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

Sebastian Wolf^{a,b,1}, Dieuwertje van der Does^c, Friederike Ladwig^d, Carsten Sticht^e, Andreas Kolbeck^a, Ann-Kathrin Schürholz^a, Sebastian Augustin^a, Nana Keinath^a, Thomas Rausch^a, Steffen Greiner^a, Karin Schumacher^a, Klaus Harter^d, Cyril Zipfel^c, and Herman Höfte^{b,1}

^aCentre for Organismal Studies, University of Heidelberg, 69120 Heidelberg, Germany; ^bSaclay Plant Science, Institut Jean-Pierre Bourgin, Unité Mixte de Recherche 1318, Institut National de la Recherche Agronomique-AgroParisTech, 78026 Versailles, France; ^cThe Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom; ^dDepartment for Plant Physiology, Center for Plant Molecular Biology, University of Tübingen, 72076 Tübingen, Germany; and ^eZentrum für Medizinische Forschung, Medizinische Fakultät Mannheim, 68167 Mannheim, Germany

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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-methylesterification 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 physico-chemical homeostasis of the cell wall during cell expansion.

brassinosteroids | cell wall integrity | pectin

All 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 yield to turgor pressure, resulting in an irreversible deformation (1, 2). The yielding of the cell wall needs to be precisely controlled to avoid rupture during growth. In addition, the abiotic and biotic environment can potentially change the cross-linking pattern of cell wall polymers and thus influence the extensibility of the cell wall. In this context, the state of the wall has to be constantly monitored and information has to be relayed to the cytosol to regulate cell wall biosynthesis and remodeling in response to developmental and environmental cues (2). Whereas cell wall integrity signaling has been extensively studied in yeast (3), in plants this process remains poorly understood (2). A small number of putative cell wall sensors have been described, including the pectin-binding wall-associated kinases (4, 5) and the receptor-like kinase THESEUS (6); however, very little is known about the signaling pathways involved. Here, we further investigated the previously

identified function of brassinosteroid (BR) hormone signaling in maintaining cell wall integrity.

The well-characterized BR signaling module is initiated by the plasma membrane-bound receptor BRI1 and its coreceptor BAK1/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK)3, which upon ligand binding transphosphorylate each other. A signal transduction cascade is initiated, starting with phosphorylation of receptor-like cytoplasmic kinases by BRI1 and which ultimately leads to transcriptional changes mediated by the transcription factors BZR1 and BES1/BZR2 (7–10). Among the transcriptional targets of BR signaling, cell wall-biosynthesis and -remodeling genes are overrepresented (7), which is consistent with the fact that BR biosynthetic and BR-insensitive mutants show pronounced dwarfism mainly caused by reduced cell elongation. Accordingly, BR activation causes changes in cell wall enzyme activities, and induces hydration and remodeling of the cell wall (11–14).

Intriguingly, in contrast to other hormones—which can act at a distance from their site of synthesis—genetic studies show that BRs seem to act more locally, on the same or adjacent cells (15–17). Whereas long-distance signaling allows growth-rate modulation, for example in response to environmental cues, the logic of an autocrine/paracrine signaling module in growth control is

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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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE48596).

¹To whom correspondence may be addressed. Email: sebastian.wolf@cos.uni-heidelberg.de or herman.hofte@versailles.inra.fr.

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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 (*rlp44-3*) also suppressed the PMEIOx 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 PMEIOx-like phenotype in transformant plants. Interestingly, some RLP44:GFP transformants even showed an enhanced PMEIOx phenotype (Fig. 1A). Thus, loss of RLP44 function was causative for the suppression of PMEIOx phenotypes in *cnu2*, whereas overexpression of RLP44 enhanced the compensatory response to PMEIOx. 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. S1I).

In summary, RLP44 represents a new putative signaling component required for the BR-mediated response to PMEIOx-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 *rlp44^{cnu2}* mutant separated from

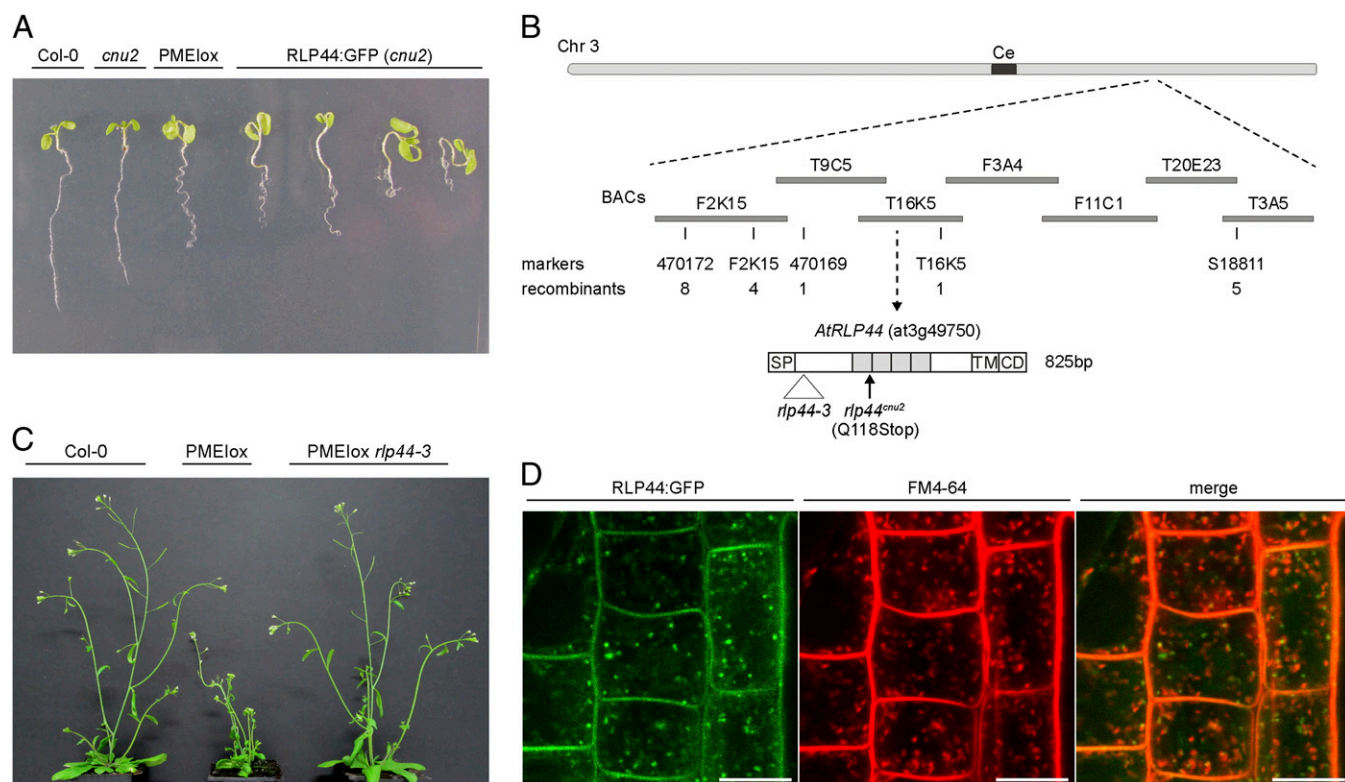


Fig. 1. Identification of RLP44, a receptor-like protein involved in cell wall signaling. (A) Wild-type-like phenotype of the suppressor mutant *cnu2* compared with the parental line, PMEIOx, in which cell wall alterations elicit a BR-mediated response resulting in a severe growth phenotype. Expression of RLP44:GFP in *cnu2* reconstitutes or enhances the PMEIOx phenotype. (B) Overview of map-based cloning procedure: 219 F2 plants showing the *cnu2* phenotype derived from a cross of *cnu2* and nontransgenic plants of the Landsberg *erecta* ecotype were analyzed with PCR-based markers. Sequencing of genes in the mapping interval revealed a C-to-T mutation, leading to a premature stop codon in *RLP44* (at3g49750). CD, short, cytosolic domain; gray shading, LRR repeats; SP, signal peptide; TM, transmembrane domain. (C) Knock-out of RLP44 in PMEIOx through T-DNA insertion reinstates the *cnu2* phenotype, confirming that RLP44 loss-of-function is causative for suppression of PMEIOx. (D) RLP44:GFP localizes to the plasma membrane in addition to intracellular punctate structures, which are partially colabeled by the endocytic tracer dye FM4-64. (Scale bars, 10 μ m.)

the PME1ox 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, *rlp44^{cnu2}* 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 *rlp44^{cnu2}* 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 PME1ox Overexpression. In our previous study, we attributed the PME1ox 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, PME1ox, *cnu2*, and the *cnu1* suppressor, which carries a hypomorphic allele of the BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (14) in a PME1ox background. In PME1ox, 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 PME1ox 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 PME1ox (14).

To further analyze the gene-expression changes in PME1ox 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 PMEases (which convert methylesterified pectin into pectate) and three putative enzymes (polygalacturonases) that degrade pectate (not pectin) were up- and down-regulated, respectively, in PME1ox (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 Ca²⁺ cross-links) in the transformant may be compensated for by increased pectin de-methylesterification and decreased pectate turn-over (Fig. S4D) (14).

In summary, these results are consistent with enhanced BR-signaling being at least in part the underlying cause for the PME1ox 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 PME1ox and epi-brassinolide (BL) treatment render dark-grown seedlings agravitropic (14, 21). Interestingly, whereas the *rlp44^{cnu2}* loss-of-function mutant suppressed this phenotype in PME1ox (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 *rlp44^{cnu2}* mutation did not further aggravate the phenotype of the weak *BRI1* allele *bri1^{cnu1}* (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 BAK1. Double-homozygous descendants of a cross between RLP44ox and *bri1^{cnu1}* had a *bri1^{cnu1}*-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

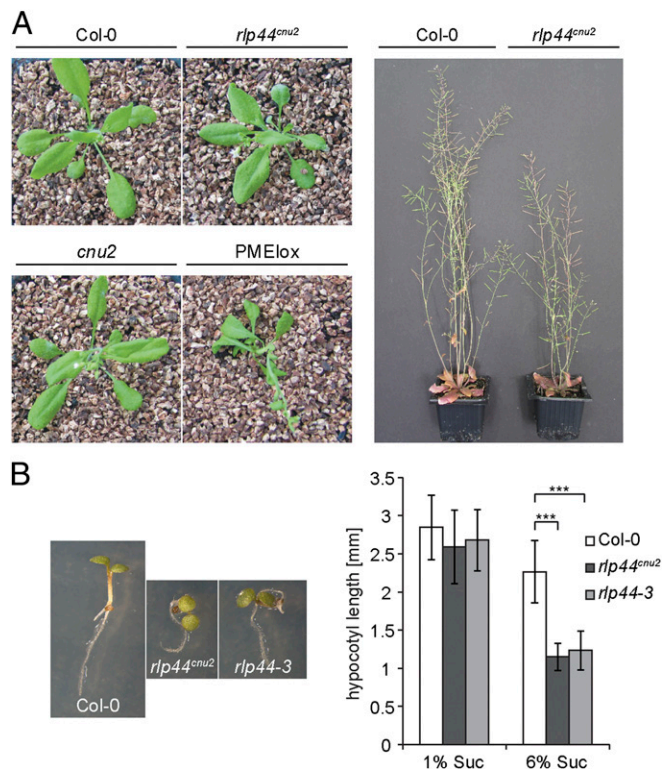


Fig. 2. RLP44 is required for normal growth. (A) Morphological phenotype of young *rlp44^{cnu2}* mutant plants compared with wild-type, PME1ox, and *cnu2* (Left). Adult plants (Right) show reduced height compared with wild-type. (B) *rlp44* mutant plants are hypersensitive toward elevated concentrations of sucrose in the medium. Bars indicate mean hypocotyl length of 7-d-old seedlings \pm SD ($n > 14$). Asterisks indicate significance as determined by Student *t* test with $P < 0.001$.

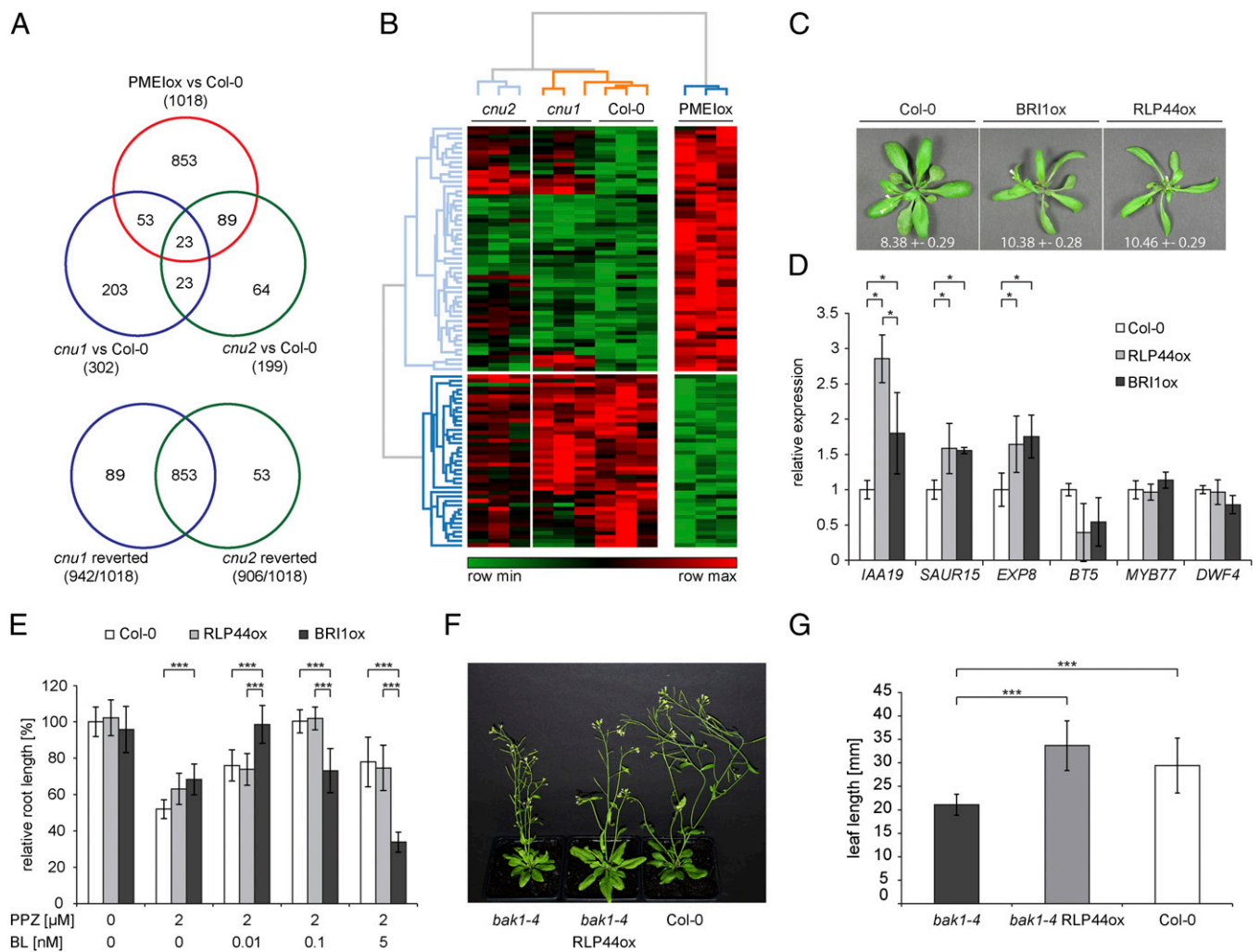


Fig. 3. RLP44 activates BR signaling. (A) Venn diagram analysis of genes with significantly altered expression compared with the Col-0 wild-type in PMElox, *cnu1* and *cnu2*, respectively. Of the 1,018 genes differentially expressed in PMElox, 906 are reverted in *cnu2* and 942 are reverted in *cnu1*. The expression of only 23 genes (including the PMElox transgene) is altered in all three genotypes (Upper). Approximately 90% of genes reverted in *cnu2* are shared with *cnu1*, indicating that the underlying mutated genes act in the same pathway. Microarray analysis was performed on 4-d-old dark-grown seedlings. (B) Hierarchical clustering analysis of genes of the GO category "response to organic substance" differentially expressed in PMElox. (C) Strong overexpression of RLP44 phenocopied BRI1 overexpressing plants with long petioles (quantification indicated by numbers, millimeter \pm SD, $n > 15$; $P < 0.001$ for both comparisons between Col-0 and transgenics according to a Student *t* test; differences between the transgenic lines are nonsignificant) and narrow, twisted leaf blades, suggesting that RLP44 can activate the BR signaling pathway similar to BRI1. (D) The expression of BR-regulated genes (19) is similar in the RLP44ox and BRI1ox lines in 5-d-old light-grown seedlings. Bars denote means of three biological replicates \pm 95% confidence interval, $n = 3$. Asterisks indicate statistical significance as determined by Student *t* test with $P < 0.05$. (E) Both BRI1ox and RLP44ox are resistant toward depletion of endogenous BRs but only BRI1ox is hypersensitive to application of BL. Bars indicate mean root length of 5-d-old seedlings \pm SD ($n > 8$). Data were analyzed using one-way ANOVA. Asterisks indicate significance with $P < 0.001$ as determined by Tukey's test. Note that significance is only indicated for comparisons within each treatment. (F and G) Expression of RLP44 rescues the *bak1-4* mutant phenotype. (G) Leaf length measurements indicate significant differences between *bak1-4* and both *bak1-4* RLP44ox and Col-0, but not between Col-0 and *bak1-4* RLP44ox. Asterisks indicate significance as determined by Student *t* test with $P < 0.001$.

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. 3 F 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. S84). Interaction of BAK1 and RLP44 was independently confirmed using FRET-FLIM in *N. benthamiana* (Fig. S8B) and the mating-based Split-Ubiquitin

system in yeast (36) (Fig. S8C). In addition, coimmunoprecipitation experiments showed that both BAK1 and BRI1 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 BRI1 (27). In addition, we noticed that RLP44 is phosphorylated, presumably in its cytoplasmic tail, and the extent of phosphorylation is affected in a PME1ox 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 PME1ox-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-methylesterified pectin in the wall (37). Importantly, the abundance of this particular cell wall polymer is affected in the PME1ox 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–BRI1 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 PME1ox) or the exogenous application of BL, *rlp44* mutations likewise suppressed the PME1ox phenotype but instead remained fully sensitive to BL. Similarly, RLP44ox phenocopied BRI1ox, but whereas BRI1ox 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

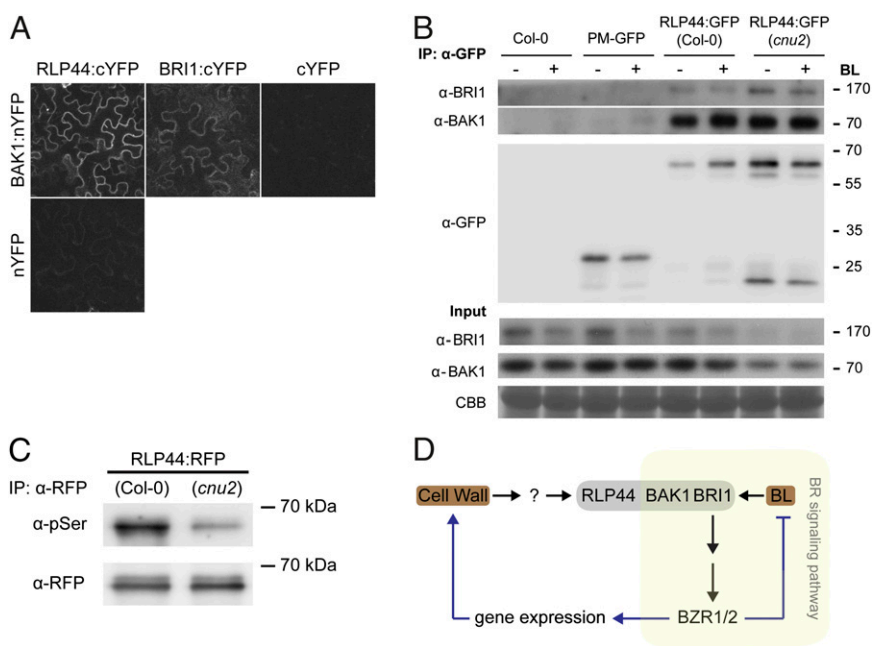


Fig. 4. RLP44 interacts with BAK1 to activate BR signaling. (A) BiFC analysis of molecular interaction between RLP44 and BAK1. YFP fluorescence is observed when RLP44:cYFP is coexpressed with BAK1:nYFP. No YFP fluorescence is detected in the control infiltration of BRI1/RLP44, BAK1:nYFP with cYFP, RLP44:cYFP with nYFP, and RLP44:cYFP and BAK1:nYFP alone, respectively. (Magnification: A, 76×.) (B) Coimmunoprecipitation reveals that both BAK1 and BRI1 are physically associated with RLP44 in RLP44:GFP overexpressing lines. Treatment with epi-BL for 90 min (40) had no effect on complex formation. PM:GFP, GFP directed to the plasma membrane by fusion with Lti6b. (C) RLP44 is phosphorylated at one or more serine residues, presumably in its cytoplasmic tail, and this post-translational modification is responsive to cell wall status. (D) Model of BR pathway activation by RLP44 upon cell wall perturbation. RLP44 is not part of BR signaling itself, but can activate the pathway laterally by interacting with the receptor complex. Transcriptional regulation is indicated by blue lines.

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 *gnu2* 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 *gnu2* with nontransgenic plants of the Landsberg *erecta* ecotype. Only plants showing a wild-type-like appearance in combination with BASTA resistance (resistance derived from PMElx transgene cassette) were selected. Strong bias was observed for the Columbia-specific band of markers CER456071 and CER470172 located at the bottom of chromosome 3 (see Table S1 for oligo sequences). CER470172 and S18811 were identified as flanking markers and 219 plants from the mapping population were analyzed with additional markers F2K15, CER470169, and T16K5. Candidate genes in the remaining interval between CER470169 and T16K5 were amplified and the PCR products were sequenced to identify the mutation.

Confocal Laser-Scanning Microscopy Analysis. Laser lines of 488 nm (GFP, FM4-64) or 514 nm (YFP) on a Zeiss LSM 510 Meta and Zeiss LSM 710 microscope systems equipped with 25 \times and 63 \times water immersion objectives were used

for excitation. Fluorescence was recorded between 530 and 600 nm for YFP and between 490 and 525 nm for GFP. FM4-64 fluorescence was recorded between 600 nm and 720 nm. Images were analyzed with Zeiss Zen software and Fiji. 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 Material 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 GSE48596. Detailed experimental procedures can be found in *SI Material and Methods*.

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