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A receptor-like protein mediates the response to pectin modification by activating brassinosteroid signaling

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The brassinosteroid (BR) signaling module is a central regulator of plant morphogenesis, as indicated by the large number of BR-responsive cell wall-related genes and the severe growth defects of BR mutants. Despite a detailed knowledge of the signaling components, the logic of this auto-/paracrine signaling module in growth control remains poorly understood. Recently, extensive cross-talk with other signaling pathways has been shown, suggesting that the outputs of BR signaling, such as gene-expression changes, are subject to complex control mechanisms. We previously provided evidence for a role of BR signaling in a feedback loop controlling the integrity of the cell wall. Here, we identify the first dedicated component of this feedback loop: a receptor-like protein (RLP44), which is essential for the compensatory triggering of BR signaling upon inhibition of pectin de-methylsterification in the cell wall. RLP44 is required for normal growth and stress responses and connects with the BR signaling pathway, presumably through a direct interaction with the regulatory receptor-like kinase BAK1. These findings corroborate a role for BR in controlling the sensitivity of a feedback signaling module involved in maintaining the physicochemical homeostasis of the cell wall during cell expansion.

brassinosteroids | cell wall integrity | pectin

A ll organisms need to integrate outside information with intrinsic cues through communication between the extracellular matrix and cell interior. This integration particularly applies to plants, the growth of which depends on biosynthesis, modification, and remodeling of their cell walls and needs to adapt to the widely varying environmental conditions inherent to a sessile lifestyle (1, 2).

The wall of growing cells is composed of diverse classes of polysaccharides and proteins forming a complex and dynamic network able to withstand the high intracellular turgor pressure. Growth occurs when cell wall extensibility is selectively increased by the activity of cell wall-modifying enzymes, so that the walls can modulate the outputs of the BR pathway, ensuring cell wall homeostasis and integrity. Here, we identified a receptor-like protein (RLP44), which mediates the activation of BR signaling through direct interaction with the BR coreceptor BAK1. Thus, RLP44 integrates cell wall surveillance with hormone signaling to control cell wall integrity and growth.

Significance

Plant growth and development depend on the biosynthesis and remodeling of the cell wall. To coordinate these two processes, surveillance mechanisms have evolved to monitor the state of the cell wall. The brassinosteroid (BR) hormone signaling pathway plays an essential role in growth control and regulates the expression of a plethora of cell wall-related genes. We have previously shown that feedback signaling from the wall can modulate the outputs of the BR pathway, ensuring cell wall homeostasis and integrity. Here, we identified a receptor-like protein (RLP44), which mediates the activation of BR signaling through direct interaction with the BR coreceptor BAK1. Thus, RLP44 integrates cell wall surveillance with hormone signaling to control cell wall integrity and growth.

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not well understood. Our recent demonstration that a pathway exists, which activates BR hormone signaling upon cell wall perturbation (14) creates a new conceptual framework for the understanding of the role of BR signaling in growth control. In this view, the BR signaling module is recruited into a feedback loop regulating the physico-chemical homeostasis of the wall during the growth process. Here, we report the identification of the first dedicated component of this pathway: the receptor-like protein (RLP)44. RLP44 is required for normal growth and its expression is necessary and sufficient to activate BR signaling downstream of ligand perception. Moreover, RLP44 seems to integrate cell wall and BR signaling by interacting with the regulatory coreceptor BAK1.

Results

Identification of RLP44 as a Novel Modifier of BR Signaling. We have recently demonstrated that upon cell wall perturbation the BR signaling pathway is activated to initiate a compensatory response (14). Thus, a pathway that relays information concerning cell wall alterations and consequences thereof to hormone signaling must exist (from here on referred to as “cell wall signaling”). To facilitate the identification of putative components involved in this pathway, we made use of plants that showed a strong BR-mediated compensatory response to cell wall perturbation induced by the ectopic expression of a pectin methylesterase inhibitor (PMEIox) (14). This response to reduced activity of pectin methylesterase (PME), which converts methylesterified pectin into (demethylesterified) pectate, includes growth retardation, root waving, agravitropism, organ fusion, and changes in gene expression (14) (see below). A forward genetic screen identified the recessive, extragenic suppressor mutant comfortably numb 2 (cnu2), which showed strong reduction of all observable BR-mediated PMEIox responses (Fig. 1A and Fig. S1A-G). Using map-based cloning, we identified a mutation associated with the cnu2 phenotype causing a premature stop codon in RLP44 (at3g49750) (18), which encodes a receptor-like protein with a predicted signal peptide, a leucine-rich repeat (LRR)-type extracellular domain, a transmembrane region, and a short cytoplasmic domain (Fig. 1B). Plants homozygous for a T-DNA insertion in the same gene (rp44-3) also suppressed the PMEox phenotype (Fig. 1C and Fig. S1H). In addition, a GFP-tagged version of RLP44 (RLP44:GFP) was able to complement cnu2, as demonstrated by a PMEox-like phenotype in transformant plants. Interestingly, some RLP44:GFP transformants even showed an enhanced PMEox phenotype (Fig. 1A). Thus, loss of RLP44 function was causative for the suppression of PMEox phenotypes in cnu2, whereas overexpression of RLP44 enhanced the compensatory response to PMEox. Confocal laser-scanning analysis of RLP44:GFP in both the wild-type and the cnu2 background showed the presence of the fusion protein in the plasma membrane as well as in intracellular compartments (Fig. 1D and Fig. S1).

In summary, RLP44 represents a new putative signaling component required for the BR-mediated response to PMEox-induced cell wall alterations.

RLP44 Is Required for Normal Growth and Stress Responses. We next assessed the role of RLP44 in normal plant development by studying the phenotype of the rp44cnu2 mutant separated from

![Fig. 1.](image-url)
the PMElox transgene. The mutant displayed a stunted growth phenotype under greenhouse conditions (Fig. 2A), indicating that RLP44 is required for normal growth. Compared with the control plants, rlp44mutant rosette leaves had shorter petioles (Fig. S2A) and decreased rosette diameter (Fig. S2B). Impaired growth was also apparent for rapidly elongating dark-grown seedlings (Fig. S2C). In addition, rlp44 mutants were hypersensitive to stress generated by elevated concentrations of sucrose (Fig. 2B) or the presence of NaCl (Fig. S2D) in the medium, conditions that also aggravate the growth phenotype of BR mutants (Fig. S2F and G) and, notably, that of mutants with altered cell wall composition or organization (19, 20). In contrast, no difference in root growth was observed between wild-type and rlp44mutant on medium supplemented with mannitol (Fig. S2E), ruling out purely osmotic effects. Consistent with the pleiotropic mutant phenotype, RLP44 has a broad expression spectrum (Fig. S3).

Loss of RLP44 Function Prevents Activation of BR Signaling by PMEI Overexpression. In our previous study, we attributed the PMElox phenotype primarily to enhanced BR signaling, which is expected to induce major changes in gene expression (14). We therefore compared the transcriptome of the Col-0 wild-type, PMElox, cnu2, and the cnu1 suppressor, which carries a hypomorphic allele of the BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (14) in a PMElox background. In PMElox, 1,018 genes were found to show significantly altered expression levels compared with the wild-type (553 up, 465 down). Interestingly, the vast majority of these transcripts reverted back to wild-type expression levels in both suppressor mutants, suggesting that RLP44 and BRI1 act in the same pathway (Fig. 3A and Dataset S1). Gene ontology (GO) enrichment analysis of the genes differentially regulated in PMElox compared with the wild-type revealed BR metabolism to be among the significantly enriched categories (Fig. S4A), and many BR biosynthetic genes known to be under negative feedback control (7) were down-regulated. This effect was alleviated or reversed in the suppressor mutants (Fig. S4B). The GO categories related to the cell wall were also enriched (Fig. S4A and C), consistent with the cell wall changes observed in PMElox (14).

To further analyze the gene-expression changes in PMElox and the suppressor mutants, we visualized, by hierarchical cluster analysis, patterns of gene expression from the significantly enriched GO categories “response to organic substance” and “cell wall organization.” Unsurprisingly, the suppressor mutants clustered with the wild-type as the majority of genes showed an expression similar to Col-0 (Fig. 3B and Fig. S4D). Notably, among the cell wall-related genes, three putative PMEs (which convert methylsterified pectin into pectate) and three putative enzymes (polygalacturonases) that degrade pectate (not pectin) were up- and down-regulated, respectively, in PMElox (Fig. S4D). This finding suggests that the initial lack of pectate (which is thought to play a role in the control of cell wall integrity through the formation of Ca++ cross-links) in the transformant may be compensated for by increased pectin de-methylsterification and decreased pectate turn-over (Fig. S4D) (14).

In summary, these observations are consistent with enhanced BR signaling being at least in part the underlying cause for the PMElox phenotype and strongly suggest that in both cnu1 and cnu2 this is prevented by interference with an early step in the signaling cascade.

RLP44 Activates BR Signaling Downstream of Ligand Perception. We next investigated whether RLP44 is a constitutive member of the BR pathway itself. To this end we took advantage of the observation that both PMElox and epi-brassinolide (BL) treatment render dark-grown seedlings agr1-like (21). Intriguingly, whereas the rlp44null loss-of-function mutant suppressed this phenotype in PMElox (in the cnu2 mutant), it remained fully sensitive to the BL treatment (Fig. S5), indicating that RLP44 was not required for the response to exogenous BL. This finding was corroborated by the finding that the rlp44null mutation did not further aggravate the phenotype of the weak BRI1 allele bri1mut (14) (see also Fig. S7A) and the analysis of RLP44 overexpression lines (RLP44ox). Indeed, RLP44ox showed a phenotype almost identical to that of BRI1 overexpressing plants (BRI1ox) (22) (i.e., elongated petioles and long, narrow, slightly epinastic leaf blades) (Fig. 3C). Accordingly, rosette diameter was increased similarly in BRI1ox and RLP44ox compared with the wild-type (Fig. 3C). In addition, expression levels of selected BR marker genes and genes reported to be BRI1ox-responsive (23) were similar in RLP44ox and BRI1ox seedlings (Fig. 3D). These observations, together with the transcriptome analysis of the loss-of-function mutants, indicate that RLP44, like BRI1, activates the BR signaling pathway. In sharp contrast to the similar behavior of BRI1ox and RLP44ox with respect to gene expression, they differed dramatically in their response to exogenous BRs. Upon depletion of endogenous BRs with the biosynthesis inhibitor propiconazole (24), root growth of BRI1ox was at least an order-of-magnitude more sensitive than the wild-type to both growth-promoting and growth-inhibiting doses of BL, whereas the RLP44ox response to BL was indistinguishable from that of the wild-type (Fig. 3E). Similar results were obtained with hypocotyl growth (Figs. S5B and S6). Thus, whereas overexpression of BRI1 strongly sensitized plants for BR, RLP44 overexpression activated the signaling pathway without increasing BR sensitivity. We next assessed whether RLP44 overexpression could rescue mutants of the BR receptors, bri1 and baks1. Double-homozygous descendants of a cross between RLP44ox and bri1mut had a bri1mut-like phenotype, indicating that RLP44 overexpression cannot rescue BRI1 deficiency and that, in turn, activation of the BR pathway by RLP44 requires functional BRI1 (Fig. S7B). Because elevated expression of BR pathway components downstream
of the receptor complex can typically rescue BR perception mutants (25–29), our results suggest that RLP44-mediated integration of cell wall and BR signaling occurs at or near the receptor complex. In contrast to the results with bri1 cnu1, RLP44 overexpression was able to rescue the phenotype of the bak1-4 mutant (30, 31) (Fig. 3F and G), possibly reflecting the different levels of genetic redundancy in the BRI1-like and BAK1/SERK gene families (32, 33): BAK1/SERK3 is partially redundant with SERK1 and SERK4, and a bri1 null-like phenotype is only observed in the absence of all three SERKs (33). Thus, RLP44-mediated rescue of bak1-4, but not of bri1 cnu1 might be explained by a possible interaction of RLP44 with the two other redundant SERKs (see below).

In conclusion, our results show that RLP44 is not a constitutive component of the BR signaling pathway itself but sensitizes this pathway in response to cell wall-derived cues.

**RLP44 Is Associated with the BR Receptor Complex.** To investigate how RLP44 might activate BR signaling, we tested candidate proteins for interaction with RLP44 using bimolecular fluorescence complementation assays (BiFC). Coexpression in *Nicotiana benthamiana* leaves of RLP44 fused to the C-terminal portion of the yellow fluorescent protein (nYFP) and BAK1 fused to nYFP gave rise to YFP fluorescence, suggesting that RLP44 interacts with BAK1 (Fig. 4A). Interestingly, the BAK1:nYFP/RLP44:cYFP signal was consistently stronger than what was observed with the BRI1/BAK1 combination used as a positive control (Fig. 4A) (28, 34), whereas control infiltrations of RLP44 and FLS2, a membrane-bound pathogen-associated molecular pattern receptor (35), did not result in detectable fluorescence (Fig. S8A). Interaction of BAK1 and RLP44 was independently confirmed using FRET-FLIM in *N. benthamiana* (Fig. S8B) and the mating-based Split-Ubiqitin

![Fig. 3. RLP44 activates BR signaling.](image-url)
system in yeast (36) (Fig. S8C). In addition, coimmunoprecipitation experiments showed that both BAK1 and BR11 were present in immunoprecipitates of RLP44:GFP from a line in a wild-type background and from a complemented cnu2 line (Fig. 4B). Consistent with the hypothesis that RLP44 exerts its effect at the level of the BR receptor complex, RLP44 overexpression was not able to rescue the subtle phenotype of bsk3-1 (Fig. S8D), which is mutated in one of the immediate downstream targets of BR11 (27). In addition, we noticed that RLP44 is phosphorylated, presumably in its cytoplasmic tail, and the extent of phosphorylation is affected in a PMElox background, substantiating our assumption that RLP44 is involved in cell wall-responsive signaling (Fig. 4C). In summary, our data indicate that RLP44 recruits the BR signaling module in response to PMElox-induced cell wall perturbation at least in part through a direct interaction with BAK1 (Fig. 4D).

Discussion

A major question in plant biology is how extrinsic cues are integrated with development to generate the phenotypic plasticity required for sessile organisms challenged by changing environments. It is assumed that cell wall surveillance is an important cellular self-perception mechanism for the control of growth and development. In addition, environmental cues, such as the presence of pathogens or neighboring cells, could be sensed by their effect on wall properties and wall integrity. Importantly, information from these cell wall surveillance pathways has to be integrated with intracellular growth regulation and defense pathways to mediate an adequate response, which includes—but is not limited to—feedback regulation of cell wall modification. In the present study, we have identified RLP44 as a positive regulator of BR signaling strength upon perturbation of the cell wall, as indicated by loss-of-function analyses, transcriptome comparison, and the effects of RLP44 overexpression. Interaction of RLP44 with the core BR signaling component BAK1 likely represents the convergence point of cell wall and BR signaling, whereas phosphorylation of RLP44 may play a role in the transduction of cell wall-derived signals.

At present, it is unclear whether the pathway harboring RLP44 is directly involved in cell wall sensing. It is conceivable that abundance or modification of cell wall polymers is monitored directly; for example, by physical interaction with the extracellular domain of RLP44, perhaps in a way similar to that of the pectin-binding wall-associated kinases (4, 5). Although there is as of yet no evidence supporting this hypothesis, it is noteworthy that at least one LRR protein, the polygalacturonase inhibitor protein, can interact with de-methylated pectin in the wall (37). Importantly, the abundance of this particular cell wall polymer is affected in the PMElox line used as genetic background for the suppressor screen, which identified RLP44 (14). However, it is also possible that changes in pectin are sensed by a different mechanism; in this scenario, RLP44 could be a downstream component, receiving information from unknown interaction partners or signaling molecules. Alternatively, derived properties, such as membrane tension, could be the relevant parameter under surveillance (38, 39). Again, in this scenario RLP44 could either be directly involved in sensing (e.g., as a part of a mechanosensor) or receive signals from upstream components. Clearly, more work is needed to distinguish between these possibilities. Here, we focused on the integration of BR and cell wall signaling. We revealed a possible mechanism with the identification of RLP44 as a modifier of BR signaling, which interacts with the regulatory receptor-like kinase BAK1. Possibly, interaction with RLP44 could favor BAK1–BR11 interaction or stabilize the interactions in the signaling complex with downstream components, such as receptor-like cytoplasmic kinases (26, 27, 40).

Interestingly, our results show that RLP44 is not a core BR signaling component but rather provides a lateral input modulating the signaling strength of the BR pathway. Indeed, whereas bri1 mutation suppressed the comparable phenotype triggered by either the inhibition of PME activity (in PMElox) or the exogenous application of BL, rlpp4 mutations likewise suppressed the PMElox phenotype but instead remained fully sensitive to BL. Similarly, RLP44ox phenocopied BR11ox, but whereas BR11ox was hypersensitive to BL, RLP44ox showed BL sensitivity indistinguishable from that of the wild-type.

Signal integration and cross-talk with other signaling pathways has been demonstrated for the BR signaling pathway before. For example, light and gibberellic acid signaling are known to converge with the BR pathway at the level of the transcription factors (41, 42), whereas cross-talk with stomatal development appears to occur more upstream in the pathway (43, 44). Cell wall-mediated feedback activation of BR signaling could help to fine-tune expression of cell wall biosynthetic and remodeling genes to ensure cell wall homeostasis during cell expansion and in response to extrinsic cues, such as environmental conditions.
that affect the physical properties of the cell wall. In accordance with this, RLP44, perhaps with other members of the large RLP gene family (14), is required for normal growth and the response to stress conditions.

Materials and Methods

Map-Based Cloning. To approximate the position of the cnu2 mutation, 23 single sequence-length polymorphism markers distributed throughout the genome were assayed (details available on request) in a population of 50 F2 individuals originating from a cross of cnu2 with nontransgenic plants of the Landsberg erecta ecotype. Only plants showing a wild-type phenotype and a wild-type banding pattern for the cnu2 mutation were selected. A strong bias was observed for the cnu2 polymorphic banding patterns, indicating that cnu2 is linked to a cDNA clone which is expressed strongly in Arabidopsis. These observations suggest that the cnu2 phenotype is caused by a mutation in a gene with a role in plant-cell wall development.

Coimmunoprecipitation (45). Mating-based split-ubiquitin systems were created by the mating-based split-ubiquitin system (mbSUS). To confirm the interaction of CER470172 and S18811, both proteins were expressed individually in yeast. The yeast strains expressing both proteins were selected by the mating-based split-ubiquitin system (mbSUS). Confocal Laser-Scanning Microscopy Analysis. Laser lines of 488 nm (YPFP, FM4-64) or 514 nm (YFP) on a Zeiss LSM 510 Meta and Zeiss LSM 710 microscope were used. Fluorescence images were obtained using a Zeiss Axiovert 40 inverted microscope equipped with a Zeiss Plan-Neofluar 40×/1.3 N.A. water immersion objective. Images were processed with Fiji and Adobe Photoshop CS for figure preparation.

Protein-Protein Interaction Analysis. Coimmunoprecipitation (45), mating-based split-ubiquitin system (36), and FRET-FLIM (46) were essentially performed as described. Details can be found in SI Materials and Methods.

Expression Analysis. Microarray data reported in this study were deposited at the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE48996. Detailed experimental procedures can be found in SI Materials and Methods.

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