Structure-Function Analysis of Immune Receptor *AtRLP23* with Its Ligand *nlp20* and Coreceptors *AtSOBIR1* and *AtBAK1*

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Pattern-triggered immunity is an inherent feature of the plant immune system. Recognition of either microbe-derived surface structures (patterns) or of plant materials released due to the deleterious impact of microbial infection is brought about by plasma membrane pattern recognition receptors (PRRs). PRRs composed of leucine-rich repeat (LRR) ectodomains are thought to mediate sensing of proteinaceous patterns and to initiate signaling cascades culminating in the activation of generic plant defenses. In contrast to LRR receptor kinases, LRR receptor proteins (LRR-RPs) lack a cytoplasmic kinase domain for initiation of downstream signal transduction. LRR-RPs form heteromeric constitutive, ligand-independent complexes with coreceptor SOBIR1. Upon ligand binding to LRR-RPs, recruitment of coreceptor SERK3/BAK1 results in formation of a ternary PRR complex. Structure-function analysis of LRR-RP-type PRRs is missing. *AtRLP23* constitutes an LRR-RP PRR that mediates recognition of a peptide motif (*nlp20*) found in numerous bacterial, fungal, and oomycete necrosis and ethylene-inducing peptide 1-like proteins (NLPs). We here report the use of a series of *AtRLP23* variants to decipher subdomains required for ligand binding and interaction with coreceptors *AtSOBIR1* and *AtBAK1*, respectively. Deletion of LRR1 or LRR3 subdomains efficiently abrogated the ability of *AtRLP23* receptor variants to confer *nlp20* pattern sensitivity, to bind *nlp20*, and to recruit *AtBAK1* into a ternary PRR complex. This suggests that the very N-terminal part of the *AtRLP23* ectodomain is crucial for receptor function. Deletion of the intracellular 17-amino-acid tail of *AtRLP23* reduced but did not abolish receptor function, suggesting an auxiliary role of this domain in receptor function. We further found that interaction of *AtRLP23* and other LRR-RP-type PRRs with *AtSOBIR1* does not require the *AtRLP23* LRR ectodomain but is brought about by a GxxxG protein dimerization motif in the transmembrane domain and a stretch of negatively charged glutamic acid residues in the outer juxta-membrane domain of the receptor. Further, *AtRLP23* levels were found to be unaltered in *Atsobir1-1* mutant genotypes,

suggesting that *AtSOBIR1* does not act as a protein scaffold in stabilizing LRR-RP-type PRRs in *Arabidopsis*.

Keywords: elicitors, MAMPs, PAMPs

Plants have evolved an innate immune system to counteract the omnipresent threat of microbial diseases (Böhm et al. 2014a; Couto and Zipfel 2016; Dodds and Rathjen 2010; Thomma et al. 2011). A fundamental step toward disease prevention and activation of immune defenses is the perception of putative invaders by the plant (Böhm et al. 2014a; Couto and Zipfel 2016; Saijo et al. 2018). For this purpose, plants have evolved plasma membrane-localized pattern recognition receptors (PRRs) that recognize danger-associated molecules derived from either pathogens directly (pathogen-associated molecular patterns [PAMPs] or microbe-associated molecular patterns) or indirectly through microbial damage-associated release of host materials (damage-associated molecular patterns) (Dangl et al. 2013; Gust et al. 2017; Jones and Dangl 2006). Recognition of such signals activates pathogen-nonspecific, generic, plant cell autonomous defense responses referred to as pattern-triggered immunity (PTI) (Boller and Felix 2009; Jones and Dangl 2006). In addition, plant immunomodulatory peptide cytokines (phytocytokines) produced upon PRR activation fine-tune the plant's immune response to attempted microbial invasion (Gust et al. 2017).

Plant PRRs can be categorized according to the molecular nature of their ectodomains (Couto and Zipfel 2016; Saijo et al. 2018). PRRs harboring lysin motif ectodomains are considered to recognize carbohydrate ligands such as fungal chitin or bacterial peptidoglycan (Kelly et al. 2017). Plasma membrane leucine-rich repeat (LRR) receptors make up a large portion of plant PRRs (Couto and Zipfel 2016; Gust and Felix 2014). Their ectodomains typically comprise variable repeats of 20 to 30 LR amino acid stretches that are implicated in recognition of predominantly proteinaceous patterns (Zhang and Thomma 2013). LRR receptors are designated as either LRR kinases (LRR-RKs) or LRR receptor proteins (LRR-RPs) depending on the presence (as in RKs) or absence (as in RPs) of an intracellular kinase domain (Couto and Zipfel 2016; Gust and Felix 2014). Initiation of intracellular signal transduction cascades through either LRR receptor type requires ligand-dependent recruitment of coreceptors belonging to the LRR-RK subfamily of SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs) that includes SERK3/BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) (Böhm et al. 2014a; Couto and Zipfel 2016; Saijo et al. 2018). Members of this protein

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family contain only five extracellular LRRs that are linked via a transmembrane domain to an intracellular kinase domain.

Arabidopsis thaliana LRR-RK FLAGELLIN SENSING 2 (*AtFLS2*) detects potential bacterial invaders by recognizing flg22, a 22-amino acid N-terminal epitope conserved within most proteobacterial flagellins (Felix et al. 1999; Gómez-Gómez and Boller 2000). *AtFLS2* recruits *AtBAK1* in a flg22-dependent manner from a complex with BAK1-INTERACTING KINASE 2 (*AtBIR2*), a negative regulator of PTI (Halter et al. 2014). Subsequently, *AtBAK1* phosphorylates BOTRYTIS-INDUCED KINASE 1 (*AtBIK1*), a receptor-like cytoplasmic kinase, and *AtBIK1* phosphorylates both *AtFLS2* and *AtBAK1*, thereby initiating further downstream signaling and immune activation (Lu et al. 2010). X-ray crystallography analysis of flg22 in complex with *AtFLS2* and *AtBAK1* ectodomains revealed ligand binding along the LRR domain of *AtFLS2* covering LRR3 to LRR16 (Sun et al. 2013). It has been suggested that flg22 acts as a molecular glue between its receptor and coreceptor, with its C terminus sandwiched between the LRR domain of *AtFLS2* and the N-terminal region of *AtBAK1* (Sun et al. 2013).

Like LRR-RKs, LRR-RP-type PRRs have been shown to require ligand-dependent interaction with BAK1 or other SERKs for downstream signaling (Albert et al. 2015; Postma et al. 2016; Zhang and Thomma 2013; Zhang et al. 2013). Unlike LRR-RKs, LRR-RPs lack a cytoplasmic signaling domain. Because LRR-RPs (*AtRLP1*, *AtRLP23*, *AtRLP30*, and *AtRLP42*) form constitutive heterodimers with SUPPRESSOR OF BIR1-1/EVERSHED (*AtSOBIR1/EVR*), an LRR-RK with five extracellular LRRs (Albert et al. 2015; Jehle et al. 2013b; Zhang et al. 2013, 2014), it has been hypothesized that LRR-RPs and *AtSOBIR1* constitute binary LRR-RKs (Gust and Felix 2014). However, a comprehensive survey of LRR-RP- and LRR-RK-mediated immune signaling pathways has recently revealed significant differences in response patterns and composition of signaling cascades mediated through either receptor type (Wan et al. 2019). For example, *AtBIK1*, previously identified as a positive regulator of PTI (Lu et al. 2010) mediated through activation of LRR-RK-type PRRs, has been shown to act as a negative regulator of PTI mediated through LRR-RP-type PRRs (Wan et al. 2019). These findings have challenged the concept of LRR-RPs serving roles merely as binary LRR-RKs, and have highlighted the fact that immune signaling networks display a rather high degree of plasticity in how plant immune activation can be achieved.

Notwithstanding, plant LRR-RP/*SOBIR1* heterodimers constitute functional PRRs (Gust and Felix 2014) with an extracellular ligand-binding domain supplied by LRR-RPs and an intracellular kinase domain supplied by *SOBIR1*. To which extent the *SOBIR1* ectodomain is implicated in ligand sensing remains open to date. Membrane dimerization motifs, termed GxxxG motifs and first reported from animal transmembrane receptor tyrosine kinases, are present in the *SOBIR1* transmembrane domain and have been shown to be important for its interaction with several immune receptors (tomato *SlCf4*, *SlEIX2*, and *SlVe1*) (Bi et al. 2016; Russ and Engelman 2000). In addition to its role in PTI signal transduction, *SOBIR1* may contribute to LRR-RP protein stability, because silencing in *Nicotiana benthamiana* of *NbSOBIR1* and *NbSOBIR1*-like resulted in reduced protein levels of heterologously expressed *SlCf4* and *SlVe1* receptors (Liebrand et al. 2013).

A significant number of LRR-RP proteins have been implicated in PTI activation (Couto and Zipfel 2016; Saijo et al. 2018). However, in most cases, the molecular identity of their ligands or a minimum immunogenic epitope within such patterns remains to be elucidated, thus impeding structural studies on ligand-receptor interactions. The PRR *AtRLP23* and its

ligand, nlp20, constitutes the only LRR-RP-type PRR/ligand pair to date, for which such an immunogenic motif has been identified and shown to physically bind via the receptor's ectodomain (Albert et al. 2015; Böhm et al. 2014b; Oome et al. 2014). Nlp20 is a conserved 20-amino-acid epitope found in numerous NECROSIS AND ETHYLENE-INDUCING PEPTIDE 1-LIKE-PROTEINS (NLPs), many of which are cytotoxic toxins that promote virulence of NLP-producing necrotrophic or hemibiotrophic fungi, oomycetes, or bacteria (Albert et al. 2015; Böhm et al. 2014b; Oome et al. 2014). NLP cytotoxicity on eudicot plants is brought about through its binding to eudicot plant-specific glycosylinositol phosphorylceramide sphingolipids (Lenarčič et al. 2017). In contrast, nlp20-induced PTI activation mediated through the LRR-RP *AtRLP23* is restricted to *Arabidopsis* and some related species of Brassicaceae (Albert et al. 2015). As shown for several LRR-RPs, nlp20/*AtRLP23* signaling requires constitutive, ligand-independent heteromeric complex formation of *AtRLP23* with *AtSOBIR1* (Albert et al. 2015).

X-ray crystallography approaches have repeatedly failed to resolve the molecular architecture of LRR-RP/*SOBIR1*/*BAK1* complexes in either ligand-bound or ligand-free form. This, and the finding that LRR-RK and LRR-RP-type PRRs differ in various aspects of PTI signaling (Wan et al. 2019), has prompted us to investigate structural requirements within *AtRLP23* for nlp20 ligand binding, and interaction with its coreceptors *AtSOBIR1* and *AtBAK1*. In this study, we have employed serial deletions of *AtRLP23* LRRs to delineate subdomains that are important for the aforementioned aspects of *AtRLP23* receptor function.

RESULTS

Structural organization of *AtRLP23*.

A combination of database analysis and manual inspection of the amino acid sequence of *AtRLP23* was used to model the structural organization of the protein. Following a 22-amino-acid signal peptide, a short N-terminal region precedes a series of 27 LRRs [consensus sequence (S/T)(G/S)x(I/L)Pxx(L/I/V/F/M)xx(L/I/V/P/F/M)xx] in the extracellular part of the receptor (Fig. 1A; Supplementary Fig. S1) (Albert et al. 2015). LRRs 22 and 23 are interrupted by an island domain (ID) of 51 amino acids. A negatively charged extracellular juxtamembrane domain (JM) enriched in glutamic acid residues separates the LRR domain from the transmembrane domain (TM). The TM stretch of 23 amino acids carries a GxxxGxxxG motif described to facilitate protein-protein interactions of membrane proteins (Bi et al. 2016; Russ and Engelman 2000). A small stretch of only 17 amino acids terminates the *RLP23* protein on the intracellular, cytoplasmic side (Fig. 1A).

The very N-terminal part of the *AtRLP23* ectodomain is crucial for conferring nlp20 sensitivity.

Our previous studies have provided evidence that the LRR ectodomain of *AtRLP23* physically binds nlp20 or derivatives thereof in vitro (Albert et al. 2015). In contrast to structural mapping approaches conducted in LRR-RKs, no particular regions of any LRR-RP PRR ectodomain have been implicated in ligand binding-coreceptor interaction. A series of *AtRLP23* deletion mutant cDNA constructs lacking either the N terminus (NT), the LRR-region, the ID, the JM, or the intracellular domain (IC) was generated in order to assess the importance of selected domains for nlp20 binding or for receptor interaction with coreceptors *AtSOBIR1* and *AtBAK1* (Fig. 1A). In addition, stretches of variable lengths within the LRR domain including or excluding the ID and the JM (Δ LRR2, Δ LRR2-3, Δ LRR2-21, Δ LRR2-ID, Δ LRR2-26, and Δ LRR2-JM) were deleted from full-length *AtRLP23* (Fig. 1A).

To assess the functionality of individual regions within *AtRLP23*, constructs encoding various mutant receptors were transformed into *Agrobacterium tumefaciens* and, upon bacterial infection, transiently expressed in *N. benthamiana*. Subsequently, transgenic *N. benthamiana* plants were tested for their ability to mount the production of the stress hormone ethylene in response to nlp20 (Fig. 1B). In addition, expression of mutant receptor proteins was monitored by protein blotting (Fig. 1C). Wild-type *N. benthamiana* is insensitive to nlp20 treatment due to the lack of a functional homolog of *AtRLP23* (Albert et al. 2015). However, *N. benthamiana* plants expressing full-length *AtRLP23* displayed a marked ethylene response following nlp20 treatment (Fig. 1B), thus confirming previous findings that transfer of a sole PRR confers pattern sensitivity to transgenic plants (Afroz et al. 2010; Albert et al. 2015; Dangl et al. 2013; Gust et al. 2010; Holton et al. 2015; Lacombe et al. 2010; Mendes et al. 2010; Schoonbeek et al. 2015; Schwessinger et al. 2015; Tripathi et al. 2014). Unchanged ethylene levels

detected in *N. benthamiana* plants treated with flg22 showed that expression of *AtRLP23* derivatives did not affect the ability of transgenic plants to respond to a pattern structurally unrelated to nlp20 (Fig. 1B).

Large deletion mutants (*AtRLP23*ΔLRR2-21 and *AtRLP23*ΔLRR2-26) or additional deletion of the extracellular JM (*AtRLP23*ΔLRR2-JM) resulted in complete loss of *AtRLP23* function (Fig. 1B). Likewise, deletion of LRRs 2-22 together with the ID (*AtRLP23*ΔLRR2-ID) yielded inactive receptor protein, suggesting strongly that most of the LRRs N terminal to the ID are required for function.

To assess the requirement of individual LRRs for receptor function, receptor mutant proteins lacking smaller fractions of the ectodomain were produced. Notably, deletion of the N-terminal region together with LRR1 (*AtRLP23*ΔNT-LRR1) already resulted in a complete loss of nlp20-induced ethylene production at concentrations as high as 1 μM nlp20 (Fig. 1B). This finding strongly suggests that this region is instrumental to *AtRLP23* receptor function. Deletion of LRR2 alone resulted in residual receptor activity (approximately 6.7% residual activity as calculated from the difference in values for elicitor concentrations required to trigger a half-maximum plant defense [EC₅₀] for activation of ethylene production) (Table 1) but deletion of LRR2 and LRR3 together (*AtRLP23*ΔLRR2-3) abolished the ability of this mutant to sense nlp20. This again hints at a fundamental importance of the very N-terminal region of the LRR domain in *AtRLP23* receptor function. In particular, the region spanning LRR1 and LRR3 defines a part of the receptor ectodomain that is critical for its function. The JM (*AtRLP23*Δ2-26) or ID (*AtRLP23*Δ2-IC) regions alone were both not sufficient to sense nlp20 (Fig. 1B).

We further assessed the functionality of 10 additional internally truncated *AtRLP23* variants that contained the first five LRRs but lacked different parts of the entire LRR domain. These studies revealed that any internal deletion also resulted in a complete loss of these mutant receptors to confer nlp20-inducible ethylene production to transgenic *N. benthamiana*. Altogether, these findings imply that any deletion within the *AtRLP23* ectodomain leads to loss of receptor function or proper topology of this PRR in the plasma membrane.

Deletion of the IC (*AtRLP23*ΔIC) resulted in no apparent reduction of this mutant protein to confer nlp20-inducible

Fig. 1. Structure function analysis of *AtRLP23*. **A**, Schematic representation of *AtRLP23* deletion mutant proteins used for structure function analysis. SP = signal peptide, NT = N-terminal domain, ID = island domain, JM = juxtamembrane domain, TM = transmembrane domain, and IC = intracellular domain; numbers indicate leucine-rich repeats. Constructs were overexpressed as green fluorescent protein (GFP) fusions in transiently transformed *Nicotiana benthamiana* plants (*p35S::AtRLP23-GFP*). Numbers in parentheses refer to amino acid residues within the *AtRLP23* sequence directly flanking deleted parts of the protein and correspond to positions in the mature protein. **B**, Ethylene accumulation after treatment with 1 μM nlp20, flg22, or water as control in *N. benthamiana* plants transiently transformed with the above constructs. Bars represent means ± standard deviation of three replicates. Asterisks indicate significant differences to water control treatments (* = P < 0.05 and ** = P < 0.01, Student's t test). Experiments were performed three times with similar results. **C**, Binding of biotinylated nlp24 ligand (nlp24-bio) to *AtRLP23* receptor deletion mutant proteins. *N. benthamiana* plants were transiently expressing RLP23-GFP as receptor, biotinylated nlp24 was used as ligand, and nonbiotinylated nlp24 as competitor of ligand binding. Images show protein blot analysis after cross-linking with ethylene glycol bis(succinimidyl succinate), protein extraction, and coimmunoprecipitation using GFP-trap beads. Blots from 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis were probed with anti-biotin antisera or GFP antisera. Representative examples selected from three independent repetitions are shown.

ethylene production to *At*RPL23-transgenic *N. benthamiana* plants at elicitor concentrations of 1 μ M (Fig. 1B). To assess a possible impact of this deletion on the efficacy of the mutant receptor, we conducted dose-response relationship studies using increasing concentrations of nlp20 to activate ethylene production. In all, 49.0 nM nlp20 triggered half-maximum ethylene production in *At*RPL23-transgenic *N. benthamiana*, whereas *At*RPL23 Δ IC-expressing plants required nlp20 concentrations of approximately 217 nM (Table 1). This suggests that deletion of the IC of *At*RPL23 caused measurable defects within the protein. However, residual activity of approximately 22.6% of this mutant protein suggested that this domain has auxiliary but no essential role in *At*RPL23 function.

The very N-terminal part of the *At*RPL23 ectodomain is crucial for nlp20 binding.

Loss of *At*RPL23 functionality in the deletion mutants tested may occur due to an inability of these mutant proteins to bind nlp20, their inability to interact with coreceptors *At*SOBIR1 and *At*BAK1, or both. To assess ligand-receptor interactions, an in vivo binding assay was performed using C-terminally biotinylated nlp24 (nlp24-bio). The nlp24 peptide contains the minimum immunogenic NLP epitope nlp20 and four additional C-terminal amino acids derived from the sequence of full-length NLP proteins. This linker region was employed to avoid possible sterical interference of the biotin tag with ligand-*At*RPL23-interaction. Nlp24-bio was incubated with *At*RPL23 receptor variants that were C-terminally tagged with green fluorescent protein (GFP). Upon ligand binding, ligand-receptor complexes were covalently linked by chemical crosslinking and, subsequently, subjected to immunoprecipitation using anti-GFP beads. Specificity of ligand binding was controlled by competing nlp24-bio binding with a large molar excess of unbiotinylated nlp24 peptide. A GFP signal at the expected molecular sizes of either *At*RPL23 mutant protein was detected in input samples in all cases (Fig. 1C). This finding suggests stable and correct expression of all proteins in *N. benthamiana*. In agreement with findings obtained from receptor function assays (Fig. 1B), nlp24-bio could only be pulled down with either the full-length *At*RPL23 receptor or with partially active deletion mutants *At*RPL23 Δ LRR2 and *At*RPL23 Δ IC (Fig. 1C). Reduced signal intensities observed for *At*RPL23 Δ LRR2 and for *At*RPL23 Δ IC may reflect lower binding affinities of nlp24-bio to these receptor mutants. This is consistent with higher EC₅₀ values (activation of ethylene production) found for these receptor mutants relative to full-length *At*RPL23 (Table 1). Δ NT-LRR1, Δ LRR2-3, Δ LRR2-21, Δ LRR2-ID, Δ LRR2-26, and Δ LRR2-JM did not bind nlp20 (Fig. 1C), thus providing an explanation for the insensitivity of plants expressing these constructs toward nlp20.

The *At*RPL23 N-terminal region is important for ligand-dependent interaction with *At*BAK1.

Upon binding of nlp20, *At*RPL23-*At*SOBIR1 heteromeric complexes recruit *At*BAK1 into a ternary receptor complex (Albert et al. 2015). The N-terminal region of *At*RPL23 (LRR1

and LRR3 domains) was found to be crucial for binding of the ligand to its receptor (Fig. 1C). GFP-tagged *At*RPL23 variants lacking those regions were next analyzed in coimmunoprecipitation experiments in order to determine whether such receptor mutants would associate with *At*BAK1 in the absence or presence of nlp20 (Fig. 2). Pull-down of GFP-tagged *At*RPL23 resulted in coimmunoprecipitation of *At*BAK1 only in the presence of nlp20 (Fig. 2), as described previously (Albert et al. 2015). The interaction of *At*RPL23 with *At*BAK1 was not detectable when *At*RPL23 Δ NT-LRR1 or *At*RPL23 Δ LRR3 were used (Fig. 2). In contrast, *At*BAK1 was recruited to a similar extent by *At*RPL23 Δ IC (lacking the cytoplasmic C-terminal tail) as by wild-type *At*RPL23. In agreement with a reduced nlp20 binding capacity observed for construct *At*RPL23 Δ LRR2, deletion of LRR2 also reduced the amount of immunoprecipitated *At*BAK1 substantially (Fig. 2). Altogether, our findings support a model in which very N-terminal regions within *At*RPL23 that are required for nlp20 ligand binding are also important for BAK1 coreceptor recruitment.

*At*RPL23 extracellular domains and ICs are not required for interaction with *At*SOBIR1.

To assess structural requirements within *At*RPL23 that are important for constitutive, ligand-independent heteromeric complex formation with coreceptor *At*SOBIR1, coimmunoprecipitation experiments were conducted. GFP-tagged *At*RPL23 or its structural mutant proteins were precipitated and coimmunoprecipitated proteins analyzed for the presence of hemagglutinin (HA)-tagged *At*SOBIR1. Surprisingly, none of the *At*RPL23 receptor proteins lost their ability to interact with *At*SOBIR1 (Fig. 3). Approximately equivalent amounts of *At*SOBIR1 were coimmunoprecipitated relative to the amounts of precipitated *At*RPL23-GFP protein (Fig. 3). We conclude that neither the extracellular domains nor the ICs of *At*RPL23 appear to be necessary for heteromeric complex formation with the coreceptor *At*SOBIR1.

Plasma membrane-associated domains support interaction of *At*RPLs with *At*SOBIR1.

GxxxG motifs are well-characterized facilitators of protein-protein interactions in animal receptor tyrosine kinases or membrane channels (Russ and Engelman 2000). Such dimerization motifs are also found in the plasma membrane-spanning region of

Table 1. Quantification of receptor activities of *At*RPL23 receptor mutants^a

Mutants	EC ₅₀ $\pm$ SD (nM)
WT	49 $\pm$ 28
Δ IC	217 $\pm$ 76
Δ LRR2	728 $\pm$ 283

^a Increasing concentrations of nlp20 elicitor were used to trigger ethylene biosynthesis in *Nicotiana benthamiana* plants transformed transiently with wild-type *At*RPL23 (WT) or receptor mutants, as indicated. Elicitor concentrations required to trigger a half-maximum plant defense (EC₅₀) are given $\pm$ standard deviation (SD).

Fig. 2. Interaction of *At*RPL23 deletion mutant proteins with *At*BAK1. Protein extracts from *Nicotiana benthamiana* transiently expressing RPL23-green fluorescent protein (GFP) and BAK1-Myc were subjected to coimmunoprecipitation by GFP-trap beads, 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blot analysis using tag-specific antisera. Representative examples selected from three independent repetitions are shown.

plant LRR-RPs (Bi et al. 2016; Gust and Felix 2014). In addition, ionic forces between oppositely charged regions in the extracellular JMs of LRR-RPs and SOBIR1 have been proposed to contribute to the formation of constitutive LRR-RP SOBIR1 heteromeric protein complexes (Gust and Felix 2014). Negatively charged glutamic acid residues in the JM sequence of LRR-RPs are predicted to interact with oppositely charged amino acid residues in the apoplastic JM of SOBIR1. To test the importance of these sequence segments more generally, four *Arabidopsis* LRR-RPs (*AtRLP1*, *AtRLP23*, *AtRLP30*, and *AtRLP42*), all of which are implicated in PAMP recognition and PTI activation (Albert et al. 2015; Jehle et al. 2013b; Zhang et al. 2013, 2014), were analyzed with regard to their ability to interact with *AtSOBIR1* (Fig. 4A). Whereas *AtRLP23*, *AtRLP30*, and *AtRLP42* harbor the aforementioned GxxxG motifs within their TMs, *AtRLP1* is an exception within the LRR-RP family because it lacks glycine residues in its TM (Supplementary Fig. S2). Despite these differences within the TM, all four proteins were capable of binding to *AtSOBIR1* in coimmunoprecipitation assays conducted in *N. benthamiana* (Fig. 4A). Coimmunoprecipitation of *AtSOBIR1* with *AtRLP1* suggests that GxxxG motifs may be dispensable in facilitating interaction of *AtSOBIR1* with LRR-RPs.

We further investigated the importance of the GxxxG motif in *AtRLP23* as well as the impact of oppositely charged amino acid residues in the extracellular JMs of *AtRLPs* and *AtSOBIR1*. Toward this end, GxxxGxxxG was changed to AxxxAxxxA (*AtRLP23* 3GA). Likewise, 12 glutamic acid residues in the *AtRLP23* JM were changed into either uncharged leucine residues (*AtRLP23* EL) or into positively charged lysine residues (*AtRLP23* EK). These *AtRLP23* mutant proteins were subsequently tested for interaction with *AtSOBIR1*. Coimmunoprecipitation assays conducted in transiently transformed *N. benthamiana* plants revealed clearly reduced levels of *AtSOBIR1* that coimmunoprecipitated together with either *AtRLP23* receptor mutant protein (Fig. 4A). Nevertheless, a faint band representing *AtSOBIR1* was found to coimmunoprecipitate along with all *AtRLP23* mutant proteins tested

(Fig. 4A). Thus, we also obtained double mutants lacking both motifs. These *AtRLP23* mutant proteins were entirely incapable of interacting with *AtSOBIR1* (Fig. 4A), suggesting that both interaction motifs act in concert in mediating *AtRLP23*–*AtSOBIR1* interaction. To investigate whether this finding holds true for another *AtRLP*, *AtRLP42*, we produced mutants lacking either one or both motifs together. Interestingly, mutation of the GxxxG motif abrogated entirely the ability of *AtRLP42* to interact with *AtSOBIR1*, which is in contrast with residual *AtSOBIR1* interaction found for the corresponding *AtRLP23* mutants (Fig. 4A). In contrast, mutation of the glutamic acid-rich stretch within *AtRLP42* affected its ability to interact with *AtSOBIR1* much less than the same mutation in *AtRLP23* did. Notably, the abilities or inabilities of all *AtRLP23* or *AtRLP42* mutant proteins to interact with *AtSOBIR1* coincide with their abilities or inabilities to confer nlp20 or PG6 sensitivity to *N. benthamiana* plants. (Fig. 4B). We conclude from these findings that the ability of *AtRLP*-type PRRs to interact with *AtSOBIR1* requires the concerted action of both protein–protein interaction motifs but that the absence of either one of such motifs may or may not abolish *AtRLP*–*AtSOBIR1* complex formation in a protein sequence-dependent manner.

AtSOBIR1 is not required for *AtRLP* protein stabilization.

AtSOBIR1 was shown to interact with a multitude of *AtRLP* proteins, including PRRs *AtRLP1*, *AtRLP23*, *AtRLP30*, and *AtRLP42* (Albert et al. 2015; Jehle et al. 2013a; Liebrand et al. 2013; Zhang et al. 2013, 2014). In all cases tested, inactivation of *AtSOBIR1* gene expression results in a complete loss of immune activation. However, whether failure to mount immune responses is due to a lack in signal transmission mediated through *AtSOBIR1* or whether *AtSOBIR1* simply stabilizes *AtRLP* receptors is unclear. In *N. benthamiana*, virus-induced gene silencing of *NbSOBIR1* and *NbSOBIR1*-like correlated with reduced levels of receptor proteins, suggesting a role of these coreceptors in stabilizing LRR-RPs *SlCf-4* and *SlVe1* RLPs in tomato (Liebrand et al. 2013).

Atsobir1-1 mutants carry a point mutation in the extracellular domain, resulting in a premature stop codon within the third LRR domain (Gao et al. 2009). Thus, *Atsobir1-1* genotypes are devoid of a functional plasma membrane-bound *AtSOBIR1* coreceptor. To assay *AtRLP* protein stability in the *Atsobir1-1* background, *Arabidopsis* Col-0 or *Atsobir1-1* mutant protoplasts were transformed with either *p35S::AtRLP1:GFP* or *pAtRLP23::AtRLP23:GFP*, or with *p35S::AtRLP30:GFP*, respectively. Receptor proteins in Col-0 and *Atsobir1-1* protoplasts accumulated at the same levels in the two genotypes (Fig. 5A). Protein abundance of *AtRLP1*-GFP or *AtRLP23*-GFP was further analyzed in stably transformed Col-0 plants and *Atsobir1-1* mutant plants. As observed in *Arabidopsis* protoplasts, signal intensity of *RLP-GFP*-expressing plants was not decreased in the *Atsobir1-1* genotype relative to that in Col-0 wild-type plants (Fig. 5B). Stability of these selected *Arabidopsis* RLPs appears to be unaffected by the absence of functional *SOBIR1* protein.

DISCUSSION

Plants employ various classes of plasma membrane PRRs to sense the presence of microbial invaders (Böhm et al. 2014a; Couto and Zipfel 2016; Saijo et al. 2018). LRR receptors are thought to have evolved a preference for recognizing proteinaceous microbial patterns (Boller and Felix 2009; Zhang and Thomma 2013). LRR-type PRRs exist in two forms as LRR-RKs and LRR-RPs (Böhm et al. 2014a; Couto and Zipfel 2016; Saijo et al. 2018). Conventional biochemical structure-function analyses have been conducted for LRR-RKs *AtFLS2*

Fig. 3. Interaction of *AtRLP23* deletion mutant proteins with *AtSOBIR1*. *AtRLP23*-green fluorescent protein (GFP) and *AtSOBIR1*-hemagglutinin (HA) proteins were transiently expressed in *Nicotiana benthamiana*, protein extracts thereof were subjected to coimmunoprecipitation using GFP-trap beads, and pelleted proteins were analyzed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and protein blotting using tag-specific antisera. Representative examples selected from three independent repetitions are shown.

and *AtEFR*, respectively, and the three-dimensional (3D) structure of a complex of *AtFLS2* and coreceptor *AtBAK1* in ligand-free and ligand-bound state has been elucidated (Sun et al. 2013). Similar analyses addressing structural requirements for ligand binding–coreceptor interaction for LRR-RP-type PRRs are missing. This is all the more desirable because anecdotally reported attempts to solve the 3D structures of LRR-RPs by X-ray crystallography have failed. In addition, LRR-RP receptor complexes reported from various plants comprise two coreceptors, *SOBIR1* and *BAK1*, suggesting a receptor complex architecture that likely differs from that found in LRR-RKs (Albert et al. 2015; Jehle et al. 2013a; Liebrand et al. 2013; Zhang et al. 2013, 2014). Likewise, comparative analyses of early signaling events and defense response patterns mediated through activation of either LRR-RK- or LRR-RP-type PRRs has revealed significant differences (Wan et al. 2019), thus challenging the prevailing concept that LRR-RP *SOBIR1* complexes constitute binary LRR-RKs (Gust and Felix 2014).

In the present report, we describe a structure-function analysis of immune receptor *AtRLP23*, an LRR-RP-type immune

receptor that mediates recognition of NLP-derived peptide nlp20. Serial deletions of LRR subdomains from the NT resulted in complete loss of nlp20 sensitivity (lack of nlp20-inducible ethylene production in *AtRLP23*-transgenic *N. benthamiana*) already when LRR1 or LRR3 subdomains were deleted. Analysis of nlp20 ligand binding revealed that all of the *AtRLP23* receptor mutant proteins that lacked the ability to confer pattern sensitivity also failed to bind its ligand nlp20. Thus, failure to bind its ligand may account for the lack of receptor activity in these mutants. This view is further corroborated by the fact that interaction of all *AtRLP23* receptor mutant proteins with coreceptor *AtSOBIR1* was not altered, thus ruling out the possibility that impaired receptor or coreceptor assembly may account for the loss of pattern sensitivity brought about by *AtRLP23* mutants lacking LRR1 or LRR3, respectively. Interestingly, deletion of subdomain LRR2 yielded partially active *AtRLP23* mutant receptor protein. This is conceivable because side chains of neighboring amino acids alternately point at opposite directions within horseshoe-shaped LRR ectodomains. Thus, we conclude that the very N-terminal portion of the *AtRLP23* ectodomain comprising LRR1 and LRR3 is crucial for ligand binding. This is

Fig. 4. Functionality of *AtRLP* immune receptors is differentially affected by mutations in membrane interaction motifs. **A**, Protein extracts from *Nicotiana benthamiana* transiently expressing *AtRLP23*-green fluorescent protein (GFP) and *AtRLP42*-GFP protein variants, *AtRLP1*-GFP, *AtRLP30*-GFP, and *AtSOBIR1*-hemagglutinin (HA) proteins were subjected to coimmunoprecipitation by GFP-trap beads, 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blot analysis using tag-specific antisera. WT = wild type. Representative examples selected from three independent repetitions are shown. **B**, Ethylene accumulation after treatment with 1 μ M nlp20, PG6, or water as control in *N. benthamiana* plants transiently transformed with the constructs indicated. Bars represent means $\pm$ standard deviation of three replicates. Asterisks indicate significant differences to water control treatments (*, **, and *** indicate $P < 0.05$, 0.01 , and 0.001 , respectively, Student's t test). Experiments were performed three times with similar results.

reminiscent of binding of flg22, a pattern of similar size to nlp20, to AtFLS2, which also starts at LRR3 and stretches out to LRR16 and keeps the ligand in a rather expanded state (Sun et al. 2013). Of note, the AtFLS2 ectodomain comprises 28 LRRs and, thus, is of similar size to the AtRLP23 ectodomain (27 LRRs) (Fig. 1A) (Albert et al. 2015; Gómez-Gómez and Boller 2000). The shared importance of AtFLS2 and AtRLP23 N termini for ligand binding may suggest a similar mode of ligand binding. This view is further substantiated by our finding that deletion of internal LRR subdomains C-terminal of LRR5 have also lost their abilities to bind nlp20, suggesting that the entire ectodomain is required for ligand binding or proper assembly of the receptor in the plasma membrane.

Both LRR-RK- and LRR-RP-type PRRs recruit BAK1 or related members of the SERK1 protein family in a ligand-dependent manner into signaling-competent PRR complexes (Böhm et al. 2014a; Couto and Zipfel 2016; Gust and Felix 2014; Saijo et al. 2018; Zhang and Thomma 2013). Thus, failure of AtRLP23 receptor mutant proteins to bind to nlp20 was expected to fail in recruiting AtBAK1 into a ternary complex comprising AtSOBIR1, AtRLP23, and AtBAK1. Indeed, we observed such a negative correlation, which again is reminiscent of flg22-induced AtFLS2–AtBAK1 interactions that were disrupted by mutations in AtFLS2 LRRs found to be important for flg22 recognition (Sun et al. 2013). Such similarities prompt us to suggest that nlp20, like flg22, may also act as “molecular glue” fostering AtRLP23 AtBAK1 heteromerization.

Like virtually all AtRLPs (Wang et al. 2008), AtRLP23 contains only a short stretch of amino acids at the intracellular

side of the plasma membrane. Here, we report the rather unexpected finding that deletion of this part leaves the shortened receptor protein functional with respect to defense activation (ethylene production), nlp20 ligand binding, and AtSOBIR1 and AtBAK1 coreceptor recruitment at elicitor concentrations routinely used. Hence, the intracellular tail region appears not to be crucial for RLP23-induced downstream signaling. However, we observed a reduced ability of the truncated AtRLP23 Δ IC mutant protein to confer activation of ethylene biosynthesis at lower elicitor concentrations, suggesting auxiliary rather than essential functions of this domain in AtRLP23 function. Because protein levels of this construct were virtually indistinguishable from those of wild-type AtRLP23 (Fig. 1C), we conclude that this tail domain may slightly affect the ability of the receptor to form intracellular signaling complexes rather than to affect receptor protein stability per se.

We have further found that deletion of large parts of the AtRLP23 ectodomain (AtRLP23 Δ LRR2–JM) or the IC (AtRLP23 Δ IC) did not affect the ability of the receptor to interact with its coreceptor AtSOBIR1 in a constitutive, ligand-independent fashion (Fig. 3). This suggests that interaction of these proteins is mediated through amino acid residues that likely reside within the transmembrane or juxtamembrane regions of the receptor. This is in agreement with complementary findings that the LRRs and ICs of the tomato coreceptor S/SOBIR1 are also dispensable for interaction with tomato LRR-RP-type receptor Cf-4 (Bi et al. 2016). Thus, constitutive heteromeric receptor/coreceptor complex formation of LRR-RP-type receptors and SOBIR1 is mainly brought about by interactions within and at the plant plasma membrane. In a search for amino acid residues responsible for formation of this complex, we deleted a GxxxG protein dimerization motif found in immune receptors AtRLP23 or AtRLP42. Unlike what was reported for mutation of such motifs in S/SOBIR1, which abrogated its interaction with tomato LRR-RP-type receptors S/Cf-4, S/Ve1, S/LeEIX2, or S/CLV2 entirely (Bi et al. 2016), we observed reduced interaction of AtRLP23 only with AtSOBIR1. Importantly, mutant receptor proteins were still capable of conferring immune activation upon nlp20 treatment. Therefore, incapacitation of GxxxG motifs alone was not sufficient to suppress receptor/coreceptor complex formation. However, inactivation of this motif in AtRLP42 abrogated its interaction with AtSOBIR1 and its ability to confer a ligand-specific ethylene response to transgenic *N. benthamiana*. Thus, we conclude that receptor protein sequence context may be crucial for the relative importance of the GxxxG motif for RLP SOBIR1 interaction ranging from essential (AtRLP42) to partial (AtRLP23) to negligible (AtRLP1, which lacks a GxxxG motif). Negatively charged glutamic acid residues within the outer JM of LRR-RPs have been suggested to mediate electrostatic interactions with positively charged residues of SOBIR (Gust and Felix 2014). Deletion of these residues resulted again in partially impaired ability of mutant AtRLP23 but not AtRLP42 to interact with AtSOBIR1 (Fig. 4A). Altogether, we conclude that both motifs contribute in concert to proper heteromeric AtRLP AtSOBIR1 complex formation in a receptor protein-specific manner that determines the relative importance of either motif for the interaction with AtSOBIR1.

Additional plant species-specific differences in the functionality of LRR-RP proteins may exist. Previous studies conducted in *N. benthamiana* plants, in which NbSOBIR1 and its close homolog NbSOBIR1-LIKE were inactivated by virus-induced gene silencing, revealed reduced levels of transiently expressed tomato LRR-RPs (Liebrand et al. 2013). This study suggested that SOBIR1 proteins may serve scaffold functions by stabilizing LRR-RP receptors in solanaceous plants. We have found that genetic inactivation of AtSOBIR1 had no

Fig. 5. Detection of AtRLP proteins in *Arabidopsis* Col-0 wild-type and *Atsobir1-1* mutant plants. **A**, Western blot (WB) analysis on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis-separated protein extracts from *Arabidopsis thaliana* protoplasts generated from leaves of Col-0 wild-type and *Atsobir1-1* mutant plants, transformed with pAtRLP23::AtRLP23-GFP, 35S::AtRLP1-GFP, or 35S::AtRLP30-GFP constructs. **B**, Western blot on proteins extracted from leaves of Col-0 wild-type plants and *Atsobir1-1* mutant plants transformed with pAtRLP23::AtRLP23-GFP and 35S::AtRLP1-GFP constructs. Blots were probed with green fluorescent protein (GFP)-specific antiserum. Representative examples selected from three independent repetitions are shown. Ponceau S staining of Rubisco large subunit (RbcL) was used as loading control (LC).

detrimental effect on the protein levels of at least three LRR-RP-type PRRs in a homologous plant background. Likewise, wild-type Col-0 and *sobir1-1* mutant genotypes stably over-expressing AtRLP-GFP proteins exhibited comparable protein levels. Thus, our findings do not support a protein scaffold function of AtSOBIR1 in stabilizing LRR-RP-type receptors.

MATERIALS AND METHODS

Plant materials and growth conditions.

All plants were grown in soil and used at an age of 4 to 6 weeks. *Arabidopsis thaliana* plants were grown under short-day conditions (22°C, diurnal cycle of 8 h of light and 16 h of darkness). *N. benthamiana* plants were grown in the greenhouse (16 h of light at 22°C and 8 h of darkness at 23°C). For stable transformation, *A. thaliana* plants were grown in the greenhouse (7 to 8 weeks old, 13 h of light and 11 h of darkness at 23°C and 14 h of light and 10 of darkness at 23°C).

Peptides.

PG6 protein (Zhang et al. 2014) was provided by J. van Kan (Wageningen University, The Netherlands). Synthetic peptides (10 mM stock solutions in 100% dimethyl sulfoxide [DMSO], further diluted in water prior to use; GenScript) were used at 1 µM for ethylene induction and for coimmunoprecipitation experiments (flg22: QRLSTGSRINSKDDAAGLQIA and nlp20: AIMYSWYFPKDSPVTGLGHR) and at 30 nM or 30 µM, as indicated, for cross-linking experiments (nlp24-K-bio: AIMYSWYFPKDSPVTGLGHRHDWEK-bio and NLP24-K: AIMYSWYFPKDSPVTGLGHRHDWEK).

Cloning of constructs.

Deletion and point-mutation constructs were generated on AtRLP23 in pCR8-Topo vector (Gateway vector; Thermo-Fisher) using the GeneArt Site-Directed Mutagenesis System (Thermo Scientific) and AccuPrime Pfx DNA Polymerase (Thermo Scientific). Mutated AtRLP23 sequences were recombined to pB7FWG2.0 (Plant Systems Biology, VIB, University of Ghent) for C-terminal GFP fusion. The coding sequence of AtRLP42 was cloned in the *SpeI* site of pLOCG (Zhang et al. 2017) for C-terminal GFP fusion. Using pLOCG::AtRLP42 as template, 5' and 3' flanks of AtRLP42 with overlap regions covering the mutations were amplified with primers listed in Supplementary Table S1. The two flanks were assembled with *SpeI*-digested pLOCG using Gibson assembly Master Mix (New England BioLabs) to generate the mutation constructs with C-terminal GFP fusion. To generate the double mutation constructs, plasmid pLOCG::AtRLP42-3GA (containing the single mutation of 3GA) was used as template. Cloning of AtRLP23, AtRLP1, AtRLP30, AtSOBIR1, and AtBAK1 constructs used in this study was described previously (Jehle et al. 2013b; Albert et al. 2015).

Plant and protoplast transformation.

For transient gene expression in *N. benthamiana*, *Agrobacterium tumefaciens* strain Gv3101 carrying the desired constructs was grown in Luria-Bertani (LB) medium for 48 h, harvested by centrifugation, resuspended in 10 mM MgCl₂ and 150 µM acetosyringone to optical density at 600 nm = 0.1 and incubated for 1 h at room temperature. The bacterial suspension was pressure infiltrated into leaves that were used for ethylene measurements, protein expression analysis, or immunoprecipitation 48 h after infiltration.

For stable transformation into *Arabidopsis*, *A. tumefaciens* strain Gv3101 harboring the constructs were grown in LB medium with appropriate antibiotics for 48 h. Cells were harvested and resuspended in 5% (wt/vol) sucrose, 10 mM

MgSO₄, and 0.01% Silwet and sprayed on buds of 6- to 8-week-old *Arabidopsis* Col-0 wild-type plants or *Atsobir1-1* mutant plants. The T1 generation was selected on 0.2% BASTA and used for protein extraction and Western blot analysis. Protoplasts from *Arabidopsis* Col-0 plants were isolated and transformed as described (Albert et al. 2015).

Immunoprecipitation assays, in vivo cross-linking, and Western blot analysis.

Leaves of transiently transformed *N. benthamiana* were harvested either directly or 3 min after infiltration of 1 µM nlp20. For in vivo cross-linking of nlp24-biotin to AtRLP23, leaves of *N. benthamiana* plants expressing p35S::AtRLP23-GFP or AtRLP23 mutant constructs were infiltrated with biotinylated nlp24 with or without unlabeled nlp24-K as competitor. For cross-linking of peptides to the receptor proteins, 2 mM ethylene glycol bis(succinimidyl succinate) (EGS) (initially dissolved in a small volume of DMSO and diluted further in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.5) was infiltrated 5 min after peptide treatment into the same leaves. At 30 min post EGS infiltration, leaf samples were harvested and frozen in liquid nitrogen.

For immunoprecipitations, 300 mg of ground leaf material from transiently transformed *N. benthamiana* was used. Membrane proteins were solubilized as described (Albert et al. 2015) and immuno-adsorbed via the GFP tag to GFP-Trap beads (ChromoTek). For detection of AtRLP23-, AtRLP1-, and AtRLP30-GFP fusion proteins in transgenic *Arabidopsis* Col-0 wild-type or *Atsobir1-1* mutant plants, two leaves per sample were ground in liquid nitrogen and proteins were extracted with loading dye for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Protein blots of 8% (immunoprecipitated proteins, protein detection in stably transformed *Arabidopsis* plants) or 10% (immunoprecipitated proteins after cross-linking to AtRLP23 deletion mutants) SDS-polyacrylamide gels were probed either directly with a Streptavidin-alkaline phosphatase conjugate (Roche) or with antibodies raised against GFP (Torrey Pines Biolabs), HA, or Myc-tags (Sigma), followed by staining with secondary antibodies coupled to alkaline phosphatase and CDP-Star (Roche) as substrates. Chemiluminescence was detected using a CCD-camera (Viber Louromat, PeqLAB).

Plant responses.

Ethylene production was performed as described (Böhm et al. 2014b). Leaf pieces were incubated in 20 mM morpholineethanesulfonic acid buffer, pH 5.7.

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LITERATURE CITED

- Afroz, A., Chaudhry, Z., Rashid, U., Ali, G. M., Nazir, F., Iqbal, J., and Khan, M. R. 2010. Enhanced resistance against bacterial wilt in transgenic tomato (*Lycopersicon esculentum*) lines expressing the *Xa21* gene. Plant Cell Tissue Organ Cult. 104:227-237.
- Albert, I., Böhm, H., Albert, M., Feiler, C. E., Imkamp, J., Wallmeroth, N., Brancato, C., Raaymakers, T. M., Oome, S., Zhang, H., Krol, E., Grefen, C., Gust, A. A., Chai, J., Hedrich, R., Van den Ackerveken, G., and Nürnberger, T. 2015. An RLP23-SOBIR1-BAK1 complex mediates NLP-triggered immunity. Nat. Plants 1: Article 15140.
- Bi, G., Liebrand, T. W., Bye, R. R., Postma, J., van der Burgh, A. M., Robatzek, S., Xu, X., and Joosten, M. H. 2016. SOBIR1 requires the GxxxG dimerization motif in its transmembrane domain to form constitutive complexes with receptor-like proteins. Mol. Plant Pathol. 17:96-107.

- Böhm, H., Albert, I., Fan, L., Reinhard, A., and Nürnberger, T. 2014a. Immune receptor complexes at the plant cell surface. *Curr. Opin. Plant Biol.* 20:47-54.
- Böhm, H., Albert, I., Oome, S., Raaymakers, T. M., Van den Ackerveken, G., and Nürnberger, T. 2014b. A conserved peptide pattern from a widespread microbial virulence factor triggers pattern-induced immunity in *Arabidopsis*. *PLoS Pathog.* 10:e1004491.
- Boller, T., and Felix, G. 2009. A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60:379-406.
- Couto, D., and Zipfel, C. 2016. Regulation of pattern recognition receptor signalling in plants. *Nat. Rev. Immunol.* 16:537-552.
- Dangl, J. L., Horvath, D. M., and Staskawicz, B. J. 2013. Pivoting the plant immune system from dissection to deployment. *Science* 341:746-751.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11: 539-548.
- Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18:265-276.
- Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y. T., Chen, S., Li, X., and Zhang, Y. 2009. Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe* 6:34-44.
- Gómez-Gómez, L., and Boller, T. 2000. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5:1003-1011.
- Gust, A. A., Brunner, F., and Nürnberger, T. 2010. Biotechnological concepts for improving plant innate immunity. *Curr. Opin. Biotechnol.* 21:204-210.
- Gust, A. A., and Felix, G. 2014. Receptor like proteins associate with SOBIR1-type of adaptors to form bimolecular receptor kinases. *Curr. Opin. Plant Biol.* 21:104-111.
- Gust, A. A., Pruitt, R., and Nürnberger, T. 2017. Sensing danger: Key to activating plant immunity. *Trends Plant Sci.* 22:779-791.
- Halter, T., Imkamp, J., Mazzotta, S., Wierzb, M., Postel, S., Bücherl, C., Kiefer, C., Stahl, M., Chinchilla, D., Wang, X., Nürnberger, T., Zipfel, C., Clouse, S., Borst, J. W., Boeren, S., de Vries, S. C., Tax, F., and Kemmerling, B. 2014. The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. *Curr. Biol.* 24:134-143.
- Holton, N., Nekrasov, V., Ronald, P. C., and Zipfel, C. 2015. The phylogenetically-related pattern recognition receptors EFR and XA21 recruit similar immune signaling components in monocots and dicots. *PLoS Pathog.* 11:e1004602.
- Jehle, A. K., Fürst, U., Lipschis, M., Albert, M., and Felix, G. 2013a. Perception of the novel MAMP eMax from different *Xanthomonas* species requires the *Arabidopsis* receptor-like protein ReMAX and the receptor kinase SOBIR. *Plant Signal. Behav.* 8: Article e27408.
- Jehle, A. K., Lipschis, M., Albert, M., Fallahzadeh-Mamaghani, V., Fürst, U., Mueller, K., and Felix, G. 2013b. The receptor-like protein ReMAX of *Arabidopsis* detects the microbe-associated molecular pattern eMax from *Xanthomonas*. *Plant Cell* 25:2330-2340.
- Jones, J. D., and Dangl, J. L. 2006. The plant immune system. *Nature* 444: 323-329.
- Kelly, S., Radutoiu, S., and Stougaard, J. 2017. Legume LysM receptors mediate symbiotic and pathogenic signalling. *Curr. Opin. Plant Biol.* 39: 152-158.
- Lacombe, S., Rougon-Cardoso, A., Sherwood, E., Peeters, N., Dahlbeck, D., van Esse, H. P., Smoker, M., Rallapalli, G., Thomma, B. P., Staskawicz, B., Jones, J. D., and Zipfel, C. 2010. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. *Nat. Biotechnol.* 28:365-369.
- Lenarčič, T., Albert, I., Böhm, H., Hodnik, V., Pirc, K., Zavec, A. B., Podobnik, M., Pahovnik, D., Žagar, E., Pruitt, R., Greimel, P., Yamaji-Hasegawa, A., Kobayashi, T., Zienkiewicz, A., Gömann, J., Mortimer, J. C., Fang, L., Mamode-Cassim, A., Deleu, M., Lins, L., Oecking, C., Feussner, I., Mongrand, S., Anderluh, G., and Nürnberger, T. 2017. Eudicot plant-specific sphingolipids determine host selectivity of microbial NLP cytolysins. *Science* 358:1431-1434.
- Liebrand, T. W., van den Berg, G. C., Zhang, Z., Smit, P., Cordewener, J. H., America, A. H., Sklenar, J., Jones, A. M., Tameling, W. I., Robatzek, S., Thomma, B. P., and Joosten, M. H. 2013. Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proc. Natl. Acad. Sci. U.S.A.* 110:10010-10015.
- Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. 2010. A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107:496-501.
- Mendes, B. M. J., Cardoso, S. C., Boscariol-Camargo, R. L., Cruz, R. B., Mourão Filho, F. A. A., and Bergamin Filho, A. 2010. Reduction in susceptibility to *Xanthomonas axonopodis* pv. *citri* in transgenic *Citrus sinensis* expressing the rice *Xa21* gene. *Plant Pathol.* 59:68-75.
- Oome, S., Raaymakers, T. M., Cabral, A., Samwel, S., Böhm, H., Albert, I., Nürnberger, T., and Van den Ackerveken, G. 2014. Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 111: 16955-16960.
- Postma, J., Liebrand, T. W., Bi, G., Evrard, A., Bye, R. R., Mbengue, M., Kuhn, H., Joosten, M. H., and Robatzek, S. 2016. Avr4 promotes Cf-4 receptor-like protein association with the BAK1/SERK3 receptor-like kinase to initiate receptor endocytosis and plant immunity. *New Phytol.* 210:627-642.
- Russ, W. P., and Engelman, D. M. 2000. The GxxxG motif: A framework for transmembrane helix-helix association. *J. Mol. Biol.* 296:911-919.
- Saijo, Y., Loo, E. P., and Yasuda, S. 2018. Pattern recognition receptors and signaling in plant-microbe interactions. *Plant J.* 93:592-613.
- Schoonbeek, H. J., Wang, H. H., Stefanato, F. L., Craze, M., Bowden, S., Wallington, E., Zipfel, C., and Ridout, C. J. 2015. *Arabidopsis* EF-Tu receptor enhances bacterial disease resistance in transgenic wheat. *New Phytol.* 206:606-613.
- Schwessinger, B., Bahar, O., Thomas, N., Holton, N., Nekrasov, V., Ruan, D., Canlas, P. E., Daudi, A., Petzold, C. J., Singan, V. R., Kuo, R., Chovatia, M., Daum, C., Heazlewood, J. L., Zipfel, C., and Ronald, P. C. 2015. Transgenic expression of the dicotyledonous pattern recognition receptor EFR in rice leads to ligand-dependent activation of defense responses. *PLoS Pathog.* 11:e1004809.
- Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., Zhou, J. M., and Chai, J. 2013. Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* 342:624-628.
- Thomma, B. P., Nürnberger, T., and Joosten, M. H. 2011. Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plant Cell* 23:4-15.
- Tripathi, J. N., Lorenzen, J., Bahar, O., Ronald, P., and Tripathi, L. 2014. Transgenic expression of the rice *Xa21* pattern-recognition receptor in banana (*Musa* sp.) confers resistance to *Xanthomonas campestris* pv. *musacearum*. *Plant Biotechnol. J.* 12:663-673.
- Wan, W.-L., Zhang, L., Pruitt, R., Zaidem, M., Brugman, R., Ma, X., Krol, E., Perraki, A., Kilian, J., Grossmann, G., Stahl, M., Shan, L., Zipfel, C., van Kan, J. A. L., Hedrich, R., Weigel, D., Gust, A. A., and Nürnberger, T. 2019. Comparing *Arabidopsis* receptor kinase and receptor protein-mediated immune signaling reveals BIK1-dependent differences. *New Phytol.* 221:2080-2095.
- Wang, G., Ellendorff, U., Kemp, B., Mansfield, J. W., Forsyth, A., Mitchell, K., Bastas, K., Liu, C. M., Woods-Tör, A., Zipfel, C., de Wit, P. J., Jones, J. D., Tör, M., and Thomma, B. P. 2008. A genome-wide functional investigation into the roles of receptor-like proteins in *Arabidopsis*. *Plant Physiol.* 147:503-517.
- Zhang, L., Kars, I., Essenstam, B., Liebrand, T. W., Wagemakers, L., Elberse, J., Tagkalaki, P., Tjoitang, D., van den Ackerveken, G., and van Kan, J. A. 2014. Fungal endopolygalacturonases are recognized as microbe-associated molecular patterns by the *Arabidopsis* receptor-like protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. *Plant Physiol.* 164:352-364.
- Zhang, L., Ni, H., Du, X., Wang, S., Ma, X. W., Nürnberger, T., Guo, H. S., and Hua, C. 2017. The Verticillium-specific protein VdSCP7 localizes to the plant nucleus and modulates immunity to fungal infections. *New Phytol.* 215:368-381.
- Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Boutrot, F. F., Tör, M., Zipfel, C., Gust, A. A., and Brunner, F. 2013. *Arabidopsis* receptor-like protein30 and receptor-like kinase suppressor of BIR1-1/EVERSHED mediate innate immunity to necrotrophic fungi. *Plant Cell* 25:4227-4241.
- Zhang, Z., and Thomma, B. P. 2013. Structure-function aspects of extracellular leucine-rich repeat-containing cell surface receptors in plants. *J. Integr. Plant Biol.* 55:1212-1223.