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Review

Immune signaling: receptor-like proteins make the difference

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To resist biotic attacks, plants have evolved a sophisticated, receptor-based immune system. Cell-surface immune receptors, which are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs), form the front line of the plant defense machinery. RLPs lack a cytoplasmic kinase domain for downstream immune signaling, and leucine-rich repeat (LRR)-containing RLPs constitutively associate with the RLK SOBIR1. The RLP/SOBIR1 complex was proposed to be the bimolecular equivalent of genuine RLKs. However, it appears that the molecular mechanisms by which RLP/SOBIR1 complexes and RLKs mount immunity show some striking differences. Here, we summarize the differences between RLP/SOBIR1 and RLK signaling, focusing on the way these receptors recruit the BAK1 co-receptor and elaborating on the negative crosstalk taking place between the two signaling networks.

Highlights

Both LRR-RLKs and LRR-RLP/SOBIR1 complexes recruit the co-receptor BAK1 to initiate downstream signaling. However, BAK1 association occurs by different mechanisms.

BAK1 is merely an activator of primary receptor kinase that perceives the corresponding ligand. Therefore, the specificity of downstream signaling is determined by the kinase domain of the primary receptor, being the kinase domain of SOBIR1 for all LRR-RLPs involved in immunity and, for example, the kinase domain of FLS2.

Some receptor-like cytoplasmic kinases (RLCKs) play opposite roles, either as positive or negative regulators downstream of LRR-RLP/SOBIR1 complexes and of LRR-RLKs.

LRR-RLP/SOBIR1 complexes employ different downstream signaling components in arabidopsis (*Arabidopsis thaliana*), when compared with the solanaceous plant *Nicotiana benthamiana*.

There is (negative) crosstalk between signaling by LRR-RLKs and LRR-RLP/SOBIR1.

Plant immunity

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 [1,2]. Cell-surface receptors are either receptor-like proteins (RLPs) or receptor-like kinases (RLKs) [3,4]. RLKs contain a ligand-binding ectodomain (ECD), a single-pass transmembrane domain (TM), and a cytoplasmic kinase domain. Despite lacking the intracellular kinase domain, RLPs share a similar overall structure with RLKs [5]. Plants deploy RLKs and RLPs to perceive **extracellular immunogenic patterns (ExIPs)** (see Glossary), leading to extracellularly triggered immunity (ExTI) [1]. The ECDs of cell-surface receptors are highly variable and they contain, for example, leucine-rich repeats (LRRs) to bind to proteins and peptides or a lysin motif (LysM) to perceive *N*-acetylglucosamine-containing ligands [5]. LRR-RLKs and LRR-RLPs form the largest family of **cell-surface immune receptors** and harbor the most extensively studied members, among which is the arabidopsis (*Arabidopsis thaliana*, *At*) LRR-RLK FLS2 [6,7]. Well-studied ExIPs are, amongst others, 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 [6–8]. Recognition of ExIPs by matching cell-surface receptors activates the frontline of plant innate immunity, which generally plays a prominent role in fending off invasion of plant tissues by a broad spectrum of pathogens and pests [1,9,10].

Typically, to overcome ExTI and facilitate invasion, host-adapted pathogens deliver intracellular effectors into plant cells [1,3,9,11]. Plants, as a result of coevolution, have developed a second layer of surveillance machinery, consisting of intracellular immune receptors formed by nucleotide-binding, leucine-rich repeat proteins (NLRs), to specifically recognize these effectors as **intracellular immunogenic patterns (InIPs)**, resulting in intracellularly triggered immunity (InTI) [1,11].

Interestingly, related to both ExTI and InTI, a series of downstream signaling outputs are activated, including an influx of extracellular calcium, the production of **reactive oxygen species (ROS)**, the

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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, a **hypersensitive response (HR)** takes place, which is a form of programmed cell death (PCD) [12–15]. Collectively, these immune responses result in disease resistance.

The last decade has witnessed major progress in deciphering how plant innate immunity is triggered and regulated. In particular, ExTI and InTI have recently been reported to mutually potentiate each other, even though they involve the activation of different classes of receptors with different subcellular localization [16–19]. There are many excellent reviews on these tremendous advances [2,6,13–15,20,21], while this review will focus on RLKs and RLPs that are responsible for the activation of the first layer of the plant immune system [5]. Previously, RLP/co-receptor complexes were proposed to be structurally and functionally equivalent to genuine RLKs, with their downstream immune signaling being identical [22,23]. However, accumulating evidence indicates that RLP-triggered immune signaling shows quantitative and qualitative differences when compared with immune signaling initiated by RLKs. Therefore, this review will summarize our current understanding concerning the activation of cell-surface immune receptors and their downstream signaling leading to plant resistance. Furthermore, the striking differences between RLP and RLK signaling will be illustrated.

The discovery of RLPs and RLKs involved in plant immunity

Tomato (*Solanum lycopersicum*, *Sl*) resistance protein Cf-9, which confers resistance to strains of the fungal pathogen *Fulvia fulva* (previously known as *Cladosporium fulvum*) that secrete avirulence protein 9 (Avr9), serving as an apoplastic effector, is the first RLP of which the encoding gene was cloned [24]. Recognition of Avr9 by Cf-9 leads, amongst others, to the activation of **receptor-like cytoplasmic kinases (RLCKs)**, MAPKs, CDPKs, and the HR [25–28]. Subsequently, additional tomato *Cf* genes, such as *Cf-2* [29], *Cf-4* [30], *Cf-5* [31], and *Hcr9-4E* [32], have been isolated, of which the protein products confer specific recognition of *F. fulva* Avr2 [33], Avr4 [34], Avr5 [35], and Avr4E [36], respectively.

Meanwhile, various RLKs that are involved in plant immunity were also isolated and characterized. Rice (*Oryza sativa*) Xa21, which confers resistance against diverse *Xanthomonas* spp., was the first RLK of which the encoding gene was cloned [37]. Well-known examples of RLKs involved in plant defense are arabidopsis **FLAGELLIN-SENSING 2 (FLS2)** and ELONGATION FACTOR-TU RECEPTOR (EFR), which perceive bacterial flagellin by recognizing its epitope flg22, and elf18, which is derived from bacterial elongation factor Tu, respectively [38–40]. In addition to its presence in arabidopsis, functional orthologs of FLS2 have been identified in many other plant species, including *Nicotiana benthamiana*, tomato, rice, and grapevine (*Vitis vinifera*) [41–44]. In tomato, an additional flagellin receptor from the RLK family, referred to as FLS3, has been characterized to perceive a second epitope of flagellin, named flgII-28 [45].

Furthermore, **BRI1-ASSOCIATED KINASE 1 (BAK1)** [46,47], CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) [48], and **SUPPRESSOR OF BIR1-1 (SOBIR1)** [49] are common co-receptors playing an important role in ExTI. These co-receptors are evolutionarily conserved in various plant species [23,50] and a recent comparative genomic analysis has suggested that RLPs are very ancient and likely pre-date the emergence of land plants. Numerous RLKs have possibly evolved directly from RLPs and this might have taken place through the integration of a kinase domain in the RLP protein sequence (Table 1) [51].

Recognition of ExIPs by RLKs and RLPs and BAK1 recruitment

Upon recognition of flg22, FLS2 recruits the LRR-RLK, BAK1 [52]. The crystal structure of the FLS2(LRR)-flg22-BAK1(LRR) complex was reported back in 2013 and revealed that FLS2

Glossary

BRI1-ASSOCIATED KINASE 1 (BAK1): also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), an LRR-RLK from the SERK family with an ectodomain consisting of five LRRs, which acts as a common regulatory co-receptor for triggering LRR-RLK- and LRR-RLP-mediated plant immunity.
Cell-surface immune receptors: plant immune receptors that are present at the plasma membrane and specifically perceive immunogenic patterns in the extracellular space (apoplast). Such receptors are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs).
Cf proteins: LRR-RLPs that provide resistance for tomato to the fungal pathogen *Fulvia fulva*.
Extracellular immunogenic pattern (ExIP): any extracellular danger signal, either externally encoded or representing a modified-self ligand, which alerts of plant attack upon its perception by a cell-surface receptor. ExIP perception results in extracellularly triggered immunity (ExTI).
FLAGELLIN-SENSING 2 (FLS2): LRR-RLK that recognizes bacterial flagellin, or its epitope flg22.
Helper NLRs: a class of nucleotide-binding, LRR proteins (NLRs) that act as regulatory nodes mediating immune signaling downstream of primary 'sensor NLRs'.
Hypersensitive response (HR): a form of localized programmed cell death at sites of attempted invasion of plant tissues, which is one of the strong immune responses mounted by resistant plants and is associated with the limitation of the proliferation of pathogenic microbes.
Intracellular immunogenic pattern (InIP): any intracellular danger signal, either externally encoded or representing a modified-self ligand, which alerts of plant attack upon its perception by an intracellular receptor. InIP perception results in intracellularly triggered immunity (InTI).
RD kinase: a class of protein kinases in which an arginine residue (Arg/R) precedes the highly conserved catalytic aspartic acid residue (Asp/D) in its catalytic loop. Non-RD kinases lack this conserved R in their catalytic loop.
Reactive oxygen species (ROS): a hallmark of the swift plant immune response, which functions not only as an

Table 1. LRR-RLPs involved in immunity, their plant origin, and their matching effectors

Gene name	Full name	Plant origin	Ligand	Refs
Cf-2	–	Tomato	Avr2 and Gr-VAP1	[29,33,118]
Cf-4	–	Tomato	Avr4	[30,34]
Cf-5	–	Tomato	Avr5	[31,35]
Cf-9	–	Tomato	Avr9	[24,119]
Hcr9-4E	–	Tomato	Avr4E	[32,36]
Ve1	–	Tomato	Ave1	[120]
EIX1/2	ETHYLENE-INDUCING XYLANASE RECEPTOR 1/2	Tomato	EIX	[121]
CuRe1	CUSCUTA RECEPTOR 1	Tomato	Cuscuta factor	[122]
I	–	Tomato	Avr1/Six4	[58]
ELR/RLP85	ELICITIN RESPONSE/RLP85	Potato	INF1	[123,124]
RLP1	–	Rice	eMAX	[125]
LYP4/6	LYSIN MOTIF-CONTAINING PROTEIN 4/6	Rice	Chitin and peptidoglycan	[126]
CEBiP	CHITIN ELICITOR BINDING PROTEIN	Rice	Chitin	[127]
CSPR1/RE02	RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS/RE02	*N. benthamiana*	csp22 and VmE02	[128–130]
RXEG1	RESPONSE TO XEG1	*N. benthamiana*	XEG1	[62]
REL	RESPONSIVE TO ELICITINS	*N. benthamiana*	Elicitins	[131]
INR	INCEPTIN RECEPTOR	Tobacco	Inceptin	[64]
RLP1	–	Arabidopsis	eMAX	[132]
RLP23	–	Arabidopsis	nlp20	[55]
RLP30	–	Arabidopsis	SCFE1	[115]
RLP32	–	Arabidopsis	Proteobacterial translation initiation factor 1 (IF1)	[133]
RLP42	–	Arabidopsis	Endopolygalacturonases	[63,134]
RLP53	–	Arabidopsis	–	[135]
LYM1/3	LYSIN-MOTIF (LYSM) DOMAIN PROTEIN 1	Arabidopsis	Peptidoglycan	[136]

antimicrobial agent but also as secondary messengers mediating endogenous immune signaling. ROS consists of an array of highly reactive molecular oxygen derivatives, such as superoxide radicals (O^{2-}) and hydrogen peroxide (H_2O_2).

Receptor-like cytoplasmic kinases (RLCKs): proteins mainly consisting of a kinase domain and which are the initial cytoplasmic transducers of the extracellular signal that is perceived by cell-surface receptors. BOTRYTIS-INDUCED KINASE 1 (BIK1) is one of the most studied RLCKs.

SUPPRESSOR OF BIR1-1 (SOBIR1): also known as EVERSHED (EVR), an RLK with an ectodomain consisting of five leucine-rich repeats (LRRs), which constitutively interacts with LRR-RLPs, thereby providing these receptors with a downstream cytoplasmic signaling domain.

directly recognizes flg22 by its LRRs and that this binding creates a novel recognition surface on FLS2(LRR) for its association with BAK1(LRR). Interestingly, the co-receptor BAK1 also binds to the C terminus of the flg22 peptide and, therefore, flg22 acts as a kind of 'molecular glue' to stabilize the FLS2-BAK1 dimerization [53] (Figure 1). This heterodimerization brings the cytoplasmic kinase domains of FLS2 and BAK1 into close proximity, allowing their reciprocal trans-phosphorylation, which initiates downstream immune signaling, resulting in resistance against bacterial invasion [54] (Figure 1).

Typically, LRR-RLPs form a ligand-independent, constitutive complex with the LRR-RLK SOBIR1 [22,23,49]. Consistent with LRR-RLKs, the LRR-RLP/SOBIR1 complex also requires the recruitment of BAK1 for the initiation of downstream signaling upon ligand perception [6,55,56]. Intriguingly, most LRR-RLPs that are involved in plant immunity contain a so-called 'loop-out' region or 'island' domain and this domain divides their extracellular LRR region into two blocks [57]. For instance, the tomato LRR-RLP I, which confers resistance against the Fusarium wilt fungus *Fusarium oxysporum* f. sp.

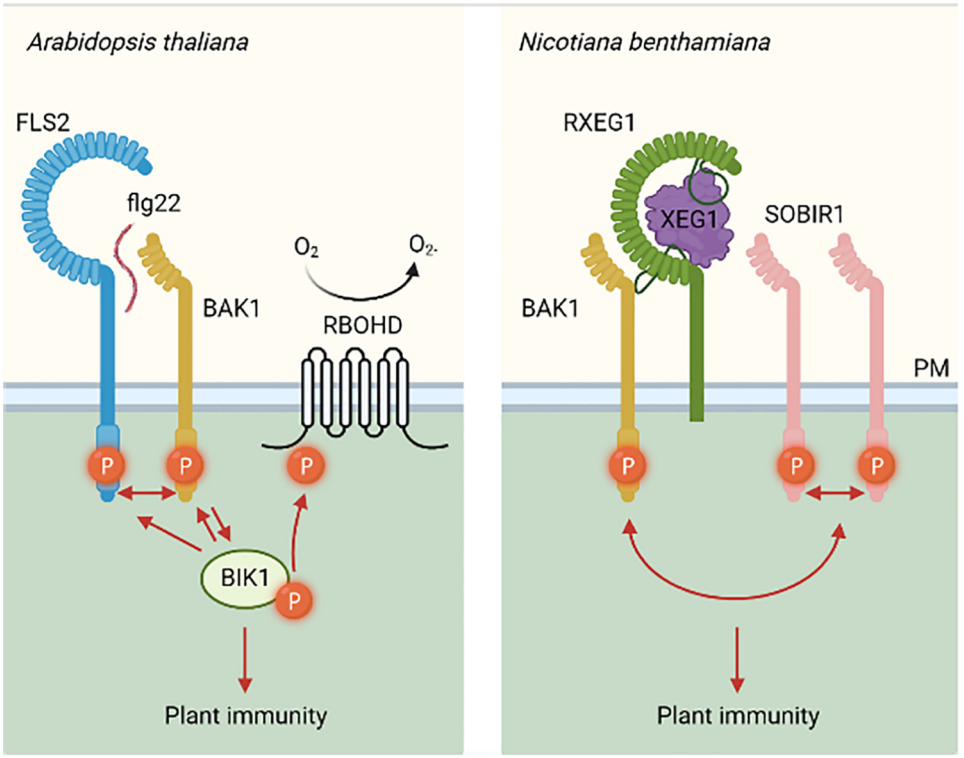

Figure 1. Activation of receptor-like kinase (RLK) and receptor-like protein (RLP) signaling. Left panel. The arabidopsis leucine-rich repeat (LRR)-RLK FLAGELLIN-SENSING 2 (FLS2) directly detects the conserved epitope flg22 derived from bacterial flagellin. Flg22 binding to the LRR domain of FLS2 generates a novel recognition surface on FLS2, which allows its interaction with the LRR domain of BRI-ASSOCIATED KINASE 1 (BAK1). In this way, flg22 is sandwiched between the LRR domains of FLS2 and BAK1. The receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1), which associates with the cytoplasmic kinase domains of both FLS2 and BAK1, is phosphorylated by activated BAK1. BIK1, in turn, phosphorylates both FLS2 and BAK1. Hereafter, activated BIK1 is released from the FLS2/BAK1 complex and phosphorylates the RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) oxidase enzyme at its N terminus, leading to the generation of extracellular reactive oxygen species (ROS). Right panel. The *Nicotiana benthamiana* LRR-RLP RESPONSE TO XEG1 (RXEG1) is the receptor of XEG1, which is an effector secreted by the soybean pathogen *Phytophthora sojae*. The LRR domain of RXEG1 contains two loop-out regions, through which RXEG1 binds XEG1. XEG1 binding leads to conformational changes in RXEG1, which result in an increased flexibility of the C-terminal loop-out region and four LRRs, thereby allowing the LRR domain of BAK1 to directly bind to this flexible region of RXEG1. SUPPRESSOR OF BIR1-1 (SOBIR1) forms homodimers in the resting state and shows low levels of auto-phosphorylation. SOBIR1 trans-phosphorylates BAK1 upon its recruitment to RXEG1, after which BAK1 in its turn phosphorylates SOBIR1. These trans-phosphorylation events lead to the full activation of the SOBIR1/BAK1-containing immune complex. The red circle with 'P' inside represents phosphorylation. Abbreviation: PM, plasma membrane.

lycopersici, possesses 31 LRRs in its ECD and these LRRs are predicted to be separated into two domains of 27 N-terminal LRRs and 4 C-terminal LRRs by a loop-out region [58,59]. The mechanism of ExIP perception and subsequent activation of the LRR-RLP-containing complex by BAK1 recruitment have remained unidentified until recently, when Sun and coworkers revealed the crystal structure of the extracellular XEG1-RXEG(LRR)-BAK1(LRR) complex [60]. The effector XEG1, which is a glycoside hydrolase 12 family protein with xyloglucanase activity, produced by the soybean pathogen *Phytophthora sojae*, acts as an important virulence factor during *P. sojae* infection [61]. The *N. benthamiana* LRR-RLP RXEG1 was later identified as the receptor of XEG1 and recognition of XEG1 by RXEG1 results in the activation of various immune responses, including ROS accumulation,

transcriptional induction of defense-related genes, and activation of a HR [62]. Notably, these immune responses depend on the co-receptors SOBIR1 and BAK1 [62]. Conceivably, the crystal structure of XEG1-RXEG1(LRR) illustrates that RXEG1 contains two loop-out regions in its ECD, named RXEG1(N-loop-out/NL) and RXEG1(island domain/ID) [60] (Figure 1). Binding of XEG1 by RXEG1 is predominantly mediated by these two loops, which form a kind of latches that are inserted into the active site groove of XEG1, thereby inhibiting its enzymatic activity and suppressing infection by *P. sojae*. XEG1 binding to RXEG1 causes structural flexibility of the C-terminal ID of RXEG1 and its last four LRRs and this increased flexibility allows an interaction to take place between RXEG1 and BAK1. Subsequent proximity of the kinase domains of SOBIR1 and BAK1 is proposed to facilitate their trans-phosphorylation, thereby initiating downstream immune signaling [60] (Figure 1).

Different from flg22, which binds to both FLS2(LRR) and BAK1(LRR), XEG1 only binds to RXEG1 (LRR) and is located far away from the site where BAK1(LRR) is recruited (Figure 1). In addition, flg22 binding does not induce a conformational change in FLS2(LRR), whereas the association with XEG1 leads to conformational changes in RXEG1(ID), which allow BAK1(LRR) binding to RXEG(ID) and the four C-terminal LRRs of RXEG1 [53,60]. This allosteric mechanism in which the ID is involved might be conserved for BAK1 recruitment by other LRR-RLPs, including the tomato **Cf proteins**, as the last four LRRs of RXEG1 and the ID are highly conserved in all RLPs. Notably, an ID is present at a very similar position in all LRR-RLPs that are involved in immunity, including arabidopsis RLP42 and INR [51]. RLP42, which is the receptor of fungal endopolygalacturonase (PG) and its epitope pg9, also requires SOBIR1 and BAK1 to trigger plant defense. Deletion of the ID in RLP42 results in the failure of pg9-induced BAK1 recruitment and, thereby, the absence of pg9-triggered immune responses in arabidopsis [63]. In addition, INR, which is an immune receptor identified in tobacco, mediates the activation of immune responses to a specific herbivore-associated molecular pattern (HAMP) from caterpillar oral secretions. These immune responses depend on SOBIR1 and BAK1 and also, in this case, the ID in INR is required for its functionality [64–66]. More interestingly, Cf-4 shares an identical ID with Cf-9 but does not respond to Avr9 and vice versa. This also holds for Cf-2 and Cf-5, which indicates that the ligand recognition specificity of Cf proteins resides at their N terminus, whereas their ID is required for BAK1 recruitment [67–69]. Indeed, ample flexibility around the region of the ID of Cf proteins was already predicted by van der Hoorn and coworkers [70], who performed homology modeling of the Cf-9 protein based on the crystal structures of bean polygalacturonase-inhibiting protein (PGIP) and the bacterial LRR protein internalin A (InlA). These findings collectively explain how BAK1 recruitment to a plethora of highly divergent ligand/RLP complexes might take place, as in all cases their ID is conserved.

Activation of RLK- and RLP-containing immune complexes, and their signaling partners, by phosphorylation

Plant cell-surface immune receptors, as well as their associated signaling partners, are subjected to tight regulation. In relation to this, phosphorylation is crucial for the activation of receptor complexes and the subsequent transduction of downstream immune signaling. Plant RLKs are classified into so-called 'RD' and 'non-RD' kinases, based on whether an arginine (Arg/R) residue precedes the catalytic aspartate (Asp/D) residue in their catalytic segment [5,71]. Most of the plant RLKs are non-**RD kinases**, which generally lack strong auto-phosphorylation activities [72]. An example of such a kinase is FLS2, which has been reported to exhibit no, or only a weak, auto-phosphorylation activity and, therefore, by itself FLS2 fails to phosphorylate downstream signaling components [73]. In contrast, RD kinases, such as CERK1 and BAK1, are activated by phosphorylation at one or more residues in their activation segment and show strong auto-phosphorylation activity [71]. Generally, non-RD kinases recruit a regulatory RD kinase, such as CERK1 or BAK1, to promote their phosphorylation and become signaling-competent

[72]. However, just recently, it was shown that EFR, having a non-RD kinase domain, uses a non-catalytic activation mechanism for the activation of its co-receptor BAK1. It was observed that BAK1 first phosphorylates the kinase domain of EFR in its activation loop, thereby stabilizing the active conformation of EFR and subsequently enabling EFR to allosterically activate BAK1 [74].

Arabidopsis BOTRYTIS-INDUCED KINASE 1 (BIK1), which is a member of the RLCK class VII subfamily 8 (RLCK-VII-8), plays a crucial role in plant immunity [75–77]. In the resting state, BIK1 associates with both BAK1 and FLS2, whereas BAK1 is sequestered from FLS2. Upon flg22 perception, BAK1 is recruited by FLS2 [73] and, meanwhile, BAK1 initiates auto-phosphorylation at tyrosine (Tyr/Y) 403; this phosphorylation is essential for BAK1 function in plant immune signaling, but not for signaling in plant development [78]. Subsequently, activated BAK1 phosphorylates BIK1 and the cytoplasmic kinase domain of FLS2, after which BIK1 phosphorylates both FLS2 and BAK1 (Figure 1). Activated BIK1 subsequently dissociates from the FLS2/BAK1 complex and activates additional downstream signaling components by phosphorylation [79].

The arabidopsis LysM-RLK LYK5, which exhibits a much higher chitin-binding affinity than the LysM-RLK CERK1, is the primary chitin receptor [80]. Chitin perception by LYK5 induces the dimerization of LYK5 and CERK1 and, after being auto-phosphorylated, activated CERK1 trans-phosphorylates LYK5 at its intracellular domain, which leads to the relocalization of LYK5 from the plasma membrane (PM) to late endocytic compartments [81]. Notably, auto-phosphorylation of threonine (Thr/T) 479 in the activation segment of *At*CERK1 is directly involved in the regulation of its kinase activity [82]. Intriguingly, reminiscent of FLS2/BAK1-mediated signaling, the LYK5/CERK1 complex also requires BIK1 for transducing downstream immune signals [75]. Activated CERK1 directly phosphorylates BIK1, leading to the dissociation of BIK1 from the kinase domain of CERK1 and subsequent phosphorylation of downstream signaling components [83].

In addition to BAK1, SOBIR1 also has a general regulatory role in plant immunity mediated by LRR-RLPs [23]. *In vitro* phosphorylation assays have shown that SOBIR1 is a dual-specificity RD kinase, as *At*SOBIR1 has been shown to auto-phosphorylate at serine (Ser/S), Thr, and Tyr residues [84]. In line with *At*SOBIR1, its orthologs *Nb*SOBIR1, *Sl*SOBIR1, and *Sl*SOBIR1-like also exhibit strong auto-phosphorylation activity [84–86]. *Nb*SOBIR1 T522, as well as its analogous residues in tomato SOBIR1 and SOBIR1-like, present in the activation segment of the kinase domain of SOBIR1, are essential for the intrinsic kinase activity of SOBIR1 [86]. In addition to auto-phosphorylation, reciprocal trans-phosphorylation events also take place between SOBIR1 and BAK1. It has been reported that upon performing *in vitro* phosphorylation assays, SOBIR1 can directly phosphorylate BAK1 and the intrinsic kinase activity of SOBIR1 is required for this trans-phosphorylation event. Accordingly, BAK1 can also directly phosphorylate SOBIR1 and this *in vitro* trans-phosphorylation event again depends on the kinase activity of BAK1 [85,86]. Trans-phosphorylation between SOBIR1 and BAK1 probably results in both obtaining a unique phosphorylation status, which is proposed to lead to the full activation of SOBIR1/BAK1-containing immune complexes [87] (Figure 1).

Strikingly, increasing evidence suggests that the regulatory RLK BAK1 is merely an activator of primary receptors that perceive their corresponding ligand, while BAK1 itself does not initiate immune signaling. Therefore, downstream signaling specificity is determined by the kinase domain of the primary receptor, which is the SOBIR1 kinase domain in the case of signaling by all LRR-RLPs involved in immunity and, for example, the kinase domain of FLS2 and EFR. This notion is reinforced by the study of Hohmann and coworkers [88], who showed that replacing the ECD of the BAK1-dependent LRR-RLKs BRASSINOSTEROID INSENSITIVE 1 (BRI1), HAESA, or ERECTA, with the ECD 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.

Although sharing their co-receptors SOBIR1 and BAK1, some LRR-RLPs induce stronger immune responses than others upon recognition of the matching ExIP. Well-known examples are the resistance proteins Cf-4 and Cf-9, which trigger a strong and fast HR in tomato, *N. benthamiana*, and tobacco, upon perception of their matching ligands, Avr4 and Avr9, respectively [67,89]. An explanation for this difference could be that LRR-RLPs constitutively associate with different amounts of SOBIR1(/SOBIR1-like) for their signaling, thereby triggering immune responses with different intensities. For instance, the HR triggered by the tomato LRR-RLP Cf-2 is much weaker and slower than the HR triggered by Cf-9 [90] and, accordingly, the amount of SOBIR1 protein that co-purifies with Cf-2 is much less than the amount co-purifying with Cf-9 [49]. Typically, LRR-RLPs interact with SOBIR1 through the GxxxGxxxG dimerization motif that is present in the TM of SOBIR1 and in the TM of the various Cf proteins [22,68]. However, the amino acid sequence of the cytoplasmic tail of Cf-2 and Cf-5, with the latter also triggering a slow and weak HR, is very similar, but very much diverges from that of Cf-4 and Cf-9 [68]. Of note, Cf-4 and Cf-9 share an identical TM and cytoplasmic tail and they both trigger a strong HR. Therefore, the intensity of the immune output might be determined by the amount of SOBIR1 protein that is constitutively recruited and the cytoplasmic tail could determine the affinity of the Cf protein for SOBIR1. Ve1 is another well-studied LRR-RLP from tomato, which specifically recognizes the Ave1 effector secreted by the fungal pathogen *Verticillium dahliae*. Truncation of the cytoplasmic tail of Ve1 or replacement of this tail with that of Ve2 results in loss of its functionality [91]. Strikingly, EFR, which confers broad-spectrum resistance to bacterial pathogens, obtains the ability to trigger a Cf-9-like HR and constitutively recruits SOBIR1 when its cytoplasmic kinase domain is replaced with the cytoplasmic tail of Cf-9 [92].

The various roles of RLCKs downstream of RLKs and RLPs

Plants have evolved numerous RLCKs that functionally and physically interact with the cytoplasmic kinase domain of RLKs to regulate plant immunity [93]. Accordingly, activation of ExTI triggers a suite of downstream signaling events, including the rapid phosphorylation of RLCKs [6,13].

BIK1 is a positive regulator of the defense response initiated by RLKs, such as FLS2, EFR, and LYK5, as flg22-, elf18-, and chitin-stimulated ROS accumulation and callose deposition are strongly dampened in arabidopsis *bik1* mutants [6,73,75]. RLP23, which is an arabidopsis 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 the common regulators SOBIR1 and BAK1 for its signaling [55]. Unexpectedly, BIK1 plays a negative role in RLP23 signaling, as arabidopsis *bik1* knockout mutants exhibit an increased nlp20/RLP23-triggered ROS burst and a higher ethylene, camalexin, and salicylic acid production [94] (Figure 2). Similar results were obtained for the combination of the LRR-RLP RLP42 and the fungal polygalacturonase PG3 [94].

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 the activation of LRR-RLP/SOBIR1-associated immune responses, but a less important role in the LRR-RLK-triggered signaling pathway [18]. Furthermore, PBL13, belonging to RLCK-VII-6, is involved in RLK signaling, whereas this RLCK is not required for RLP function [18,95]. These observations suggest that LRR-RLPs and LRR-RLKs have different, maybe even opposite, requirements concerning their downstream signaling by RLCKs and that negative crosstalk might take place between the two signaling pathways.

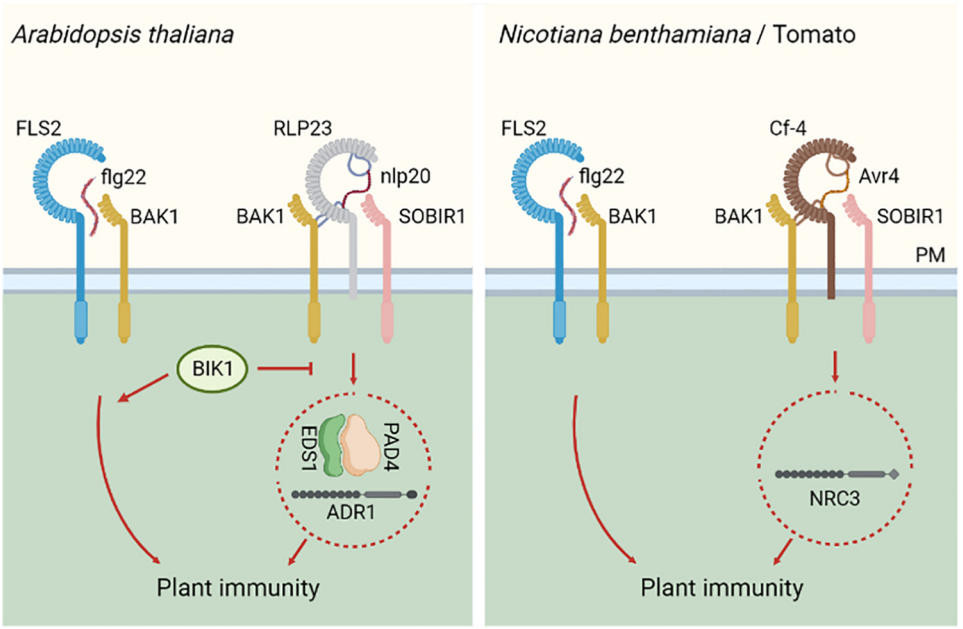

Figure 2. Extracellularly triggered immunity (ExTI) mediated by leucine-rich repeat (LRR)-receptor-like proteins (RLPs) requires components of the intracellularly triggered immunity (InTI) signaling pathway. In arabidopsis (left panel), LRR-RLPs such as RLP23 constitutively associate with the LRR-receptor-like kinase (RLK) SUPPRESSOR OF BIR1-1 (SOBIR1) in the resting state and recruit the LRR-RLK BRI-ASSOCIATED KINASE 1 (BAK1) upon ligand perception. Downstream signaling requires the lipase-like proteins ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN-DEFICIENT (PAD4) and the helper nucleotide-binding, leucine-rich repeat protein (NLR) ACTIVATED DISEASE RESISTANCE 1 (ADR1), which are important signaling components of InTI. Nevertheless, the EDS1/PAD4/ADR1 node appears to have a less prominent role in signaling by LRR-RLKs, such as FLAGELLIN-SENSING 2 (FLS2). The receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1) is a positive regulator of FLS2 signaling, whereas BIK1 is a negative regulator of RLP23 signaling. In *Nicotiana benthamiana* and tomato, and possibly also in other solanaceous plants in which the LRR-RLP Cf-4 is functional (right panel), the helper NLR NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 3 (NRC3) plays an essential role in downstream signaling mediated by Cf-4. However, NRC3 is not required for FLS2 signaling. Note that NRCs are absent in arabidopsis. Abbreviation: PM, plasma membrane.

Regulation of RLK and RLP signaling by InTI

Although cell-surface receptors and NLRs are activated through distinct mechanisms at different subcellular locations, ExTI and InTI eventually converge into several common downstream defense outputs, including MAPK activation, ROS production, calcium influx, and the accumulation of pathogenesis-related (PR) proteins [15].

In arabidopsis, RLP23 has been reported to require the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/PHYTOALEXIN-DEFICIENT 4 (PAD4)/ACTIVATED DISEASE RESISTANCE 1 (ADR1) node for its signaling (Figure 2). EDS1 is a well-known signaling component of InTI and plays a pivotal role in basal immunity of not only the model plant arabidopsis but also of important crop plants [96,97]. Arabidopsis EDS1 physically associates with PAD4, and the EDS1/PAD4 complex requires the **helper NLR** ADR1 to mediate basal immunity [98,99] (Figure 2). The production of ROS and ethylene, in addition to callose deposition triggered by nlp20/RLP23, is strongly dampened in arabidopsis *pad4* and *eds1* mutants, whereas in these mutants, upon activation of FLS2 by flg22, these responses are only reduced. This indicates that the EDS1/PAD4/ADR1 node plays an essential role in LRR-RLP signaling (Figure 2) but has a less important role in LRR-RLK signaling [18].

It has been unveiled that Cf-4/SOBIR1-initiated ExTI also requires InTI-associated components for downstream signaling. Different from ExTI-related responses that activate basal and weak immunity, Avr4/Cf-4 signaling is associated with a strong HR [67]. This strong HR is significantly compromised in *N. benthamiana* when the genes encoding the NB-LRR PROTEIN REQUIRED FOR HR-ASSOCIATED CELL DEATH 2 and 3 (NRC2/3) are knocked out. Transient expression of NRC3 complements the Avr4/Cf-4-triggered HR in the knockout line, indicating that NRC3 is the main NLR participating in innate immunity activated by LRR-RLP/SOBIR1 complexes in solanaceous plants [100] (Figure 2). Notably, NRCs are not present in arabidopsis but have been identified in kiwifruit, coffee, monkey flower, ash tree, and some solanaceous species, including *N. benthamiana*, tomato, pepper, and potato [101]. It is worth noting that NRC3 is not required for flg22/FLS2-induced ROS accumulation and MAPK activation in *N. benthamiana* [102]. These observations reinforce the notion that LRR-RLPs employ different downstream signaling components when compared with LRR-RLKs. Surprisingly, the Cf-4/Avr4-triggered HR is neither impaired in *N. benthamiana eds1*, *nrg1*, or *pad4* single mutants, nor in an *eds1 pad4 sag101a sag101b* (*epss*) quadruple mutant [103]. These results lead to the suggestion that, unlike for LRR-RLP signaling in arabidopsis, the EDS1/PAD4/ADR1 node does not play a role, or only a minor role, in LRR-RLP signaling in the Solanaceae. Possibly in this family the NRCs play the role of this node (Figure 2). Still, it has been reported by Wang and coworkers that ADR1, EDS1, and PAD4 mediate stomatal immunity in both *N. benthamiana* and arabidopsis, providing evidence for some role of these proteins in *N. benthamiana* immunity [104].

A recent study has reported that in arabidopsis the HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) resistosome, which is formed by the nucleotide-binding (NB)-LRR protein ZAR1, RESISTANCE-RELATED KINASE 1 (RKS1), and the RLCK PBL2 upon bacterial infection, functions as a calcium-permeable channel in the PM to trigger immune signaling [105–107]. Calcium influx is a fast regulatory response and contributes to the activation of plant immunity [13,15]. ZAR1 carries a so-called MADA motif in its N-terminal CC domain and, intriguingly, this motif is also conserved in the NRCs and is required for NRC4 auto-activity, which leads to a HR in *N. benthamiana* [108]. 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 PM [109]. This raises the possibility that, similar to ZAR1, NRCs form a cation channel to allow Ca^{2+} to enter the cytoplasm, thereby triggering PCD. 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 *F. fulva* that secrete Avr4.

Taken together, emerging evidence has pinpointed that the molecular mechanism of LRR-RLP/SOBIR1-mediated ExTI differs from that of LRR-RLK-mediated ExTI, as there is a differential requirement of RLCKs and InTI-related components downstream of these receptors. Importantly, recent evolutionary analyses on the signaling partners of plant cell-surface receptors involved in immunity have indicated that cytoplasmic kinases, such as RLCKs and MAPKs, are very ancient and evolved before the emergence of land plants, whereas EDS1 and helper NLRs have emerged more recently and have likely been integrated into LRR-RLP signaling pathways during evolution [51].

Crosstalk between immune signaling pathways initiated by RLKs and RLPs

Crosstalk between various cell-surface receptor-triggered immune signaling pathways has recently been recognized as a common mechanism to modulate plant immunity [13,20,76]. In arabidopsis, the LysM-RLK LYK5 recruits the co-receptor CERK1 to activate plant immune responses upon the perception of chitin. However, FLS2 forms a heterodimer with BAK1 in

response to the binding of bacterial flagellin or flg22, leading to the activation of downstream intracellular signaling [38,39,80,81,110]. Intriguingly, recent work has shown that the activation of FLS2 by bacterial pathogens or by the flg22 peptide 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 occurs 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 upon flg22 elicitation turns CERK1 into a primed state, which prepares the host for future fungal attacks [111] (Figure 3).

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 [112]. Activation of FLS2 by flg22 induces the phosphorylation of NIK1 at T474 by BAK1, which further facilitates mounting host resistance to viruses in an NIK1-dependent manner [113]. 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 (Figure 3). The mechanism by which NIK1 inversely regulates the different pathways remains to be explored.

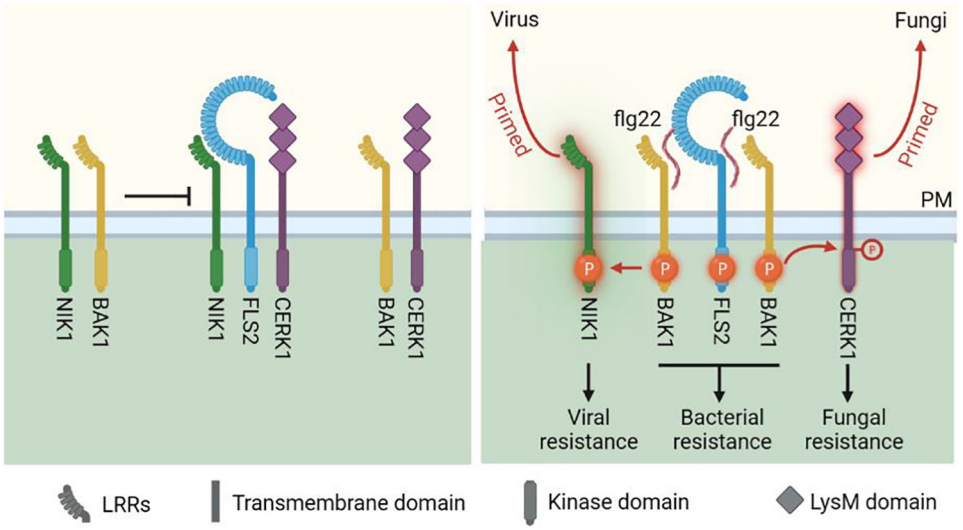

Figure 3. Crosstalk between immune signaling pathways initiated by receptor-like kinases (RLKs). In arabidopsis, the chitin co-receptor CERK1 physically interacts with both FLAGELLIN-SENSING 2 (FLS2) and BRI-ASSOCIATED KINASE 1 (BAK1) in the resting state (left panel). Likewise, NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE1 (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 to the leucine-rich repeats (LRRs) of FLS2 (right panel), BAK1 is recruited by FLS2, leading to the 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 CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) only in the intracellular juxtamembrane region and this phosphorylation primes the host for its response to potential fungal attacks. However, 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 an NIK1-dependent manner. The red open and filled circles with 'P' inside represent low-level and high-level phosphorylation, respectively. Receptors with red boundaries are activated in such a way that they prime the plant. Abbreviations: LysM, lysin motif; PM, plasma membrane.

As RLP, Cf-4 lacks an intracellular kinase domain and constitutively interacts with SOBIR1 [22,49]. Reminiscent of FLS2 signaling, the Cf-4/SOBIR1 complex recruits BAK1 upon detection of Avr4 by Cf-4 [56]. Recently, it has been 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 burst that is induced by this flagellin epitope, but similar to the ROS burst induced by Avr4 in *N. benthamiana:Cf-4* [114]. Notably, FLS2 neither interacts with SOBIR1 nor requires SOBIR1 for its functionality [49,114,115]. RLCKs are key players in linking cell-surface receptors with downstream signaling components, including RBOHs [93]. Therefore, a possible crosstalk between RLP/SOBIR1- and FLS2-triggered immune signaling in *N. benthamiana* might take place through yet unidentified RLCKs. One speculation could be that some RLCKs that, for example, transcriptionally regulate the second ROS burst, have a higher affinity for the cytoplasmic kinase domain of SOBIR1 than for this domain of FLS2 and that these RLCKs will only interact with the kinase domain of FLS2 to mediate the generation of the second ROS peak upon flg22 ligand perception, when the SOBIR1 protein is absent (Figure 4). This suggests that the RLP/SOBIR1 immune signaling pathway might at least partially negatively regulate the FLS2-triggered pathway, leading to resistance. As mentioned earlier, BIK1 appears to promote FLS2 signaling, whereas this RLCK suppresses RLP/SOBIR1 signaling. Further studies on identifying the possible differential interactors of FLS2 in *N. benthamiana:Cf-4* and in the *N. benthamiana:Cf-4 sobir1/sobir1-like* mutant plants, should shed light on how this potential crosstalk between FLS2/BAK1- and RLP/SOBIR1/BAK1-mediated signaling pathways actually takes place.

Outstanding questions

Why did plants evolve numerous LRR-RLKs, next to a plethora of LRR-RLPs requiring SOBIR1, to cope with microbial infection?

Why do plant genomes encode such a large number of RLCKs? Why are there so many different RLCKs involved in immune signaling and how is their specific functioning and subcellular localization determined?

Which RLCKs play a role downstream of LRR-RLP/SOBIR1 complexes and what kind of (non-redundant) functions do they have in the cellular machinery to mount resistance to pathogens?

How is (negative) crosstalk between LRR-RLP/SOBIR1 complexes and RLKs established?

Concluding remarks and future perspectives

Over the last two decades, several lines of research have largely contributed to our further understanding of the interaction between plants and pathogenic microbes [6,13,20]. Particularly, the publication of the zigzag model in 2006 has resulted in the formulation of a general concept

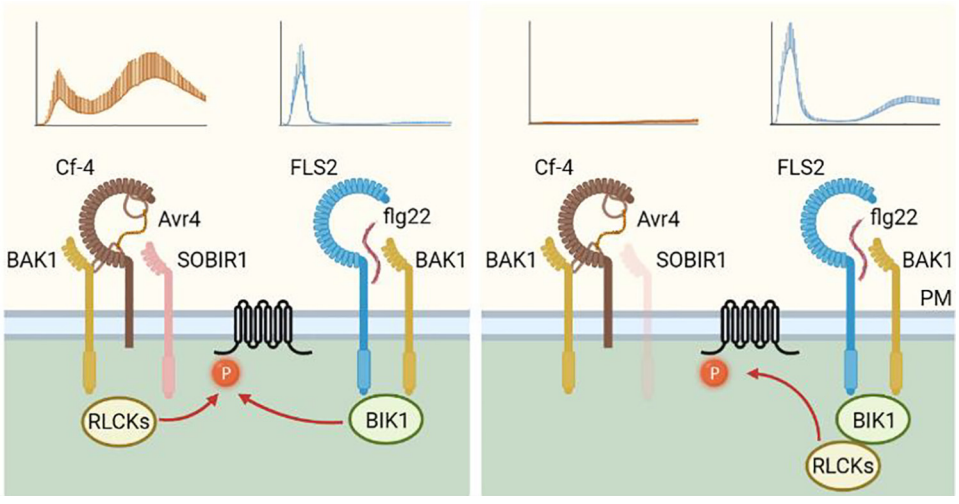

Figure 4. Crosstalk between immune signaling pathways initiated by receptor-like proteins (RLPs) and receptor-like kinases (RLKs). In *Nicotiana benthamiana*, the matching Avr4/Cf-4 combination triggers a biphasic reactive oxygen species (ROS) burst, while flg22/FLAGELLIN-SENSING 2 (FLS2) triggers a rapid and monophasic ROS burst (left panel). The Avr4/Cf-4-induced ROS burst is completely abolished when *SOBIR1* is knocked out (right panel). Intriguingly, an unexpected second sustained ROS burst, triggered by flg22/FLS2, appears in *N. benthamiana sobir1* knockout plants. This crosstalk between Cf-4/SUPPRESSOR OF BIR1-1 (SOBIR1) signaling and FLS2 signaling might take place via yet unidentified receptor-like cytoplasmic kinases (RLCKs). Abbreviations: BAK1, BRI-ASSOCIATED KINASE 1; BIK1, BOTRYTIS-INDUCED KINASE 1; PM, plasma membrane.

concerning resistance and susceptibility in plant–pathogen interactions, which has further stimulated numerous research efforts on understanding the fundamentals of plant innate immunity [1,3,10,116,117]. Interestingly, the LRR-RLP/SOBIR1 complex, which was initially considered to be structurally and functionally identical to LRR-RLKs, albeit consisting of two components [22,23], has now been demonstrated to initiate an immune signaling pathway that is intrinsically different from the pathway that is employed by canonical LRR-RLKs, as both types of cell-surface immune receptors activate signaling networks that overlap but differ in certain aspects [18,94]. This difference might be due to a differential involvement of downstream RLCKs and helper NLRs and allows mutual (negative) crosstalk between the two pathways to take place. Further investigation of how plants utilize groups of RLPs and RLKs simultaneously to modulate and transduce immune signals from the extracellular space into the cytoplasm, and how exactly these signals are further potentiated by NLR-triggered responses, will lead to a more complete understanding of the plant immune system (see Outstanding questions).

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Declaration of interests

The authors declare no competing interests.

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