S/SOBIR1*-like T526A, which fail to restore the Avr4-triggered HR in *N. benthamiana*:*Cf-4 sobir1* mutant plants, were transiently co-expressed in *benthamiana*:*Cf-4 sobir1* plants with Avr4 (both at an OD₆₀₀ of 0.8). Transient expression of their respective WT and kinase-dead D to N version, in combination with Avr4, was included as positive and negative controls, respectively. The leaf samples were collected at 2 dpi. Total protein extracts were subjected to immuno-purification (IP) using GFP-affinity beads, followed by western blotting (WB) with αGFP antibody (upper panels). The amount of total protein that was used for the IP is reflected by the Rubisco band present in the total protein extracts loaded on SDS gel (lower panels). Arrowheads indicate the band corresponding to SOBIR1-eGFP. Note that transient expression of WT *SOBIR1* in combination with Avr4 results in cell death, resulting in very low WT *SOBIR1* accumulation levels.

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SOBIR1 homologs

<i>Nicotiana benthamiana</i> _SOBIR1 (<i>Nb</i> SOBIR1)	DFGLAKA--L---PDAH-----THV TTS NVAGTVGYIAPE
<i>Solanum lycopersicum</i> _SOBIR1 (<i>Sl</i> SOBIR1)	DFGLAKA--V---PDAH-----TH ITTS NVAGTVGFIAPE
<i>Solanum lycopersicum</i> _SOBIR1-like (<i>Sl</i> SOBIR1-like)	DFGLAKA--V---PDAH-----TH ITTS NVAGTIGYIAPE
<i>Arabidopsis thaliana</i> _SOBIR1 (<i>At</i> SOBIR1)	DFGLAKA--M---PDAV-----TH ITTS SHVAGTVGYIAPE
<i>Solanum tuberosum</i> _SOBIR1	DFGLAKA--V---PDAH-----TH ITTS NVAGTMGYIAPE
<i>Capsicum chinense</i> _SOBIR1_A0A2G3BFP7	DFGLAKA--V---PDAH-----THV TTS NVAGTVGYIAPE
<i>Capsicum baccatum</i> _SOBIR1	DFGLAKA--V---PDAH-----THV TTS NVAGTVGYIAPE
<i>Nicotiana tabacum</i> _SOBIR1_A0A1S3ZD83	DFGLAKA--V---PDAH-----THV TTS NVAGTVGYIAPE
<i>Nicotiana tabacum</i> _SOBIR1_A0A1S3XP18	DFGLAKA--L---PDAH-----TH ITTS NVAGTVGYIAPE
<i>Nicotiana sylvestris</i> _SOBIR1	DFGLAKA--L---PDAH-----TH ITTS NVAGTVGYIAPE
<i>Capsicum annuum</i> _SOBIR1_A0A2G2Y0L5	DFGLAKA--V---PDAH-----THV TTS NVAGTVGYIAPE
<i>Nicotiana attenuata</i> _SOBIR1_A0A1J6INR1	DFGLAKA--L---PDAH-----TH ITTS NVAGTVGYIAPE
<i>Capsicum annuum</i> _SOBIR1_A0A1U8G6T0	DFGLAKA--V---PDAH-----TH ITTS NVAGTIGYIAPE
<i>Capsicum chinense</i> _SOBIR1_A0A2G3CCQ8	DFGLAKA--V---PDAH-----TH ITTS NVAGTIGYIAPE
<i>Sesamum indicum</i> _SOBIR1_A0A6I9SRZ7	DFGLAKA--M---PDAN-----THV TTS NVAGTVGYIAPE
<i>Juglans regia</i> _SOBIR1	DFGLAKA--M---PDAN-----TH IST SNVAGTVGYIAPE
<i>Vigna radiata</i> _SOBIR1	DFGLAKA--M---PDYK-----THV TTS NVAGTVGYIAPE
<i>Cicer arietinum</i> _SOBIR1	DFGLAKA--M---PDAG-----TH IST SNVAGTVGYIAPE
<i>Glycine soja</i> _SOBIR1_A0A445L292	DFGLAKA--M---PDYK-----TH ITTS NVAGTVGYIAPE
<i>Prunus yedoensis</i> var. <i>nudiflora</i> _SOBIR1	DFGLAKA--V---PEYH-----TH ITTS NVAGTVGYIAPE
<i>Hibiscus syriacus</i> _SOBIR1_A0A6A2Y1P7	DFGLAKA--M---PDAN-----THV TTS NLAGTVGYIAPE
<i>Glycine soja</i> _SOBIR1_A0A445KB68	DFGLAKA--M---PDYK-----TH ITTS NVAGTVGYIAPE
<i>Hibiscus syriacus</i> _SOBIR1_A0A6A3CRM4	DFGLAKA--M---PDAC-----THV TTS NLAGTVGYIAPE
<i>Sesamum indicum</i> _SOBIR1_A0A6I9TZP4	DFGLAKA--L---PDAN-----THV TTS NVAGTVGYIAPE
<i>Gossypium hirsutum</i> _SOBIR1	DFGLAKA--M---PDAQ-----TH ITTS NLAGTVGYIAPE

Other RLKs

<i>Nicotiana benthamiana</i> _BAK1	DFGLAKL--M---DYKD-----THV TTA -VRG T IGHIAPE
<i>Solanum lycopersicum</i> _BAK1	DFGLAKL--M---DYKD-----THV TTA -VRG T IGHIAPE
<i>Arabidopsis thaliana</i> _BAK1	DFGLAKL--M---DYKD-----THV TTA -VRG T IGHIAPE
<i>Arabidopsis thaliana</i> _CERK1	DFGL T KL T EV---GG SA -----T R GMAG T FGYMAPE
<i>Arabidopsis thaliana</i> _NIK1	DFGLAKL--L---DHQD-----SHV TTA -VRG T VGHIAPE
<i>Arabidopsis thaliana</i> _BRI1	DFGMARL--M---S AM D-----THL SVST LAGTPGYVPE
<i>Arabidopsis thaliana</i> _HAESA	DFGLAKV G QM---S G SK-----T PE AM S G I AG SC GYIAPE
<i>Arabidopsis thaliana</i> _EFR	DFGLAQL--L---YKYD RES FL NQ F SS AGVRG T IGYAAPE
<i>Arabidopsis thaliana</i> _FLS2	DFG T ARI--LGFRED S -----T T AST S AFEG T IGYLAPE

Figure S2. *Nb*SOBIR1^{T522} is evolutionarily conserved in the kinase domain of various RLKs from different plant species. The protein sequences of SOBIR1 homologs from various plants species were obtained from the UniProt database (<https://www.uniprot.org/>), while the protein sequences of other RLKs were retrieved from TAIR (<https://www.arabidopsis.org/index.jsp>) and the Sol Genomics Network (<https://solgenomics.net/>). The alignment was visualized using JalView and only the amino acid sequences of the activation segment of the kinase domain are shown. All the putatively phosphorylatable Ser (S) and Thr (T) residues are highlighted in orange. The positions of the five Ser/Thr residues of *Nb*SOBIR1 that are subject of this study are indicated on top.

Supplemental Table S1. Nucleotide sequences of the primers used in this study.

Primer code	Primer name	Sequence (5' - 3')
Primers used for introducing point mutations in the SOBIR1 kinase domain (the introduced mutations are indicated with a capital)		
ho113	NbSOBIR1_T512A fw*	gcactcccagatgccatGcacatgttacgacttc
ho114	NbSOBIR1_T512A rev	gaagtcgtaacatgtgCatgggcatctgggagtg
ho115	NbSOBIR1_T515A fw	gatgccatacacatgttGcgacttcaaagtgttcagg
ho116	NbSOBIR1_T515A rev	cctgcaacatttgaagtcgCaacatgtgtatgggcatc
ho117	NbSOBIR1_T516A fw	gccatacacatgttacgGcttcaaatgttcaggaaac
ho118	NbSOBIR1_T516A rev	gttcctgcaacatttgaagCcgtaacatgtgtatgggc
ho119	NbSOBIR1_S517A fw	gccatacacatgttacgactGcaaatgttcagg
ho120	NbSOBIR1_S517A rev	cctgcaacatttgCagtcgtaacatgtgtatgggc
ho121	NbSOBIR1_T522A fw	cttcaaatgttcagggaGctgtgggatattgcacc
ho122	NbSOBIR1_T522A rev	ggtgcaatatacccacagCctctgcaacatttgaag
ao1	SISOBIR1_T503A fw	gcagttccagatgctcatGcacatatcaccacttc
ao2	SISOBIR1_T503A rev	gaagtggtgatgtgCatgagcatctggaactgc
ao3	SISOBIR1_T506A fw	gctcatacacatcGccacttcaaagtgttcag
ao4	SISOBIR1_T506A rev	ctgcaacatttgaagtgGgatgtgtatgagc
ao5	SISOBIR1_T507A fw	caccGcttcaaatgttcaggaaactgttgattattg
ao6	SISOBIR1_T507A rev	caataaatccaacagttcctgcaacatttgaagCgggtg
ao7	SISOBIR1_S508A fw	gctcatacacatcaccactGcaaatgttcagg
ao8	SISOBIR1_S508A rev	cctgcaacatttgCagtggtgatgtgtatgagc
ao9	SISOBIR1_T513A fw	caaatgttcagggaGctgttgattattgc
ao10	SISOBIR1_T513A rev	gcaataaatccaacagCtctgcaacatttg
ho166	SISOB-like_T516A_fw	gctgtcccagatgctcatGcacattatacaacttc
ho167	SISOB-like_T516A_rev	gaagttgtaatatgtgCatgagcatctgggacagc
ho168	SISOB-like_T519A_fw	cagatgctcatacacatattGcaacttcaaagtggc
ho169	SISOB-like_T519A_rev	gccacatttgaagtgCaatatgtgtatgagcatctg
ho170	SISOB-like_T520A_fw	gatgctcatacacatattacaGcttcaaatgttcagg
ho171	SISOB-like_T520A_rev	ctgccacatttgaagCtghtaatgtgtatgagcatc
ho172	SISOB-like_S521A_fw	ctcatacacatattacaactGcaaatgttcaggaaac
ho173	SISOB-like_S521A_rev	gttcctgccacatttgCagttgtaatatgtgtatgag
ho174	SISOB-like_T526A_fw	cttcaaatgttcagggaGctataggatacatcgctc
ho175	SISOB-like_T526A_rev	gagcgatgtatcctatagCtctgccacatttgaag
Primers used for generating <i>E. coli</i> expression constructs of the SOBIR1 kinase domain (sequences that are identical to the expression vectors are underlined)		
ho201	pET-GST_fw	aagcttgcggccgcactcgag
ho202	pET-GST_rev	cagggggccctggaacagaacttc
ho203	NbSOBIR1_KD+90bp_fw	<u>gttctgttcagggggccctg</u> cgaagggaagactgatggaac
ho204	NbSOBIR1_KD+90bp_rev	<u>ctcgagtgcggccgcaagctt</u> ctaagtcttgatctgagttaacatac

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ho215	SISOBIR1_KD+90bp_fw	<u>gttctgttccaggggccctg</u> agaagaggggaataacgattcaag
ho216	SISOBIR1_KD+90bp_rev	<u>ctcgagtgcggccgcaagctt</u> taatgcttgatctgagttaacatgc
ho217	SISOBIR1-like_KD+90bp_fw	<u>gttctgttccaggggccctg</u> agagggatcagaaatgatccagg
ho218	SISOBIR1-like_KD+90bp_rev	<u>ctcgagtgcggccgcaagctt</u> taatgcttgatctgcatcaacatgc
ho219	pET-15b_fw	cggatcctcgagcatatggctg
ho220	pET-15b_rev	gctgctaacaagcccgaaggaag
ho221	NbSO_KD+90bp_fw	<u>gccatatgctcgaggatccgc</u> gaaagggaaagactgatggaac
ho222	NbSO_KD+90bp_rev	<u>ctttcgggctttgttagcagc</u> taatgcttgatctgagttaacatac
ho223	SISO_KD+90bp_fw	<u>gccatatgctcgaggatccgc</u> agaagaggggaataacgattcaag
ho224	SISO_KD+90bp_rev	<u>ctttcgggctttgttagcagc</u> taatgcttgatctgagttaacatgc
ho225	SISO-like_KD+90bp_fw	<u>gccatatgctcgaggatccgc</u> agagggatcagaaatgatccagg
ho226	SISO-like_KD+90bp_rev	<u>ctttcgggctttgttagcagc</u> taatgcttgatctgcatcaacatgc
ho282	NbSERK3b_kd_fw	<u>gccatatgctcgaggatccggg</u> acaactcaagaggttttccttg
ho283	NbSERK3b_kd_rev	<u>ctttcgggctttgttagcagc</u> tcatcttgccccgacaattcatc
ho286	SISERK3a_kd_fw	<u>gccatatgctcgaggatccggg</u> acaactcaaaaggttttccttg
ho287	SISERK3a_kd_rev	<u>ctttcgggctttgttagcagc</u> tcatcttgccctgacaactcatc

* fw, forward; rev, reverse.

Chapter 4

A particular tyrosine residue, located in the kinase domain of SOBIR1, is essential for LRR-RLPs to trigger plant immunity

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Abstract

The first layer of plant innate immunity relies on cell-surface receptors that perceive extracellular immunogenic patterns (ExIPs). Leucine-rich repeat (LRR) receptor-like kinases (LRR-RLKs) and LRR receptor-like proteins (LRR-RLPs) are the most extensively characterized plant cell-surface receptors. In contrast to LRR-RLKs, LRR-RLPs lack a cytoplasmic kinase domain. The LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1) has been identified to constitutively associate with LRR-RLPs, thereby providing them with an intracellular kinase domain for downstream signaling. Therefore, SOBIR1 is well-recognized to play a broad and essential role in LRR-RLP-mediated plant immunity against various microbes. For instance, the well-studied tomato LRR-RLP Cf-4, which specifically detects the apoplastic effector Avr4 that is secreted by the pathogenic intercellular fungus *Cladosporium fulvum*, requires SOBIR1 to mediate host resistance against *C. fulvum*. In addition, SOBIR1 is also essential for the hypersensitive response (HR) induced by the xyloglucanase BcXYG1, which is an ExIP secreted by the necrotrophic fungal pathogen *Botrytis cinerea*. However, how SOBIR1 initiates downstream immune responses remains largely unknown. Here, we report that the tyrosine (Tyr/Y) residue Y469, present in the kinase domain of *Nicotiana benthamiana* (*Nb*) SOBIR1, as well as its analogous Tyr residues in both tomato SOBIR1 and SOBIR1-like, is essential for the Avr4/Cf-4-triggered activation of a mitogen-activated protein kinase (MAPK) cascade and the HR. Interestingly, this particular Tyr residue is not required for Avr4/Cf-4-induced reactive oxygen species (ROS) production and also not for the intrinsic kinase activity of SOBIR1. Moreover, this particular Tyr residue, and two additional residues (*Nb*SOBIR1^{Y429} and *Nb*SOBIR1^{Y530}), as well as their analogous residues in both tomato SOBIR1s, play a crucial role in mediating the BcXYG1-triggered HR. This work sheds light on the importance of Tyr residues in the kinase domain of SOBIR1 in regulating plant immunity.

Introduction

Plants are sessile organisms that lack an adaptive immune system, and therefore they largely rely on an extensive two-layered innate immune system to confront diverse biotic attacks (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Zhou and Zhang, 2020). The first layer is activated by sensing extracellular immunogenic patterns (ExIPs), leading to extracellularly-triggered immunity (ExTI) (van der Burgh and Joosten, 2019). This sensing depends on cell-surface receptors, which are in most cases either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel, 2014; Gust et al., 2017). ExIPs can be any extracellular danger signal, including pathogen-derived immunogenic patterns and effectors, and host-derived damage-associated molecular patterns (van der Burgh and Joosten, 2019). The second layer of plant innate immunity is activated upon sensing of intracellular immunogenic patterns (InIPs), leading to intracellularly-triggered immunity (InTI) (van der Burgh and Joosten, 2019). This sensing is mediated by cytoplasmic receptors, which are mainly nucleotide-binding, leucine-rich repeat (NB-LRR) receptors, also referred to as NLRs (Lolle et al., 2020; Zhou and Zhang, 2020). InIPs can be any intracellular danger signal, such as effectors that are secreted into the plant cells by host-adapted microbial pathogens to promote colonization of the host (van der Burgh and Joosten, 2019). Both ExTI and InTI are associated with various downstream immune outputs, including a swift reactive oxygen species (ROS) burst, the activation of a mitogen-activated protein kinase (MAPK) cascade, calcium influx, and, in some cases, the induction of programmed cell death, referred to as the hypersensitive response (HR) (van der Burgh and Joosten, 2019; Zhou and Zhang, 2020; Chang et al., 2021; Lu and Tsuda, 2021; Ngou et al., 2021).

RLKs and RLPs share the same overall structure (Monaghan and Zipfel, 2012; Macho and Zipfel, 2014; Steinbrenner, 2020). They both are composed of an ectodomain that is potentially involved in ligand binding and a single-pass transmembrane domain. However, in contrast to RLKs, RLPs lack an intracellular kinase domain for downstream signaling (Chisholm et al., 2006; Monaghan and Zipfel, 2012; Wang and Chai, 2020; Lee et al., 2021). The ectodomains of both RLKs and RLPs are highly variable, thereby providing several means to perceive a wide range of ligands (Monaghan and Zipfel, 2012; Ranf, 2017; Tang et al., 2017). For example, cell-surface receptors that contain LRRs preferentially bind proteins or peptides, such as bacterial flagellin or elongation factor Tu. In addition, lysin motif (LysM)-containing ectodomains are involved in binding of carbohydrate-based ligands, such as fungal chitin, whereas lectin-type motifs are implicated in the perception of extracellular ATP or bacterial lipopolysaccharides (LPS) (Monaghan and Zipfel, 2012; Böhm et al., 2014; Macho and Zipfel, 2014; Zipfel, 2014; Couto and Zipfel, 2016; Wan et al., 2019). To date, the most extensively studied plant cell-surface receptors are LRR-RLKs and LRR-RLPs (Kong et al., 2021; Lee et al., 2021). Typically, LRR-RLKs form a complex with the regulatory LRR-RLK BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE (BAK1/SERK3, further referred to as

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BAK1), in a ligand-dependent manner (Heese et al., 2007; Chinchilla et al., 2009; Ranf, 2017; Wan et al., 2019; Albert et al., 2020). Interestingly, LRR-RLPs have been found to constitutively interact with the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1), and thereby SOBIR1 provides LRR-RLPs with an intracellular kinase domain (Liebrand et al., 2013; Liebrand et al., 2014). Consistent with LRR-RLKs, the LRR-RLP/SOBIR1 complex also requires the recruitment of BAK1 for the initiation of downstream signaling upon ligand perception (Postma et al., 2016; DeFalco and Zipfel, 2021).

Phosphorylation of signaling partners is crucial for initiating defense signaling and regulating protein function in both animals and plants. Unlike the majority of mammalian receptor kinases that possess tyrosine (Tyr/Y) kinase activity in addition to serine (Ser/S)/threonine (Thr/T) kinase activity, plant RLKs were traditionally annotated as serine/threonine-specific kinases (Bleecker, 2001; Manning, 2002; Lim and Pawson, 2010). However, this knowledge has been revised over the past twenty years, as emerging evidence shows that plant RLKs can also undergo tyrosine phosphorylation, in addition to serine/threonine phosphorylation. Besides, it is proposed that tyrosine phosphorylation even plays a predominant role in RLK-mediated immunity in plants (de la Fuente van Bentem and Hirt, 2009; Macho et al., 2015). A good example is *Arabidopsis* (*Arabidopsis thaliana*, At) BAK1, which is a dual-specificity kinase (Wang et al., 2005; Wang et al., 2008; Lin et al., 2014). Both FLAGELLIN-SENSING 2 (FLS2) and ELONGATION FACTOR-TU RECEPTOR (EFR) require BAK1 to mediate plant immune signaling upon recognition of the conserved 22-amino acid epitope (flg22) of bacterial flagellin and the conserved 18-amino acid peptide (elf18) of bacterial elongation factor Tu, respectively. Phosphorylation of T455 in the activation segment of BAK1 is essential for obtaining its kinase activity (Wang et al., 2005; Wang et al., 2008), while phosphorylation of Y403 is required for BAK1 function in downstream immune signaling, as an amino acid change at Y403 strongly reduced flg22- and elf18-triggered ROS production and flg22-triggered MAPK activation, but does not impair the function of BAK1 in plant development (Perraki et al., 2018).

As mentioned above, in addition to BAK1, SOBIR1 is another common co-regulator of LRR-RLPs and plays an essential role in the initiation of plant immunity by LRR-RLPs (Gust and Felix, 2014; Liebrand et al., 2014). The tomato (*Solanum lycopersicum*, Sl) resistance protein Cf-4 is one of the best-studied LRR-RLPs, and specifically recognizes the apoplastic effector Avr4 that is secreted by the pathogenic intercellular fungus *Cladosporium fulvum* (Joosten et al., 1994; Thomas et al., 1997). Cf-4 also constitutively associates with SOBIR1 and recruits BAK1 in response to the perception of Avr4, leading to the rapid phosphorylation of SOBIR1 and BAK1 at their kinase domains (Liebrand et al., 2013; Postma et al., 2016; van der Burgh et al., 2019). AtSOBIR1 has been demonstrated to auto-phosphorylate at Ser, Thr, and Tyr residues (Leslie et al., 2010). Recently, we showed that T522, present in the activation segment of *Nicotiana benthamiana* (Nb) SOBIR1, as well as its analogous residues in tomato

A tyrosine residue in SOBIR1 is essential for plant immunity

SOBIR1 and SOBIR1-like, is required for the intrinsic kinase activity of SOBIR1 (Chapter 3). However, whether tyrosine residue(s) that is (are) present in the kinase domain of SOBIR1 is (are) essential for its signaling function and whether tyrosine phosphorylation of SOBIR1 plays a role in regulating Cf-4/SOBIR1-mediated signaling, remain to be elucidated.

Here, we analyze the importance of all Tyr residues in the kinase domain of *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1*-like, by combining a site-directed mutagenesis screen and a complementation study in either *N. benthamiana sobir1* or *N. benthamiana:Cf-4 sobir1* knock-out plants. Interestingly, *NbSOBIR1*^{Y469}, as well as its analogous residues in *S/SOBIR1* and *S/SOBIR1*-like, is identified to be essential for Avr4/Cf-4-triggered MAPK activation and the HR, and also the BcXYG1-triggered HR. Strikingly, we found that this particular Tyr residue is not required for the intrinsic kinase activity of SOBIR1 and also not for the Avr4/Cf-4-induced ROS production. In addition, additional Tyr residues were found to play a crucial role in regulating BcXYG1-mediated immune signaling. Our study illustrates the importance of particular Tyr residues that are present in the kinase domain of SOBIR1 in LRR-RLP-triggered plant innate immunity.

Results

***NbSOBIR1*^{Y469}, as well as its analogous residues in both tomato SOBIR1s, is essential for mounting the full Avr4/Cf-4-triggered HR**

Although being a tomato protein, Cf-4 is also functional in *N. benthamiana*, as the transient expression of *Avr4* triggers a typical HR in *Cf-4*-transgenic *N. benthamiana* plants (Gabriëls et al., 2007). We previously reported that the functionality of *Cf-4* in *N. benthamiana* is abolished by knocking out *SOBIR1*, which is reflected by the loss of the Avr4-induced HR in *N. benthamiana:Cf-4 sobir1* knock-out plants (Huang et al., 2021) (Chapter 2). Interestingly, this HR can be restored by complementation consisting of transient expression of *NbSOBIR1*, *S/SOBIR1* or *S/SOBIR1*-like, which makes these knock-out plants excellent material for studying the fundamentals of SOBIR1-mediated plant immunity.

To determine whether particular Tyr residues that are present in the kinase domain of SOBIR1 are specifically required for the Avr4/Cf-4-triggered HR, all the eight to nine Tyr residues present in the kinase domain of *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1*-like, were selected to be studied. Strikingly, most of these Tyr residues are highly conserved in SOBIR1 from many different plant species (Figure 1A and S1). We conducted a site-directed mutagenesis involving the substitution of each of the eight Tyr residues of *NbSOBIR1*, nine Tyr residues of *S/SOBIR1*, and eight Tyr residues of *S/SOBIR1*-like, by phenylalanine (Phe/F), which lacks the phosphorylatable hydroxyl group at the aromatic ring. Thus, in total 25 SOBIR1 Tyr-to-Phe mutants were obtained that were subsequently subjected to a complementation study. Each SOBIR1 mutant was co-expressed with *Avr4* in *N. benthamiana:Cf-4 sobir1* knock-out plants, including their

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corresponding wild-types (WTs) as a positive control and kinase-dead mutants (*NbSOBIR1* D482N, *SISOBIR1* D473N or *SISOBIR1*-like D486N) as a negative control. Consistent with our previous observations, complementation with *NbSOBIR1* WT, *SISOBIR1* WT or *SISOBIR1*-like WT rescued the Avr4-triggered HR in *N. benthamiana*:*Cf-4 sobir1* knock-out plants, whereas complementation with their respective kinase-dead mutants did not (Figure 1) (Huang et al., 2021) (Chapter 2 and 3). Intriguingly, in contrast to their WTs, but very similar to their respective kinase-dead mutants, transient expression of *NbSOBIR1* Y469F, *SISOBIR1* Y460F, and *SISOBIR1*-like Y473F failed to fully complement the Avr4-triggered HR in *N. benthamiana* *sobir1* knock-out plants (Figure 1B, 1D, and 1F). Quantification of the HR intensity that was obtained upon complementation by red light imaging (Landeo Villanueva et al., 2021), showed that the differences in the HR intensity between *NbSOBIR1* WT and *NbSOBIR1* Y469F, between *SISOBIR1* WT and *SISOBIR1* Y460F, and between *SISOBIR1*-like WT and *SISOBIR1*-like Y473F, were significant (Figure 1C, 1E, and 1G). Furthermore, the remaining Tyr mutants that we generated showed a similar complementation capacity in *N. benthamiana*:*Cf-4 sobir1* knock-out plants as their corresponding WT (Figure 1).

Notably, *NbSOBIR1*^{Y469} is analogous to *SISOBIR1*^{Y460} and *SISOBIR1*-like^{Y473} (Figure 1A and S1). And more importantly, the loss of full complementation capacity of *NbSOBIR1* Y469F, *SISOBIR1* Y460F, and *SISOBIR1*-like Y473F, was not caused by a lack of protein accumulation, as we observed that all these mutant versions of SOBIR1 properly accumulated *in planta* (Figure S2). Taken together, these observations demonstrate that this particular conserved Tyr residue in the kinase domain of SOBIR1 plays an essential role in mediating the Avr4/Cf-4-specific HR.

A tyrosine residue in SOBIR1 is essential for plant immunity

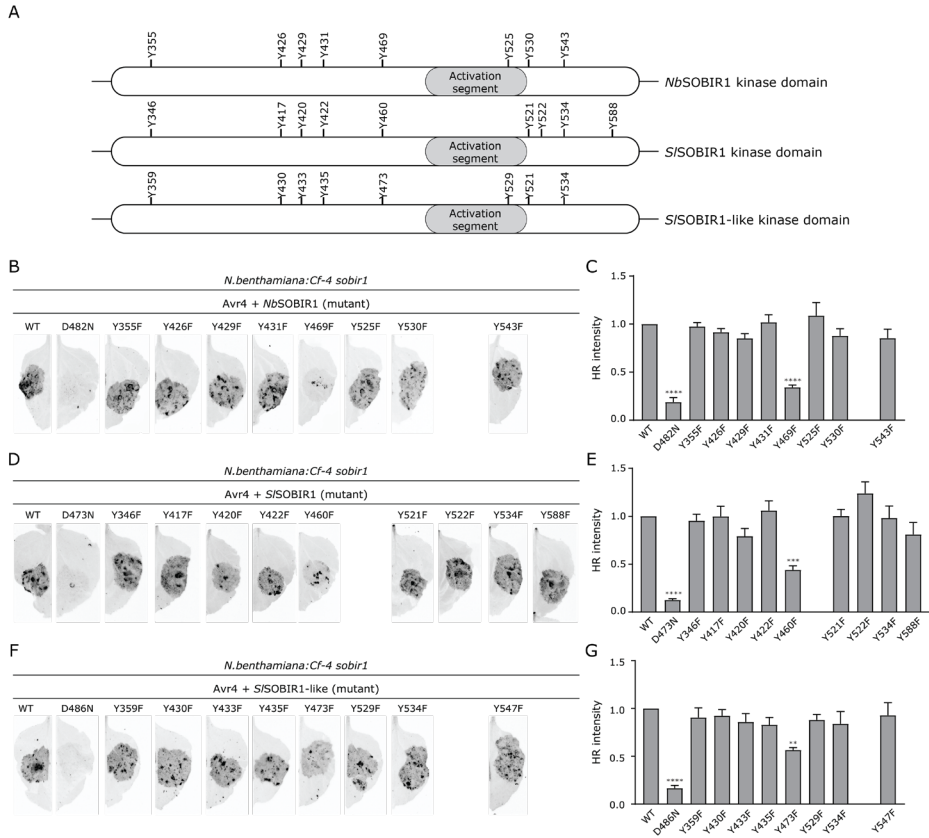


Figure 1. A site-directed mutagenesis screen identifies the analogous Tyr residues *NbSOBIR1*^{Y469}, *S/SOBIR1*^{Y460} and *S/SOBIR1-like*^{Y473}, present in the kinase domain of SOBIR1, to be essential for the Avr4/Cf-4-triggered HR in *N. benthamiana*. (A) Schematic diagrams of the kinase domains of *NbSOBIR1*, *S/SOBIR1* and *S/SOBIR1-like*, with the location of the activation segment and all Tyr (Y) residues indicated. (B to G) Mutagenesis screen of all putative Tyr phosphorylation sites in *NbSOBIR1* (B and C), *S/SOBIR1* (D and E), and *S/SOBIR1-like* (F and G), to determine their importance in immune signaling by complementation. All generated SOBIR1 Tyr-to-Phe (F, which is a variant of Tyr that cannot be phosphorylated) mutants were transiently co-expressed with *Avr4* by agro-infiltration (both at an OD₆₀₀ of 0.8) in the leaves of *N. benthamiana*:Cf-4 *sobir1* knock-out plants, with their corresponding wild types (WTs) as positive controls and their kinase-dead mutants (*NbSOBIR1* D482N, *S/SOBIR1* D473N, and *S/SOBIR1-like* D486N) as negative controls. The presence of an HR was determined at 5 days post infiltration (dpi), using the red light imaging system (Filter: 605/50; Light: Green Epi Illumination; Exposure time: 2 seconds) (B, D and F). The intensity of the HR was subsequently quantified by Image Lab. Data shown are the average relative intensities of the HR + standard error of the mean (SEM) (n≥6) that was triggered upon complementation with the various SOBIR1 mutants, when compared to complementation with the respective WT SOBIR1 construct, of which the HR that was triggered was set to 1 (C, E and G). Statistical significance was determined by a one-way ANOVA test. ** p<0.001; *** p<0.0001; **** p<0.0001. Experiments were repeated at least three times with similar results, and representative results are shown. *Nb*, *Nicotiana benthamiana*; *Sl*, *Solanum lycopersicum*; D, aspartic acid/Asp; N, asparagine/Asn; Y, tyrosine/Tyr; F, phenylalanine/Phe. Note that the position of the various Tyr-to-Phe mutants in B, C, D, E, F, and G is aligned with that in A.

***NbSOBIR1*^{Y469}, as well as its analogous residues in both tomato *SOBIR1*s, is not required for *Avr4/Cf-4*-triggered ROS production but does play a role in MAPK activation**

In addition to an HR, *Avr4* also triggers a rapid ROS production in *N. benthamiana:Cf-4*, which is a well-known feature of ExTI (van der Burgh and Joosten, 2019; Albert et al., 2020; Huang et al., 2021). The apoplastic ROS are produced mainly by plasma membrane-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Lamb and Dixon, 1997; Torres et al., 2005; Qi et al., 2017). In *N. benthamiana* and tomato, the RESPIRATORY BURST OXIDASE HOMOLOGUE B (RBOHB) plays a major role in producing apoplastic ROS (Yoshioka et al., 2003). To investigate whether *NbSOBIR1*^{Y469}, *SISOBIR1*^{Y460} and *SISOBIR1-like*^{Y473} are also required for regulating the *Avr4/Cf-4*-triggered ROS burst, we monitored the ROS accumulation in the leaves of *N. benthamiana:Cf-4 sobir1* knock-out plants in which we transiently expressed the individual *SOBIR1* mutants, upon adding *Avr4* protein. In line with our previous observations, the *Avr4*-triggered biphasic ROS burst was completely abolished in *N. benthamiana:Cf-4 sobir1* knock-out plants and was at least partially complemented by transiently expressing *NbSOBIR1* WT, *SISOBIR1* WT or *SISOBIR1-like* WT, but not upon expressing their corresponding kinase-dead mutants (Figure 2A, 2B, and 2C) (Chapter 3). Surprisingly, similar to their WTs and all other Tyr-to-Phe mutants, transient expression of *NbSOBIR1* Y469F, *SISOBIR1* Y460F, or *SISOBIR1-like* Y473F also partially restored the *Avr4*-triggered ROS burst in *N. benthamiana:Cf-4 sobir1* knock-out plants (Figure 2A, 2B, and 2C). These results suggest that *NbSOBIR1*^{Y469}, *SISOBIR1*^{Y460} and *SISOBIR1-like*^{Y473} are not essential for the *Avr4/Cf-4*-triggered ROS burst.

MAPK activation is another critical early signaling event related to ExTI (Zhang et al., 2018; Zhou and Zhang, 2020). To further explore the importance of *NbSOBIR1*^{Y469}, as well as its analogous Tyr residues in *SISOBIR1* and *SISOBIR1-like*, in *Cf-4/SOBIR1*-mediated immune signaling, we determined the occurrence of *Avr4/Cf-4*-induced MAPK activation in *N. benthamiana:Cf-4 sobir1* knock-out plants, after co-expressing *NbSOBIR1* Y469F, *SISOBIR1* Y460F or *SISOBIR1-like* Y473F with *Avr4*. Again, *NbSOBIR1* WT, *SISOBIR1* WT, and *SISOBIR1-like* WT were taken along as positive controls, and their kinase-dead mutants were taken along as negative controls. In contrast to the WTs, but similar to the kinase-dead mutants, co-expression of *NbSOBIR1* Y469F, *SISOBIR1* Y460F, or *SISOBIR1-like* Y473F with *Avr4* failed to restore the *Avr4/Cf-4*-triggered MAPK activation in *N. benthamiana:Cf-4 sobir1* knock-out plants (Figure 2D). Again, the lack of MAPK activation was not correlated with their protein accumulation, as mentioned earlier, all tested *SOBIR1* Tyr mutants properly accumulated *in planta* (Figure S2). Collectively, the aforementioned results indicate that, in addition to their importance in the *Avr4/Cf-4*-triggered HR, *NbSOBIR1*^{Y469}, *SISOBIR1*^{Y460} and *SISOBIR1-like*^{Y473} are also required for the *Avr4/Cf-4*-induced MAPK activation, but not for ROS accumulation.

A tyrosine residue in SOBIR1 is essential for plant immunity

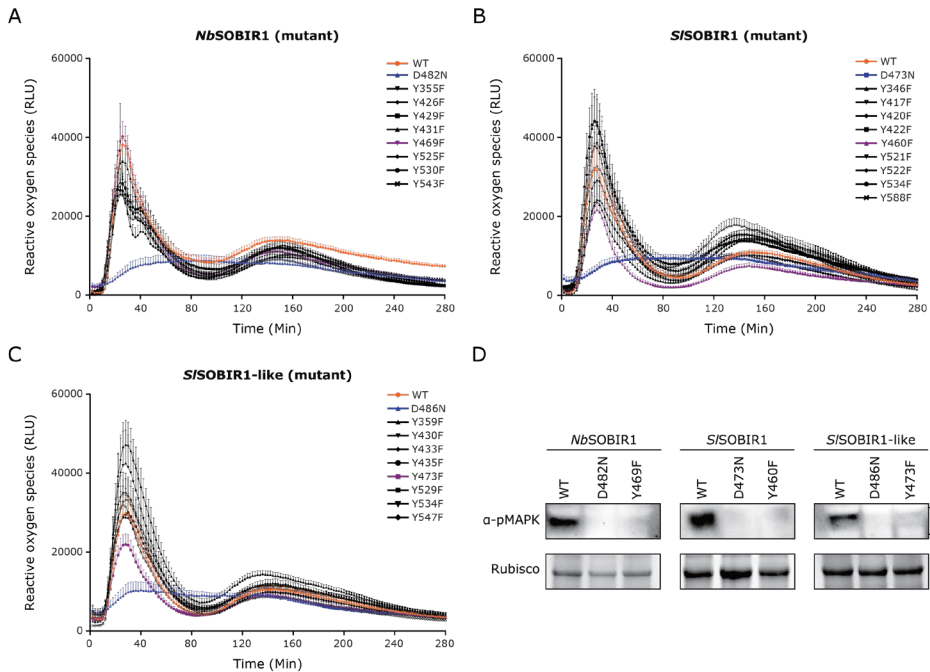


Figure 2. Complementation with *NbSOBIR1*^{Y469F}, *S/SOBIR1*^{Y460F} or *S/SOBIR1*-like^{Y473F} restores the *Avr4*/*Cf-4*-induced ROS burst but does not rescue MAPK activation in the *N. benthamiana*:*Cf-4 sobir1* mutant line. The different Tyr mutants of *NbSOBIR1* (A), *S/SOBIR1* (B), and *S/SOBIR1*-like (C) that were generated, were transiently expressed in leaves of the *N. benthamiana*:*Cf-4 sobir1* mutant line, with their corresponding WT as positive controls and kinase-dead mutants as negative controls. Leaf discs were taken from these plants at 24 hours after agro-infiltration, followed by adding 0.1 μ M *Avr4* protein and measuring ROS accumulation over time. ROS production is expressed as relative light units (RLUs) and the data are represented as mean + SEM. (D) *NbSOBIR1*^{Y469F} (left panel), *S/SOBIR1*^{Y460F} (middle panel) and *S/SOBIR1*-like^{Y473F} (right panel), as well as their corresponding WT and kinase-dead mutants, were transiently co-expressed with *Avr4* in leaves of *N. benthamiana*:*Cf-4 sobir1* mutant plants. The leaf samples were collected at 2 dpi. Hereafter, total protein extracts were subjected to immunoblotting with a p42/p44-erk antibody to determine the activation of downstream MAPKs by phosphorylation. Experiments were repeated at least three times and similar results were obtained. Representative pictures are shown.

NbSOBIR1*^{Y469}, as well as its analogous residues in both tomato *SOBIR1*s, is dispensable for the intrinsic kinase activity of *SOBIR1

We next sought to determine whether a mutation at *NbSOBIR1*^{Y469}, *S/SOBIR1*^{Y460} and *S/SOBIR1*-like^{Y473}, which are all Tyr residues present in the kinase domain of *SOBIR1*, would affect the intrinsic kinase activity of *SOBIR1*. Therefore, we performed an *in vitro* phosphorylation assay, for which we expressed the cytoplasmic kinase domain of *NbSOBIR1*^{Y469}, *S/SOBIR1*^{Y460} and *S/SOBIR1*-like^{Y473}, as well as their corresponding WT and kinase-dead mutants, in *Escherichia coli*, followed by SDS-PAGE of boiled cell lysate. Hereafter, the protein gels were stained by Pro-Q diamond to determine the

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phosphorylation levels of the various kinase domains and gels were also stained by Coomassie brilliant blue (CBB) to determine the total protein content of the lysates (Taylor et al., 2013).

Transient expression of *NbSOBIR1* Y469F, *SISOBIR1* Y460F or *SISOBIR1*-like Y473F at least partially restored the Avr4-triggered ROS production in *N. benthamiana*:*Cf-4 sobir1* knock-out plants (Figure 2A, 2B, and 2C), which led us to speculate that these three Tyr-to-Phe mutants would still exhibit intrinsic kinase activity. As expected, the recombinant *NbSOBIR1* Y469F, *SISOBIR1* Y460F and *SISOBIR1*-like Y473F kinase domains all showed strong auto-phosphorylation activity *in vitro* (Figure 3). It is worth noting that we did not observe obvious differences between the staining intensities of the bands upon Pro-Q staining or between the protein mobilities of these three mutants and their corresponding WT (Figure 3). This suggests that in all cases the overall phosphorylation levels are similar. Therefore, we conclude that *NbSOBIR1*^{Y469}, as well as its analogous residues in *SISOBIR1* and *SISOBIR1*-like, play no, or only a minor role, in determining the intrinsic kinase activity of SOBIR1.

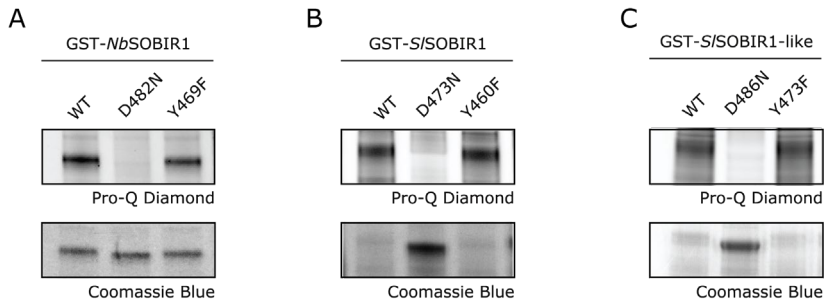


Figure 3. *NbSOBIR1*^{Y469F}, *SISOBIR1*^{Y460F} and *SISOBIR1*-like^{Y473F} all exhibit intrinsic kinase activity. The N-terminally GST-tagged cytoplasmic kinase domains of *NbSOBIR1*^{Y469F} (A), *SISOBIR1*^{Y460F} (B) and *SISOBIR1*-like^{Y473F} (C), were produced in *E. coli*, with their corresponding WT as positive controls and their kinase-dead mutants as negative controls. After SDS-PAGE of the *E. coli* lysates, the recombinant proteins were stained with Coomassie brilliant blue (lower panel), whereas the phosphorylation status of the kinase domains was determined by performing a Pro-Q Diamond stain (upper panel). Experiments were repeated at least three times with similar results, and representative results are shown.

***NbSOBIR1*^{Y469}, as well as its analogous residues in both tomato SOBIR1s, is crucial for BcXYG1-triggered, SOBIR1-dependent immune signaling**

SOBIR1 was initially identified to interact with the tomato LRR-RLPs Cf-4 and Ve1, conferring resistance against the fungal pathogens *C. fulvum* and *Verticillium dahliae*, respectively (Liebrand et al., 2013). Later, SOBIR1 was found to be present throughout the plant kingdom and to play a broad role in regulating LRR-RLP-mediated immunity against bacteria, fungi, oomycetes, viruses, and herbivores in many different plants (Liebrand et al., 2013; Zhang et al., 2013; Bi et al., 2014; Liebrand et al., 2014; Albert et al., 2015; Peng et al., 2015; Catanzariti et al., 2017; Gui et al., 2017; Zhu et al.,

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2017; Domazakis et al., 2018; Steinbrenner et al., 2020; Zhang et al., 2021; Zhang et al., 2021; Zhang et al., 2021). We therefore next asked whether this particular Tyr residue present in the kinase domain of SOBIR1 also plays a role in additional SOBIR1-dependent immune signaling events. To answer this question, we employed BcXYG1, which is a Glycoside Hydrolase Family 12 (GH12) xyloglucanase that is secreted by the necrotrophic pathogen *Botrytis cinerea* (Zhu et al., 2017), as an ExIP that induces an HR in leaves of *N. benthamiana*. This HR was found to be strongly reduced in either SOBIR1- or BAK1-silenced *N. benthamiana* plants, suggesting that the HR is likely triggered upon activation of an RLP and therefore requires both SOBIR1 and BAK1 (Zhu et al., 2017).

Consistently, we observed that transient expression of BcXYG1 in wild-type *N. benthamiana* induced an obvious HR at the infiltration sites and that this HR was abolished in two independent *N. benthamiana sobir1* knock-out lines (Figure 4A). These observations further support that the BcXYG1-triggered HR is indeed dependent on SOBIR1. Subsequently, we transiently co-expressed all individual SOBIR1 Tyr-to-Phe mutants that we generated with BcXYG1 in leaves of *N. benthamiana sobir1* knock-out plants, followed by quantifying the HR intensity at 5 dpi using the red light imaging system (Landeo Villanueva et al., 2021). Interestingly, the intensity of the HR obtained upon transient co-expression of NbSOBIR1 WT with BcXYG1 was significantly higher than that obtained upon transient co-expression of NbSOBIR1 Y469F or NbSOBIR1 D482N with BcXYG1 (Figure 4B), which demonstrates that, in addition to its requirement for the Avr4/Cf-4-induced HR, NbSOBIR1^{Y469} is also required for the BcXYG1-induced HR in *N. benthamiana*. In agreement with these findings, the analogous residues of NbSOBIR1^{Y469} that are present in S/SOBIR1 and S/SOBIR1-like were also identified to be required for the BcXYG1-triggered HR (Figure 4C and 4D), suggesting a broad role of this particular Tyr residue in plant immunity.

Furthermore, two additional Tyr-to-Phe mutants, being NbSOBIR1 Y429F and Y530F, did not fully rescue the BcXYG1-triggered HR as well when overexpressed (Figure 4B), suggesting that NbSOBIR1^{Y429} and NbSOBIR1^{Y530} also play a pivotal role in BcXYG1-triggered immune signaling. Similarly, complementation with constructs having the analogous residues mutated in S/SOBIR1 and S/SOBIR1-like in leaves of *N. benthamiana sobir1* knock-out plants also failed to fully restore the BcXYG1-induced HR (Figure 4C and 4D). Moreover, we also observed that the HR intensity obtained upon co-expression of S/SOBIR1 Y534F with BcXYG1 was significantly lower when compared to the positive control (Figure 4C), nevertheless, this observation did not hold for its analogous residues in NbSOBIR1 and S/SOBIR1-like (Figure 4B and 4D). Altogether, we conclude that the particular Tyr residue in the kinase domain of SOBIR1 that is essential for Avr4/Cf-4/SOBIR1-triggered signaling is also required for BcXYG1-triggered signaling in *N. benthamiana*. Furthermore, additional Tyr residues in the kinase domain of SOBIR1 also appear to contribute to immune signaling triggered by BcXYG1.

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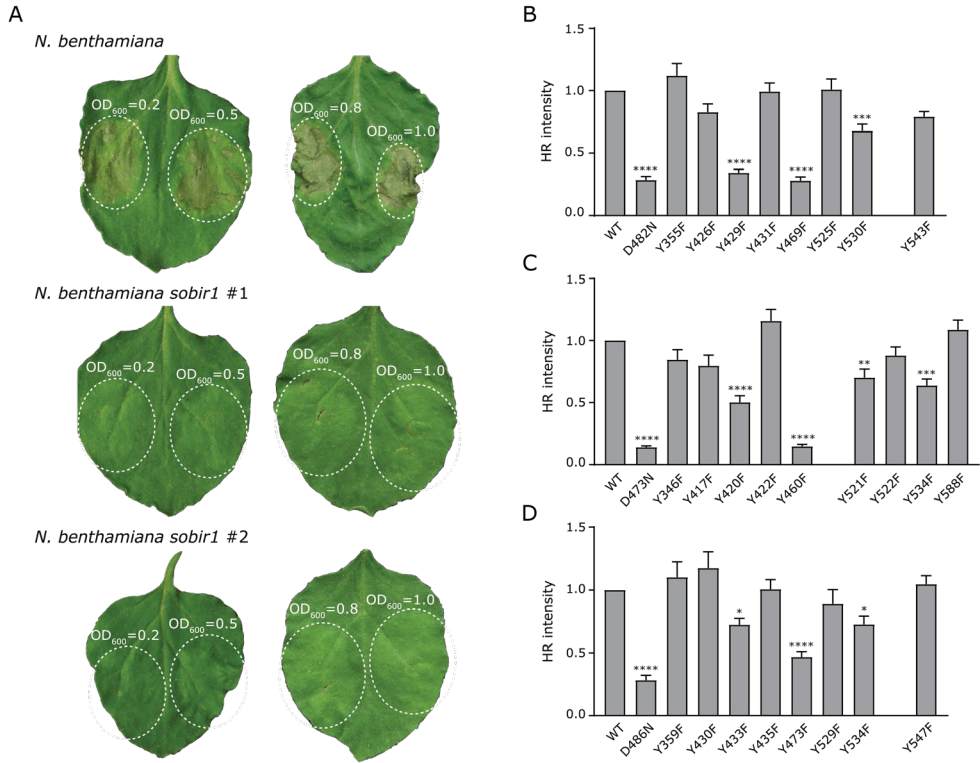


Figure 4. *NbSOBIR1*^{Y469}, *S/SOBIR1*^{Y460} and *S/SOBIR1*-like^{Y473}, present in the kinase domain of *SOBIR1*, play a role in the *BcXYG1*-triggered *SOBIR1*-dependent signaling pathway. (A) *BcXYG1* was agro-infiltrated in leaves of wild-type *N. benthamiana* and two independent *sobir1* mutant lines, at the indicated OD₆₀₀. The leaves were photographed at 4 dpi. All generated Tyr-to-Phe mutants of *NbSOBIR1* (B), *S/SOBIR1* (C), and *S/SOBIR1*-like (D) were transiently co-expressed with *BcXYG1* by agro-infiltration (OD₆₀₀ = 0.8), in leaves of *N. benthamiana sobir1* knock-out plants, with their corresponding WT as positive controls and kinase-dead mutants (*NbSOBIR1* D482N, *S/SOBIR1* D473N, and *S/SOBIR1*-like D486N) as negative controls. The formation of HR was detected at 5 dpi using the red light imaging system (Filter: 605/50; Light: Green Epi Illumination; Exposure time: 2 seconds). The intensity of the HR was subsequently quantified by Image Lab. Data shown are the average relative intensities of the HR + SEM (n≥6) that was triggered upon complementation with the various *SOBIR1* mutants, when compared to complementation with the respective WT *SOBIR1* construct, of which the HR that was triggered was set to 1. Statistical significance was determined by a one-way ANOVA test. * p<0.01, ** p<0.001; *** p<0.0001; **** p<0.0001. Experiments were repeated at least three times with similar results, and representative results are shown. Note that the position of the various Tyr-to-Phe mutants is aligned with their positions in Figure 1A.

Discussion

RLKs and RLPs, present at the cell surface, are at the basis of the first line of plant innate immunity (Monaghan and Zipfel, 2012; Böhm et al., 2014; Macho and Zipfel, 2014; Tang et al., 2017; Kanyuka and Rudd, 2019; Wan et al., 2019; Zhou and Zhang, 2020; DeFalco and Zipfel, 2021). Due to the lack of a cytoplasmic kinase domain, RLPs constitutively associate with the RLK SOBIR1 and require the RLK BAK1 to transduce the immune signal from the extracellular space into the plant cell (Zhou and Zhang, 2020). Phosphorylation of the ligand-activated receptor complex is a critical molecular switch for the initiation of immune signaling, both in plants and in mammals (Monaghan and Zipfel, 2012; Tang et al., 2017; Mithoe and Menke, 2018; Kong et al., 2021). Unlike in mammals, where Tyr phosphorylation of receptor kinases plays an important role in their activation and subsequent initiation of signaling, plant genomes do not specifically encode Tyr kinases (Pawson and Scott, 2005; Afzal et al., 2008). However, in the past two decades, various plant RLKs have been identified to phosphorylate not only at Ser/Thr residues but also at Tyr residues (de la Fuente van Bentem and Hirt, 2009; Macho et al., 2015). Therefore, Tyr phosphorylation is now also recognized as a common feature taking part in the activation of RLK-mediated signaling in plants.

SOBIR1 is a well-known LRR-RLK, which constitutively interacts with LRR-RLPs involved in immunity and which is essential for their function in the activation of plant immune responses (Gust and Felix, 2014; Liebrand et al., 2014). To determine whether the Tyr residues that are present in the kinase domain of SOBIR1 play an important role in SOBIR1 function, we here performed a site-directed mutagenesis screen, consisting of complementation studies with SOBIR1 Tyr-to-Phe mutants, in either *N. benthamiana*:Cf-4 *sobir1* or in *N. benthamiana* *sobir1* knock-out plants. We observed that *NbSOBIR1*^{Y469}, as well as its analogous residues in *S/SOBIR1* and *S/SOBIR1*-like, play a vital role in both the Avr4/Cf-4- and BcXYG1-triggered HR, as well as in the Avr4/Cf-4-induced activation of a MAPK cascade (Figure 1, 2, and 4). Strikingly, a vital role in regulating plant immunity has recently been assigned to this particular Tyr residue present in the kinase domain of several well-known RLKs. For instance, upon the perception of elf18, the Arabidopsis LRR-RLK EFR phosphorylates at Y836, which is equivalent to *NbSOBIR1*^{Y469} (Figure 5A), and this phosphorylation is required for the activation of EFR itself and the initiation of sequential downstream immune responses. A mutation at EFR^{Y836} in Arabidopsis leads to a strong reduction of elf18-stimulated ROS production, suppression of both MAPK activation and trans-phosphorylation of the receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1), and resistance against the pathogenic bacterium *Pseudomonas syringae* is compromised (Macho et al., 2014). The Arabidopsis LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) is the co-receptor of the fungal cell wall component chitin (Miya et al., 2007; Wan et al., 2008). Phosphorylation of CERK1^{Y428}, which is also analogous to *NbSOBIR1*^{Y469} (Figure 5A), is essential for chitin-triggered CERK1 activation, ROS production, MAPK activation, downstream RLCK phosphorylation, and resistance to the

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fungal pathogen *B. cinerea* (Liu et al., 2018). Recently, the Arabidopsis lectin RLK LIPO-OLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) has been reported to phosphorylate at Y600, which is also the analogous residue of *NbSOBIR1*^{Y469} (Figure 5A), upon its activation. A mutation at *LORE*^{Y600} results in a highly compromised LORE-mediated ROS accumulation and reduced resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (Luo et al., 2020). These results collectively demonstrate the importance of this particular Tyr residue in the kinase domain of cell surface RLKs that are involved in plant immunity.

SOBIR1 is also required for LRR-RLP-mediated plant immunity against bacteria (Gao et al., 2009), and to examine whether mutating this particular Tyr residue in SOBIR1 into a non-phosphorylatable Phe residue has effects on SOBIR1-mediated resistance to different *P. syringae* strains, a newly established method, referred to as Agromonas, can be employed. This method involves transient expression of, in our case, different SOBIR1 Tyr-to-Phe mutants in leaves of *N. benthamiana sobir1* knock-out plants, followed by either infiltration or spray inoculation of *P. syringae*, and then monitoring the bacterial growth (Buscaill et al., 2021). SOBIR1 was initially identified to positively regulate tomato resistance against *C. fulvum* and *V. dahliae* (Liebrand et al., 2013), and it will be interesting to mutate this particular Tyr residue in *S/SOBIR1* and *S/SOBIR1*-like in resistant Cf-4 and Ve1 tomato by the CRISPR/Cas9 gene-editing technique, followed by challenging the plants with *C. fulvum* and *V. dahliae*, respectively.

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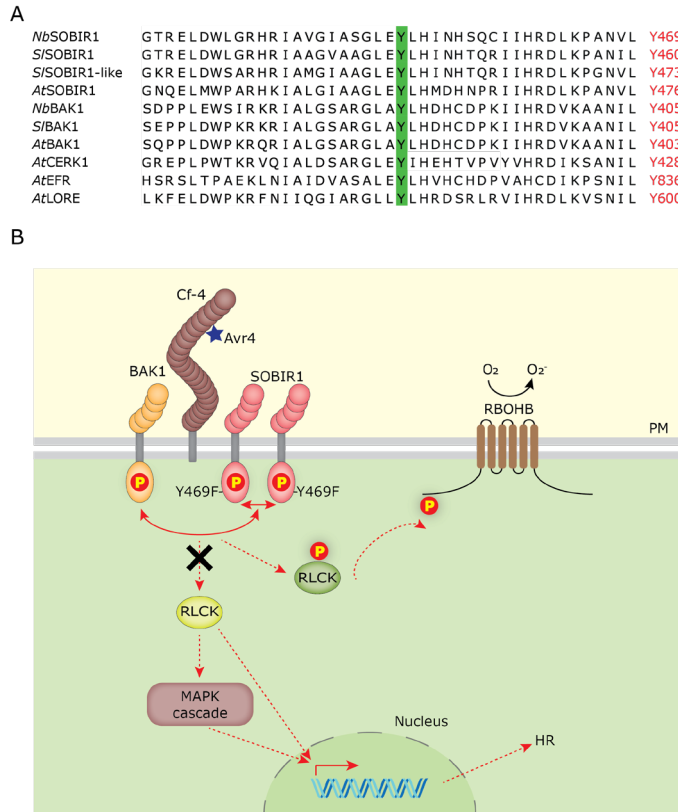


Figure 5. *NbSOBIR1*^{Y469} is evolutionarily conserved in various cell surface RLKs that play a role in plant immunity. (A) Protein sequence alignment of part of the kinase domain of SOBIR1 with the kinase domain of related RLKs. The protein sequences of *NbSOBIR1*, *SlSOBIR1* and *SlSOBIR1-like* were obtained from the Sol Genomics Network (<https://solgenomics.net/>), while the protein sequences of the Arabidopsis RLKs were retrieved from TAIR (<https://www.arabidopsis.org/index.jsp>). The alignment was visualized using JalView, and the Tyr (Y) residues that are analogous to *NbSOBIR1*^{Y469} are highlighted in green, with their positions in the complete protein indicated on the right in red. Note that only the amino acids surrounding this particular Tyr residue are shown. *Nb*, *Nicotiana benthamiana*; *Sl*, *Solanum lycopersicum*; *At*, *Arabidopsis thaliana*. (B) A model of *NbSOBIR1* Y469F-regulated Avr4/Cf-4 signaling. Upon recognition of Avr4 by Cf-4, the *NbSOBIR1* Y469F mutant initiates strong auto-phosphorylation, followed by trans-phosphorylation between SOBIR1 and BAK1 upon BAK1 recruitment, leading to the full activation of the *NbSOBIR1* Y469F/BAK1 complex. Likely, receptor-like cytoplasmic kinases (RLCKs) are further phosphorylated by this complex that subsequently trigger the production of ROS. Interestingly, the *NbSOBIR1* Y469F/BAK1 complex fails to activate a downstream MAPK cascade, as well as the Avr4/Cf-4-triggered HR. This suggests that the complex either cannot directly activate a MAPK cascade anymore or is not able to phosphorylate RLCKs involved in this MAPK activation. PM, plasma membrane. The red circle with 'P' inside represents phosphorylation.

Despite its important role in the Avr4/Cf-4- and BcXYG1-triggered HR, *NbSOBIR1*^{Y469}, as well as its analogous residues in *SlSOBIR1* and *SlSOBIR1-like*, is dispensable for the intrinsic kinase activity of SOBIR1 and the Avr4/Cf-4-induced ROS production (Figure 2 and 3). It has been reported that silencing of *RBOHB* in *N. benthamiana* results in a

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reduction and delay of the HR induced by INF1, which is an ExIP secreted by the oomycete pathogen *Phytophthora infestans*. In addition, the constitutive HR caused by overexpressing the MAPKK mutant *MEK^{DD}*, was also impaired in *RBOHB*-silenced *N. benthamiana* (Yoshioka et al., 2003). Thus, the apoplastic ROS might positively regulate the formation of the HR that results from the MAPK activation. However, the apoplastic ROS is by itself likely not sufficient to trigger an HR (Figure 1 and 2). Altogether, based on the findings of this study, we envision that the perception of Avr4 by Cf-4 still leads to a strong auto-phosphorylation of the kinase domain of *NbSOBIR1* Y469F (and also of *S/SOBIR1* Y460F and *S/SOBIR1*-like Y473F) (Figure 3), and that the subsequent trans-phosphorylation between *NbSOBIR1* Y469F and recruited BAK1 will fully activate the *NbSOBIR1* Y469F/BAK1 complex (Figure 5B). An increasing amount of RLCKs has been identified to bridge plant immune receptors and downstream signaling components (Bi et al., 2018; Cui et al., 2018; Liang and Zhou, 2018; DeFalco and Zipfel, 2021). Therefore, we presume that particular RLCKs are being phosphorylated, and thereby activated, by this receptor complex and that these RLCKs are then released from this complex to further phosphorylate plasma membrane-resident RBOHB, which then mediates the production of apoplastic ROS. The *NbSOBIR1* Y469F/BAK1 complex is not able to activate a downstream MAPK cascade and the subsequent HR (Figure 1 and 2D) and therefore we speculate that this failure is due to the lack of activation of some particular downstream RLCKs (Figure 5B). The recently developed method, consisting of TurboID-based proximity-dependent labeling, coupled with mass spectrometry (MS) (Branon et al., 2018; Larochelle et al., 2019; Mair et al., 2019), by which for example signaling partners that are in close proximity of SOBIR1 can be labeled by irreversible biotinylation, should be used to identify the interactors of *NbSOBIR1* WT. It should subsequently be tested whether the Y469F mutant maybe only shows a subset of these interactors. This work should eventually provide some clues on how this particular Tyr residue specifically regulates plant immune signaling.

In addition to *NbSOBIR1*^{Y469}, two additional Tyr residues, *NbSOBIR1*^{Y429} and *NbSOBIR1*^{Y530}, were found to be essential for BcXYG1-mediated immune signaling (Figure 4). Our previous work has shown that overexpression of *AtSOBIR1* in *N. benthamiana* induces constitutive activation of a MAPK cascade and an HR (Wu et al., 2017; van der Burgh et al., 2019), and mutation of either *AtSOBIR1*^{Y436} or *AtSOBIR1*^{Y538} strongly dampens the ability of *AtSOBIR1* to trigger this auto-immune response (van der Burgh et al., 2018). Intriguingly, *AtSOBIR1*^{Y436} is the equivalent Tyr residue of *NbSOBIR1*^{Y429}, and even though the equivalent residue of *AtSOBIR1*^{Y538} in *NbSOBIR1* is not a Tyr residue, the residue just before it is *NbSOBIR1*^{Y530} (Figure S1). Hence, the aforementioned Tyr residues are all important for regulating plant immunity. The structure of the inactive *AtSOBIR1* kinase domain has been determined recently (Wei et al., 2022). *AtSOBIR1*^{Y436} is the critical gatekeeper residue which locates in the center of the ATP-binding pocket (Figure S3). In activated plant RLCKs, the gatekeeper residue forms a hydrogen bond with the conserved lysine (Lys/K)/glutamic acid (Glu/E) salt bridge. Phosphorylation or mutation of this specific residue is likely to completely

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abolish the kinase activity of RLKs (Bojar et al., 2014). AtSOBIR1^{Y476} and AtSOBIR1^{Y538} are located on the α E loop and α EF loop, respectively, which are away from the catalytic center (Figure S3), implying that these two Tyr residues are not required for the intrinsic kinase activity of AtSOBIR1. In line with this hypothesis, our *in vitro* phosphorylation assays showed that NbSOBIR1^{Y469}, S/SOBIR1^{Y460} and S/SOBIR1-like^{Y473}, which are analogous to AtSOBIR1^{Y476}, are dispensable for the intrinsic kinase activity of SOBIR1 (Figure S3). Although AtSOBIR1 has been reported to auto-phosphorylate at Tyr residues (Leslie et al., 2010), no phosphorylated Tyr residues have been detected by MS in the kinase domain of AtSOBIR1, when auto-phosphorylated, or after being trans-phosphorylated by BAK1 *in vitro* (Wei et al., 2022), or when produced *in planta* (van der Burgh et al., 2018). Additionally, based on our previous MS analysis of recombinant GST-NbSOBIR1, we did not identify any phosphorylated Tyr residue in the kinase domain of NbSOBIR1 *in vitro* (Chapter 3). Nonetheless, based on the structure of the AtSOBIR1 kinase domain, AtSOBIR1^{Y476} and AtSOBIR1^{Y538} are solvent-exposed, therefore, they might be easier to access by downstream components or other regulatory proteins. Collectively, it is likely that the important Tyr residues in the kinase domain of SOBIR1 do not regulate plant immune responses by phosphorylation, but by interacting with specific downstream signaling partners, such as RLCKs.

Materials and Methods

Plant growth conditions

N. benthamiana sobir1/sobir1-like and *N. benthamiana:Cf-4 sobir1/sobir1-like* knock-out plants were used in this study, as *SOBIR1-like* is not functional in *N. benthamiana*, we further referred to these mutant plants as *N. benthamiana sobir1* and *N. benthamiana:Cf-4 sobir1* knock-out plants respectively (Huang et al., 2021) (Chapter 2). All *N. benthamiana* plants were grown under 15 h of light at 21 °C and 9 h of darkness at 19 °C in a climate chamber, with a relative humidity of ~70%.

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

The constructs pBIN-KS-35S::*NbSOBIR1*-eGFP (SOL2911), pBIN-KS-35S::*NbSOBIR1*^{D482N}-eGFP (kinase-dead mutant) (SOL7928), pBIN-KS-35S::*S/SOBIR1*-eGFP (SOL2774), pBIN-KS-35S::*S/SOBIR1*^{D473N}-eGFP (kinase-dead mutant) (SOL2875), pBIN-KS-35S::*S/SOBIR1-like*-eGFP (SOL2773), pBIN-KS-35S::*S/SOBIR1-like*^{D486N}-eGFP (kinase-dead mutant) (SOL2876), pMOG800-Avr4 and pH2G-35S::*BcXYG1*-HA (SOL8006), have been described previously (Liebrand et al., 2013; Zhu et al., 2017). Mutations were introduced by performing overlap extension PCRs using Phusion Hot Start II DNA Polymerase (Thermo Scientific), using the primers that are listed in Supplemental Table S1 (Liu and Naismith, 2008). The plasmids pENTR/D-Topo::*NbSOBIR1* (SOL4064), pENTR/D-Topo::*S/SOBIR1* (SOL2746) and

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pENTR/D-Topo::S/SOBIR1-like (SOL2745) were used as templates (Liebrand et al., 2013). DpnI (NEB) was employed to digest the methylated template plasmids remaining in the PCR products, which were then transformed into *E. coli* DH5a. Hereafter, the required SOBIR1 variants carrying the individual mutations were selected by Sanger sequencing, and then introduced into pBIN-KS-35S::GWY-eGFP (SOL2095; for C-terminally tagging with eGFP), by using Gateway LR Clonase II (Invitrogen). Following this procedure, the binary vectors pBIN-KS-35S::NbSOBIR1^{Y355F}-eGFP (SOL7914), pBIN-KS-35S::NbSOBIR1^{Y426F}-eGFP (SOL7915), pBIN-KS-35S::NbSOBIR1^{Y429F}-eGFP (SOL7916), pBIN-KS-35S::NbSOBIR1^{Y431F}-eGFP (SOL7917), pBIN-KS-35S::NbSOBIR1^{Y469F}-eGFP (SOL7918), pBIN-KS-35S::NbSOBIR1^{Y525F}-eGFP (SOL7919), pBIN-KS-35S::NbSOBIR1^{Y530F}-eGFP (SOL7920), pBIN-KS-35S::NbSOBIR1^{Y543F}-eGFP (SOL7921), pBIN-KS-35S::S/SOBIR1^{Y346F}-eGFP (SOL7974), pBIN-KS-35S::S/SOBIR1^{Y417F}-eGFP (SOL7975), pBIN-KS-35S::S/SOBIR1^{Y420F}-eGFP (SOL7976), pBIN-KS-35S::S/SOBIR1^{Y422F}-eGFP (SOL7977), pBIN-KS-35S::S/SOBIR1^{Y460F}-eGFP (SOL7978), pBIN-KS-35S::S/SOBIR1^{Y521F}-eGFP (SOL7979), pBIN-KS-35S::S/SOBIR1^{Y522F}-eGFP (SOL7980), pBIN-KS-35S::S/SOBIR1^{Y534F}-eGFP (SOL7981), pBIN-KS-35S::S/SOBIR1^{Y588F}-eGFP (SOL7982), pBIN-KS-35S::S/SOBIR1-like^{Y359F}-eGFP (SOL7942), pBIN-KS-35S::S/SOBIR1-like^{Y430F}-eGFP (SOL7943), pBIN-KS-35S::S/SOBIR1-like^{Y433F}-eGFP (SOL7944), pBIN-KS-35S::S/SOBIR1-like^{Y435F}-eGFP (SOL7945), pBIN-KS-35S::S/SOBIR1-like^{Y473F}-eGFP (SOL7946), pBIN-KS-35S::S/SOBIR1-like^{Y529F}-eGFP (SOL7947), pBIN-KS-35S::S/SOBIR1-like^{Y534F}-eGFP (SOL7948) and pBIN-KS-35S::S/SOBIR1-like^{Y547F}-eGFP (SOL7949), for transient *in planta* expression were obtained.

A. *tumefaciens*-mediated transient transformation

The insert sequence of all generated binary plasmids was confirmed by Sanger sequencing, followed by transformation into *A. tumefaciens* (further referred to as Agrobacterium) strain C58C1, carrying the helper plasmid pCH32. Hereafter, each SOBIR1 variant was transiently co-expressed with *Avr4*, at an optical density at 600 nm (OD₆₀₀) of 0.8 for both SOBIR1 and *Avr4*, in leaves of 4- to 5-week old *N. benthamiana*:*Cf-4 sobir1* knock-out plants, as previously described (van der Hoorn et al., 2000). Similarly, each SOBIR1 variant was also transiently co-expressed with *BcXYG1* (OD₆₀₀=0.8 for both of them) in leaves of 4- to 5-week-old *N. benthamiana* *sobir1* knock-out plants. The development of an HR was photographed and quantified at 5 days post infiltration (dpi) by red light imaging using the ChemiDoc (Bio-Rad), with an exposure time of 2 seconds (Landeo Villanueva et al., 2021). Statistical analysis was performed using a one-way ANOVA test by GraphPad Prism 9.

Reactive oxygen species (ROS) assay

Various SOBIR1 Tyr-to-Phe mutant constructs were agro-infiltrated (OD₆₀₀=0.8) in the youngest fully expanded leaves of *N. benthamiana*:*Cf-4 sobir1* knock-out plants, over the complete leaf surface. *NbSOBIR1* WT, *S/SOBIR1* WT and *S/SOBIR1*-like WT binary constructs were taken along as positive controls, while their kinase-dead mutant binary constructs (*NbSOBIR1* D482N, *S/SOBIR1* D473N and *S/SOBIR1*-like D486N) were taken along as negative controls. Leaf discs were collected at 24 h after agro-infiltration by using biopsy punches (Ø 5 mm, Robbins Instruments) and floated on 80 µL of sterile water in a 96-well plate. After overnight incubation, the water was replaced by 50 µL of fresh sterile water and the discs were incubated for another 1 h. Hereafter, 50 µL of the reaction solution was added, making each well contain 50 µM of luminol (L-012, Fujifilm, Japan), 10 µg/mL of horseradish peroxidase (Sigma) and 0.1 µM of Avr4 protein, in a total volume of 100 µL. Subsequently, the amount of luminescence was monitored using a CLARIOstar plate reader (BMG Labtech).

Immunoprecipitation (IP) and MAPK assay

eGFP-tagged *NbSOBIR1* WT, *NbSOBIR1*^{D482N}, *NbSOBIR1*^{Y469F}, *S/SOBIR1* WT, *S/SOBIR1*^{D473N}, *S/SOBIR1*^{Y460F}, *S/SOBIR1*-like WT, *S/SOBIR1*-like^{D486N} and *S/SOBIR1*-like^{Y473F}, were transiently co-expressed with *Avr4* (OD₆₀₀=0.8 for each binary vector), in the youngest fully expanded leaves of *N. benthamiana*:*Cf-4 sobir1* plants. Leaf samples were collected at 2 dpi and subsequently used to perform an IP with GFP_Trap beads, as described previously (Liebrand et al., 2013). After SDS-PAGE of the soluble total protein extract (input), the activated MAPKs were detected by immunoblotting with anti-p42/p44-erk antibodies (NEB). Concurrently, the immunoprecipitated protein samples were also separated on an SDS-PAGE gel, followed by subjecting the gel to immunoblot analysis with αGFP-HRP antibodies (Milenyi Biotec GmbH).

Recombinant protein expression and *in vitro* phosphorylation assay

To produce the recombinant cytoplasmic kinase domain of *NbSOBIR1*^{Y469F}, *S/SOBIR1*^{Y460F} and *S/SOBIR1*-like^{Y473F} in *E. coli*, the vector pET-GST (Addgene No. 42049) was employed. Firstly, this vector was linearized by PCR amplification with the primer pairs pET-GST_fw/rev (Supplemental Table S1), and meanwhile, the coding sequence of the kinase domain of *NbSOBIR1*^{Y469F}, *S/SOBIR1*^{Y460F} and *S/SOBIR1*-like^{Y473F} was amplified from the corresponding pENTR/D-Topo plasmids by PCR, using primers containing the identical sequence of pET-GST (Supplemental Table S1). Hereafter, the linearized vector and amplified insert were recombined by using the ClonExpress II One Step Cloning Kit (Vazyme, China). After transformation to *E. coli* DH5α, the correct expression constructs were selected by performing colony PCRs and Sanger sequencing.

Recombinant protein expression *in vitro* was conducted as described (Chapter 3), and the sequential *in vitro* phosphorylation assays were performed as previously described (Taylor et al., 2013).

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Supplementary Data

[illegible]

A tyrosine residue in SOBIR1 is essential for plant immunity

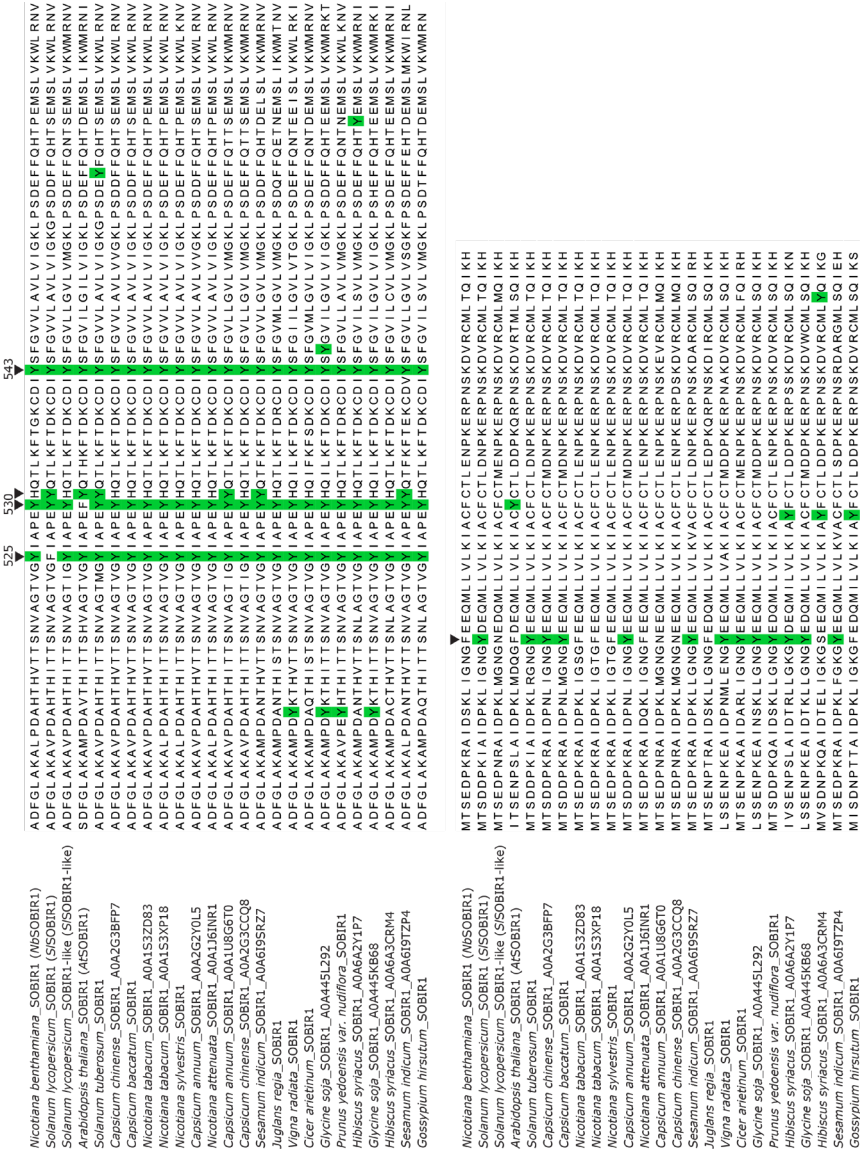


Figure S1. Alignment of the protein sequences of the SOBIR1 kinase domain from various plant species. Amino acid sequences of the SOBIR1 homologs from various plant species were obtained from the UniProt database (<https://www.uniprot.org/>). The alignment was visualized using JalView and only the amino acid sequences of the kinase domain are shown, with all the Tyr (Y) residues highlighted in green. The Tyr residues that are subject of this study are marked with arrowheads on the top, and their position in *NbSOBIR1* is indicated on the top. The RD motif (in which a conserved arginine (R) precedes the highly conserved catalytic aspartate (D)), present in all SOBIR1 kinase domains, is indicated by a box.

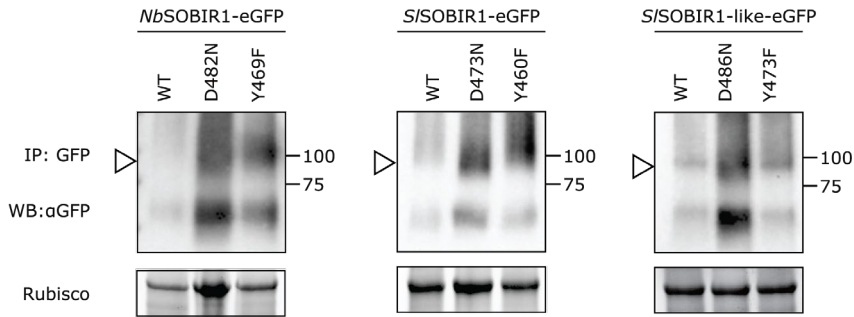


Figure S2. Accumulation levels of *NbSOBIR1*^{Y469F}, *S/SOBIR1*^{Y460F} and *S/SOBIR1-like*^{Y473F}, as well as their corresponding wild-type (WT) and kinase-dead (D to N) versions, *in planta*. *NbSOBIR1*^{Y469F}, *S/SOBIR1*^{Y460F} and *S/SOBIR1-like*^{Y473F}, which fail to fully restore the *Avr4*-triggered HR in *N. benthamiana*:*Cf-4 sobir1* mutant plants, were transiently expressed in *N. benthamiana*:*Cf-4 sobir1* plants in combination with *Avr4* (both at an OD₆₀₀ of 0.8), next to their respective WT that was combined with *Avr4* as a positive control, and their kinase-dead D to N version that was combined with *Avr4* as a negative control. Leaf samples were collected at 2 dpi. Total protein extracts were subjected to immunopurification (IP) of the GFP-tagged SOBIR1 mutants using GFP-affinity beads, followed by western blotting (WB) with αGFP antibody (upper panels). The amount of total protein that was used for the IP is reflected by the Rubisco band present in the stain-free gel (lower panels). Arrowheads indicate the band representing SOBIR1-eGFP. Note that transient expression of *SOBIR1* WT in combination with *Avr4* triggers an HR in *N. benthamiana*:*Cf-4 sobir1* plants, which explains the low accumulation levels of SOBIR1 WT.

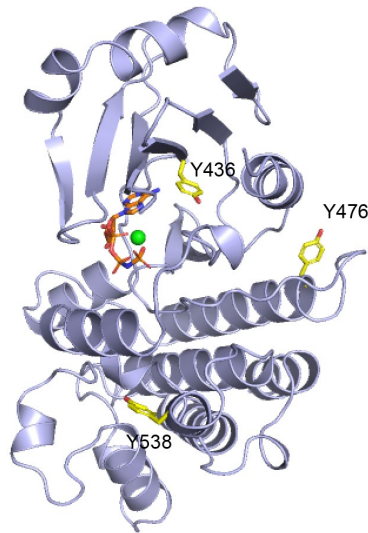


Figure S3. Overall structure of the inactive *AtSOBIR1* kinase domain and positions of the three important Tyr residues. The ribbon diagram of the SOBIR1 kinase domain is colored in light blue. The nonhydrolyzable ATP analog AMP-PNP and Mg²⁺ are presented as an orange stick and a green sphere, respectively. *AtSOBIR1*^{Y436}, *AtSOBIR1*^{Y476} and *AtSOBIR1*^{Y538} are highlighted as yellow sticks.

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Supplemental Table S1. Nucleotide sequences of the primers used in this study.

Primer code	Primer name	Sequence (5'-3')
Primers used for introducing point mutations (the introduced mutations are indicated with a capital)		
ho123	NbSOBIR1_Y355F fw*	ggtggatgCGGagaagtttTtagagctgagttaccggg
ho124	NbSOBIR1_Y355F rev	cccggttaactcagctctaAaaacttctccgcatccacc
ho125	NbSOBIR1_Y426F fw	ctagggcagactgccattTcttggtatatgaatatatg
ho126	NbSOBIR1_Y426F rev	catatattcatataccaagAaatggcagctggcctag
ho127	NbSOBIR1_Y429F fw	gactgccattacttggtatTgaatatatgaaaaatgg
ho128	NbSOBIR1_Y429F rev	ccatttttcatatattcaAataccaagtaatggcagctc
ho129	NbSOBIR1_Y431F fw	gccattacttggtatatgaatTtatgaaaaatgggagc
ho130	NbSOBIR1_Y431F rev	gtccccatttttcataAattcatataccaagtaatggc
ho131	NbSOBIR1_Y469F fw	gatagcttctggacttgagtTtctccatataaaccac
ho132	NbSOBIR1_Y469F rev	gtggtttatatggagaAactcaagtcagaagctatc
ho133	NbSOBIR1_Y525F fw	gcaggaaactgtgggatTattgaccagataaccatc
ho134	NbSOBIR1_Y525F rev	gatggtattctggtgcaataAatcccacagttctctgc
ho135	NbSOBIR1_Y530F fw	gatatattgcaccagaatTccatcagacactgaag
ho136	NbSOBIR1_Y530F rev	cttcagtgctgatggAattctggtgcaatatatc
ho137	NbSOBIR1_Y543F fw	cgggtaagtgtgatataTcagctttggtgtggtg
ho138	NbSOBIR1_Y543F rev	caccacaccaaagctgAatatatcacacttaccgg
ao11	SISOBIR1_Y346F fw	gtggctgtggagaagttTtagagcagagctac
ao12	SISOBIR1_Y346F rev	gtagctctgctctaAaaacttctccacagccac
ao13	SISOBIR1_Y417F fw	gccagactgtcattTcttggtctcgaatacatg
ao14	SISOBIR1_Y417F rev	catgtattcgtagaccaagAaatgacagcttggc
ao15	SISOBIR1_Y420F fw	cttggtctTcgaatacatgaaaaatgggagtttacag
ao16	SISOBIR1_Y420F rev	ctgtaaactcccatttttcatgtattcgAagaccaag
ao17	SISOBIR1_Y422F fw	cgaatTcatgaaaaatgggagtttacaggatatcc
ao18	SISOBIR1_Y422F rev	ggatatcctgtaaactcccatttttcatgAattcg
ao19	SISOBIR1_Y460F fw	gctgctggtctcagagtTtctccatataaaccatac
ao20	SISOBIR1_Y460F rev	gtatggtttatatggagaAactcgagaccagcagc
ao21	SISOBIR1_Y521F fw	gcaccagaatTtatcagacactgaagtttacag
ao22	SISOBIR1_Y521F rev	ctgtaaactcagtgctgataaAattctggtgc
ao23	SISOBIR1_Y522F fw	gcaccagaatattTcagacactgaagtttac
ao24	SISOBIR1_Y522F rev	gtaaactcagtgctgaAaatattctggtgc
ao25	SISOBIR1_Y534F fw	gataagtgtgatataTcagctttggtgtggtgc
ao26	SISOBIR1_Y534F rev	gcaccacaccaaagctgAatatatcacacttatc
ao27	SISOBIR1_Y588F fw	gcttataggaaatggatTcgacgaacaatgc
ao28	SISOBIR1_Y588F rev	gcatttgctcgtcgAatccatttctataagc
ho142	SISOB-like_Y359F_fw	cattgggcaagggtgatgtggaaaagtttTaaagctgc
ho143	SISOB-like_Y359F_rev	ctttccgtcactccaggtaatgcagctttaAaaactttcc

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ho144	SISOB-like_Y430F_fw	gccaagaccagactgccactTcttggtctatgagtacatg
ho145	SISOB-like_Y430F_rev	catgtactcatagaccaagAagtggcagctcgtgcttggc
ho146	SISOB-like_Y433F_fw	ctgccactacttggtctTtgagtacatgaaaaatgggagc
ho147	SISOB-like_Y433F_rev	gctccccattttcatgtactcaAagaccaagtagtggcag
ho148	SISOB-like_Y435F_fw	gccactacttggtctatgagTcatgaaaaatgggagc
ho149	SISOB-like_Y435F_rev	gctccccattttcatgAactcatagaccaagtagtggc
ho150	SISOB-like_Y473F_fw	gctgctggactcgagTtctccatataaatcatactcagcg
ho151	SISOB-like_Y473F_rev	cgctgagtatgatttatatggagaAactcgagtcaggcagc
ho152	SISOB-like_Y529F_fw	ggcaggaaactataggatTcatcgctccagaatatcatcag
ho153	SISOB-like_Y529F_rev	ctgatgatattctggagcgatAatcctatagttctgcc
ho154	SISOB-like_Y534F_fw	ggatacatcgctccagaatTtcatcagacactgaagtccactg
ho155	SISOB-like_Y534F_rev	cagtgaacttcagtgctgatgaAattctggagcgatgatcc
ho156	SISOB-like_Y547F_fw	ctgataagtgatataTcagctttgggggtgctg
ho157	SISOB-like_Y547F_rev	cagcaccccaagctgAatatatcacactatcag
Primers used for generating <i>E. coli</i> expression constructs (sequences that are identical to those of the expression vectors are underlined)		
ho201	pET-GST_fw	aagcttgcggccgcactcgag
ho202	pET-GST_rev	cagggggccctggaacagaacttc
ho203	NbSOBIR1_KD+90bp_fw	<u>gttctgttccaggggccct</u> gcgaaagggaaagactgatggaac
ho204	NbSOBIR1_KD+90bp_rev	<u>ctcgagtgcgccgcaagctt</u> ctaagtcttgatctgagttaacatac
ho215	SISOBIR1_KD+90bp_fw	<u>gttctgttccaggggccct</u> gagaagagggaataacgattcaag
ho216	SISOBIR1_KD+90bp_rev	<u>ctcgagtgcgccgcaagctt</u> ctaagtcttgatctgagttaacatgc
ho217	SISOBIR1-like_KD+90bp_fw	<u>gttctgttccaggggccct</u> gagagggatcagaatgatccagg
ho218	SISOBIR1-like_KD+90bp_rev	<u>ctcgagtgcgccgcaagctt</u> taagtcttgatctgcatcaacatgc

* fw, forward; rev, reverse.

Chapter 5

Receptor-like cytoplasmic kinases from class VII, subfamily 6, redundantly and positively regulate the Avr4/Cf-4-triggered oxidative burst in *Nicotiana benthamiana*

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Abstract

Cell-surface receptors, which are either receptor-like proteins (RLPs) or receptor-like kinases (RLKs), form the first layer of the plant innate immune system. Upon recognition of the matching extracellular immunogenic pattern (ExIP), the activated cell-surface receptor complexes trigger a battery of downstream signaling events. Receptor-like cytoplasmic kinases (RLCKs) are well-recognized to act as the initial cytoplasmic transducers, bridging the cell-surface receptor complexes with the downstream signaling components. Arabidopsis (*Arabidopsis thaliana*, At) RLCK class VII subfamilies 4, 5, 6, 7 and 8 have been reported to be generally involved in immunity and in the accumulation of reactive oxygen species (ROS) that is induced by different ExIPs. However, it is largely unknown whether different RLCK-VII subfamily members play a specific or redundant role downstream of the tomato RLP Cf-4 in Solanaceous plants. Resistance protein Cf-4 is the receptor of Avr4, which is an apoplastic effector secreted by the pathogenic intercellular fungus *Cladosporium fulvum*. Here, by knocking out multiple candidate genes belonging to different RLCK-VII subfamilies in *Nicotiana benthamiana* plants stably expressing Cf-4, we show that members of the RLCK-VII subfamilies 6, 7 and 8 are required for the Avr4/Cf-4-triggered ROS production. Further studies show that members of RLCK-VII-6 play an important role in regulating the ROS production triggered by multiple ExIPs, in addition to ROS triggered by Avr4, but not in regulating the Avr4/Cf-4-triggered mitogen-activated protein kinase (MAPK) activation and the hypersensitive response (HR), which refers to the programmed cell death that is eventually taking place upon recognition of Avr4 by the Cf-4 resistance protein. Subsequent complementation studies with the individual RLCKs that were knocked out indicate that the different members from subfamily 6 function redundantly to positively regulate Avr4/Cf-4-triggered ROS production, even though they appear to have a different subcellular localization.

Introduction

Plants have evolved a two-layered innate immune system to fend off invading microbes, of which the first layer is mediated by cell-surface receptors (van der Burgh and Joosten, 2019). The two largest families of such receptors are formed by receptor-like proteins (RLPs) and receptor-like kinases (RLKs) (Monaghan and Zipfel, 2012; Macho and Zipfel, 2014; Ranf, 2017; DeFalco and Zipfel, 2021). These receptor families both possess an extracellular domain and a single-pass transmembrane domain, however, RLKs contain an additional intracellular kinase domain that is absent in RLPs (Jones and Dangl, 2006; Zipfel, 2008; Monaghan and Zipfel, 2012; Macho and Zipfel, 2014; Zipfel, 2014). Both RLKs and RLPs detect extracellular immunogenic patterns (ExIPs), leading to extracellularly-triggered immunity (ExTI) (van der Burgh and Joosten, 2019). ExIPs include, but are not limited to, pathogen-derived microbe-associated molecular patterns (MAMPs), extracellular effectors, and host-derived damage-associated molecular patterns (DAMPs). Extracellular effectors are secreted proteins from adapted pathogens that promote host colonization (Boller and Felix, 2009; Dodds and Rathjen, 2010; van der Burgh and Joosten, 2019). Bacterial flagellin (or its N-terminal 22-amino acid epitope, flg22), bacterial elongation factor TU (EF-Tu) (or its N-terminal 18-amino acid epitope, elf18), and fungal chitin, which all are conserved microbial structural components and well-studied MAMPs, are specifically recognized by the leucine-rich repeat (LRR)-RLK FLAGELLIN-SENSING 2 (FLS2), the LRR-RLK EF-Tu RECEPTOR (EFR), and the lysin motif (LysM)-RLK LYSIN MOTIF-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) of *Arabidopsis* (*Arabidopsis thaliana*, *At*), respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004; Cao et al., 2014). Representative examples of extracellular effectors are Avr4 and Avr9, which are small, cysteine-rich proteins that are secreted by the biotrophic extracellular fungal pathogen *Cladosporium fulvum*, and which are perceived by the matching tomato (*Solanum lycopersicum*, *Sl*) LRR-RLPs Cf-4 and Cf-9, respectively (van Kan et al., 1991; van den Ackerveken et al., 1992; Jones et al., 1994; Joosten et al., 1994; Thomas et al., 1997). Generally, DAMPs are plant degradation products that are passively released during pathogen attacks or actively generated upon host tissue damage caused by pathogen ingress. Perception of DAMPs by cell-surface receptors also results in the initiation of plant immunity (Macho and Zipfel, 2014; Bigeard et al., 2015; Boutrot and Zipfel, 2017).

Upon recognition of the matching ExIPs, LRR-RLKs, such as FLS2, recruit the common regulator BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1) to initiate immune signaling (Heese et al., 2007; Chinchilla et al., 2009). LRR-RLPs, such as Cf-4 and Cf-9, lack an intracellular kinase domain and constitutively interact with the LRR-RLK SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1) (Gao et al., 2009; Liebrand et al., 2013; Liebrand et al., 2014). Interestingly, the LRR-RLP/SOBIR1 complex, similar to an LRR-RLK carrying a cytoplasmic kinase domain itself, also undergoes complex formation with the RLK BAK1 in response to

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recognition of the corresponding ExIP. BAK1 recruitment allows activation of the cell-surface receptor complex by trans-phosphorylation events that take place between the two cytoplasmic kinase domains that have now come into close proximity (Liebrand et al., 2014; Postma et al., 2016). Activation of these cell-surface receptor complexes subsequently triggers a suite of downstream signaling events and immune outputs, including the rapid phosphorylation of downstream receptor-like cytoplasmic kinases (RLCKs), the swift production of apoplastic reactive oxygen species (ROS), the activation of mitogen-activated protein kinase (MAPK) cascades, large-scale transcriptional reprogramming, and in some cases a hypersensitive response (HR) that consists of a rapid programmed cell death (Tsuda and Katagiri, 2010; Lu and Tsuda, 2021; Yuan et al., 2021).

Swift production of ROS, which is the hallmark of ExTI, not only functions as an antimicrobial agent but also plays an essential role in endogenous immune signaling (Mittler, 2017; Qi et al., 2017; Waszczak et al., 2018). A recent study has for example reported that apoplastic ROS can be sensed by HYDROGEN-PEROXIDE-INDUCED Ca^{2+} INCREASES 1 (HPCA1), which is an LRR-RLK that is localized to the plasma membrane (PM). This sensing further activates plant immunity by regulating Ca^{2+} -channel gating and Ca^{2+} influx (Wu et al., 2020). Apoplastic ROS production is mainly mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, which are also termed RESPIRATORY BURST OXIDASE HOMOLOGS; RBOHs (Qi et al., 2017; Waszczak et al., 2018). The NADPH oxidase RBOHD and, to a lesser extent RBOHF, both play a key role in ROS generation related to ExTI in Arabidopsis, whereas RBOHB is required for ROS accumulation in both *Nicotiana benthamiana* (*Nb*) and tomato upon pathogen challenge (Torres et al., 2002; Yoshioka et al., 2003; Adachi et al., 2015; Li et al., 2015). In addition to the ROS burst, transient activation of MAPK cascades is another well-known feature of ExTI (Yu et al., 2017; van der Burgh and Joosten, 2019; Zhou and Zhang, 2020). A MAPK cascade typically consists of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK) module, by which phosphorylation signals are transmitted linearly from MAPKKKs to downstream MAPKs (Dodds and Rathjen, 2010; Bigeard et al., 2015). The activated MAPK cascade further either positively or negatively regulates plant immune responses, by altering the expression of defense-related genes through the phosphorylation of certain transcription factors, the accumulation of ROS, and/or regulating plant hormone signaling (Zhang and Klessig, 2001; Asai et al., 2008; Ishihama et al., 2011; Kong et al., 2012; Tsuda et al., 2013; Su et al., 2018).

Increasing evidence suggests that RLCKs are the direct downstream cytoplasmic substrates of activated receptor complexes and that they fill the gap between receptors present at the cell surface and downstream signaling components, such as the MAPK modules (Lin et al., 2013; Macho and Zipfel, 2014; Tang et al., 2017; Bi et al., 2018; Zhou and Zhang, 2020). Arabidopsis BOTRYTIS-INDUCED KINASE 1 (BIK1) is one of the best-characterized RLCKs and is required for triggering ROS production upon the

perception of multiple ExIPs, such as flg22, elf18, and chitin (Lu et al., 2010; Zhang et al., 2010; Wan et al., 2019). Importantly, BIK1 has been shown to directly phosphorylate RBOHD, thereby stimulating ROS production (Li et al., 2014). In addition to BIK1, Arabidopsis AvrPphB SUSCEPTIBLE 1-LIKE 27 (PBL27) has been reported to connect chitin perception to MAPK cascade activation, as MAPKKK5 is directly phosphorylated by PBL27 (Yamada et al., 2016). BIK1, PBL27 and some other RLCKs that have been identified as central positive regulatory components that relay immune signaling from the cell surface to the intracellular space, all belong to Arabidopsis RLCK class VII (Zhang et al., 2010; Lin et al., 2015; Luo et al., 2020; Pruitt et al., 2021). Class RLCK-VII is composed of 46 members, and according to their sequence similarity, these members can be further divided into nine subfamilies, termed RLCK-VII-1 to RLCK-VII-9 (Rao et al., 2018). Interestingly, flg22-, elf18- and chitin-triggered ROS production is significantly reduced in *rlck-vii-5*, *-7*, and *-8* mutants, whereas in contrast, *rlck-vii-6* mutants exhibit higher flg22-induced ROS accumulation levels when compared to wild-type plants. Notably, the members of RLCK-VII-4 are particularly required for both the chitin-triggered ROS burst and MAPK activation in Arabidopsis (Bi et al., 2018; Rao et al., 2018).

Compared to the model plant Arabidopsis, little is known about the function of the various classes and subfamilies of RLCKs in Solanaceous plants, and it is thus far not known which RLCKs are involved in the LRR-RLP/SOBIR1-triggered immune signaling pathway. Cf-4 and Cf-9 are well-characterized LRR-RLPs of tomato, and the RLCK AVR9-Cf-9-INDUCED KINASE 1 (ACIK1) has been reported to play a role as a positive regulator in the Avr4/Cf-4- and Avr9/Cf-9-triggered HR, as well as in resistance of tomato against *C. fulvum* (Rowland et al., 2005). Nevertheless, it is presently unknown which particular RLCKs are essential for the LRR-RLP/SOBIR1/BAK1-triggered ROS burst and MAPK activation, as well as how these RLCKs eventually activate immunity. In this study, by knocking out multiple candidate genes belonging to different RLCK-VII subfamilies in transgenic *N. benthamiana* plants stably expressing *Cf-4*, we show that members from RLCK-VII-6, *-7* and *-8* are required for full Avr4/Cf-4-triggered ROS production. Three different homozygous *rlck-vii-6* mutant lines were obtained, in which eight *RLCK* family members were targeted by multiplex CRISPR/Cas9, and these plants were further characterized. We reveal that these eight members from *N. benthamiana* RLCK-VII-6 together play a positive regulatory role in activating ROS production, but not in the Avr4/Cf-4-triggered MAPK activation and initiation of the HR. Subsequent complementation studies with the individual *RLCKs* that had been knocked out indicate that the different members from subfamily 6 function redundantly to positively regulate ROS production, even though they appear to have different subcellular localizations. Finally, we show that despite their homology to *AtBIK1*, members of *N. benthamiana* RLCK-VII-6 are not required for resistance to *Botrytis cinerea*.

Results

Members of *N. benthamiana* RLCK-VII-6, -7 and -8 are all required for the Avr4/Cf-4/SOBIR1-triggered ROS burst

Increasing evidence has shown that RLCKs play an important role in regulating plant defense responses (Lin et al., 2013; Liang and Zhou, 2018; DeFalco and Zipfel, 2021). The genome of Arabidopsis contains 149 RLCK-encoding genes, which are divided into 17 classes (Shiu and Bleecker, 2001; Shiu et al., 2004). Some members from RLCK-VII, such as BIK1, PBL1, PBL13, RPM1-INDUCED PROTEIN KINASE (RIPK) and PBL27, have been well described (Lu et al., 2010; Zhang et al., 2010; Shinya et al., 2014; Lin et al., 2015; Li et al., 2021). The 46 members of RLCK-VII can be further classified into nine subfamilies, of which subfamilies 4, 5, 6, 7, and 8 were found to be involved in the ROS accumulation induced by different ExIPs (Rao et al., 2018; Luo et al., 2020; Pruitt et al., 2021). In addition to Arabidopsis, the Solanaceous plant *N. benthamiana* is another versatile experimental host plant (Goodin et al., 2008; Bombarely et al., 2012). In our previous studies, silencing of some individual RLCK-encoding genes in *N. benthamiana*, followed by monitoring the intensity of the Avr4/Cf-4-triggered HR, did not result in the identification of any promising individual RLCK candidate (van der Burgh et al., 2018). This could be the result of redundancy, as the family of RLCKs in *N. benthamiana* is large and thus multiple RLCK proteins might perform the same function (Goodin et al., 2008; Kourelis et al., 2019). Another reason might be that the selected candidates are only involved in regulating ROS production and/or MAPK activation, but not in the activation of the HR.

To search for RLCKs in Solanaceous plants, we, first of all, performed a phylogenetic analysis to identify Arabidopsis BIK1 homologs in Arabidopsis, tomato, and *N. benthamiana* (Figure S1). All the RLCKs were further assigned to six subfamilies, which are referred to as subfamily 4, 5, 6, 7, 8, and 9, and are corresponding to the RLCK-VII subfamilies in Arabidopsis that were previously reported by Rao and colleagues (Figure S1B) (2018). Thanks to the fast development of the CRISPR/Cas9 technology, it is now possible to simultaneously target multiple homologous genes with high efficiency in various plant species (Cong et al., 2013; Nekrasov et al., 2013; Zhou et al., 2018; Stuttmann et al., 2021). To identify the RLCKs that play a role in cytoplasmic immune signaling downstream of the activated Avr4/Cf-4/SOBIR1/BAK1 complex, and to cope with the consequences of functional gene redundancy, we attempted to implement the CRISPR/Cas9 system in *N. benthamiana*, stably expressing the *Cf-4* transgene, to simultaneously target multiple RLCK-encoding genes belonging to the same subfamily (Table S1). *N. benthamiana* RLCK-VII-6 contains 14 members, which might be beyond the limit of the current multiplex CRISPR/Cas9 system (Figure S1B). Hence, the genes that are up-regulated or remain unchanged in their expression in *N. benthamiana*:*Cf-4* upon transient expression of *Avr4*, *NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 1 (NRC1) D481V*, which triggers an elicitor-independent

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HR in *N. benthamiana*, and *COAT PROTEIN (CP)*, which also triggers an HR in *N. benthamiana* when being overexpressed, were selected to be knocked out (Figure S2 and Table S1) (Gabriëls et al., 2007; Tameling et al., 2010).

After obtaining the primary transformants (the T1 generation), in which all targeted *RLCK* members of each subfamily were anticipated to be knocked out, we performed ROS burst assays with the mutants to identify those *RLCK* subfamilies that are required for the ROS production triggered upon activation of the Cf-4/SOBIR1 complex by Avr4. As we observed before, the Avr4 protein triggered a biphasic ROS burst in leaf discs obtained from *N. benthamiana*:Cf-4 plants (Huang et al., 2021) (Chapter 2) (Figure 1). Strikingly, the intensity of the first, early phase of the ROS burst, which generally reaches its peak at around 20 minutes after the addition of the Avr4 protein, was only slightly reduced, whereas the second, more sustained phase of the ROS burst, generally reaching its peak around 140 minutes after Avr4 addition, was completely absent in all the independent *N. benthamiana*:Cf-4 *RLCK-VII-6* knock-out lines, referred to as *rlck-vii-6*, that had been generated (Figure 1). This observation indicates that members of *RLCK-VII-6* play an important role in regulating the Avr4-triggered ROS burst in Cf-4-transgenic *N. benthamiana*, especially concerning the second phase, which is the more sustained phase of the ROS burst. Intriguingly, the Avr4-induced ROS production was overall strongly compromised in all the *rlck-vii-7* mutant lines, whereas two of the *rlck-vii-8* mutant lines showed a similar reduction of the overall ROS burst when treated with Avr4 (Figure 1). These findings suggest that also the members of *RLCK-VII-7* and -8 are redundantly required for the Avr4/Cf-4-triggered ROS burst. Additionally, one *rlck-vii-4* mutant line exhibited an increased ROS production induced by Avr4/Cf-4, while several *rlck-vii-5* and *rlck-vii-9* knock-out lines only displayed a mildly compromised Avr4/Cf-4-triggered ROS accumulation (Figure 1). Therefore, the members from *RLCK-VII-4*, -5 and -9 might also play a minor role in regulating the Avr4/Cf-4-induced ROS burst. However, as these mutant lines were all from the T1 generation and their genotypes were not exactly determined, it will be necessary to repeat these experiments with homozygous mutant lines, before we can draw solid conclusions.

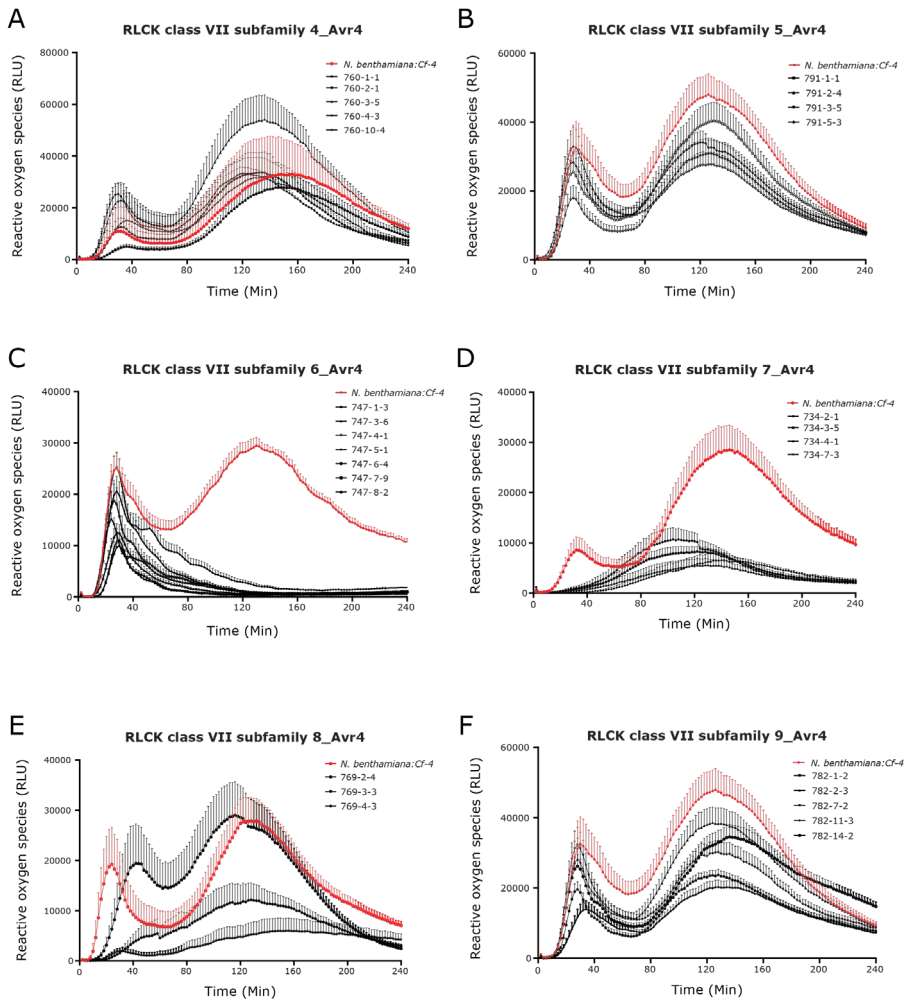


Figure 1. Various subfamilies from the RLCK clade VII play an important role in the Avr4/Cf-4/SOBIR1-triggered ROS burst in Cf-4-transgenic *N. benthamiana*. Selected members from each RLCK class VII subfamily, being 4, 5, 6, 7, 8, 9, of *N. benthamiana* were targeted for knock-out in *N. benthamiana*:Cf-4 by CRISPR/Cas technology. Subsequently, ROS accumulation induced upon treatment of leaf discs obtained from five individual *rlck-vii-4* transformants (A), four *rlck-vii-5* transformants (B), seven *rlck-vii-6* transformants (C), four *rlck-vii-7* transformants (D), three *rlck-vii-8* transformants (E) and five *rlck-vii-9* transformants (F), with the Avr4 protein, was determined. For this, leaf discs obtained from the different mutant plants, as well as from *N. benthamiana*:Cf-4 itself (the positive control), were treated with a final concentration of 0.1 μ M Avr4 protein, and the generation of ROS was monitored. Of note, all transformants tested were from the T1 generation. ROS production is expressed as relative light units (RLUs) and the data are represented as mean, plus the standard error of the mean (SEM) ($n \geq 6$). The ROS profiles of the positive control that was included in all the assays are indicated in red in all the line charts. Similar results were obtained in replicates and data from one representative experiment are shown.

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To further explore whether these RLCK-VII subfamilies in *N. benthamiana* also play a role in LRR-RLK-triggered immune signaling, we treated all the mutant lines with the flg22 peptide to activate the endogenous FLS2 receptor of *N. benthamiana*. *N. benthamiana*:Cf-4 was used as a positive control, which showed a rapid and typical monophasic FLS2-mediated ROS burst upon flg22 peptide treatment, reaching its peak at about 25 minutes after the addition of the peptide (Huang et al., 2021) (Chapter 2) (Figure S3). Similar to our previous observation when performing ROS assays with the pure Avr4 protein, the flg22-induced ROS accumulation was dampened to different levels in all the *rlck-vii-6* mutants and was almost completely eliminated in some of the *rlck-vii-7* and *rlck-vii-8* mutants (Figure S3). Surprisingly, several *rlck-vii-4* mutant lines displayed an increased ROS burst when challenged with flg22, which suggests that members of RLCK-VII-4 may negatively regulate the flg22/FLS2-triggered ROS production. As observed previously upon treatment with Avr4, the remaining *rlck-vii* mutants did not show obvious phenotypes in these flg22 assays. Our observations indicate that, in addition to a role in Avr4/Cf-4/SOBIR1-triggered immune signaling, the members of the RLCK-VII-6, -7 and -8 also play a positive role in FLS2-mediated immune signaling.

Generation and characterization of homozygous *rlck-vii-6* mutant plants

As seeds of the *rlck-vii-6* mutant lines were the first to arrive in our lab, we decided to continue with this subfamily in this study. To knock out eight members from RLCK-VII-6 simultaneously, nine single-guide RNAs (sgRNAs) were designed (Figure 2A, S2 and Table S1). The genotypes of four T1 mutant plants, which originated from two independent transgene-free lines in which *rlck-vii-6* was targeted, were determined and the result was described by Stuttmann et al. (2021). Briefly, all the targeted genes in all the four tested plants were mutated as expected, and most of the mutations were found to be biallelic (Stuttmann et al., 2021). Based on these results, we amplified the targeted gene regions of the plants in the T2 generation, followed by Sanger sequencing of the generated fragments. Importantly, three independent homozygous mutant plants, named 747-3-6-2, 747-3-6-7 and 747-7-9-6, in which all eight candidate genes were knocked out, were obtained (Figure 2B, S4, S5 and S6). However, for 747-3-6-2 and 747-3-6-7, only a 3-bp deletion was introduced in *Niben101Scf03268g03002*, and therefore this particular gene might not be actually knocked out. Notably, these mutant lines did not exhibit any significant changes in plant growth and morphology, despite the fact that the mutant plants were slightly smaller when compared to the original *N. benthamiana*:Cf-4 plants in which the mutagenesis was performed (Figure 2C).

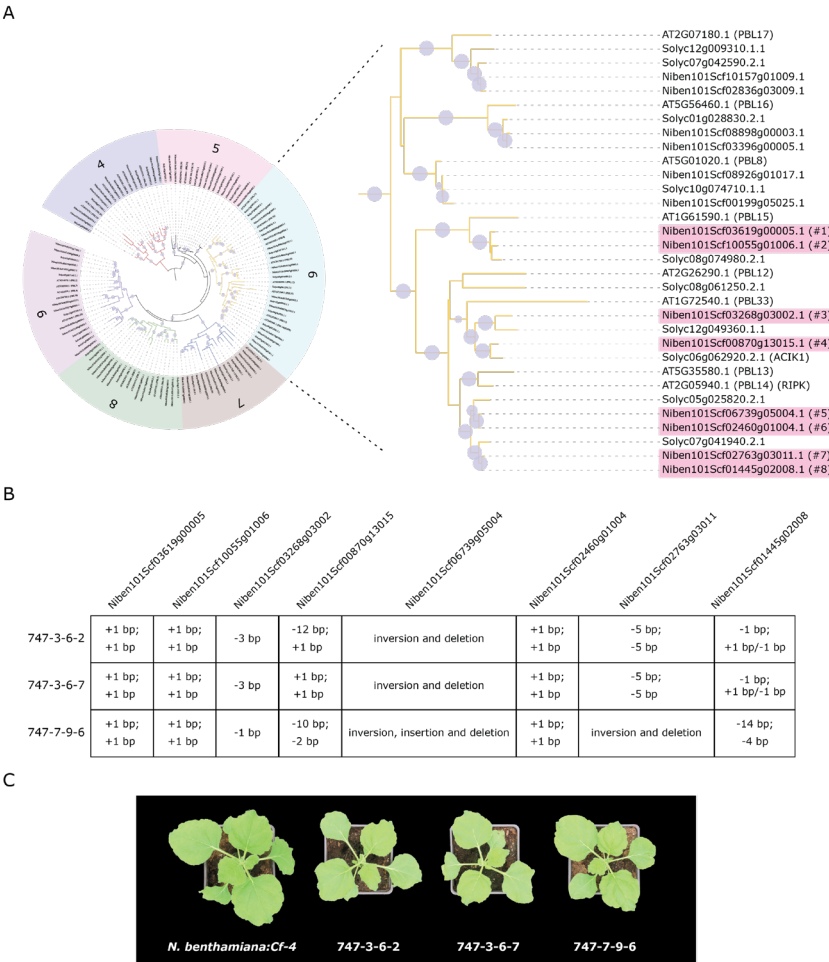


Figure 2. CRISPR/Cas9-mediated knockout of members from RLCK-VII-6 in *N. benthamiana*:*Cf-4*. (A) Phylogenetic tree of BIK1 homologs from Arabidopsis, tomato, and *N. benthamiana* (left) (as shown in Figure S1), of which subfamily 6 is shown in detail on the right. The genes that were selected to be knocked out are highlighted in pink (see also Figure S2). (B) Overview of the types of mutations present in all the RLCK-VII-6 members in the three homozygous mutant lines (see also Figure S4-S6). Note that two single-guide RNAs (sgRNAs) were designed to target each gene, except for *Niben101Scf03268g03002*, which was only targeted by one sgRNA (Table S1). (C) Morphological phenotypes of *N. benthamiana*:*Cf-4* and the three independent *rlck-vii-6* mutant lines. All plants were grown in soil under the same conditions, at the same time and were photographed when they were four to five weeks old. Note that the pictures of the plants were taken under the same conditions and are placed on a black background.

N. benthamiana RLCK-VII-6 members contribute to the ROS production triggered by multiple ExIPs

Both the Avr4- and flg22-induced ROS burst was attenuated in the *rlck-vii-6* mutant plants from the T1 generation (Figure 1C and S3C). To further verify these results, leaf

discs taken from the homozygous T2 mutant lines were again treated with either the Avr4 protein or flg22 peptide, followed by monitoring ROS accumulation, with *N. benthamiana*:Cf-4 plants taken along as a positive control. Consistent with the aforementioned results obtained with the T1 plants, ROS production triggered by the Avr4 protein and flg22 peptide was strongly compromised when compared to *N. benthamiana*:Cf-4, thereby confirming that the members of RLCK-VII-6 are indeed required for both Cf-4/SOBIR1- and FLS2-triggered immune signaling (Figure 3A and 3B). The importance of this RLCK subfamily in regulating ROS accumulation led us to determine possible changes in the ROS burst induced by other ExIPs, such as chitin, in the 747-3-6-2, 747-3-6-7 and 747-7-9-6 lines. Strikingly, these three independent knock-out lines also exhibited an obvious reduction in chitin-triggered ROS production, when compared to *N. benthamiana*:Cf-4 (Figure 3C).

The Arabidopsis LRR-RLPs RLP23 and RLP42 perceive a conserved 20-amino-acid peptide from necrosis and ethylene-inducing peptide 1 (NEP1)-like proteins (nlp20) and *B. cinerea* endo-polygalacturonases (PGs and their derived peptide, pg13), respectively (Zhang et al., 2014; Albert et al., 2015; Zhang et al., 2021). Similar to Cf-4, both RLP23 and RLP42 require the recruitment of SOBIR1 and BAK1 to initiate immune signaling upon recognition of their matching elicitor (Liebrand et al., 2013; Zhang et al., 2014; Albert et al., 2015; Zhang et al., 2021). Interestingly, although *RLP23* and *RLP42* are Arabidopsis genes, *N. benthamiana* plants that overexpress either *RLP23* or *RLP42* show sensitivity to the corresponding ExIP. This suggests that RLP23 and RLP42 can employ endogenous *N. benthamiana* SOBIR1 and BAK1 and additional, probably functionally conserved, downstream partners to activate immune signaling in response to treatment with nlp20 and pg13, respectively (Albert et al., 2015; Zhang et al., 2021). To decipher whether the *N. benthamiana* RLCK-VII-6 family members also play a role downstream of RLP23 and RLP42, we transiently overexpressed either one of them in the different *rlck-vii-6* mutant lines, as well as in *N. benthamiana*:Cf-4, and collected leaf discs of the infiltrated area at 24 hours after infiltration. Subsequently, after adding the corresponding elicitors, we monitored ROS production. Strikingly, similar to the situation with Avr4/Cf-4, both the nlp20/RLP23- and pg13/RLP42-mediated ROS burst in *N. benthamiana* plants was biphasic, of which the first burst was rapid with a relatively high amplitude, whereas the second burst was sustained, with relatively low amplitude (Figure 3D and 3E). Both the nlp20/RLP23- and the pg13/RLP42-triggered ROS production was significantly impaired in all *rlck-vii-6* mutant plants, when compared to *N. benthamiana*:Cf-4. This was especially the case for the second burst, which was completely abolished (Figure 3D and 3E). Collectively, these results indicate that the members of the RLCK-VII-6 subfamily are required for regulating the ROS production triggered by a broad spectrum of ExIPs.

Chapter 5

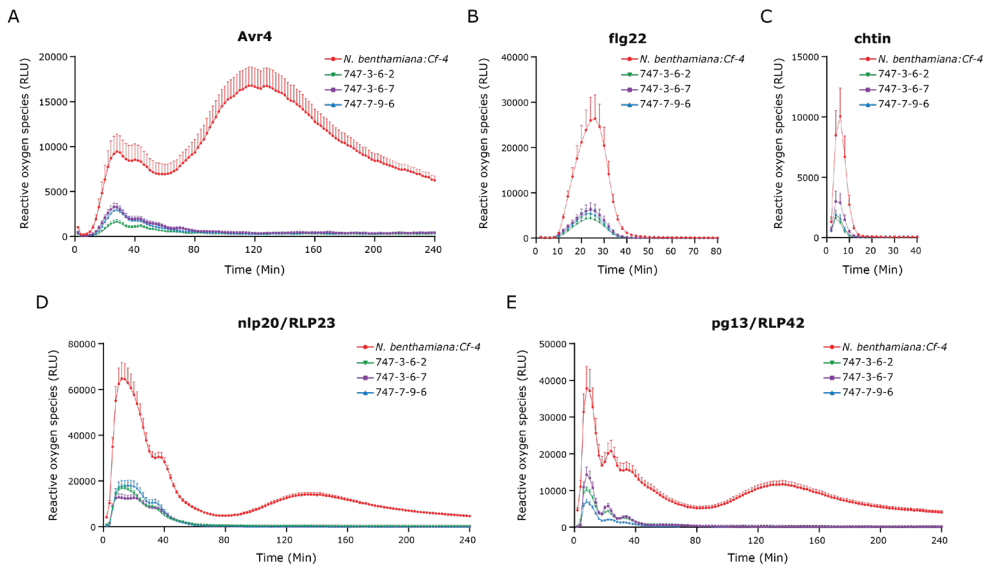


Figure 3. Members of the *N. benthamiana* RLCK-VII-6 subfamily are required for ROS production triggered by multiple ExIPs. Leaf discs obtained from three independent *rlck-vii-6* homozygous T2 mutant lines, as well as from *N. benthamiana*:Cf-4 (the positive control), were treated with a final concentration of 0.1 μ M pure Avr4 protein (A), a final concentration of 0.1 μ M flg22 (B), or a final concentration of 10 μ M chitohexaose (C), and ROS production was monitored. Leaf discs taken from the independent *N. benthamiana*:Cf-4 *rlck-vii-6* lines, as well as from *N. benthamiana*:Cf-4, transiently expressing either RLP23 (D) or RLP42 (E), were treated with the corresponding elicitors (at a final concentration of 1 μ M nlp20 and 1 μ M pg13, respectively), and the accumulation of ROS was again monitored. ROS production is expressed as RLUs and the data are represented as mean + SEM ($n \geq 6$). The ROS profiles of the positive control that was included in all the assays are indicated in red in all the line charts. All experiments were repeated at least three times and data from one representative experiment are shown.

***N. benthamiana* RLCK-VII-6 is dispensable for Avr4/Cf-4-triggered MAPK and HR activation**

In addition to ROS production, the activation of a downstream MAPK cascade is another key signaling event of ExTI, which generally can be detected within minutes upon ExIP recognition (Yu et al., 2017; Zhang et al., 2018). We observed that infiltration of the Avr4 protein in leaves of *N. benthamiana*:Cf-4 induces the activation of a MAPK cascade within five minutes (Figure S7). To explore whether this Avr4/Cf-4-triggered MAPK activation is affected in the *rlck-vii-6* mutant plants, we infiltrated pure Avr4 protein in leaves of the different knock-out mutants as well as in leaves of *N. benthamiana*:Cf-4 plants. The leaf samples were harvested at 15 min after Avr4 infiltration, followed by performing a western blotting assay revealing MAPK activation. WOUND-INDUCED PROTEIN KINASE (WIPK) and SA-INDUCED PROTEIN KINASE (SIPK) are two MAPKs that are activated as a result of phosphorylation in *N. benthamiana* (Sharma et al., 2003) and that can be detected by specific antibodies. Intriguingly, no obvious changes in MAPK activation in the three mutant lines were observed when compared to *N.*

benthamiana:Cf-4, suggesting that while the RLCK-VII-6 members are important for the ROS burst, these are not essential for the Avr4/Cf-4-triggered MAPK activation (Figure 4A).

Unlike some ExIPs such as flg22, elf18 or chitin that only induce weak ExTI responses in plants, Avr4 triggers a typical HR in leaves of *N. benthamiana*:Cf-4 and MM-Cf-4 tomato (Cai et al., 2001; Gabriëls et al., 2006; Gabriëls et al., 2007; de Wit, 2016). To examine whether members of RLCK-VII-6 contribute to the Cf-4/Avr4-triggered HR in *N. benthamiana*, we infiltrated either pure Avr4 protein or *Agrobacterium tumefaciens* harboring the binary constitutive Avr4 expression construct in leaves of *rlck-vii-6* mutant lines and of *N. benthamiana*:Cf-4 as a control. Neither the Avr4 protein infiltration nor the agro-infiltration revealed significant changes in the capacity to mount an Avr4/Cf-4-triggered HR of the knock-out plants when compared to *N. benthamiana*:Cf-4 (Figure 4B and 4C). Altogether, these results demonstrate that the members of RLCK-VII-6 play no, or only a minor role, in regulating the Avr4/Cf-4-triggered MAPK and HR activation.

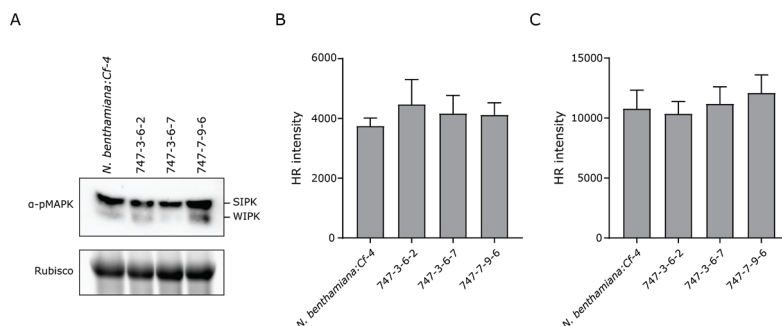


Figure 4. Members of *N. benthamiana* RLCK-VII-6 are dispensable for the Avr4/Cf-4-triggered MAPK and HR activation. (A) The three independent *rlck-vii-6* homozygous T2 mutant lines do not exhibit an altered Avr4/Cf-4-triggered MAPK activation when compared to *N. benthamiana*:Cf-4 plants. A solution of 5 μ M pure Avr4 protein was infiltrated in leaves of mutant plants, as well as in *N. benthamiana*:Cf-4 plants. Leaf samples were taken 15 min later and total protein extracts were run on SDS gel and subjected to immunoblotting employing a p42/p44-erk antibody specifically detecting MAPKs that are activated by phosphorylation (α -pMAPK). The intensity of the Rubisco band in the stain-free gel indicates equal loading. Experiments were repeated at least three times and similar results were obtained. Representative pictures are shown. (B and C) The intensity of the Avr4/Cf-4-triggered HR in leaves of the three independent *rlck-vii-6* mutant plants is similar when compared to *N. benthamiana*:Cf-4. (B) A solution of 5 μ M pure Avr4 protein was infiltrated in leaves of the different plants, which were subsequently imaged using the ChemiDoc, with the Red Fluorescent Protein (RFP) channel, at 2 days post infiltration (dpi). (C) Avr4 was transiently expressed in leaves of the different plants (at an OD₆₀₀ of 0.5), which were subsequently imaged using the ChemiDoc at 4 dpi. The intensity of the HR obtained upon either Avr4 protein infiltration or its transient expression was subsequently quantified by Image Lab. Statistical significance was determined by a one-way ANOVA test. Data are represented as mean + SEM. Experiments were repeated at least three times with similar results, and representative results are shown. Note that the HR intensity obtained upon transient expression of Avr4 is more than two-fold higher than upon infiltration of the Avr4 protein.

Transient complementation with individual RLCK-VII-6 members in *rlck-vii-6* mutant plants partially restores the Avr4/Cf-4-triggered ROS burst

In each independent *rlck-vii-6* mutant line, seven or eight RLCK-encoding genes were knocked out simultaneously (Figure 2B, S4, S5 and S6). To further pinpoint the members from RLCK-VII-6 that actually play a role in regulating ROS production upon elicitor challenge, we performed a complementation study by transiently overexpressing the individual genes that were knocked out in leaves of the different *N. benthamiana* *rlck-vii-6* homozygous T2 mutant plants, followed by monitoring whether the Avr4-triggered ROS burst was (partially) restored. Furthermore, the protein accumulation level of each candidate that was transiently expressed *in planta*, was also determined.

Apart from *Niben101Scf03619g00005* (#1, see Figure 2A for the numbering), for which we failed to clone the open reading frame (ORF) from cDNA of *N. benthamiana*, the ORFs of the remaining candidates were inserted into a binary overexpression vector providing a C-terminal GFP tag. Complementation with *Niben101Scf10055g01006* (#2) or *Niben101Scf02763g03011* (#7) did not restore ROS accumulation in the knock-out plants after Avr4 protein treatment, which might be because of their low protein accumulation levels and/or because of these RLCKs not having a role in the ROS burst (Figure 5). Transient overexpression of *GUS* was included as a negative control, and importantly, overexpressing *GUS* did not result in any background ROS accumulation, even though the accumulation level of the GUS protein was extremely high when compared to the accumulation levels of the various RLCKs (Figure 5). Interestingly, we observed that transient overexpression of *Niben101Scf03268g03002* (#3), *Niben101Scf00870g13015* (#4), *Niben101Scf06739g05004* (#5), *Niben101Scf02460g01004* (#6), or *Niben101Scf01445g02008* (#8), at least partially restored the ROS production triggered by Avr4/Cf-4 in the knock-out plants. The Avr4/Cf-4-triggered ROS burst observed upon complementation with *Niben101Scf03268g03002* (#3) or *Niben101Scf00870g13015* (#4) was more prolonged when compared to complementation with *Niben101Scf06739g05004* (#5), *Niben101Scf02460g01004* (#6), or *Niben101Scf01445g02008* (#8). In the latter case, the kinetics of the restored ROS burst was reminiscent of the first phase of the ROS burst triggered by Avr4 in *N. benthamiana*:Cf-4 plants. Notably, ROS accumulation was also detected without the addition of Avr4, except for *Niben101Scf02460g01004* (#6) (Figure S8). Nonetheless, the amplitude of the ROS burst was in all cases higher upon treatment with Avr4 protein, when compared to the water treatment (Figure 5 and S8). Constitutive ROS accumulation might be the result of RLCK overexpression, as, for example, the accumulation of *Niben101Scf02460g01004* (#6) was relatively low and this RLCK did not cause such a background ROS production.

Importantly, we observed that the capability of RLCK members to complement the Avr4/Cf-4-triggered ROS production in *rlck-vii-6* mutant lines was positively correlated

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with their accumulation level. Taken together, we conclude that members #3 to #6, #8 and probably also #2 and #7 from RLCK-VII-6, redundantly and positively regulate the Avr4/Cf-4-triggered ROS production in *N. benthamiana*.

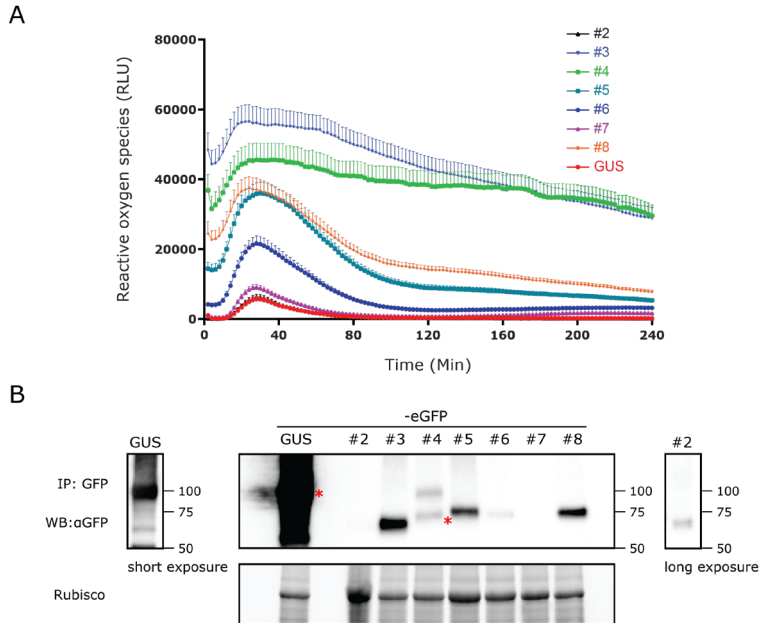


Figure 5. Avr4/Cf-4-induced ROS production in *rlck-vii-6* mutants is partially complemented by transient overexpression of individual RLCK genes belonging to this subfamily and that were knocked out in *N. benthamiana*:Cf-4. (A) The cDNAs of seven members from *N. benthamiana* class VII subfamily 6 were cloned and transiently overexpressed in leaves of *rlck-vii-6* mutant plants, in which all these RLCK members were knocked-out (at an OD₆₀₀ of 0.8), with GUS as a negative control. Discs taken from these leaves at 1 dpi, were treated with 0.1 μ M pure Avr4 protein (final concentration), followed by monitoring ROS production using a chemiluminescence assay. ROS production is expressed as RLUs and the data are represented as mean + SEM (n \geq 6). (B) Protein accumulation levels of the various RLCKs when overexpressed in *rlck-vii-6* mutant plants. Leaf samples were taken at 2 dpi, and total protein extracts were subjected to immuno-purification by using GFP-affinity beads. After SDS-PAGE, the immunopurified proteins were detected by immunoblotting using an α GFP antibody (upper panel), while the amount of total protein that was used for the IP is reflected by the Rubisco band (lower panel). The numbers indicated in (A) and (B) are corresponding to the numbers indicated in Figure 2A. IP, immunoprecipitation; WB, western blot. Representative results from three independent experiments are shown. Red stars indicate the expected sizes of GUS and RLCK #4. #2, Niben101Scf10055g01006; #3, Niben101Scf03268g03002, #4, Niben101Scf00870g13015, #5, Niben101Scf06739g05004, #6, Niben101Scf02460g01004, #7, Niben101Scf02763g03011, #8, Niben101Scf01445g02008.

The various RLCK-VII-6 members have a different subcellular localization

Typically, RLCKs are cytoplasmic proteins that for the greater part consist of a kinase domain, thereby functionally and physically associating with the kinase domain of RLKs (Lin et al., 2013; Liang and Zhou, 2018). Some RLCKs were described to anchor to the PM via a transmembrane helix or through the presence of N-terminal myristoylation and/or palmitoylation motifs (DeFalco and Zipfel, 2021). We therefore next asked what the subcellular localization of the different members of *N. benthamiana* RLCK-VII-6 is. To address this question, we conducted a confocal microscopy study of transiently expressed members of this subfamily in leaves of *N. benthamiana*. All members, fused to GFP at their C terminus, were co-expressed in leaves of *N. benthamiana* with *AUTOINHIBITED* CA^{2+} -ATP-ASE, *ISOFORM 8* (ACA8), fused to mCherry, which is a PM marker protein (Postma et al., 2016; van der Burgh et al., 2019). Probably due to the low levels of their accumulation, we did not observe green fluorescence signals for the Niben101Scf10055g01006 (#2), Niben101Scf00870g13015 (#4), and Niben101Scf02763g03011 (#7) proteins in the epidermal cells of the infiltrated leaves of *N. benthamiana* (Figure 5 and 6). Nevertheless, ACA8-mCherry, showing a red fluorescence signal, was in all cases detected at the PM (Figure 6).

Strikingly, green fluorescence for Niben101Scf03268g03002 (#3) was observed mainly at the chloroplasts, and possibly also at low levels at the PM. Even more interestingly, in addition to their localization at the PM, Niben101Scf06739g05004 (#5) and Niben101Scf01445g02008 (#8) appeared to also localize at the nucleus, as green fluorescence signals were detected in the nucleus only for these two RLCKs, and not for the other candidates. Furthermore, Niben101Scf02460g01004 (#6) was found to localize only at the PM (Figure 6). Collectively, although these RLCKs are all homologs belonging to the same subfamily, they appear to have different subcellular localizations, implying that they might play different roles in regulating plant immunity.

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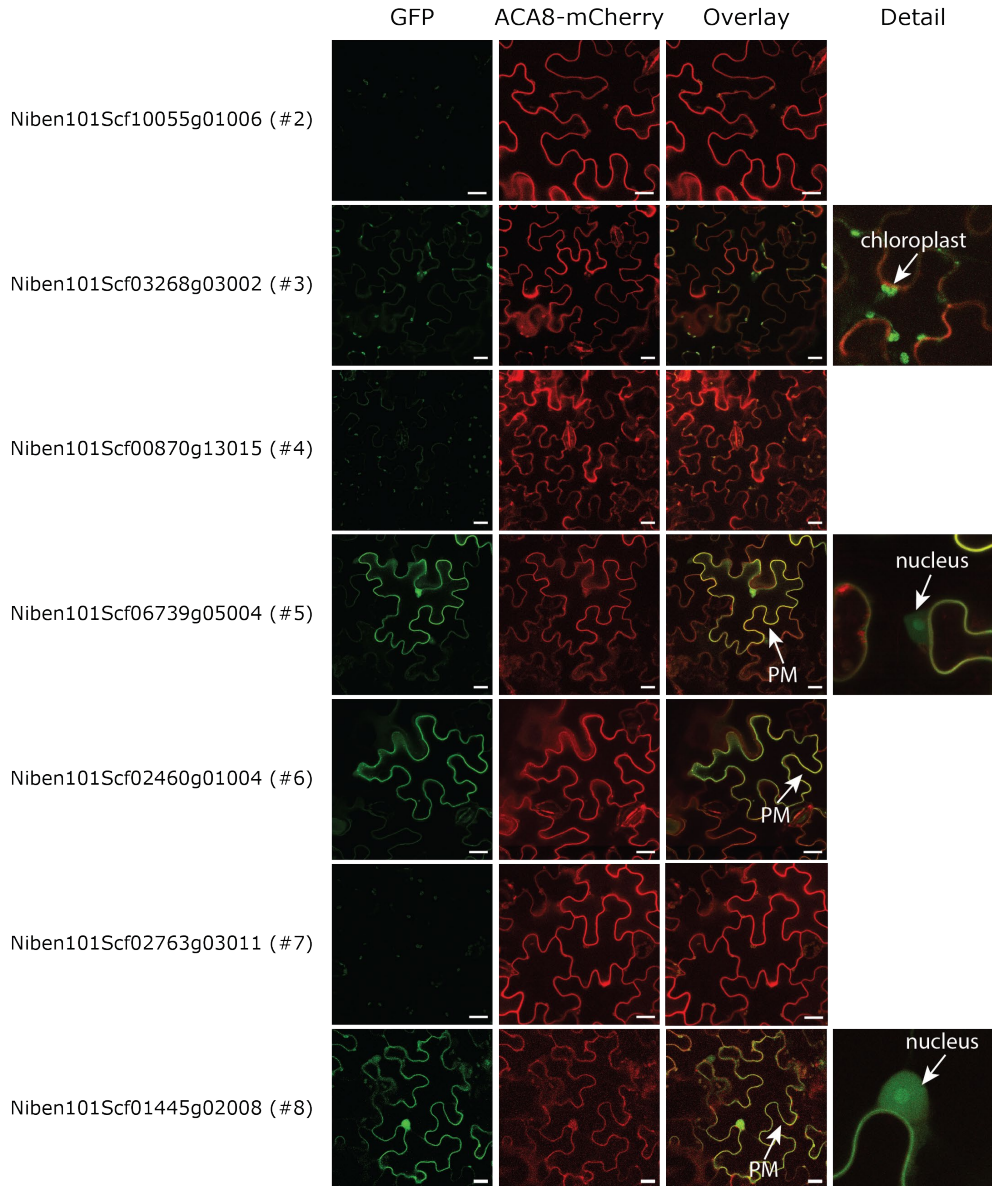


Figure 6. Subcellular localization of various members of RLCK-VII-6 from *N. benthamiana*. C-terminally GFP-tagged versions of the various members of RLCK-VII-6 from *N. benthamiana* were transiently co-expressed with the plasma membrane (PM) marker ACA8, fused to mCherry, in leaves of *N. benthamiana* (at an OD₆₀₀ of 0.3). Confocal microscopy images were taken at 2 dpi. From left to right, the first column of panels shows the green fluorescence, the second column shows the mCherry fluorescence at the PM, while the overlay images of the third column show a possible co-localization at the PM of the RLCK proteins fused to GFP and ACA8-mCherry, by the production of a yellow color. For some images, details are magnified in the fourth column. The experiment was performed two times and representative photographs are shown. Scale bars, 25 μ m.

rlck-vii-6* mutant plants do not exhibit altered susceptibility to *B. cinerea

Arabidopsis BIK1 is the best-studied RLCK, and this particular kinase has been reported to be required for basal resistance of *Arabidopsis* to *B. cinerea* (Veronese et al., 2006). All RLCKs characterized in this study are closely related to AtBIK1 (Figure S1B), and to determine a possible role of the members of RLCK-VII-6 in resistance of *N. benthamiana* to *B. cinerea*, leaves from the different *rlck-vii-6* mutant plants, as well as from *N. benthamiana*:Cf-4, were inoculated with a *B. cinerea* spore suspension, followed by evaluating the lesion development over a period of three days. Quantification revealed that the sizes of the lesions that developed on the leaves of the various mutant plants were similar to the size of those developed on *N. benthamiana*:Cf-4 plants, and no significant differences were observed (Figure 7). Therefore, it can be concluded that knocking out RLCK-VII-6 members in *N. benthamiana* does not affect the proliferation of *B. cinerea* on this plant, demonstrating that, unlike AtBIK1 itself, *N. benthamiana* AtBIK1 homologs belonging to RLCK-VII-6 are not essential for resistance against *B. cinerea*.

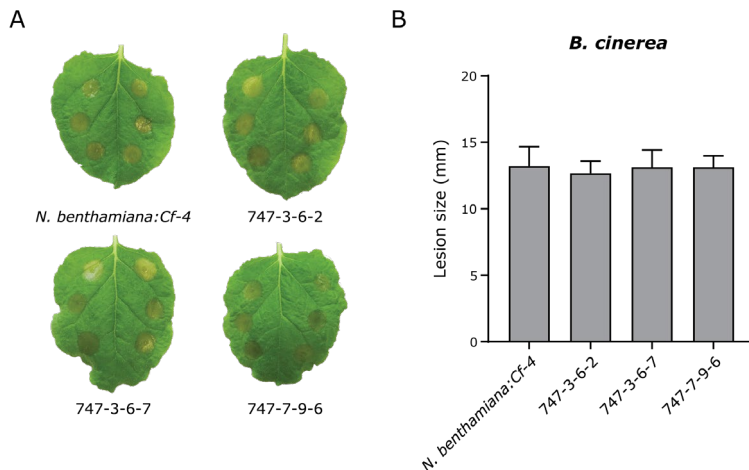


Figure 7. *N. benthamiana* *rlck-vii-6* mutant plants are not impaired in their resistance to *B. cinerea*. Leaves of the various *N. benthamiana*:Cf-4 *rlck-vii-6* mutant plants and *N. benthamiana*:Cf-4 (control) were droplet-inoculated with a spore suspension of *B. cinerea* strain B05.10. The lesions that developed, indicative of an infection, were photographed at 3 dpi (A), and the lesion sizes were measured (B). Bars represent mean lesion sizes + SEM. Experiments were repeated at least three times and similar results were obtained.

Discussion

Plants deploy numerous cell-surface receptors to sense the presence of ExIPs in the extracellular space (Zipfel, 2008; Dodds and Rathjen, 2010; Böhm et al., 2014; Zipfel, 2014; van der Burgh and Joosten, 2019; Kanyuka and Rudd, 2019). Activation of cell-surface receptor complexes upon ExIP perception activates a battery of key

downstream signaling modules, among which is the rapid phosphorylation of RLCKs (Yu et al., 2017; Wan et al., 2019; Albert et al., 2020). It is hypothesized that RLCKs act as the initial cytoplasmic transducers of the extracellular signal that is perceived by cell-surface receptors (DeFalco and Zipfel, 2021). The RLCK family in the plant kingdom is extremely large and highly diverse, thereby providing a potential mechanism to determine the specificity of many different plant immune outputs and developmental processes upon activation of cell surface receptors (Albert et al., 2020; Zhou and Zhang, 2020).

The LRR-RLP Cf-4 is one of the most studied cell-surface receptors (Thomas et al., 1997; Colwyn M. Thomas, 2000; van der Hoorn et al., 2000). Cf-4 constitutively associates with the LRR-RLK SOBIR1 and recruits the LRR-RLK BAK1 in an Avr4 ligand-dependent manner (Liebrand et al., 2013; Liebrand et al., 2014). To identify the RLCKs that function as a core hub to evoke immune signaling and to subsequently trigger defense responses downstream of the activated Avr4/Cf-4/SOBIR1/BAK1 complex, we implemented a highly efficient multiplex gene-editing system to simultaneously knock out multiple *RLCK* genes in *N. benthamiana*:Cf-4 plants. We have obtained three homozygous *rlck-vii-6* mutant lines, which all exhibit a strongly impaired Avr4/Cf-4-induced ROS burst, when compared to the original *N. benthamiana*:Cf-4 plants (Figure 2 and 3). Even though two knock-out lines, 747-3-6-2 and 747-3-6-7, display only a 3-bp deletion in *Niben101Scf03268g03002*, which suggests that this gene might not be knocked out, we did not observe obvious differences between these two lines and the 747-7-9-6 line, in which this particular gene is completely knocked out, in respect to the ROS burst induced by multiple ExIPs, MAPK activation, and the HR triggered by Avr4/Cf-4 (Figure 3 and 4). As the expression of this particular gene remains unchanged in *N. benthamiana*:Cf-4 upon treatment with Avr4 (Figure S2), it is likely that this particular RLCK plays no, or only a minor role, in the Avr4/Cf-4-triggered immune signaling pathway.

Intriguingly, *AtBIK1* has been reported to play opposite roles in LRR-RLK and LRR-RLP/SOBIR1 signaling, as *Arabidopsis bik1* mutants show compromised ROS accumulation when challenged with flg22 or elf18, but an enhanced ROS production upon challenge with nlp20 or PG3, which is perceived by the LRR-RLPs RLP23 and RLP42, respectively (Wan et al., 2019). Nonetheless, the ROS burst in the *N. benthamiana rlck-vii-6* knock-out plants, which is stimulated by multiple ExIPs in addition to Avr4, is in all cases strongly dampened (Figure 3). These observations demonstrate that, unlike *AtBIK1*, *N. benthamiana* RLCK-VII-6 does not appear to have a function downstream of LRR-RLPs that is opposite to the function of RLCK-VII-6 downstream of LRR-RLKs. Furthermore, our results are reminiscent of the role of the RLCK-VII-6 member PBL14 (RIPK) in *Arabidopsis* which is also required for broad-spectrum positive regulation of ROS production in plant immunity (Li et al., 2021). *Arabidopsis* PBL13 and PBL14 are both members of the RLCK-VII-6, but play opposite roles in regulating innate immunity (Figure S1B and 2) (Rao et al., 2018). PBL13 has

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been reported to act as a negative regulator, as *pbl13* mutants display increased ROS accumulation and MAPK activation in response to flg22, whereas in contrast, a recent study shows that PBL14 positively contributes to ROS production induced by multiple ExIPs and by intracellular immunogenic patterns (InIPs) (Lin et al., 2015; Lee et al., 2020; Li et al., 2021). However, the selected RLCK members from subfamily 6 in this study do not appear to play opposite regulatory roles, as our complementation studies with the individual RLCK-encoding genes reveal that they only show a positive role in regulating the Avr4/Cf-4-triggered ROS burst (Figure 5). In contrast to *N. benthamiana* *rlck-vii-6*, Arabidopsis *rlck-vii-6* mutant plants show significantly increased ROS accumulation triggered by flg22, either suggesting a different role of the various RLCKs in different plant species or indicating that each subfamily does not contain the identical functional RLCK homologs in different plant species (Rao et al., 2018).

Despite the importance of strictly regulating the ROS burst, RLCK-VII-6 does not show a detectable role in the Avr4/Cf-4-triggered downstream MAPK activation and in the mounting of the associated HR in *N. benthamiana* (Figure 4). Consistently, multiple Arabidopsis RLCKs, such as BIK1, PBL1, and PBL14 have also been shown to all play an essential role in ROS production upon elicitation, but not in MAPK activation (Lu et al., 2010; Zhang et al., 2010; Li et al., 2014; Li et al., 2021). Altogether, our data support the conclusion that *N. benthamiana* RLCK-VII-6 is specifically involved in regulating the ROS burst upon LRR-RLK and LRR-RLP activation. To further support this conclusion, it will be necessary to investigate the possible changes in MAPK activation in *N. benthamiana* *rlck-vii-6* mutant plants triggered by flg22 and by chitin.

We observed that the kinetics of the Avr4/Cf-4-induced ROS burst in *N. benthamiana* *rlck-vii-6* is different from that in *rlck-vii-7* and *rlck-vii-8* mutant lines. For the *rlck-vii-6* mutant lines, the second burst is specifically and completely inhibited, whereas, for the *rlck-vii-7* and *rlck-vii-8* mutant lines, the overall ROS production is strongly attenuated (Figure 1). This raises the possibility that there are different downstream ROS regulatory mechanisms in *N. benthamiana*, which together determine the ROS profile. In Arabidopsis, RBOHD is engaged in extracellular ROS production, and growing evidence has suggested that RLCKs differentially regulate RBOHD activation through the differential phosphorylation of various sites in the RBOHD enzyme (Kadota et al., 2019). For instance, BIK1 directly phosphorylates the N-terminus of RBOHD to positively regulate ROS production in Arabidopsis, whereas PBL13, which negatively regulates RBOHD activation, directly phosphorylates the C-terminus of RBOHD. In *N. benthamiana*, the RBOHB homolog is responsible for the fast apoplastic ROS production during the establishment of immunity, and therefore we speculate that members from RLCK-VII-6 and RLCK-VII-7/8 phosphorylate RBOHB at different sites, thereby causing different ROS kinetics. Furthermore, it has been reported that in *N. benthamiana* the first burst of the biphasic apoplastic ROS burst is mediated by swift RBOHB phosphorylation, whereas the second burst is the result of transcriptional upregulation of the *RBOHB* gene, a process that is mediated by activated WRKY transcription factors

RLCK-VII-6 positively regulates ROS in *N. benthamiana*

(Adachi et al., 2015). Hence, we hypothesize that members from RLCK-VII-6, upon their activation by the upstream cell-surface complex, phosphorylate RBOHB at specific sites for the swift ROS burst, and meanwhile directly or indirectly phosphorylating certain transcription factors to regulate the later phase of the ROS burst.

By performing complementation studies, we show that, aside from #2 and #7, transiently expressing individual RLCK members from subfamily 6, at least partially restores the Avr4/Cf-4-triggered ROS burst in the *N. benthamiana*:Cf-4 *rlck-vii-6* knock-out plants (Figure 5). However, due to their low protein accumulation levels, we cannot exclude the possibility that also #2 and #7 play a role in regulating the ROS burst. Hence, in general, these RLCKs function redundantly and they all positively regulate ROS production. To further verify this conclusion, kinase-dead variants of selected RLCKs should be included in the complementation studies in the future, as such variants are expected not to be able to complement the observed phenotypes. These observations are in line with the findings of a recent study, in which it was shown that in Arabidopsis knocking out both *PBL30* and *PBL31* simultaneously, instead of only the individual genes one by one, significantly reduces the ethylene production that is triggered by multiple ExIPs (Pruitt et al., 2021). Thus, there is indeed gene redundancy within each RLCK subfamily. Notably, our recent studies involving TurboID-based proximity-dependent labeling by biotinylation, in combination with mass spectrometry, have revealed that RLCK #5 and #6 are specifically biotinylated, when using the *NbSOBIR1*-TurboID fusion as a bait that is transiently expressed in leaves of *N. benthamiana* plants (Landeo Villanueva, unpublished data). Interestingly, these RLCKs cluster with Arabidopsis PBL13 and PBL14 and tomato ACIK1 in the phylogenetic tree (Figure 2A). Accordingly, previous split-luciferase assays have shown that Solyc06g062920 (the homolog of #4), Solyc05g025820 (the homolog of #5 and #6), and Solyc07g041940 (the homolog of #7 and #8), are likely to specifically interact with the kinase domain of *S*/SOBIR1 and *At*FLS2 (van der Burgh et al., 2018) (Figure S1B and 2A). These data collectively demonstrate that several RLCKs are interesting subjects for further studies. Research aiming to determine their location and dynamic interactions, as well as deciphering the phosphorylation events that take place between the various RLCKs and the activated Cf-4/SOBIR1/BAK1 complex, will be needed to determine their exact role in LRR-RLP-triggered immune signaling.

The tomato RLCK ACIK1 belongs to RLCK-VII-6 and was previously identified as an essential signaling component downstream of Cf-4 and Cf-9, as silencing of *ACIK1* compromised the Avr4/Cf-4- and Avr9/Cf-9-triggered HR in *N. benthamiana* and also suppressed Cf-9-mediated resistance of tomato against a strain of *C. fulvum* secreting Avr9 (Rowland et al., 2005). However, we failed to reproduce this result by knocking out several homologs of ACIK1 in *N. benthamiana* (Figure 2A), as in addition to a clearly suppressed ROS burst, we did not observe any changes in the Avr4/Cf-4-triggered MAPK activation and the intensity of the HR in the *rlck-vii-6* knock-out plants (Figure 4). A possible explanation for this observation could be that the previous

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studies were performed based on virus-induced gene silencing, and therefore the construct that was used might also knock down some other *RLCK* members that do play a role in the Avr4/Cf-4-induced HR.

A number of Arabidopsis *RLCK*-VII members, such as PBL27 and PBL34, have been shown to localize to the PM, which is proposed to be a prerequisite for their function in plant immunity (Yamaguchi et al., 2013; Lin et al., 2015; Li et al., 2021). For example, PBL27, which has been shown to bridge the events of extracellular chitin perception and downstream cytoplasmic MAPK activation, interacts with both the chitin co-receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) and MAPKKK5 on the PM (Shinya et al., 2014; Yamada et al., 2016). Furthermore, PBL34 is associated with the lectin domain-containing RLK LIPO-OLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) on the PM and plays an important role in LORE-mediated downstream immune responses (Luo et al., 2020). Additionally, some Arabidopsis *RLCK*-XII members have also been reported to localize to the PM, and some typical examples of these are BRASSINOSTEROID-SIGNALING KINASE 5 (BSK5), BSK7, and BSK8 (Majhi et al., 2019; Majhi et al., 2021). Consistently, we observed that the *N. benthamiana* *RLCK*-VII-6 members #3, #5, #6, and #8 all localize to the PM, which provides the possibility that they can indeed physically interact with SOBIR1, as SOBIR1 is also present at the PM (Figure 6) (Liebrand et al., 2013; Bi et al., 2016; Postma et al., 2016; van der Burgh et al., 2019). Moreover, we also observed green fluorescence signals of #3 from the chloroplasts (Figure 6). ROS can be produced in various parts and organelles of plant cells, including the PM and chloroplasts (Shapiguzov et al., 2012; Sierla et al., 2013; Waszczak et al., 2018) and the role of this particular *RLCK* in the chloroplasts remains to be studied. In addition to their localization at the PM, #5 and #8 are also likely to localize to the nucleus (Figure 6). These findings are in agreement with an earlier report, showing that AtBIK1 also localizes to the nucleus, in addition to its PM localization, and where it plays a direct role in regulating jasmonic acid (JA) signaling through trans-phosphorylating particular transcription factors (Lal et al., 2018). Therefore, it is possible that #5 and #8 also have a function inside the nucleus to directly regulate plant immune signaling, such as contributing to the activation of some WRKY transcription factors that mediate *RBOHB* transcriptional upregulation, resulting in the subsequent second burst of apoplastic ROS (Adachi et al., 2015). It will be interesting to determine the localization of these *RLCKs* upon activation of the Cf-4/SOBIR1 complex by Avr4 and to verify whether their localization is determined by particular amino acid motifs present in the various *RLCKs*, or by the signaling partners that they interact with.

Some Arabidopsis *RLCKs*, such as BIK1, BSK5, BSK7, and BSK8, have been previously demonstrated to play an important role in positively regulating host resistance to the necrotrophic fungal pathogen *B. cinerea* (Veronese et al., 2006; Majhi et al., 2019; Majhi et al., 2021). Accordingly, here, we also determined the susceptibility of *N. benthamiana* *rlck-vii-6* knock-out plants to *B. cinerea*, but no obvious differences

between the mutant plants and the control were detected (Figure 7). Hence, we conclude that members from *N. benthamiana* RLCK-VII-6 are not required for basal resistance to *B. cinerea*. Further work will be needed to examine whether other *N. benthamiana* RLCK (sub)families are required for this type of resistance to necrotrophic pathogens. Furthermore, we aim to determine whether knocking out various RLCKs in tomato will affect the gene-for-gene resistance to the biotrophic fungal pathogen *C. fulvum*.

Materials and Methods

Generation of the different *N. benthamiana* mutant lines

A highly efficient multiplex editing technique employed to knock out multiple RLCK-VII subfamily members in *N. benthamiana*:*Cf-4* has been described previously (Stuttman et al., 2021). To screen for homozygous transformants of *rlck-vii-6*, genomic DNA from each mutant plant was isolated by using the Phire Tissue Direct PCR Master Mix (Thermo Fisher Scientific), followed by amplifying the sgRNA-targeted regions and subsequent Sanger sequencing of the obtained PCR fragments. Primers used for genotyping can be found in Table S2.

Plant growth conditions

All *N. benthamiana* plants used in this study were cultivated in a climate chamber under 15 h of light at 21 °C and 9 h of darkness at 19 °C, with a relative humidity of ~70%.

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

The coding sequences of eight candidate genes from *N. benthamiana* RLCK-VII-6 were amplified by PCR with *N. benthamiana* cDNA as a template, and then cloned into the pENTR/D-Topo vector (Invitrogen). After sequence confirmation by Sanger sequencing, the coding regions were subsequently introduced into the pBIN-KS-35S::GWY-eGFP vector (SOL2095; for C-terminally tagging with eGFP), by using Gateway LR Clonase II (Invitrogen). The *C. fulvum* Avr4 effector was transiently expressed using the pMOG800 construct (van der Hoorn et al., 2000). Primers used for generating overexpression constructs are listed in Table S2.

A. *tumefaciens*-mediated transient transformation

All binary plasmids were transformed into *A. tumefaciens* (further referred to as *Agrobacterium*) strain C58C1, carrying the helper plasmid pCH32. *Agrobacterium* strains harboring the transient expression constructs of RLP23 and RLP42 were received from Lisha Zhang and Thorsten Nürnberger (Albert et al., 2015; Zhang et al., 2021). Infiltration of *Agrobacterium* into leaves of *N. benthamiana* (agro-infiltration) was performed as described before, at an OD₆₀₀ of 0.8 (van der Hoorn et al., 2000).

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For the HR assays, *Agrobacterium* harboring the Avr4 expression construct was infiltrated in leaves of 4-week-old *N. benthamiana*:*Cf-4* and *rlck-vii-6* mutant plants, at an OD₆₀₀ of 0.8. The development of an HR was imaged at 4 dpi by the ChemiDoc (Bio-Rad), with the Red Fluorescent Protein (RFP) channel (Filter: 605/50; Light: Green Epi Illumination; Exposure time: 2 seconds) (Landeo Villanueva et al., 2021). Hereafter, the intensity of the HR was quantified using ImageLab. Statistical analysis was performed using one-way ANOVA by GraphPad Prism 9.

For checking the protein accumulation level of each RLCK candidate after its transient expression in leaves of *N. benthamiana*, a protein immunoprecipitation assay, followed by immunoblotting, was performed as described before (Liebrand et al., 2012).

Reactive oxygen species (ROS) assay

For ROS burst assays, leaf discs were taken from 4-week-old *N. benthamiana*:*Cf-4* and *rlck* mutant plants, while for plants transiently expressing RLCK members for the complementation studies, leaf discs were collected at 24 h after ago-infiltration. Leaf discs were then floated on 80 μ L of sterile water in a 96-wells plate overnight and hereafter, the water in each well was replaced carefully by 50 μ L of fresh sterile water. After another 1 h of incubation, 50 μ L of the reaction solution, containing 100 μ M of luminol (L-012, Fujifilm, Japan), 20 μ g/mL horseradish peroxidase (Sigma), and the elicitor to be tested (being 0.2 μ M Avr4 protein, 0.2 μ M flg22, 20 μ M chitohexaose, 2 μ M nlp20 or 2 μ M pg13), was added to each well. Subsequently, the production of luminescence was monitored with a CLARIOstar plate reader (BMG Labtech). The line charts showing the detected values of ROS were created using GraphPad Prism 9.

MAPK activation assay

N. benthamiana leaf samples, infiltrated with a solution of 5 μ M Avr4 protein, were harvested at different time points after treatment and total protein was extracted and subjected to SDS-PAGE, after which an anti-p42/p44-erk antibody (NEB) was employed to detect the activated MAPKs on western blots.

Confocal microscopy and image analysis

To visualize the subcellular localization of different members from RLCK class VII subfamily 6, each RLCK coding sequence, C-terminally tagged with GFP, was transiently co-expressed with ACA8-mCherry in *N. benthamiana* leaves, at an OD₆₀₀ of 0.3. Leaf samples were collected at 2 dpi and processed by confocal microscopy analysis. Firstly, a small piece was sliced from the leaf sample and treated with perfluorodecalin, which is an infiltrative imaging medium and enables high-resolution laser scanning confocal imaging. The abaxial side of the infiltrated leaf piece was examined by a Zeiss LSM 510 META confocal laser scanning microscope (CLSM). Images were acquired either with a 40x objective for overviews or a 63x objective for detailed pictures. A 488 nm argon laser was employed to capture the GFP fluorophore, of which the emission was detected through a 505-530 nm band-pass filter. On the

other hand, a 543 nm helium-neon laser was used to excite the fluorophore of mCherry, of which the emission was detected through a 560-615 nm band-pass filter. Images were further analyzed and processed with Zen (blue edition) software (Zeiss).

***B. cinerea* inoculation assay**

A *B. cinerea* inoculation assay was performed as described previously (Zhang and van Kan, 2013). In brief, 2 μ L of a conidial suspension of strain B05.10 (10^6 conidia/mL in potato dextrose broth) was droplet-inoculated onto leaves of *N. benthamiana*. Lesion sizes were determined at 3 dpi and obtained data were further analyzed statistically using one-way ANOVA by GraphPad Prism 9.

Phylogenetic analysis of the RLCKs from Arabidopsis, *N. benthamiana*, and tomato

To conduct a phylogenetic analysis of the AtBIK1 homologs in *N. benthamiana*, tomato, and Arabidopsis, their predicted proteomes were obtained from www.solgenomics.net and www.arabidopsis.org. Hereafter, the three predicted proteomes were independently queried for Pfam domains by using HMMER (v3.1b2; gathering cut-off) (Eddy, 1998). Sequences that contain annotated Pfam domains aside from cytoplasmic kinases (PF00069 or PF07714) were removed, and the sequences of the annotated kinase domains were extracted. Then, we took the domain PF07714 as a lead and removed the sequences that deviated in length from the kinase domain of AtBIK1. The remaining 1,455 kinase domain sequences were aligned using MAFFT (v7.271) (Katoh and Standley, 2013), the alignment was subsequently trimmed using ClipKIT (v1.3.0; smart-gap) (Steenwyk et al., 2020) and a neighbor-joining phylogenetic tree was built using QuickTree (1000) (Howe et al., 2002) (Figure S1A). Next, a well-supported (>92% bootstrap support) sub-clade of putative BIK1 homologs, which comprised 123 sequences including AtBIK1, was extracted from this guide tree. Subsequently, a refined phylogenetic tree was generated with these sequences by using the maximum-likelihood (ML) phylogeny as implemented in IQ-Tree (v2.2.0) (Nguyen et al., 2015). The 123 extracted kinase domains were re-aligned using MAFFT and trimmed as described above, and the ML phylogeny was constructed in IQ-Tree, using automatic amino acid substitution model selection (optimal model: Q.plant with five categories of rate heterogeneity) (Kalyaanamoorthy et al., 2017; Minh et al., 2021). Branch support for the phylogenetic tree was obtained using ultrafast bootstrap, as well as SH-aLRT implemented in IQ-tree (Hoang et al., 2018). Protein motifs in the complete amino acid sequences of the various RLCKs were identified using the MEME software suite (v5.4.1; mode = any number of repetitions, number of motifs 20, minimum and maximum length 10 and 200, respectively) (Bailey et al., 2009).

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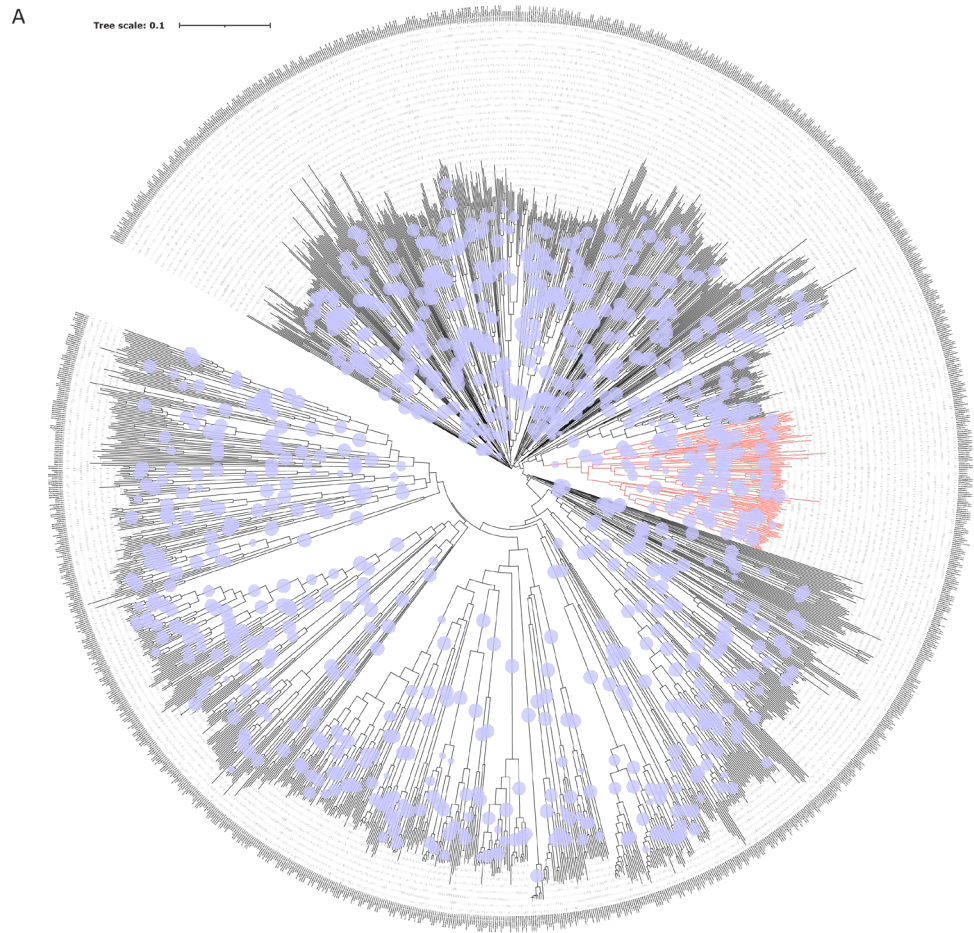
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Supplementary Data



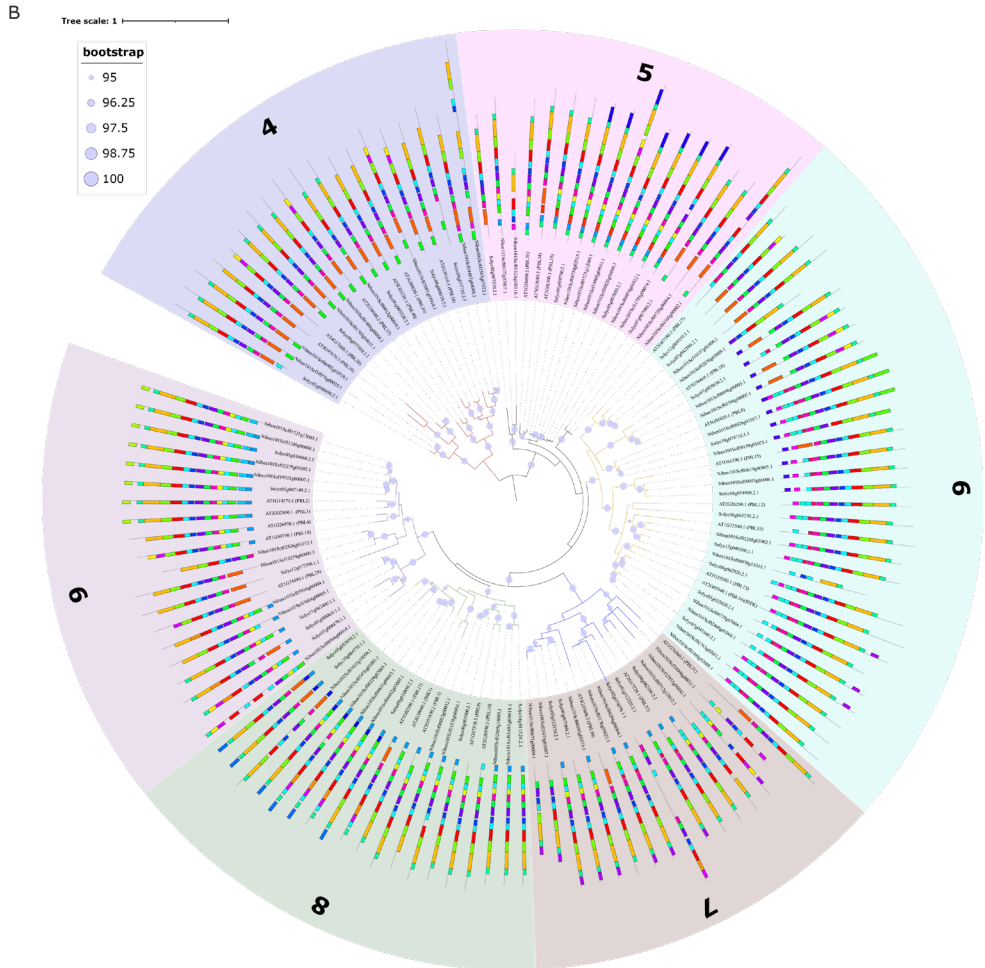


Figure S1. Phylogenetic analysis of BIK1 homologs from Arabidopsis, tomato, and *N. benthamiana*. (A) The amino acid sequences of only the kinase domain were extracted from all RLCK members from Arabidopsis, tomato, and *N. benthamiana* and aligned to subsequently generate a neighbor-joining phylogenetic tree using QuickTree (Howe et al., 2002). The sub-clade of putative BIK1 homologs, which comprised 123 sequences including AtBIK1 (bootstrap support higher than 92%) is shown in red. (B) Phylogenetic analysis of the RLCK-VII subfamily members from Arabidopsis, tomato, and *N. benthamiana*. Amino acid motifs identified in the complete protein sequence using MEME are shown (Bailey et al., 2009). All the members present in this tree were further assigned to 6 subfamilies, which are depicted in different colors. These subfamilies are referred to as subfamily 4, 5, 6, 7, 8, and 9, according to the RLCK-VII subfamilies in Arabidopsis reported previously by Rao et al. (2018).

RLCK-VII-6 positively regulates ROS in *N. benthamiana*

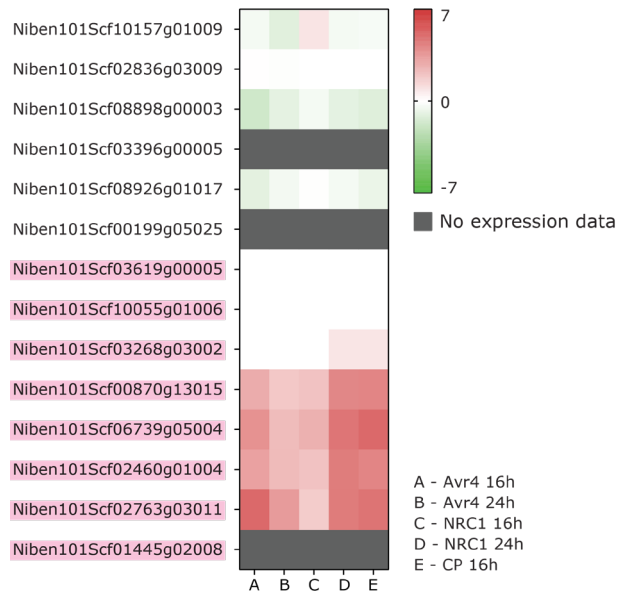


Figure S2. Gene expression of the 14 members from *N. benthamiana* RLCK-VII-6. Heat map of the relative expression (log2) of all 14 RLCK members of subfamily 6, as determined upon transient expression of *Avr4*, constitutively active *NRC1* (Gabriëls et al., 2007), or *CP* of potato virus X (Tameling et al., 2010), in leaves of both *Cf-4*- and *Rx*-transgenic *N. benthamiana* plants. The genes that were selected to be knocked out are highlighted in pink. NRC1, NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 1; CP, COAT PROTEIN.

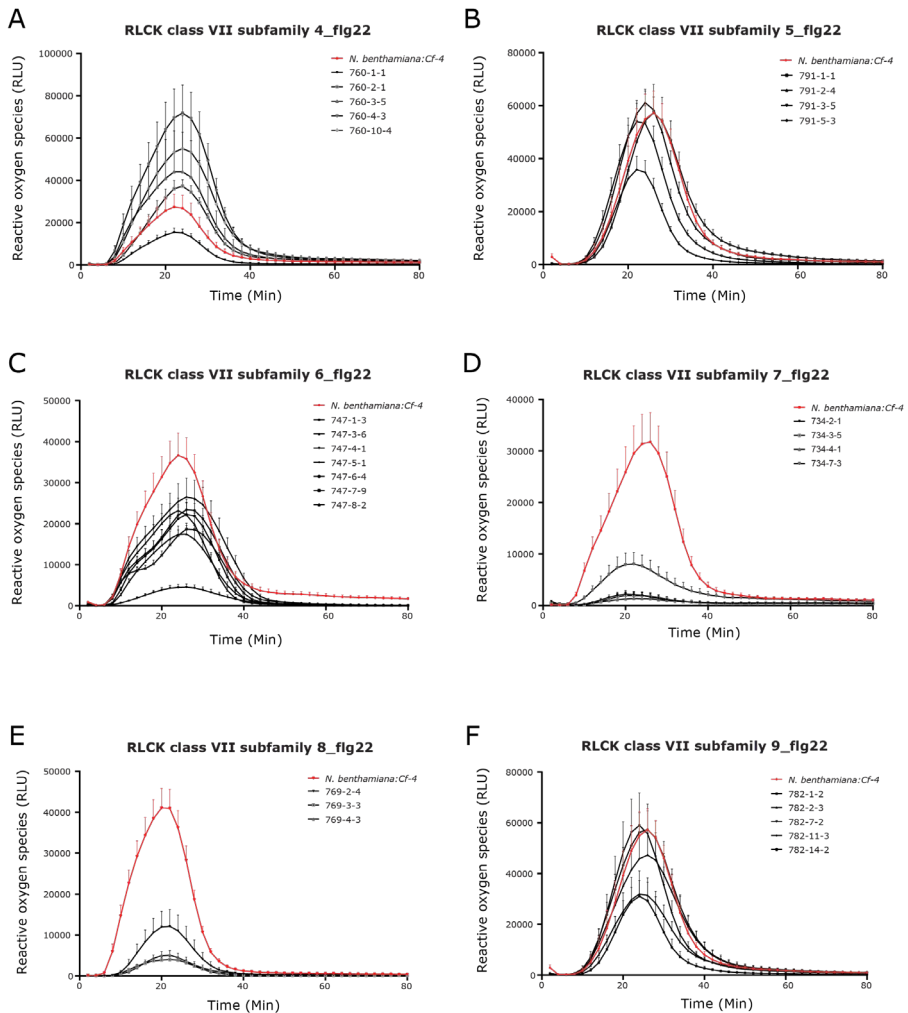


Figure S3. RLCK-VII-6, -7 and -8 from *N. benthamiana* also play a positive role in the flg22/FLS2-triggered ROS burst. ROS production, triggered upon treatment with flg22, by discs taken from leaves of *rlck-vii-4* (A), *rlck-vii-5* (B), *rlck-vii-6* (C), *rlck-vii-7* (D), *rlck-vii-8* (E) and *rlck-vii-9* (F) *N. benthamiana*:*Cf-4* mutant plants from the T1 generation, was measured. For this, leaf discs were taken from the different mutant plants, as well as from *N. benthamiana*:*Cf-4* (the positive control), followed by treatment with a final concentration of 0.1 μ M flg22 peptide and subsequent monitoring of the accumulation of ROS. ROS production is expressed as relative light units (RLUs) and the data are represented as mean plus the standard error of the mean (SEM) ($n \geq 6$). The ROS traces of the positive control are indicated in red in all the line charts. All experiments were repeated at least three times and data from one representative experiment are shown.

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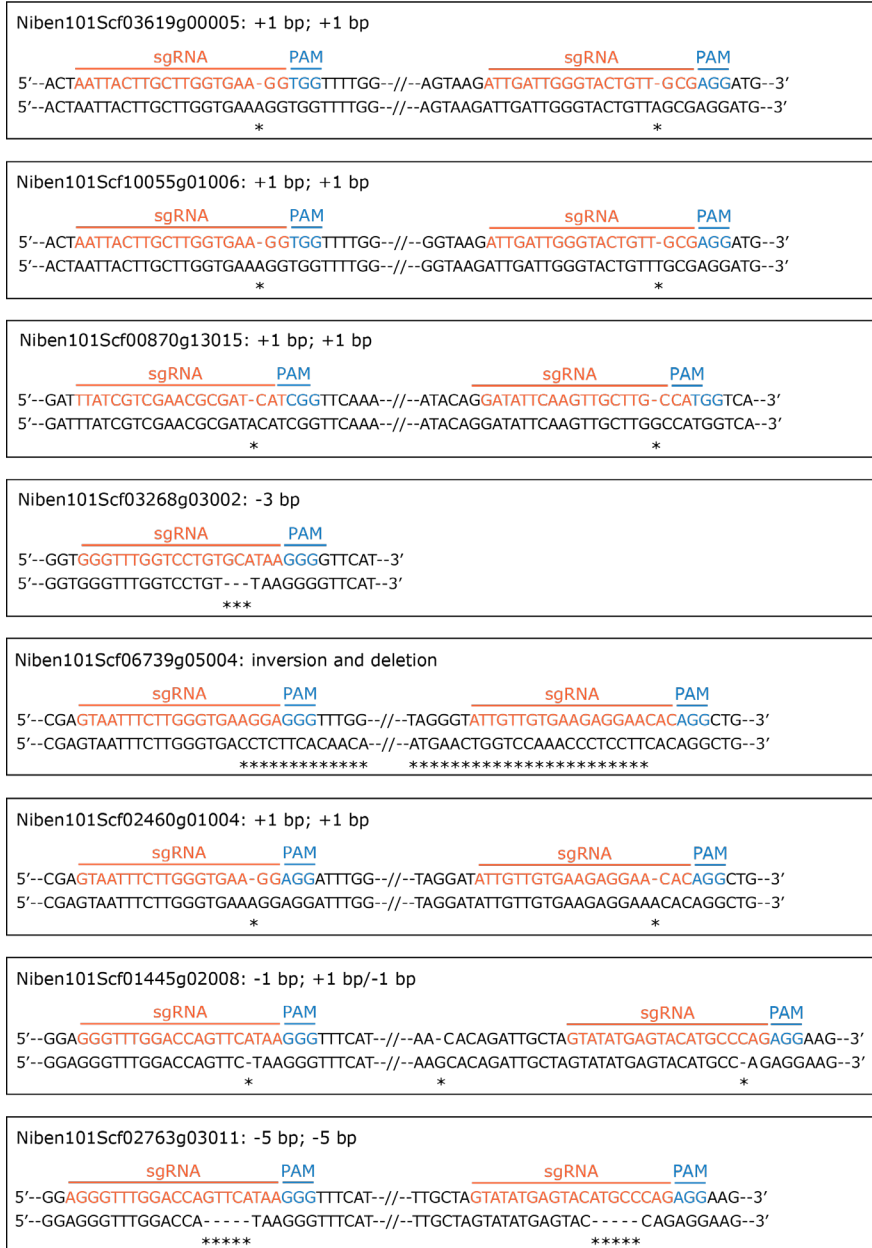


Figure S5. The genotype of T2 *rlck-vii-6* mutant line 747-3-6-7. In *rlck-vii-6* mutant line 747-3-6-7, which was among the offspring of the T1 line 747/1-6 generated by Stuttmann et al. (2021), the gene regions targeted by various sgRNAs were amplified by PCR and the obtained fragments were subjected to Sanger sequencing. Hereafter, the nucleotide sequences that were obtained were aligned with the wild-type sequence, which in each panel is shown as the upper strand. The sgRNA sequences are shown in orange, and the protospacer-adjacent motifs (PAMs) are shown in blue. The type of mutations and the numbers of deleted or inserted nucleotides are indicated after the gene name.

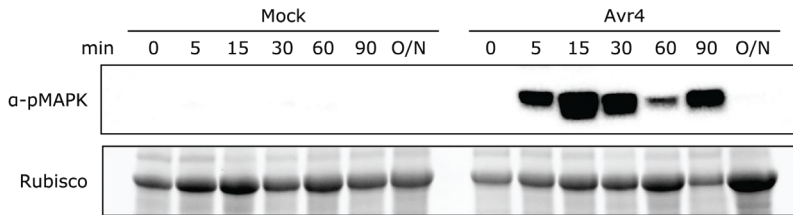


Figure S7. The Avr4 protein triggers a swift MAPK activation in *N. benthamiana*:Cf-4. Water (mock) or 5 μ M of the pure Avr4 protein was infiltrated in leaves of *N. benthamiana*:Cf-4. Leaf samples were taken at the indicated time points after Avr4 infiltration, and total protein extracts were subjected to immunoblotting using a p42/p44-erk antibody specifically detecting MAPKs that are activated by phosphorylation (α -pMAPK). Rubisco is shown as a total protein loading control. O/N, overnight.

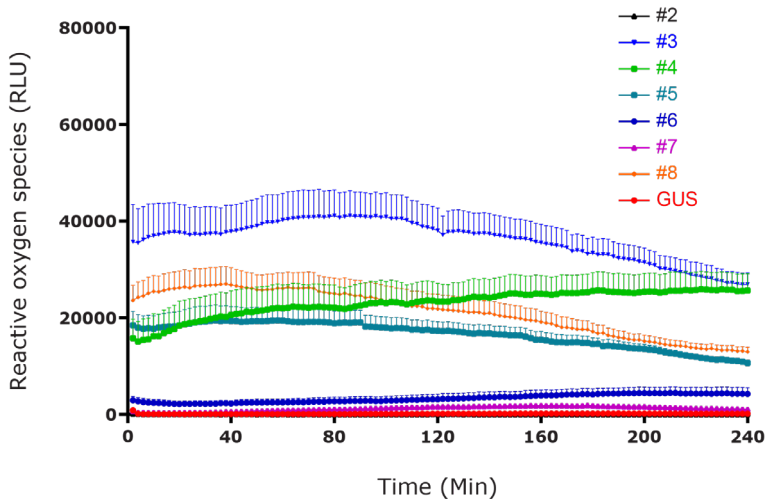


Figure S8. Transient overexpression of several individual RLCK genes from *N. benthamiana* RLCK-VII-6 leads to constitutive ROS accumulation in *rlck-vii-6* mutants, in which these RLCK genes were knocked out. The cDNAs of seven members from *N. benthamiana* RLCK-VII-6 were cloned and transiently overexpressed in leaves of *N. benthamiana*:Cf-4 *rlck-vii-6* homozygous T2 mutant plants in which all these RLCK members were knocked-out (at an OD₆₀₀ of 0.8), with GUS as a negative control. Discs taken from these leaves at 1 dpi, were treated with water, followed by monitoring ROS production using a chemiluminescence assay. ROS production is expressed as RLUs and the data are represented as mean + SEM (n \geq 6). #2, Niben101Scf10055g01006; #3, Niben101Scf03268g03002, #4, Niben101Scf00870g13015, #5, Niben101Scf06739g05004, #6, Niben101Scf02460g01004, #7, Niben101Scf02763g03011, #8, Niben101Scf01445g02008.

RLCK-VII-6 positively regulates ROS in *N. benthamiana*

Table S1. RLCK genes selected to be targeted by sgRNAs.

Subfamily	Gene name	Number of sgRNAs	Sequence of sgRNAs (5' - 3')	Note
4	<i>Niben101Scf10970g00025</i>	2	TTACTTAAGATTGGAGAAGGAGG; ATGGGGAAAGAGGTATTCAGCGG	
	<i>Niben101Scf06902g02019</i>			
	<i>Niben101Scf01745g04011</i>	3	ATTAAGAAGCTCAATACACTTGG; GGAATGGCTTATTTCACGACGG	Not present in the phylogeny, but clustered to this subfamily according to iTAK
	<i>Niben101Scf01489g00004</i>			
	<i>Niben101Scf01071g03021</i>			
	<i>Niben101Scf00012g00010</i>	2	GTAGTGCCAGCGTTGATACGCGG; GTGCGGAGGATGATGAAAGGGGG	
	<i>Niben101Scf03565g07022</i>	2	GGATCGGCTAAAGTTCTGTGTGG; GCTCATAGGTTATTGCGCACAGG	
	<i>Niben101Scf04487g04008</i>			
<i>Niben101Scf05081g01018</i>		GTTCTACGTTGGCGACAAGAAGG; GATACCAGCACAGAGTCCAGAGG		
5	<i>Niben101Scf06172g03007</i>	2	GCTTTGGCCCTGTTTACAAAGGG; CTAATTTAACCAAATCTGATGG	Not expressed according to QUT
	<i>Niben101Scf01326g08016</i>			
	<i>Niben101Scf06750g02015</i>	2	CCAGAATCCCTTCTTGGTGAAGG; ACCGCATCTAGCAGAATGTTGG	
	<i>Niben101Scf01521g12006</i>			
	<i>Niben101Scf08874g01012</i>			
	<i>Niben101Scf11795g00014</i>	2	TGGAGTCTTGTGAATCAAAGGGG; GTTACTTCCGCATTACTTGTGG	
	<i>Niben101Scf14805g04011</i>			
	<i>Niben101Scf10820g01005</i>	2	AAGAGCTAAAAGCTGCAACGGGG; GTTTATGAGTTCATGACCCGTGG	
	<i>Niben101Scf05720g00004</i>			
<i>Niben101Scf03100g00002</i>				
6	<i>Niben101Scf10157g01009</i>			Not selected, see Figure S2
	<i>Niben101Scf02836g03009</i>			Not selected, see Figure S2
	<i>Niben101Scf08898g00003</i>			Not selected, see Figure S2
	<i>Niben101Scf03396g00005</i>			Not selected, see Figure S2
	<i>Niben101Scf08926g01017</i>			Not selected, see Figure S2
	<i>Niben101Scf00199g05025</i>			Not selected, see Figure S2
	<i>Niben101Scf03619g00005</i>	2	AATTACTTGCTTGGTGAAGGTGG; ATTGATTGGGTACTGTTGCGAGG	
	<i>Niben101Scf10055g01006</i>			
	<i>Niben101Scf03268g03002</i>	1	GGGTTTGGTCCTGTGCATAAGGG	
	<i>Niben101Scf00870g13015</i>	2	TTATCGTCGAACGCGATCATCGG; GATATTCAAGTTGCTTGCCATGG	
	<i>Niben101Scf06739g05004</i>	2		

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	<i>Niben101Scf02460g01004</i>		GTAATTTCTGGGTGAAGGAGG; ATTGTTGTGAAGAGGAACACAGG	
	<i>Niben101Scf02763g03011</i>	2	GGGTTTGGACCAGTTTCATAAGGG; GTATATGAGTACATGCCAGAGG	
	<i>Niben101Scf01445g02008</i>			
	<i>Niben101Scf14996g00011</i>			Not expressed according to QUT
	<i>Niben101Scf12935g00042</i>			Not expressed according to QUT
	<i>Niben101Scf00712g13012</i>	2	GAGCTAGGAAGATTATCAACAGG; CCTAGTGGAGATGGAGGAGTGG	
	<i>Niben101Scf04294g06004</i>	2	AAGACTTCTCATCCTAACCTGG; CTTGCTTCTCCGATGCATGTAGG	
7	<i>Niben101Scf01176g01025</i>			
	<i>Niben101Scf00585g02015</i>	2	CGCTCGCGGTAGCTGAAAAACGG; CTTGACACAAAACGACCTAGTGG	
	<i>Niben101Scf03673g03007</i>	2	ATTATGCAGAAGGGGAAGCTTGG; AGCCGCAAGTCCCAAGAAAGTGG	
	<i>Niben101Scf00671g00009</i>			
	<i>Niben101Scf02413g03005</i>	1	CTTGACACAAAACGACCTAGTGG	Not present in the phylogeny, but clustered to this subfamily according to iTAK
	<i>Niben101Scf03493g00018</i>	2	ATGAACTCCGAGTAGCCACGAGG; GCTATAACCAAACAGTTCCCGG	
	<i>Niben101Scf02869g18003</i>			
	<i>Niben101Scf01378g00004</i>	2	ATCGGGACGAAAATTCCTAGTGG; GATAGCAATCCAAGCTCGAGGG	
	<i>Niben101Scf00012g00012</i>			
8	<i>Niben101Scf06482g03003</i>	3	GTCAGGACGGAAGTTTCTAGTGG; GTAGTAGGAGAAGGAGGTTTTGG	
	<i>Niben101Scf00635g04002</i>			
	<i>Niben101Scf00229g07003</i>			
	<i>Niben101Scf05476g01001</i>	2	TTAAACCAAGAAGGGTGGCAGGG; CTCCTACAACATATCAGGACGG	
	<i>Niben101Scf01025g10006</i>			
	<i>Niben101Scf09004g00018</i>	2	GTTTATGAGGTCATGACAAGAGG; GCCCATAGTACCTATAACCCCTGG	
	<i>Niben101Scf11684g00005</i>	2	TTAAGCCAGAAAGCTTTCAGGGG; GTATATGACCAAAGGAAGCTTGG	
	<i>Niben101Scf05916g00004</i>			
9	<i>Niben101Scf10274g00001</i>	2	TTATGGCCCTGCAAACCTCGAGG; GTATATGAATTTATGCCAAGAGG	
	<i>Niben101Scf02826g01012</i>			
	<i>Niben101Scf19533g00007</i>	2	GTCAAGAAATTGAAGCCGGAAGG; GTGTATGAGTTCATGCCTAAAGG	
	<i>Niben101Scf02279g05002</i>			
	<i>Niben101Scf11389g00006</i>	3	GGTTCGTGGGAATCCTAGAGG; GCAACTTCGTATCCAAACCTGG; GTGTATGAGTTTCATGCCTAAAGG	
	<i>Niben101Scf01521g13003</i>			

iTAK (<http://itak.feilab.net/>); QUT (<http://www.benthgenome.qut.edu.au/>). The genes that are not expressed according to the QUT genome browser were not chosen to be targeted by CRISPR/Cas9; while some genes that are not present in the phylogenetic tree (Figure S1B) but were found to be clustered in a specific subfamily by using iTAK, were selected to be knocked out.

RLCK-VII-6 positively regulates ROS in *N. benthamiana*

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

Primer code	Primer name	Sequence (5' - 3')
Primers for genotyping the <i>rlck-vii-6</i> mutant plants		
ho228	747_geno_1-F_JS2419*	CCAAAACCAAAGGCAAAGGAG
ho229	747_geno_1-R1004_JS2420	GATTAGCCAGGTAGATAGTGG
ho230	747_geno_1-R5004_JS2421	GACTGAAGTTCCAAAAGCC
ho231	747_geno_2-F_JS2422	GTGATGAAGATTGCATGGG
ho232	747_geno_2-R2008_JS2423	GTTTACGAGTTATTGCACGAT
ho233	747_geno_2-R3011_JS2424	CACCAACTCCCAAAAGCC
ho234	747_geno_3-F3002_JS2425	GTGGTCATTCAAGGCTAGC
ho235	747_geno_3-R3002_JS2426	TTTTCTTAAACTCCGTGCCG
ho236	747_geno_4-F13015_JS2427	TCATCATCATAGTAGGCTAGCC
ho237	747_geno_4-R13015_JS2428	TAGTCCTTTTGCAGCACCTA
ho238	747_geno_5-F_JS2429	CGTAGGTTGTCTTCTCGGA
ho239	747_geno_5-R1006_JS2430	TGTAACCTCTGGGGTACGT
ho240	747_geno_5-R0005_JS2431	TGCGTACCATCAAAACAACC
ho241	747_R1-13015_JS2486	TGTTTTCTCTTACACACCCC
ho242	747_F2-13015_JS2487	TGAATACATGGCAAGGGGAA
Primers for generating <i>RLCK</i> overexpression constructs (Gateway sites for directional cloning are underlined)		
ho249	13015_fw	<u>cacc</u> ATGGTGGCTTCCAAAATTG
ho250	13015_(nostop) rev	CTTAAGTATATGTTTTTATTGTTC
ho251	3002_fw	<u>cacc</u> ATGGCTTCAAGATTACCTTGAAAG
ho252	3002_(nostop) rev	CTTGTTTTCTCTCTTTTCTTCAC
ho253	2008/3011_fw	<u>cacc</u> ATGACAGTGATGAAGATTGCATG
ho254	2008_(nostop) rev	AACTCTCTTAAACCAATTGTGCAATG
ho255	3011_(nostop) rev	ATCTCTCTTAAACCAATTGTGCAATG
ho256	1004/5004_fw	<u>cacc</u> ATGAAGATTACATGGGAATCTCTAGTTCC
ho257	1004_(nostop) rev	AGCTCTATGGAAATCATTGTGCAATG
ho258	5004_(nostop) rev	AGCTCTATGGAAATTATTGCGCAATG
ho259	0005_fw	<u>cacc</u> ATGAAAGACTCAAAATCTTGAGACCC
ho260	0005_(nostop) rev	CTTGCTCTTACTATGAACAGCCATACG
ho261	1006_fw	<u>cacc</u> ATGAAAGACCCAAAACCTTGAGAC
ho262	1006_(nostop) rev	CTTGCCCTTACTATGAACAGCCATAC

* fw, forward; rev, reverse.

Chapter 6

General Discussion



Introduction

To monitor the diverse biotic threats in the surrounding environment, plants have developed a two-layered innate immune system; the first layer is mediated by cell-surface receptors, while the second layer is mediated by intracellular receptors (Dangl and Jones, 2001; Jones and Dangl, 2006; Dodds and Rathjen, 2010; van der Burgh and Joosten, 2019). Cell-surface receptors are either receptor-like proteins (RLPs) or receptor-like kinases (RLKs) (Jones and Dangl, 2006; Zipfel, 2008; Monaghan and Zipfel, 2012). RLKs contain a ligand-binding ectodomain (ECD), a single-pass transmembrane domain, and a cytoplasmic kinase domain. Despite lacking the intracellular domain, RLPs share a similar overall structure with RLKs (Monaghan and Zipfel, 2012; Böhm et al., 2014; Macho and Zipfel, 2014). Plants deploy RLKs and RLPs to perceive 'danger signals' in the extracellular space, termed extracellular immunogenic patterns (ExIPs), leading to extracellularly-triggered immunity (ExTI) (Zipfel, 2014; Gust et al., 2017; van der Burgh and Joosten, 2019; DeFalco and Zipfel, 2021). ExIPs can be conserved microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), and extracellular effector proteins. Of which, well-studied MAMPs are the N-terminal 22-amino acid epitope of bacterial flagellin (flg22), the conserved 18-amino acid peptide of bacterial elongation factor Tu (elf18), and fungal chitin (Li et al., 2020; DeFalco and Zipfel, 2021; Lee et al., 2021).

Typically, to overcome ExTI and facilitate invasion, host-adapted pathogens deliver effector proteins, referred to as intracellular immunogenic patterns (InIPs), into plant cells (Jones and Dangl, 2006; Dodds and Rathjen, 2010; Varden et al., 2017; van der Burgh and Joosten, 2019). Plants, in their turn, have evolved intracellular nucleotide-binding, leucine-rich repeat (NB-LRR) receptors (NLRs) to specifically recognize these InIPs, resulting in intracellularly-triggered immunity (InTI) (Cui et al., 2015; Varden et al., 2017; van der Burgh and Joosten, 2019; Lolfe et al., 2020). Plant NLRs have a modular domain architecture, harboring a variable N-terminal domain that functions in signaling, a conserved central nucleotide-binding oligomerization domain (NOD) that binds ADP in the resting state, and ATP in the active state, and a C-terminal LRR domain that functions in effector recognition (Song et al., 2020; Wang and Chai, 2020). Broadly, based on their N-terminus, plant NLRs can be divided into three major groups, the coiled-coil (CC)-type NLRs (CNLs), the Toll/interleukin-1 receptor-like (TIR)-type NLRs (TNLs), and the resistance to powdery mildew 8-like domain (RPW8)-type NLRs (RNLs) (Jones et al., 2016; Jubic et al., 2019).

Interestingly, both with ExTI and InTI a series of downstream signaling outputs is activated, including an influx of extracellular calcium, the production of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase (MAPK) cascades, and Ca^{2+} -dependent protein kinases (CDPKs), callose deposition, a global transcriptional reprogramming, and in some cases, the hypersensitive response (HR)

which is a form of programmed cell death (PCD) (Peng et al., 2018; Zhou and Zhang, 2020; Lu and Tsuda, 2021; Yuan et al., 2021).

RLP signaling is fundamentally different from RLK signaling

In general, both RLKs and receptor-like cytoplasmic kinases (RLCKs) are classified as serine/threonine kinases, but RLCKs lack an ectodomain for ligand perception (Shiu and Bleecker, 2001; Lin et al., 2013; DeFalco and Zipfel, 2021). Plants have evolved numerous RLCKs that functionally and physically interact with RLKs to regulate plant immunity (Liang and Zhou, 2018). Accordingly, activation of ExTI triggers a suite of downstream key signaling modules, including the rapid phosphorylation of RLCKs (Zhou and Zhang, 2020; DeFalco and Zipfel, 2021).

The best-characterized RLCK of the model plant *Arabidopsis thaliana*, *At*) is BOTRYTIS-INDUCED KINASE 1 (BIK1) from the RLCK class VII subfamily 8 (RLCK-VII-8) (Rao et al., 2018; DeFalco and Zipfel, 2021). BIK1 is a positive regulator of the defense response initiated by RLKs, such as FLAGELLIN-SENSING 2 (FLS2), ELONGATION FACTOR-TU RECEPTOR (EFR), and LYSIN MOTIF RECEPTOR KINASE 5 (LYK5), as their corresponding ExIPs flg22-, elf18-, and chitin-stimulated ROS accumulation and callose deposition are strongly dampened in *Arabidopsis bik1* mutants (Lu et al., 2010; Zhang et al., 2010). RLP23, an *Arabidopsis* leucine-rich repeat (LRR)-RLP, perceives necrosis and ethylene-inducing peptide 1-like proteins (NLPs and their derived peptide, nlp20) from various bacteria, fungi, and oomycetes and requires common regulators SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1) and BRI-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1) for its signaling (Albert et al., 2015). Unexpectedly, BIK1 appeared to play a negative role in LRR-RLP signaling, as *Arabidopsis bik1* knock-out mutants exhibit increased nlp20/RLP23-triggered ROS burst and ethylene, camalexin, and salicylic acid (SA) production (Wan et al., 2019). Similar results were obtained for the combination of the LRR-RLP RLP42 and the fungal polygalacturonase PG3 (Wan et al., 2019). Previously, the RLP/SOBIR1 complex was postulated to be structurally and functionally identical to a genuine RLK. However, surprisingly, the aforementioned observations unveil unexpected differences between RLK- and RLP/SOBIR1-mediated signaling (Gust and Felix, 2014; Liebrand et al., 2014; Wan et al., 2019).

In agreement with these findings, a recent study has reported that *Arabidopsis* AvrPphB SUSCEPTIBLE1-LIKE 30 (PBL30) and PBL31, belonging to RLCK-VII-7, play an essential positive regulatory role in LRR-RLP/SOBIR1-associated immune responses, but a less important role in the LRR-RLK-triggered signaling pathway (Pruitt et al., 2021). More interestingly, the authors further determined that RLP23 requires the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/PHYTOALEXIN-DEFICIENT 4 (PAD4)/ACTIVATED DISEASE RESISTANCE 1 (ADR1) node for its signaling. EDS1 is a well-known signaling component of InTI and plays a pivotal role in basal immunity of

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not only the model plant *Arabidopsis* but also of important crop plants (Parker et al., 1996; Aarts et al., 1998; Hu et al., 2005). *Arabidopsis* EDS1 physically associates with two signaling partners, PAD4 and SENESCENCE-ASSOCIATED GENE 101 (SAG101) to form distinct protein complexes, of which the EDS1/PAD4 complex requires the helper NLR ADR1 to mediate basal immunity, whereas EDS1/SAG101 form a dimer with another helper NLR, N REQUIREMENT GENE 1 (NRG1), to promote InTI and host resistance (Wagner et al., 2013; Wu et al., 2019; Dongus and Parker, 2021). The production of ROS and ethylene, in addition to callose deposition triggered by nlp20/RLP23, but not by flg22/FLS2, is strongly dampened in *Arabidopsis pad4* and *eds1* mutants, indicating that the EDS1/PAD4/ADR1 node plays an essential role in LRR-RLP signaling, but a less important role in LRR-RLK signaling (Pruitt et al., 2021).

Taken together, emerging evidence has pinpointed that the molecular mechanism of LRR-RLP/SOBIR1-triggered ExTI is fundamentally different from that of LRR-RLK-mediated ExTI, as there is a differential requirement of RLCKs and NLRs downstream of these receptors. Nonetheless, the evolutionary mechanisms behind these observations remain to be addressed.

Crosstalk between immune signaling pathways initiated by cell-surface receptors

Crosstalk between various cell-surface receptor-triggered immune signaling pathways has recently been recognized as a common mechanism to modulate plant immunity (Zhou and Zhang, 2020; Kong et al., 2021; Ngou et al., 2021). In *Arabidopsis*, LYK5, which is the primary receptor of fungal chitin, recruits the co-receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) to activate plant immune responses upon the perception of chitin. Of note, both LYK5 and CERK1 contain lysin motif (LysM) domains in their ECDs. On the other hand, the *Arabidopsis* LRR-RLK FLS2 forms a heterodimer with the LRR-RLK BAK1 in response to the binding of bacterial flagellin and/or flg22, leading to the activation of downstream intracellular signaling (Gómez-Gómez and Boller, 2000; Gómez-Gómez et al., 2001; Chinchilla et al., 2007; Cao et al., 2014; Erwig et al., 2017). Intriguingly, recent work has shown that in *Arabidopsis* the activation of FLS2 by bacterial pathogens or flg22 induces the phosphorylation of CERK1 and that this phosphorylation is mediated by the common regulator BAK1. Different from the chitin-triggered phosphorylation of CERK1, which is throughout its cytoplasmic kinase domain, flg22 only triggers the phosphorylation of CERK1 in its cytoplasmic juxtamembrane region, resulting in an intermediate phosphorylation level. Interestingly, the phosphorylation of CERK1 by BAK1 under flg22 elicitation turns CERK1 into a primed state, which allows the host to be prepared for future fungal attacks (Figure 1) (Gong et al., 2019).

Moreover, the *Arabidopsis* LRR-RLK NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE1 (NIK1), which is an essential regulator of plant antiviral immunity, physically interacts with both FLS2 and BAK1, and these interactions are enhanced upon flg22

elicitation (Machado et al., 2015; Zorzatto et al., 2015). Activation of FLS2 by flg22 induces the phosphorylation of NIK1 at T474 by BAK1, which further facilitates mounting host resistance to viruses in a NIK1-dependent manner (Figure 1) (Li et al., 2019). Interestingly, NIK1 plays a negative role in the complex assembly of FLS2/BAK1, as well as in the subsequent activation of antibacterial immune responses. Therefore, there is an inhibitory interplay between the NIK1-mediated antiviral signaling pathway and the FLS2/BAK1-mediated antibacterial signaling pathway. The mechanism by which NIK1 inversely regulates different pathways remains to be explored.

FLS2 is an extensively studied LRR-RLK. In addition to its presence in Arabidopsis, functional orthologs of FLS2 have been identified in many other plant species, including *Nicotiana benthamiana* (Nb), tomato (*Solanum lycopersicum*, Sl), rice (*Oryza sativum*, Os) and grapevine (*Vitis vinifera*) (Hann and Rathjen, 2007; Robatzek et al., 2007; Ryota Takai et al., 2008; Trda et al., 2014). In tomato, an additional flagellin receptor from the LRR-RLK family, referred to as FLS3, has been characterized to perceive a second epitope of flagellin, named flgII-28. This perception is S/FLS2-independent and enhances host resistance to bacterial pathogens (Hind et al., 2016). Strikingly, the RLK WALL-ASSOCIATED KINASE 1 (WAK1), which perceives plant cell wall pectin or its break-down products, has recently been reported to form a complex with S/FLS2/S/FLS3 in an flg22/flgII-28- and S/BAK1-independent manner, and S/WAK1 was found to play an important positive regulatory role in flg22- and flgII-28-triggered immune responses (Zhang et al., 2020).

Cf-4 is a well-studied LRR-RLP of tomato, which specifically recognizes the matching effector Avr4 from the pathogenic fungus *Cladosporium fulvum* (Joosten et al., 1994; Thomas et al., 1997). Being an RLP, Cf-4 lacks an intracellular kinase domain and thus constitutively interacts with the LRR-RLK SOBIR1, thereby forming a ligand-independent bimolecular RLK (Gao et al., 2009; Liebrand et al., 2013; Gust and Felix, 2014). Consistent with FLS2 signaling, the Cf-4/SOBIR1 complex recruits BAK1 upon detection of Avr4 by Cf-4 (Postma et al., 2016). Recently, we have shown that flg22 triggers an unexpected biphasic ROS burst in *N. benthamiana*:Cf-4 *sobir1/sobir1-like* mutant plants, unlike the normally rapid and monophasic ROS induced by flg22, but similar to the ROS burst induced by Avr4 in *N. benthamiana*:Cf-4 (Huang et al., 2021) (Chapter 2). Notably, FLS2 neither interacts with SOBIR1 nor requires SOBIR1 for its functionality. Further studies on identifying possible differential interactors of FLS2 in *N. benthamiana*:Cf-4 and in the *N. benthamiana*:Cf-4 *sobir1/sobir1-like* mutant plants by TurboID-based proximity labeling coupled with mass spectrometry (MS) should shed light on how the potential crosstalk occurs between FLS2/BAK1- and RLP/SOBIR1/BAK1-mediated signaling pathways.

Collectively, there is diverse potential crosstalk between different cell-surface receptors. Further investigation of how plants utilize groups of RLPs and RLKs simultaneously to modulate and transduce immune signals from the extracellular space into the plant cell, will lead to a more complete understanding of the plant immune system.

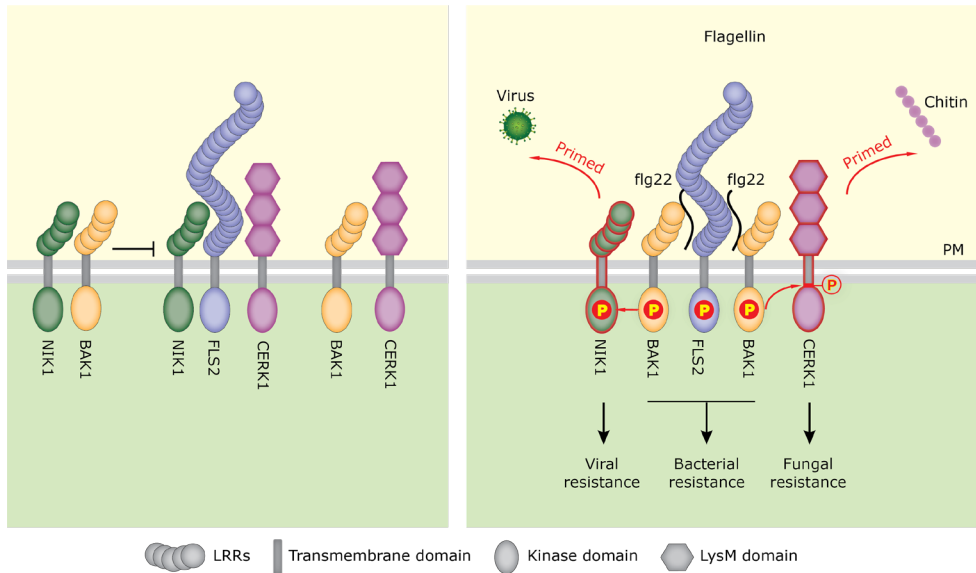


Figure 1. Examples of crosstalk between different cell-surface receptors in Arabidopsis. In the resting state (left panel), the chitin co-receptor CERK1 physically interacts with both FLS2 and BAK1. Likewise, NIK1, which is an essential regulator in plant antiviral immunity, also associates with both FLS2 and BAK1, and acts as a negative regulator by sequestering BAK1 from FLS2. In response to the binding of flg22 (right panel), BAK1 is recruited by FLS2, leading to the rapid phosphorylation of the kinase domains of BAK1 and FLS2. Subsequently, plant antibacterial immunity mediated by the FLS2/BAK1 complex is initiated. Meanwhile, activated BAK1 phosphorylates CERK1 only in the intracellular juxtamembrane region, and this phosphorylation primes the host for potential fungal attacks. On the other hand, the association of NIK1 with FLS2/BAK1 is enhanced by flg22 treatment. Activated BAK1 phosphorylates NIK1 at its kinase domain, which enhances host resistance to viruses in a NIK1-dependent manner. The red open and filled circles with 'P' inside represent a low-level and a high-level of phosphorylation, respectively. Receptors with red boundaries are activated in such a way that they prime the plant. PM, plasma membrane.

Mutual potentiation of plant immunity by cell-surface and intracellular receptors

Although cell-surface receptors and NLRs are activated through distinct mechanisms, ExTI and InTI eventually converge into several common downstream outputs, including MAPK activation, ROS production, calcium influx, and the accumulation of pathogenesis-related (PR) proteins (Tsuda and Katagiri, 2010; 2018; Lu and Tsuda, 2021; Yuan et al., 2021). Perception of ExIPs by cell-surface receptors triggers a transient MAPK activation within several minutes, while recognition of InIPs by NLRs leads to a slower, but more sustained, MAPK activation (Tsuda et al., 2013; Yu et al., 2017). Moreover, ROS is a hallmark of the very early response following the activation of ExTI, whereas, on the other hand, ROS is also crucial for InTI. Quantitative phosphoproteomics analysis in *Arabidopsis* has unveiled that serine (Ser/S) 343 and S347 of RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), which is the key enzyme catalyzing the production of apoplastic ROS, are common phosphorylation sites required for both ExTI- and InTI-associated ROS burst, indicating that there is an intricate interplay between ExTI and InTI, and that RBOHD might be one of the integration points of these immune responses (Torres et al., 2002; Kadota et al., 2019).

Notably, a recent study based on flg22/FLS2-triggered ExTI and AvrRps4/RRS1/RPS4-triggered InTI has revealed that the activation of InTI enhances ExTI-related immune responses, as pre-activation of InTI^{AvrRps4} promotes the flg22-triggered ROS burst and callose deposition, as well as the expression of several immune-responsive genes. Meanwhile, InTI also requires ExTI for its function, as multiple cell-surface receptor loss-of-function mutants show a higher susceptibility to *P. syringae* pv *tomato* (*Pst*) DC3000 carrying AvrRps4. ExTI, in turn, also potentiates InTI-associated HR. Consequently, the mutual potentiation of plant immunity triggered by cell-surface receptors and NLRs, eventually leads to stronger immune responses against pathogens (Ngou et al., 2021). Interestingly, this conclusion is reinforced by a back-to-back published study, which shows that in *Arabidopsis* the InTI responses that are induced by avirulent strains of *P. syringae* are markedly impaired in *fls2 efr cerk1* and *bak1 bkk1 cerk1* triple knock-out mutants, in which ExTI is suppressed. Furthermore, the RLCK BIK1 plays a crucial role in the full activation of RBOHD, suggesting that BIK1 could be another integration point of ExTI and InTI (Yuan et al., 2021).

Earlier work from our group has unveiled that the Cf-4/SOBIR1-initiated ExTI also requires some NLRs for downstream signaling. Different from some described ExTI responses that activate a basal and weak immunity, Avr4/Cf-4 signaling is associated with a swift ROS burst and a strong HR (van der Burgh et al., 2019). Importantly, silencing of *EDS1* or *NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 1* (*NRC1*) in transgenic *N. benthamiana* expressing Cf-4 compromises Avr4-induced HR (Gabriëls et al., 2006; Gabriëls et al., 2007). Consistently, *NRC1*-silenced tomato plants also exhibit a reduced Cf-4/Avr4-mediated HR and show a compromised

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resistance to *C. fulvum* (Gabriëls et al., 2007). Later, another tomato LRR-RLP, Ve1, which confers resistance to the fungal pathogen *Verticillium dahliae* and also relies on SOBIR1 for its function, has also been reported to require EDS1 and NRC1, in addition to the NLR NON-RACE-SPECIFIC DISEASE RESISTANCE (NDR1), for its immune signaling (Fradin et al., 2009; Liebrand et al., 2013). Surprisingly, the Cf-4/Avr4-triggered HR is not impaired in *N. benthamiana* *eds1* or *pad4* single mutants, or in an *eds1 pad4* double mutant (unpublished data) (Gantner et al., 2019). These results lead to the suggestion that maybe, unlike for LRR-RLP signaling in Arabidopsis, the EDS1/PAD4/ADR1 node does not play a role, or only plays a minor role, in LRR-RLP signaling in Solanaceae. Additionally, NRC1 was reported to be missing in the recently updated *N. benthamiana* genome sequence, and NRC3, instead of NRC1, appeared to be required for the HR in *N. benthamiana* triggered by Cf-4/Avr4 (Figure 2) (Wu et al., 2016; Kourelis et al., 2021). Notably, NRCs do not exist in the model plant Arabidopsis but have been identified in kiwifruit (*Actinidia deliciosa*), coffee (*Coffea arabica*), monkey flower (*Erythranthe guttata*), ash tree (*Fraxinus excelsior*), and some Solanaceous species, including *N. benthamiana*, tomato, pepper (*Capsicum annuum*), and potato (*Solanum tuberosum*) (Wu et al., 2017).

A recent study has reported that in Arabidopsis the HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) resistosome, which is formed by the CNL ZAR1, RESISTANCE-RELATED KINASE 1 (RKS1), and the RLCK PBL2, upon bacterial infection, functions as a calcium-permeable cation channel in the plasma membrane to trigger immune signaling (Wang et al., 2019; Wang et al., 2019; Bi et al., 2021; Ngou et al., 2021). Calcium influx is one of the fast regulatory responses and contributes to the activation of plant immunity (Zhou and Zhang, 2020; Yuan et al., 2021). ZAR1 carries a so-called MADA motif in its N-terminal CC domain, and intriguingly, this motif is conserved in the NRCs and is required for NRC4 auto-activity, which leads to an HR in *N. benthamiana* (Adachi et al., 2019). In response to a *Phytophthora infestans* infection, non-activated NRC4 accumulates at the extrahaustorial membrane (EHM), which is the site where this oomycete delivers its InIPs. Upon its activation, NRC4 oligomerizes into resistosomes that target the EHM, while some of the resistosomes get released from the EHM to target the plasma membrane (Duggan et al., 2021). This raises the possibility that, similar to ZAR1, NRCs form a cation channel to allow Ca^{2+} to enter the cytoplasm, thereby triggering PCD. Transient expression of tomato *NRC1* (*SINRC1*) does not complement the Cf-4/Avr4-induced cell death in *N. benthamiana* *nrc2/3/4* triple mutant line, and therefore it should be verified whether complementation with *S/NRC3* will restore the Cf-4/Avr4-mediated HR in this mutant line. In addition, further studies are needed to investigate whether knocking out *NRC3* in Cf-4-containing tomato suppresses the Avr4-triggered HR and compromises resistance against strains of *C. fulvum* that secrete Avr4.

Altogether, the results of the aforementioned studies indicate that the immune pathways activated by cell-surface receptors and intracellular NLRs show mutual

potentiation and convergence, which is required for full activation of plant innate immunity and the mounting of a robust resistance response to pathogens (Ngou et al., 2021; Yuan et al., 2021; Yuan et al., 2021).

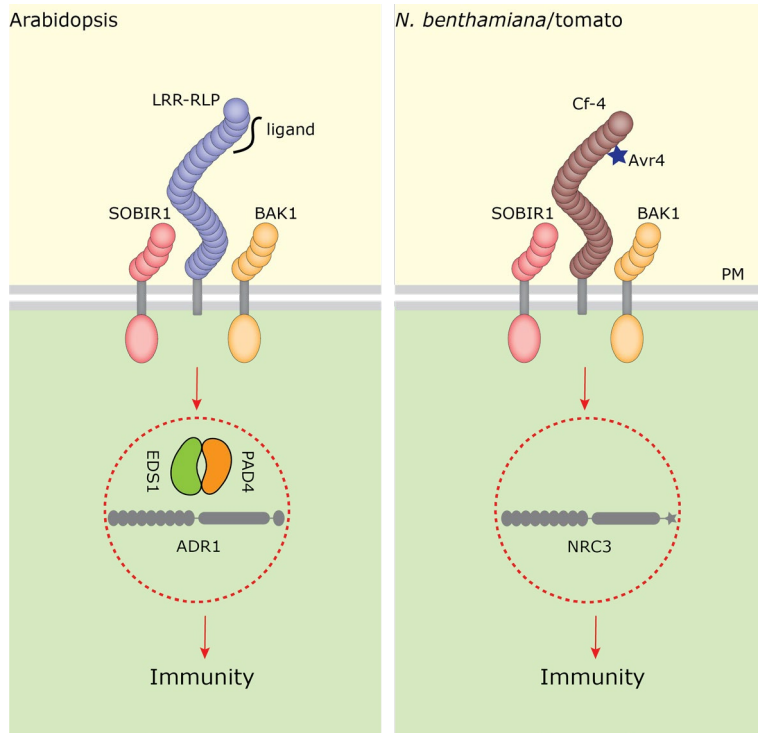


Figure 2. ExTI mediated by LRR-RLPs might require different components of the InTI signaling pathway in Arabidopsis when compared to the Solanaceous plants *N. benthamiana* and tomato. In Arabidopsis (left panel), LRR-RLPs, such as RLP23, constitutively associate with the LRR-RLK SOBIR1 in the resting state and recruit another LRR-RLK, BAK1, upon ligand perception. Downstream signaling requires the lipase-like proteins EDS1 and PAD4, and the helper NLR ADR1, which are important signaling components of InTI. It should be noted that NRCs are absent in Arabidopsis. In *N. benthamiana* (and possibly also in other Solanaceous plants like tomato) (right panel), the helper NLR NRC3 appears to play an essential role in downstream signaling mediated by the LRR-RLP Cf-4. Consistent with the requirements of RLP23 for the initiation of downstream signaling, Cf-4 also requires SOBIR1 and BAK1 for its function. PM, plasma membrane.

Regulation of plant immunity by phosphorylation of signaling proteins

Plant cell-surface receptors, as well as their associated regulators and signaling partners, are subjected to tight regulation. Protein phosphorylation is crucial for the activation of receptor complexes and the subsequent transduction of the immune signaling to eventually mount resistance. Unlike their mammalian counterparts, among

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which are numerous receptor tyrosine (Tyr/Y) kinases that typically mediate Tyr phosphorylation upon ligand perception, plants do not have such specific Tyr kinases but do contain Ser/threonine (Thr/T) kinases. Nevertheless, accumulating evidence has shown that plant protein kinases do not only phosphorylate at Ser and Thr residues but also at Tyr residues (Afzal et al., 2008; de la Fuente van Bentem and Hirt, 2009; Mithoe and Menke, 2018).

Auto-phosphorylation of RLKs

Plant RLKs are classified into RD and non-RD kinases, based on whether an arginine (Arg/R) residue precedes the catalytic aspartate (Asp/D) residue in the catalytic segment (Johnson et al., 1996; Macho and Zipfel, 2014). Most of the plant RLKs are non-RD kinases, which generally lack strong auto-phosphorylation activities (Dardick et al., 2012). A good example is the Arabidopsis RLK FLS2, which has been reported to exhibit no, or only a weak auto-phosphorylation activity, at such a level that it fails to phosphorylate the RLCK BIK1 (Lu et al., 2010). In contrast, RD kinases, such as CERK1 and BAK1, are activated by phosphorylation on one or more residues in their activation segment and show strong auto-phosphorylation activity (Johnson et al., 1996). Generally, non-RD kinases recruit a regulatory RD kinase, such as CERK1 or BAK1, to promote their phosphorylation and become signaling-competent (Dardick et al., 2012).

CERK1 is an indispensable LysM-RLK for fungal chitin-triggered immune signaling in Arabidopsis, and auto-phosphorylation of T479 in the activation segment of Arabidopsis CERK1 is directly involved in the regulation of its kinase activity (Suzuki et al., 2016). Moreover, the common regulator of LRR-RLKs and LRR-RLPs/SOBIR1 in Arabidopsis, BAK1, contains 4 phosphorylation sites in its activation segment, T446, T449, T450, and T455 (Yun et al., 2009). It was observed that a multiple substitution of these Thr residues by an alanine (Ala/A) residue at position 446, 449, and 450, results in the loss of intrinsic BAK1 kinase activity, whereas the single mutations T446A, T449A, or T450A and the double mutations T446A/T449A, T446A/T450A or T449A/T450A do not affect BAK1 intrinsic kinase activity. Nonetheless, the single T455A mutant of BAK1 has completely lost its kinase activity, indicating that T455 is crucial for the activation of BAK1, while the other three Thr residues might play a minor regulatory role or possibly play a role in the recruitment of downstream signaling partners (Yun et al., 2009).

In addition to BAK1, SOBIR1 also has a general regulatory role in plant immunity mediated by LRR-RLPs (Liebrand et al., 2014). As far as we know, SOBIR1 is conserved in all plant species, including Arabidopsis, *N. benthamiana*, tomato, tobacco (*Nicotiana tabacum*), and lettuce (*Lactuca sativa*) (Liebrand et al., 2014; Bi et al., 2016). SOBIR1 is a dual-specificity RD kinase, as AtSOBIR1 has been shown to auto-phosphorylate at Ser, Thr, and Tyr residues (Leslie et al., 2010). Consistent with what has been shown for AtSOBIR1 (Leslie et al., 2010; Wei et al., 2022), NbSOBIR1, S/SOBIR1, and

S/SOBIR1-like also exhibit strong auto-phosphorylation activity. *NbSOBIR1*^{T522}, as well as its analogous residues in tomato *SOBIR1* and *SOBIR1*-like, present in the activation segment of the kinase domain of *SOBIR1*, has been identified to be essential for the intrinsic kinase activity of *SOBIR1* and for the initiation of the various immune responses by *Avr4/Cf-4* (Chapter 3). Intriguingly, *NbSOBIR1*^{T522} is equivalent to *Arabidopsis* *BAK1*^{T455} and *CERK1*^{T479}, which reveals the importance of this particular Thr residue in the activation of RD kinases.

Recently, some well-studied *Arabidopsis* RLKs have been reported to undergo Tyr phosphorylation, in addition to Ser/Thr phosphorylation (Oh et al., 2009; Lin et al., 2014). Moreover, Tyr phosphorylation is proposed to play a predominant role in regulating RLK-mediated plant immunity (de la Fuente van Bentem and Hirt, 2009; Macho et al., 2015). A good example is *CERK1*, which auto-phosphorylates at Y428, and this phosphorylation is essential for chitin-stimulated *CERK1* activation (Liu et al., 2018). Additionally, auto-phosphorylation at Y836 is required for the activation of *EFR* upon recognition of *elf18*, and also for the initiation of subsequent downstream immune responses, including ROS production and MAPK activation (Macho et al., 2014). *BAK1* has also been shown to auto-phosphorylate at Y403, and this phosphorylation is essential for *BAK1* function in plant immune signaling but not in plant development (Perraki et al., 2018). Of note, *CERK1*^{Y428} is equivalent to *EFR*^{Y836} and *BAK1*^{Y403}. Strikingly, their analogous Tyr residue in *NbSOBIR1*, as well as in *S/SOBIR1* and *S/SOBIR1*-like, has been identified to play a crucial role in *Avr4/Cf-4*-triggered MAPK activation and the HR, but not in triggering ROS accumulation (Chapter 4). Nevertheless, no phosphorylated Tyr residue in the kinase domain of *NbSOBIR1* or *AtSOBIR1* was detected by MS (Chapter 3) (van der Burgh et al., 2018; Wei et al., 2022). Therefore, unlike the aforementioned RLKs, *SOBIR1* might employ its Tyr residues that are located in the kinase domain to regulate plant immunity by interacting with specific immune signaling substrates, instead of playing a role in differential phosphorylation.

Trans-phosphorylation of RLKs

In addition to the auto-phosphorylation of RLKs, sequential trans-phosphorylation between cell-surface receptors is pivotal for the specific activation of receptor complexes upon ligand perception, and the subsequent initiation and transduction of immune signaling. For example, the binding of bacterial flagellin, or the *flg22* peptide, by *FLS2* induces the heterodimerization of *FLS2* and *BAK1* within seconds, whereby *flg22* acts as a kind of molecular glue between the LRRs of *FLS2* and *BAK1* (Zipfel et al., 2004; Chinchilla et al., 2007; Sun et al., 2013). Subsequent trans-phosphorylation of the kinase domains of *FLS2* and *BAK1*, which have now come in close proximity, triggers the full activation of this receptor complex (Sun et al., 2013). Intriguingly, activated *BAK1* can also phosphorylate *CERK1*, in this case at S268, S282, and S283 of its juxtamembrane region. As mentioned earlier, this trans-phosphorylation renders

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CERK1 into a primed state and further potentiates CERK1-mediated chitin responses (Gong et al., 2019). Furthermore, bacteria and flg22 also induce the phosphorylation of NIK1 at T474 in an FLS2/BAK1-dependent manner. This trans-phosphorylation event elicits the NIK1-mediated antiviral defense (Li et al., 2019).

Although Arabidopsis LYK5 lacks an essential subdomain for having kinase activity, LYK5 is required for chitin-stimulated CERK1 homodimerization and subsequent auto-phosphorylation (Cao et al., 2014). Activated CERK1 further trans-phosphorylates LYK5, which leads to the re-localization of LYK5 from the plasma membrane into late endocytic compartments (Erwig et al., 2017). Similar to the role of CERK1 in the function of LysM-RLKs, SOBIR1 and BAK1 are the common regulators for triggering LRR-RLP-mediated plant immunity (Liebrand et al., 2014). Kinase activity of both SOBIR1 and BAK1 is required for LRR-RLP-mediated immune signaling upon ligand perception and it is proposed that in addition to SOBIR1 auto-phosphorylation, reciprocal trans-phosphorylation events take place between SOBIR1 and BAK1 (Liebrand et al., 2013; van der Burgh et al., 2019). Here, we provide *in vitro* support for this model and show that SOBIR1 is able to directly phosphorylate BAK1, whereas the intrinsic kinase activity of SOBIR1 is required for this trans-phosphorylation event. Accordingly, BAK1 is also able to directly phosphorylate SOBIR1, and this trans-phosphorylation event again depends on the kinase activity of BAK1 (Chapter 3). Trans-phosphorylation between SOBIR1 and BAK1 probably results in both to obtain a unique phosphorylation status, which is proposed to lead to the full activation of SOBIR1/BAK1-containing immune complexes (Chapter 3).

Strikingly, increasing evidence suggests that the regulatory RLK BAK1 is merely an activator of primary receptors that perceive their corresponding ligand. Therefore, downstream signaling specificity is determined by the kinase domain of the primary receptor, which is SOBIR1 in the case of LRR-RLP signaling. This notion is reinforced by the study of Hohmann et al. (2020), which shows that replacing the ectodomain of the BAK1-dependent LRR-RLKs BRASSINOSTEROID INSENSITIVE 1 (BRI1), HAESA and ERECTA, with the ectodomain of BAK1-INTERACTING RECEPTOR-LIKE KINASE 3 (BIR3), which is a pseudokinase and forms a constitutive complex with BAK1 in a ligand-independent manner, does not affect their signaling specificity.

Trans-phosphorylation of RLCKs

Upon their activation, RLKs mediate the phosphorylation of numerous downstream signaling partners, including the swift trans-phosphorylation of RLK-tethered RLCKs. Flg22 perception by FLS2 induces the phosphorylation of BIK1 by recruited BAK1, mainly at T237, which is a post-translational modification that is required for BIK1 activation and its release from the FLS2/BAK1 complex (Figure 3) (Lin et al., 2014). Of note, BAK1 T455, which is essential for the activation of BAK1 itself, is also an important residue for the trans-phosphorylation of BAK1 by BIK1, as a T455N mutation in the kinase domain of BAK1 dramatically reduces the ability of BAK1 to be trans-

phosphorylated by BIK1 (Lin et al., 2014). Moreover, a combination of MS analysis and site-directed mutagenesis has revealed that tyrosine phosphorylation plays a major regulatory role in plant innate immunity (de la Fuente van Bentem and Hirt, 2009; Lin et al., 2014; Macho et al., 2014; Macho et al., 2015). For example, BIK1 Y150 is important for the intrinsic kinase activity of BIK1, while Y243 and Y250 are phosphorylation sites that are targeted by BAK1 and more specifically contribute to the overall tyrosine phosphorylation of BIK1, which determines its functioning as a signaling partner (Lin et al., 2014). Interestingly, EFR has been shown to phosphorylate BIK1 at S89 and T90 (Figure 3), and a phosphorylation-mimic mutation of S89 and T90 indeed promotes resistance to bacterial pathogens (Lal et al., 2018). In addition, BIK1 constitutively forms a complex with CERK1/LYK5 in the resting state and dissociates from this complex in response to chitin (Figure 3), a phenomenon which is reminiscent of the dynamic interaction between BIK1 and FLS2/BAK1 and EFR/BAK1 (Zhang et al., 2010). It is worth noting that the phosphorylation of CERK1 at Y428 and Y557 is indispensable for triggering the chitin-induced dissociation of CERK1 and BIK1 (Liu et al., 2018).

Recently, the Arabidopsis G-type lectin RLK LIPO-OLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) has been reported to be the receptor of medium-chain 3-hydroxy fatty acids (mc-3-OH-FAs). Interestingly, three closely related RLCKs (PBL34, PBL35, and PBL36) from the RLCK-VII are components of the constitutive LORE complex. Upon elicitation by 3-OH-C10:0 (one of the mc-3-OH-FAs), LORE is phosphorylated at Y600, which is required for LORE-mediated plant immunity (Luo et al., 2020). Of note, activated LORE directly phosphorylates PBL34 at T306 and T310 (Figure 3). This trans-phosphorylation is pivotal for the subsequent initiation of downstream immune responses. Interestingly, LORE Y600 is targeted by the bacterial effector HopAO1, which promotes the dephosphorylation of LORE (Luo et al., 2020).

Arabidopsis BRASSINOSTEROID-SIGNALING KINASE 5 (BSK5), which is a signaling component from the RLCK-XII acting downstream of multiple RLKs, specifically associates with EFR and PEP1 RECEPTOR 1 (PEPR1). Unlike the closely related member BSK6, BSK5 is phosphorylated by EFR and PEPR1 in its kinase activation loop (Figure 3), with S209 and T210 as the major phosphorylation sites. This trans-phosphorylation, as well as the intrinsic kinase activity of BSK5, plays an essential role in BSK5-mediated immune activation (Majhi et al., 2019). In contrast to BSK5, which does not physically interact with FLS2, BSK7 and BSK8 interact with FLS2 but not with other RLKs (Figure 3). Consistently, *bsk7* and *bsk8* single and *bsk7,8* double mutants display impaired flg22-triggered immune responses (Majhi et al., 2021).

Trans-phosphorylation of additional downstream signaling components by RLCKs

Accumulating evidence has pinpointed that RLCKs play a role as central kinases to relay immune signals perceived in the extracellular space to downstream cytoplasmic

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signaling components (Li et al., 2014; Bi et al., 2018; Lal et al., 2018; Liang and Zhou, 2018; Yuan et al., 2021). As mentioned above, upon the perception of flg22 by FLS2, BIK1 is phosphorylated by BAK1, followed by its release from the FLS/BAK1 complex. Activated BIK1 subsequently initiates ROS production by directly phosphorylating RBOHD at S39 and S343 at its N-terminus (Figure 3) (Kadota et al., 2014). On the other hand, being the direct substrate protein of EFR, BIK1 is phosphorylated by EFR at S89 and T90. In addition to its presence at the plasma membrane, BIK1 also localizes to the nucleus, where BIK1 selectively interacts with and phosphorylates the transcription factors WRKY33, 50, and 57. These phosphorylation events regulate the intrinsic levels of jasmonic acid (JA) and SA, as well as the expression of JA- and SA-responsive genes (Figure 3) (Lal et al., 2018). In addition to BIK1, the Arabidopsis RLCK-VII member PBL13, which is a negative regulator of plant innate immunity, has been reported to directly interact with and phosphorylate the C-terminus of RBOHD at both S862 and T912, in the resting state (Lin et al., 2015). To avoid detrimental effects on the host cells, phosphorylation of the RBOHs and the production of ROS should be tightly controlled (Kadota et al., 2015; Kong et al., 2021). Concerning RBOHD, the E3 ubiquitin ligase PBL13-INTERACTING RING DOMAIN E3 LIGASE (PIRE) constitutively associates with both RBOHD and PBL13. Phosphorylation of RBOHD by PBL13 promotes its ubiquitination by PIRE, resulting in the degradation of RBOHD (Figure 3) (Lee et al., 2020). Therefore, RLCKs and ubiquitin ligases act in concert to regulate RBOHD activation and degradation, thereby guaranteeing the swift initiation of proper and robust immune responses, but also again a fast suppression of these responses after having been effective against invading pathogens.

The Arabidopsis RLCK-VII contains 46 members, which are further divided into nine subfamilies, and of which subfamilies 4, 5, 6, 7, and 8 are generally implicated in ExTI, especially in the production of ROS triggered by various ExIPs (Rao et al., 2018). When compared to the model plant Arabidopsis, it is unknown whether similar RLCK-VII subfamilies play a role downstream of the Cf-4/SOBIR1/BAK1 complex in Solanaceous plants, or whether different subfamilies are involved. By simultaneously knocking out eight candidate genes belonging to RLCK-VII-6 in *N. benthamiana* plants stably expressing *Cf-4*, we show that members of this subfamily are indeed also required for ROS accumulation induced by multiple ExIPs, in addition to Avr4, in *N. benthamiana* plants. However, these particular RLCKs are dispensable for the Avr4/Cf-4-triggered MAPK activation and the HR. Intriguingly, despite their different subcellular localization, these eight members from RLCK-VII-6 appear to function redundantly to positively regulate Avr4/Cf-4-triggered ROS production (Chapter 5). Furthermore, we also observed that the Avr4-stimulated ROS burst was strongly compromised in *N. benthamiana*:*Cf-4 rlck-vii-7* and *rlck-vii-8* knock-out lines. We will soon determine their genotypes in order to identify the RLCKs from these two subfamilies that contribute to the ROS burst. Thus, members of the RLCK-VII-7 and -8 subfamilies also likely play a redundant role in regulating the Avr4/Cf-4-triggered ROS production in *N. benthamiana* (Chapter 5). In both *N. benthamiana* and tomato, RBOHB is responsible

for the apoplastic ROS production (Yoshioka et al., 2003; Li et al., 2015). Whether members from RLCK-VII-6, -7, and/or -8 can directly phosphorylate RBOHB to regulate the production of ROS upon their activation by the RLP/SOBIR1/BAK1 complex, remains to be further explored.

Arabidopsis PBL27, which is a member of RLCK-VII-1, associates with MAPKKK5 at the plasma membrane, and chitin perception stimulates the phosphorylation and thereby activation of PBL27 by CERK1 (Figure 3). Subsequently, activated PBL27 trans-phosphorylates the C-terminal domain of MAPKKK5 mainly at S622 and, to a lesser extent, at S617, S658, S660, T677, and S685. This trans-phosphorylation event is required for the MAPKKK5-induced activation of the downstream MAP kinases MPK3 and MPK6 of the MAPK module. In addition, MAPKKK5 has been shown to associate with and phosphorylate the MAPKKs MKK4 and MKK5 (Figure 3) (Yamada et al., 2016). Accordingly, the homolog of PBL27 in rice, *Os*RLCK185, physically interacts with and directly phosphorylates *Os*MAPKKK18, also leading to the activation of a MAPK cascade (Wang et al., 2017; Yamada et al., 2017). In addition, members from *Arabidopsis* RLCK-VII-4 are particularly crucial for chitin-triggered activation of the same downstream MAPK cascade, which consists of MAPKKK5 (and its close homolog MAPKKK3), MKK4/5, and MPK3/6 (Bi et al., 2018; Rao et al., 2018) (Figure 3). Surprisingly, the study of Bi et al. (2018) has shown that MPK6 further phosphorylates MAPKKK5 at S682 and S692, this trans-phosphorylation enhances MPK3/6 activation and host disease resistance. However, which specific RLCK(s) from this subfamily directly regulate CERK1/LYK5-mediated MAPK activation, remains to be elucidated (Rao et al., 2018).

Interestingly, MAPKKK5 is also the substrate of the RLCK BSK1, which is a member of RLCK-XII that plays a role in flg22-triggered immunity (Figure 3). MAPKKK5 contains N- and C-terminal domains of which the function is not clear, and a kinase domain. BSK1 associates with MAPKKK5 at its kinase domain, while it trans-phosphorylates MAPKKK5 at S289 in its N terminus (Yan et al., 2018).

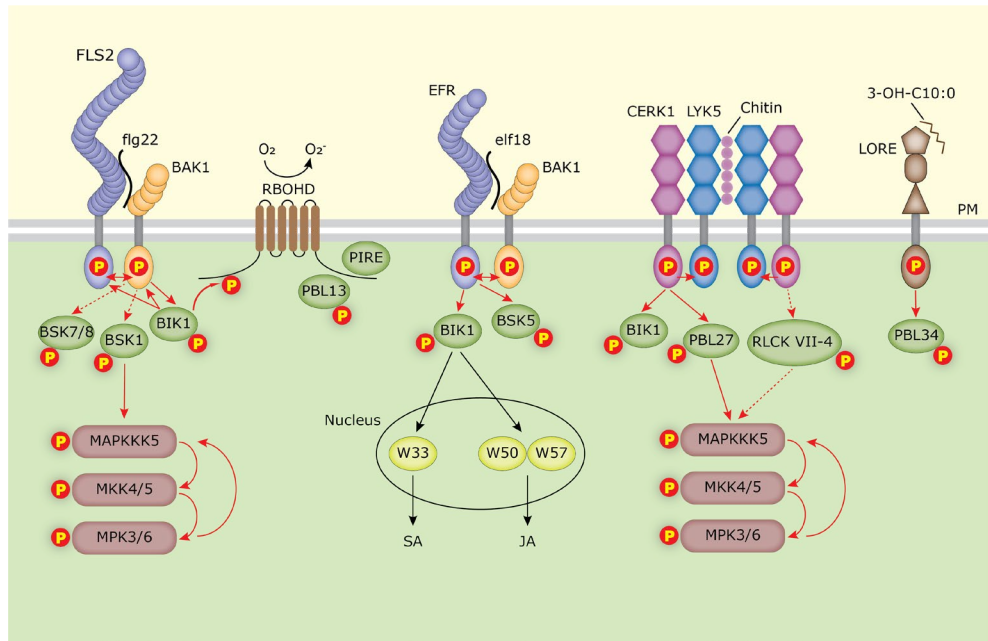


Figure 3. Overview of the cell-surface receptor complexes involved in initiating defense signaling in Arabidopsis. The RLCK BIK1, which associates with the cytoplasmic kinase domains of both FLS2 and BAK1, is directly phosphorylated by activated BAK1 in response to flg22 binding to the extracellular LRRs of FLS2. BIK1, in turn, phosphorylates both FLS2 and BAK1. Hereafter, activated BIK1 is released from the FLS2/BAK1 complex and phosphorylates the RBOHD oxidase enzyme at its N-terminus directly, leading to the generation of ROS. In addition to BIK1, RLCKs BSK1, BSK7, and BSK8 are also required for FLS2/BAK1-mediated immune responses. BSK1 interacts with and phosphorylates MAPKKK5, which in its turn induces the activation of a MAPK cascade. In contrast to BIK1, the RLCK PBL13 negatively regulates plant defense responses by associating with and phosphorylating RBOHD at its C-terminus in the resting state. The ubiquitin ligase PIRE subsequently initiates ubiquitin-triggered degradation of phosphorylated RBOHD, thereby keeping ROS production in check. EFR directly phosphorylates the RLCKs BIK1 and BSK5 upon the perception of elf18 by EFR and subsequent recruitment of BAK1. Activated BIK1 promotes an increase in the levels of JA and SA, through interacting with and phosphorylating various WRKY transcription factors (indicated as W33, W50, and W57) in the nucleus. Furthermore, BIK1, PBL27, and members from RLCK-VII-4 are involved in chitin-induced signaling. The co-receptor CERK1 forms heterodimers with the chitin receptor LYK5 and undergoes auto-phosphorylation upon the perception of chitin. The activation of CERK1 further stimulates the phosphorylation of PBL27 and BIK1. PBL27 selectively bridges the gap between the CERK1/LYK5 complex and the associated downstream MAPK cascade, consisting of MAPKKK5, MKK4/5, and MPK3/6, by directly phosphorylating MAPKKK5. MPK6 further phosphorylates MAPKKK5 to enhance the activation of MPK3/6. The G-type lectin RLK LORE is the receptor of medium-chain 3-hydroxy fatty acids (mc-3-OH-FAs), among which 3-OH-C10:0 triggers the phosphorylation of LORE upon its binding. Furthermore, activated LORE phosphorylates the RLCK PBL34 that is required for the functionality of LORE, which triggers the dissociation of PBL34 from the kinase domain of LORE and subsequent immune activation. Solid arrows indicate signaling events with known mechanisms supported by literature, whereas the anticipated signaling processes that have not been experimentally revealed are denoted with dashed arrows. The red circle with 'P' inside represents phosphorylation. PM, plasma membrane.

Concluding remarks

Several lines of research have over the last two decades largely contributed to our further understanding of the interaction between plants and pathogenic microbes (Zhou and Zhang, 2020; DeFalco and Zipfel, 2021; Ngou et al., 2021). Particularly, publication of the zigzag model in 2006 has resulted in the formulation of a general concept concerning resistance and susceptibility in plant-pathogen interactions, which has further stimulated numerous research efforts on understanding the fundamentals of plant innate immunity (Jones and Dangl, 2006; Thomma et al., 2011; Cook et al., 2015; van der Burgh and Joosten, 2019). Nevertheless, this knowledge is now again being revisited, as several recent studies have provided experimental evidence for the mutual potentiation of plant innate immunity by first-line cell-surface receptors and second-line intracellular receptors (Ngou et al., 2021; Pruitt et al., 2021; Tian et al., 2021; Yuan et al., 2021). It is likely that numerous RLCKs present in plants form the key players to regulate the crosstalk between these two lines of defense. Meanwhile, the LRR-RLP/SOBIR1 complex, which was initially considered to be structurally and functionally identical to an LRR-RLK, albeit consisting of two components (Gust and Felix, 2014; Liebrand et al., 2014), has now been demonstrated to initiate an immune signaling pathway that is fundamentally different from the pathway that is triggered by canonic LRR-RLKs (Wan et al., 2019; Pruitt et al., 2021). Interestingly, this difference might be due to a differential involvement of RLCKs. General questions to guide future studies should include:

- Why do plants evolve numerous RLPs, in addition to a plethora of RLKs, to cope with microbial infection?
- Why do plants require such a large number of RLCKs for initiating downstream immune signaling?
- Does the mutual potentiation between the signaling mediated by cell-surface receptors and by intracellular receptors, which was recently discovered by studying the model plant *Arabidopsis*, also apply to Solanaceous plants?

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Summary

Plants have evolved a two-layered innate immune system to cope with invading microbes from the surrounding environment. Receptors present at the plasma membrane, which are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs), form the first layer of the plant innate immune system and play an important role in mediating host resistance against various pathogens. Our research focuses on the interaction between the extracellular pathogenic fungus *Cladosporium fulvum* and tomato (*Solanum lycopersicum*, *Sl*). Previously, we have shown that the cell-surface receptor Cf-4, which is a leucine-rich repeat (LRR)-RLP, specifically recognizes the apoplastic effector Avr4 secreted by *C. fulvum*. This recognition leads to resistance of tomato to *C. fulvum*. In contrast to RLKs, RLPs lack an intracellular kinase domain for downstream signaling. Interestingly, our subsequent research has found SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR, further referred to as SOBIR1), which is an LRR-RLK, to constitutively interact with Cf-4 and to thereby provide Cf-4 with a kinase domain. Consistent with LRR-RLK-mediated immune signaling, the Cf-4/SOBIR1 complex recruits the LRR-RLK BRI-ASSOCIATED KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3, further referred to as BAK1) to initiate plant immunity, upon the perception of Avr4 by Cf-4. Notably, BAK1 recruitment was later found to be a general phenomenon upon ligand-mediated activation of the LRR-RLP/SOBIR1 complex. The research described in this thesis was aimed to elucidate the molecular mechanisms behind the activation of the Cf-4/SOBIR1/BAK1 complex and to identify essential receptor-like cytoplasmic kinases (RLCKs) that play a role in immune signaling downstream of the Cf-4/SOBIR1 complex.

Chapter 1 introduces the general background of the plant innate immune system, with a focus on cell-surface receptors and the immune responses that they trigger upon recognition of an invading pathogen. In addition, an overview of the general signaling events that take place downstream of the Cf-4/SOBIR1/BAK1 complex upon its activation is provided, as well as an inventory of the known signaling components involved in Cf-4 signaling.

SOBIR1 is a positive regulator of LRR-RLP-mediated immune signaling and appears to be present throughout the plant kingdom. Overexpression of Arabidopsis (*Arabidopsis thaliana*, *At*) *SOBIR1* results in constitutive activation of cell death and associated defense responses in both Arabidopsis and the model Solanaceous plant *Nicotiana benthamiana* (*Nb*). Nevertheless, no symptoms of auto-immunity were observed when *NbSOBIR1*, *SISOBIR1*, or *SISOBIR1-like* was overexpressed in leaves of *N. benthamiana* plants. To generate the material that can be used to characterize the function of SOBIR1 *in planta* and to study the fundamentals of plant immunity triggered by Avr4/Cf-4, we employed the CRISPR/Cas9 system to knock out *SOBIR1* in *N. benthamiana*. In **Chapter 2**, we show that we successfully knocked out *SOBIR1* and its homolog *SOBIR1-like* both in wild-type *N. benthamiana* and in *N. benthamiana*

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stably expressing the *Cf-4* transgene. Strikingly, *N. benthamiana sobir1* (*/sobir1-like*) knock-out plants are non-responsive to the Avr4/*Cf-4* combination, and consistently, *N. benthamiana:Cf-4 sobir1* (*/sobir1-like*) knock-out plants are also non-responsive to Avr4. Interestingly, flg22, which is a conserved 22-amino acid epitope of bacterial flagellin and can be recognized by the LRR-RLK FLAGELLIN-SENSING 2 (FLS2), triggers a monophasic reactive oxygen species (ROS) burst in *N. benthamiana*, but a biphasic ROS burst in *sobir1* (*/sobir1-like*) knock-out plants. This observation implies that there is crosstalk taking place between RLP/SOBIR1 signaling and FLS2 signaling.

The *sobir1* (*/sobir1-like*) knock-out plants described in Chapter 2 were subsequently implemented for complementation studies, combined with a site-directed mutagenesis screen of putative phosphorylation sites present in SOBIR1. One serine (Ser/S) and four threonine (Thr/T) residues that are present in the activation segment of the kinase domain of SOBIR1 were studied in **Chapter 3**. *NbSOBIR1*^{T522}, as well as its analogous residues in *S/SOBIR1* and *S/SOBIR1-like*, was identified to be essential for Avr4/*Cf-4*-induced ROS accumulation, the activation of a mitogen-activated protein kinase (MAPK) cascade, and the hypersensitive response (HR). Further *in vitro* phosphorylation assays demonstrated that this particular Thr residue is required for the intrinsic kinase activity of SOBIR1. Additionally, we provide *in vitro* evidence to support the SOBIR1/BAK1 activation model that was proposed before.

Recently, Tyr phosphorylation has been recognized to be a common feature of RLK activation in plants. Therefore, all Tyr residues present in the kinase domain of *NbSOBIR1*, *S/SOBIR1*, and *S/SOBIR1-like* were individually changed into the non-phosphorylatable amino acid phenylalanine (Phe/F) and the obtained mutants were included in the complementation study, as described in **Chapter 4**. We show that *NbSOBIR1*^{Y469}, as well as its analogous residues in *S/SOBIR1* and *S/SOBIR1-like*, plays a crucial role in Avr4/*Cf-4*-triggered MAPK activation and the HR, whereas this residue is not essential for the Avr4/*Cf-4*-induced ROS production or the intrinsic kinase activity of SOBIR1. In addition, this particular residue is also required for the BcXYG1-triggered HR in *N. benthamiana* plants. However, no phosphorylated Tyr residue was detected in the kinase domain of either *AtSOBIR1* or *NbSOBIR1* by mass spectrometry. Therefore, we propose that SOBIR1 employs this important Tyr residue in its kinase domain to trigger plant immunity by binding a specific substrate, instead of being important in a phosphorylated state.

Cell-surface receptors deploy a large number of downstream RLCKs to relay the immune signals from the extracellular space into the plant cells. **Chapter 5** describes the identification and characterization of RLCKs that play a role downstream of the *Cf-4/SOBIR1/BAK1* complex. We show that eight members from the RLCK class VII subfamily 6 (RLCK-VII-6) in *N. benthamiana:Cf-4* plants play a pivotal role in regulating the production of ROS that is stimulated by multiple extracellular immunogenic patterns (ExIPs), including the Avr4 protein. Strikingly, these eight members are dispensable for Avr/*Cf-4*-triggered MAPK activation and the HR. More

importantly, despite their different subcellular localization, these eight members appear to function redundantly as positive regulators of the Avr4/Cf-4-induced ROS accumulation. Furthermore, members from the RLCK-VII-7 and -8 subfamilies might also be essential for the Avr4/Cf-4-triggered ROS burst. However, the molecular mechanisms by which the various members from the RLCK-VII-6 and RLCK-VII-7/8 subfamilies regulate the production of ROS in *N. benthamiana* are not known.

Finally, **Chapter 6** provides a comprehensive overview of the recent advances in our understanding of plant innate immunity. We summarize and discuss the major findings obtained in this thesis and place them into a broader perspective. Moreover, general questions that should guide further research are also listed.

Acknowledgments

When it almost comes to the end of my PhD journey, I would like to take this opportunity to express my great appreciation for people who have ever provided me with either scientific or mental support. Without your help and dedicated involvement in every step of the process, this thesis would have never been accomplished.

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Words cannot express my countless gratitude to my paranymphs. **Ciska**, thanks for being such a reliable colleague and sincere friend! I appreciate all the constructs you generated and all experiments you conducted for this project; this thesis would not have been completed without your help. Thanks for always being around to listen to me, encourage me, and support me, I cannot imagine how difficult my life in the Netherlands would be without you. You are amazing just the way you are. **Laurens**, thanks for being the Lab hero and the real lifesaver at Phytopathology! I was so lucky to have you around during my PhD study. You are always calm, patient, positive, optimistic, and willing to help everyone. How can you always have solutions for the problems we face? Thanks for always telling me 'this is not the end of the world', I appreciate all your technical and mental support.

Next, I would like to extend my sincere thanks to all the (Solanaceae) **SOL team** members! Thank you everyone for creating this warm atmosphere. **Thomas**, thanks for making time to discuss my experimental results with me and for providing valuable input into my PhD project. **Aranka**, **Jinbin**, **Sergio**, and **Christiaan**, thanks for helping me to get started at Phytopathology. I appreciate that you are always so friendly and patient. **Aranka**, thanks for your excellent work on determining the

Acknowledgments

activation and signaling transduction of the SOBIR1/BAK1-containing immune complexes, which constitutes a solid basis for my research. I am so delighted that you are still around and I look forward to more fruitful discussions with you in the near future. **Jinbin**, I am so grateful for your support and encouragement when I was in times of trouble. I wish you a lot of success in China. **Sergio**, I appreciate all the help I received from you and wish you good luck with finishing your PhD thesis. **Christiaan**, even though you were doing your BSc thesis in our lab when I just started my PhD, I have learned so much from you. Collaborating with you speeded up my adaptation to the new environment. I am glad that you are back on the SOL team as a PhD candidate. I appreciate your contributions at the starting phase of my PhD as the expert on plant CRISPR/Cas9 and also in the final phase of my PhD as the expert on confocal microscopy. Let us see what exciting discoveries we will make together in the future. **Esratur, Huan, and Nicat**, you joined the SOL team when we needed more hands and I am confident that you will do great. I hope you will enjoy your PhD journey and wish you all the best. Special thanks to **Jim** and **Deny**, who did their MSc theses at the SOL lab in the first year of my PhD. Your support and encouragement helped me regain my confidence. Now you are both doing your PhD, I wish you lots of success.

I'm extremely grateful to our collaborators who have contributed a lot to the work presented in this thesis. **Johannes**, we are still surprised by how efficient your CRISPR/Cas9 system was and how many *N. benthamiana rldk* mutant lines you have generated for us. Thanks for doing this great job, I appreciate your contribution. **Michael**, I contacted you when I was most in need of help, thanks for being so supportive. I appreciate the bioinformatics-related work you have done for us and your critical feedback on my thesis. I look forward to continuing to collaborate with you! **Aalt-Jan**, it was a great experience to co-supervise Daniël (an MSc student) with you, thanks for being so interested in our research. I also look forward to an intensified collaboration on exploring the application of AlphaFold in our project. **Norbert**, I am thankful for your help with the usage of the confocal microscopy and cannot wait to see what more surprises it will bring to us.

I would like to continue by thanking colleagues from Phytopathology, who made my PhD journey so enjoyable and memorable. **Gert**, thanks for being such an excellent chair and for caring about everyone in our chair group. I appreciate your effort to make our group a warm, safe, cooperative, and supportive place to work. **Francine**, how can I express my countless gratitude to you? I admire your endless enthusiasm for science and your eagerness to learn. You are amiable, frank, considerate, diligent, and gregarious. You set an example of how outstanding a female scientist could be. Thanks for always being willing to share your knowledge and wisdom with us and giving advice whenever needed. Especially, thanks for teaching me how to review a manuscript and how to write a report to the editor. It is always a great pleasure to co-review manuscripts with you. **Jan**, you are a great teacher, I admire your enthusiasm for teaching and research. I am also thankful for your effort to push my defense to happen.

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Special thanks to China Scholarship Council (**CSC**) and The Graduate School Experimental Plant Sciences (**EPS**). Without CSC, I would not be able to come to Wageningen; while without EPS, I could not be able to complete my PhD study and obtain my doctoral degree.

My dearest friend, **Hua**, I cannot thank you enough for your company, encouragement, and help. Thanks for always making time to listen to me and being so supportive no matter what. I appreciate all the happiness and difficulties we experienced together, which made us get better and stronger. You are so courageous, compassionate, and passionate. I wish you lots of success in the future! Go, girl! **Xu**, thanks for sharing your experience and providing insightful advice to me. **Meixin**, my little sister, thanks for taking care of me! I am happy that you are back to Wageningen again. You are doing great! I wish you an enjoyable PhD study here. **Wenqing**, **Ying**, and **Jiayu**, thanks for always being so friendly to me. I wish you all the best!

Acknowledgments

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Last but not least, I would like to express my special appreciation for my family. Thank you for your unconditional love and support. I miss you as much as you miss me, I am proud of you as much as you are proud of me, and I love you as much as you love me.

About the author

Wen Huang (黄雯) was born on February 24th, 1991 in Hebei, China. After graduation from high school in 2010, she started a four-year bachelor study in Plant Sciences at Southwest University, Chongqing, China. She conducted her BSc thesis at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China, where she focused on the cloning and expression profiling of the aminopeptidase N encoding gene of the rice leafroller moth *Cnaphalocrocis medinalis*.

After completing her BSc, she enrolled in a joint MSc program between Southwest University and the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, under the supervision of Prof. Wei Ding, Prof. Jie Feng, and Jin Xu. She performed her MSc study on the functional analysis of strain-specific effectors from *Ralstonia solanacearum* strain Po82.

The research on the interaction between plants and microbes evoked her great interest, therefore, in the final year of her MSc study, she wrote a PhD proposal together with Dr. Matthieu Joosten, which was successfully granted by the China Scholarship Council (CSC). In December 2017, she started her PhD research at the Laboratory of Phytopathology, Wageningen University and Research, the Netherlands. Under the supervision of Dr. Matthieu Joosten, she investigated the activation and signal transduction of SOBIR1/BAK1-containing immune complexes present at the plant cell surface. The results of the conducted research are described in this thesis.

Since January 2022, she continues her research on plant immune signaling as a Postdoc at the Laboratory of Phytopathology, Wageningen University and Research, the Netherlands.

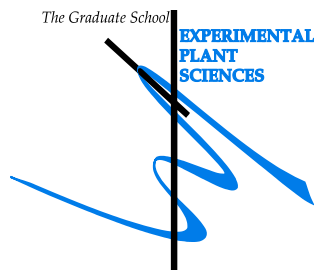


Photo taken by Betsie van Ojik

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Wen Huang
Date: 28 June 2022
Group: Laboratory of Phytopathology
University: Wageningen University & Research



1) Start-Up Phase	<u>date</u>	<u>cp</u>
► First presentation of your project The role of SOBIR1 and its signalling partners in plant immunity to microbial pathogens	30 Nov 2018	1.5
► Writing or rewriting a project proposal Elucidating the role of SOBIR1 and signalling partners of this regulatory cell surface receptor-like kinase in plant immunity to microbial pathogens.	24 Sep 2018	3.0
► MSc courses Plant-Microbe Interactions (PHP-30306), plus exam	14 May-1 Jun 2018	6.0
<i>Subtotal Start-Up Phase</i>		10.5

2) Scientific Exposure	<u>date</u>	<u>cp</u>
► EPS PhD student days EPS PhD student days 'Get2Gether', Soest, NL EPS PhD student days 'Get2Gether', Soest, NL	15-16 Feb 2018 11-12 Feb 2019	0.6 0.6
► EPS theme symposia EPS Theme 2 & Willie Commelin Scholten Day 'Interactions between Plants and Biotic Agents', Amsterdam, NL EPS Theme 2 & Willie Commelin Scholten Day 'Interactions between Plants and Biotic Agents', Utrecht, NL EPS Theme 2 & Willie Commelin Scholten Day 'Interactions between Plants and Biotic Agents', online EPS Theme 2 & Willie Commelin Scholten Day 'Interactions between Plants and Biotic Agents', online	24 Jan 2018 4 Feb 2020 9 Feb 2021 8 Feb 2022	0.3 0.3 0.2 0.2
► Lunteren Days and other national platforms Annual Meeting 'Experimental Plant Sciences', Lunteren, NL Annual Meeting 'Experimental Plant Sciences', Lunteren, NL Annual Meeting 'Experimental Plant Sciences', online NWO Life2021, online	9-10 Apr 2018 8-9 Apr 2019 12-13 Apr 2021 26-28 May 2021	0.6 0.6 0.5 0.6
► Seminars (series), workshops and symposia Seminar: Dr. Timothy Friesen, USDA-ARS, Fargo, ND, US Seminar: Dr. Melvin Bolton, USDA, Fargo, US Seminar: Dr. Yang Wang, Nanjing Agricultural University, CN Seminar: Prof. Volker Lipka, Georg-August University, Göttingen, DE Seminar: Dr. Andrea Gust, Tübingen University, DE Seminar: Prof. Antonio Di Pietro, Department of Genetics, Cordoba, ES Seminar: Prof. David Geiser, Department of Plant Pathology and Environmental Microbiology, Penn State University, US Seminar: Dr. Theo van der Lee, Biointeractions and Plant Health, WUR, NL Seminar: Dr. Daniela Sueldo, Plant Chemetics Laboratory, University of Oxford, UK	18 Apr 2018 7 Sep 2018 10 Sep 2018 14 Sep 2018 21 Sep 2018 17 Oct 2018 29 Oct 2018 18 Jan 2019 8 Feb 2019	0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1

Education statement

Seminar: Dr. Jaap Wolters, Plant Breeding, WUR, NL	15 Feb 2019	0.1
Seminar: Dr. Luigi Faino, University of Rome "Sapienza", IT	22 Feb 2019	0.1
Seminar: Prof. Guido van den Ackerveken, Translational Plant & Microbial Biology, Utrecht University, NL	8 Mar 2019	0.1
Seminar: Dr. Rays Jiang, University of South Florida, US	5 Jul 2019	0.1
Seminar: Dr. Elwira Smakowska-Luzan, Gregor Mendel Institute, Austrian Academy of Sciences, AT	6 Dec 2019	0.1
Seminar: Dr. Eva Stukenbrock, Max Planck Institute Plön and Christian-Albrechts University of Kiel, DE	13 Dec 2019	0.1
Seminar: Matthew Neubauer, Department of Biology, Indiana University, US	10 Jun 2020	0.1
Seminar: Prof. Sarah Gurr, chair in Food Security at Exeter University, UK	15 Jan 2021	0.1
Seminar: Dr. Stefan Geisen, Laboratory of Nematology, WUR, NL	20 Jan 2021	0.1
Seminar: Dr. Daniel Croll, Institute of Biology, University of Neuchâtel, CH	18 Feb 2021	0.1
Seminar: Prof. James Brown, the John Innes Centre, UK	5 Mar 2021	0.1
Seminar: Prof. Dr. Haswell, Washington University-Saint Louis, US	11 May 2021	0.1
► Seminar plus		
Nick Talbot - PhD students Laboratory of Phytopathology	2 Apr 2019	0.1
Harrold van den Burg - PhD students Laboratory of Phytopathology	25 Mar 2021	0.1
Antonio Di Pietro - PhD students Laboratory of Phytopathology	21 Oct 2021	0.1
Gero Steinberg - PhD students Laboratory of Phytopathology	11 Nov 2021	0.1
Frank Takken - PhD students Laboratory of Phytopathology	30 Nov 2021	0.1
► International symposia and congresses		
European Plant Science Retreat (EPSR), Utrecht, NL	3-6 Jul 2018	1.0
XVIII International Congress on Molecular Plant-Microbe Interactions (IS-MPMI XVIII), Glasgow, UK	14-18 Jul 2019	1.5
► Presentations		
Poster presentation: Phosphorylation of specific residues of the kinase domain of SOBIR1 is essential for its role in immune signalling, EPSR, Utrecht, NL	3-6 Jul 2018	1.0
Poster presentation: Phosphorylation of specific residues of the kinase domain of SOBIR1 is essential for its role in immune signalling, IS-MPMI XVIII Congress, Glasgow, UK	14-18 Jul 2019	1.0
Oral presentation: The role of SOBIR1 phosphorylation in RLP-triggered immunity, EPS theme 2 & Willie Commelin Scholten Day, Utrecht, NL	4 Feb 2020	1.0
Poster presentation: The role of SOBIR1 phosphorylation in RLP-triggered immunity, Annual Meeting 'Experimental Plant Sciences', online	12 Apr 2021	1.0
Oral presentation: The role of SOBIR1 phosphorylation in RLP-triggered immunity, Annual Meeting 'Experimental Plant Sciences', online	13 Apr 2021	1.0
Oral presentation: SOBIR1 phosphorylation and immune signalling downstream of the Cf-4/SOBIR1/BAK1 complex, BASF-Nunhems, online	6 May 2021	1.0
► 3rd year interview		
► Excursions		
Online Networking Event - Bejo Zaden, organized by Seed Valley and EPS PhD council	14 Dec 2020	0.2
<i>Subtotal Scientific Exposure</i>		15.8

3) In-Depth Studies	<u>date</u>	<u>cp</u>
► Advanced scientific courses & workshops		
EPS PhD course: Transcription Factors and Transcriptional Regulation, Wageningen, NL	10-12 Dec 2018	1.0
EPS PhD course: Bioinformatic Introduction Course, online	5-9 Jul 2021	1.5
► Journal club		
SOL group literature discussion sessions	2017-2022	1.5
Laboratory of Phytopathology expertise meeting literature discussion sessions	2020-2021	1.0
► Individual research training		
<i>Subtotal In-Depth Studies</i>		5.0

Education statement

4) Personal Development	<u>date</u>	<u>cp</u>
► General skill training courses WGS course: Scientific Artwork-Vector graphics and images, Wageningen, NL EPS Introduction course, Wageningen, NL WGS course: Searching and Organising Literature for PhD, Wageningen, NL EPS workshop: Scientific Paper Writing, Wageningen, NL WGS course: The Essentials of Scientific Writing and Presenting, Wageningen, NL EPS workshop: Applying for a Marie Skłodowska-Curie Fellowship: from proposal to project, Wageningen, NL edX course: Write 101x: English Grammar and Style (provided by the University of Queensland), online WGS course: Adobe InDesign Essential Training, Wageningen, NL WGS workshop: Reviewing a Scientific Manuscript, Wageningen, NL YoungWUR workshop: How to Create Impactful Infographics and Data Visuals, online EPS workshop: Marie Curie fellowship, online ► Organisation of meetings, PhD courses or outreach activities PhD representative in the Laboratory of Phytopathology Management Team ► Membership of EPS PhD Council	19-20 Mar 2018 27 Mar 2018 11-12 Jun 2019 24 Oct 2019 29 Nov-9 Dec 2019 16 Dec 2019 27 Jul-22 Sep 2020 9-10 Nov 2020 12 Nov 2020 24 Nov 2020 10 Dec 2020 18 Jan-12 Apr 2021	0.6 0.3 0.6 0.2 1.2 0.2 1.2 0.6 0.1 0.3 0.2 0.4
<i>Subtotal Personal Development</i>		5.9

5) Teaching & Supervision Duties	<u>date</u>	<u>cp</u>
► Courses Molecular Aspects of Bio-interactions Ecological Aspects of Bio-interactions ► Supervision of BSc/MSc students Bsc internship - Victor Knooren Msc thesis - Torsten van der Schriek Msc thesis - Agnes Omabour Hagan Msc thesis - Daniël Davidse Msc thesis - Mark Koppies	Dec 2018 Oct 2019 Aug 2018-Oct 2018 Sep 2018-May 2019 Jul 2019-Mar 2020 Jun 2020-Nov 2020 Aug 2020-Mar 2021	1.5 1.5 3.0
<i>Subtotal Teaching & Supervision Duties</i>		6.0

TOTAL NUMBER OF CREDIT POINTS*	43.2
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Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

* A credit represents a normative study load of 28 hours of study.

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