Polar recruitment of RLD by LAZY1-like protein during gravity signaling in root branch angle control

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In many plant species, roots maintain specific growth angles relative to the direction of gravity, known as gravitropic set point angles (GSAs). These contribute to the efficient acquisition of water and nutrients. AtLAZY1/LAZY1-LIKE (LZY) genes are involved in GSA control by regulating auxin flow toward the direction of gravity in Arabidopsis. Here, we demonstrate that RCC1-like domain (RLD) proteins, identified as LZY interactors, are essential regulators of polar auxin transport. We show that interaction of the CCL domain of LZY with the BRX domain of RLD is important for the recruitment of RLD from the cytoplasm to the plasma membrane by LZY. A structural analysis reveals the mode of the interaction as an intermolecular β-sheet in addition to the structure of the BRX domain. Our results offer a molecular framework in which gravity signal first emerges as polarized LZY3 localization in gravity-sensing cells, followed by polar RLD1 localization and PIN3 relocalization to modulate auxin flow.
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ravity is a fundamental environmental signal that affects all organisms on Earth. Plant organs sense gravity as a directional cue to control their growth orientation and shoots typically grow upward while roots grow downward. This growth response to gravity is known as gravitropism. Plant roots and shoots are generally maintained at specific angles relative to the direction of gravity, referred to as the gravitropic setpoint angle (GSA), which is one aspect of gravitropism. Proper positioning of leaves or roots enables efficient light reception and reproduction or water and nutrient uptake, respectively. Thus, the regulation of branch angles is an important trait in crop breeding to increase yield.

When plant organs incline and turn away from GSA, a change in their orientation relative to gravity is perceived in specialized gravity-sensing cells, known as statocytes. In Arabidopsis thaliana, shoot endodermal cells and root columella cells are statocytes that contain high-density starch-accumulating amyloplasts. Amyloplasts play a role as statoliths; that is, they relocate according to the direction of gravity, triggering intracellular signaling. Subsequently, the signal promotes the transport of the plant hormone auxin toward the direction of gravity in the responsive organ, resulting in the differential growth of the organ. Thus, gravity signaling in statocytes is a key process in which physical information derived from amyloplast sedimentation is converted to regulation of auxin transport. PIN-FORMED3 (PIN3), a member of auxin efflux facilitator PIN family, is uniformly localized to the plasma membrane (PM) of statocytes in vertically growing Arabidopsis organs. Upon gravistimulation by reorientation, PIN3 undergoes polar localization to the lower side (the direction of gravity) of the statocytes of the roots and hypocotyls, which could contribute to the directional transport of auxin to the lower flank of the organs. Meanwhile, LAZY1 family genes are involved in gravitropism in many plant species. At least four of the six LAZY1-LIKE (LZY) genes are redundantly required for gravitropism of roots and shoots and LAZY1, LAZY2, and LAZY3 play a key role in gravity signaling in statocytes. PIN3 and LZY have also been shown to be involved in the GSA control of lateral roots (LRs) and vascular development where the promoter activity of ATHB8 is conserved among land plants and share a similar domain composition containing a pleckstrin homology (PH) domain, regulator of chromosome condensation 1 (RCC1)-like motif repeats, a Fab1/YGL023/Vps27/EEA1 (FYVE) domain, and a Brevis radix (BRX) domain. The results demonstrated that at least RLD1 and RLD4 are involved in GSA control of LRs. The GSA phenotype of rld1 rld4 was rescued by expressing RLD1-GFP under the control of its own promoter RLD1p and the statocyte-specific promoter of ACTIN DEPOLYMERIZING FACTOR9 (ADF9), indicating that functional RLD1-GFP in the root statocytes is responsible for the GSA control of LRs. In addition, the PRs of the rld1 rld2 double mutant displayed reduced gravitropic responses. These phenotypes were mild, suggesting that remaining RLD genes, RLD2 and RLD3, could function in GSA control and root gravitropism. To investigate this possibility, a rld1 rld2 rld3 quadruple mutant was constructed. Severe defects in organ formation were observed in rld1 rld2 rld3 rld4 quadruple mutant embryos and seedlings. Since it is considered that these severe phenotypes of the quadruple mutant are due to impaired vascular development where the promoter activity of RLD genes was detected (Supplementary Fig. 2), vascular-specific complementation analysis was performed. The expression of RLD1-mCherry driven by the provascular- and vascular-specific promoter of ARABIDOPSIS THALIANA HOMEBOX8 (ATHB8) rescued embryo development and root formation in rld1 rld2 rld3 rld4 seedlings as expected, but it was not sufficient for gravitropic growth of PRs. RLD2 and RLD3 are also required for root gravitropism. Our data suggest that RLD1–4 are redundantly involved in GSA control of LRs and root gravitropism in columella cells.

RLD regulates PIN-dependent auxin transport. An asymmetric expression pattern of the auxin-responsive promoter driving GFP (DR5rev-GFP) toward the direction of gravity was distinctly observable in wild-type stage 3 LRs, whereas it was hardly observed in rld1 rld4 (Fig. 2a, b). This result indicates that RLD1 and RLD4 regulate LR GSA through the control of auxin flow. Loss of function of RLD1 and RLD4 caused a significant reduction in the signal intensity of PIN3-GFP in statocytes of the LRs throughout stages 1, 2, and 3 (Fig. 2c–e). In addition, rld1 rld2 rld3 rld4 mutant embryos displayed severe reduction of PIN1-GFP expression together with defective expression pattern of DR5rev-GFP (Fig. 2f–m). These results suggest that RLD1–4 modulates auxin transport through regulation of PIN localization in the GSA control of LRs and during embryogenesis. Striking
mRNA level decreased to wild type level, similar to the case of gnom mutants. The decrease of gnom resulted in the decrease of PIN protein level, which can be restored by gnom-1

in the vasculature. Arrow marked with "g" represents the direction of gravity. Scale bar, 1 cm (a–g). In addition, the 1 μM BFA treatment had a greater negative impact on LR development in rld1 rld4 than in the wild type (Supplementary Fig. 7b, c, i, j).

These findings suggest that the RLDs could regulate auxin flow in the same pathway as GNOM to control PIN proteins not only in root gravitropism but also in plant development.

**LZY recruits RLD to the PM.** Previous studies showed that LZY proteins were localized mainly in the PM of protoplast cells. In contrast, RLD proteins were not localized in the PM of protoplast cells, but in the cytoplasm and punctate structures (Fig. 3a and Supplementary Fig. 8a–c). Interestingly, when co-expressed with LZY2 or LZY3 in protoplast cells, RLD proteins were localized in the PM along with LZY proteins, indicating that LZYs recruit RLDs from the cytoplasm and punctate structures to the PM (Fig. 2b and Supplementary Fig. 8d–h). To confirm LZY-dependent RLD recruitment to the PM in plants, LZY2-mCherry and RLD1-mCherry were co-expressed in seedlings harboring both 35S:RLD1-GFP and G10-90p:XVE»LZY2-mCherry. In
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central line and box boundaries. Whiskers show min to max values.

expression in WT-like (a, c) and rld1-2 rld4-1 (b, d). Arrowheads indicate DR5rev:GFP expression in lateral root tips of Col (a, c) and rld1-2 rld4-1 (b, d). Arrowheads indicate DR5rev:GFP expression in lateral root cap cells (upper side, blue; lower side, yellow). e GFP intensity in the central columella cells of lateral roots of Col and rld1-2 rld4-1 harboring PIN3p:PIN3-GFP. Median and quartile values are provided by the central line and box boundaries. Whiskers show min to max values. n, sample number of three biologically independent experiments. Asterisks indicate significant differences by Student’s t test (P < 0.05). Source data are provided as a Source Data file. f-m DR5rev:GFP (f, g, h, i) and PIN1-GFP (j, k, l, m) expression in WT-like (f, g, j, k) and rld1-2 rld2-2 rld3-2 rld4-1 (h, i, l, m) embryos dissected from ovules of plant homozygous for rld1-2, rld3-2, and rld4-1 and heterozygous for rld2-2 at the globular (f, h, j, l) and heart stage (g, i, k, m). Arrowheads and brackets indicate strong GFP signals in the hypophysis/ radicle tip and provascularis of basal region, respectively, while blank arrowheads and dashed brackets represent disappeared GFP signals in respective regions. Arrow marked with “g” represents the direction of gravity. Scale bars, 20 μm (a-d, f-m).

root cap cells, ectopically expressed RLD1-GFP was localized in the cytoplasm (Fig. 3c). When co-expressed with LZY2-mCherry, the RLD1-GFP signal was detected mainly in the PM. Next, the domains of both LZY and RLD responsible for LZY-dependent RLD recruitment to the PM were identified, using transient assays in protoplast cells. The CCL domain of LZY and the BRX domain of all RLDs are necessary and sufficient for LZY-RLD interaction, which is necessary and sufficient for the recruitment of RLD from the cytosol to the PM.

Structural analysis of the CCL-BRX complex. To characterize the interaction between RLDs and LZY3, the RLD2 BRX domain and the LZY3 CCL peptide were purified and subjected to a binding assay using isothermal titration calorimetry (ITC). We found that the BRX domain and the CCL peptide form a 1:1 complex with a K_D value of 9.7 nM (Fig. 4a). To elucidate the structural basis of the interaction, we determined the crystal structures of the RLD2 BRX domain bound to the LZY3 CCL peptide (referred to as the RLD2-LZY3 complex) at 1.35 Å
Fig. 3 LZY recruits RLD to the plasma membrane via the CCL-BRX interaction. a, b Co-expression of RLD1-GFP with mCherry (a) and LZY3-mCherry (b) in Arabidopsis protoplast cells. Arrows indicate plasma membrane-localized signals. c Localization of RLD1-GFP (left) and LZY2-mCherry (middle) in primary root of 8-day-old seedling harboring 35S:RLD1-GFP and G10-90p:XVE:LZY2-mCherry, transferred to MS medium plate containing 1 μM estradiol at 7 days old. Merged image of RLD1-GFP and LZY2-mCherry (right). Arrowheads indicate LZY2-mCherry-expressing cells, where RLD1-GFP was co-localized with LZY2-mCherry in the plasma membrane. Scale bars, 10 μm. d, e Co-expression of RLD1-GFP and CCL-deleted LZY3-mCherry (LZY3ΔCCL) (d), and of the BRX domain-deleted RLD1-GFP (RLD1ΔBRX) and LZY3-mCherry (e) in Arabidopsis protoplast cells. f, g Co-expression of the BRX domain (BRXd)-mCherry with GFP (f) and LZY3-GFP (g) in Arabidopsis protoplast cells. h, i Co-expression of RLD1-GFP with mCherry-LT16b (h) and CCL-mCherry-LT16b (i) in Arabidopsis protoplast cells. Scale bars, 10 μm (a, b, d-i) and 20 μm (j). j, k Interaction between LZY3 and truncated forms of RLD1 and RLD2 (j), and between C terminus of RLDs and truncated forms of LZY3 (k) in the Y2H system. Interaction was indicated by growth on selection medium lacking leucine, tryptophan, histidine, and adenine.

resolution (Supplementary Table 3). The RLD2 BRX domain adopts a compact α + β structure comprising an N-terminal three-stranded antiparallel β-sheet and C-terminal α1- and α2-helices (Fig. 4b, c). The LZY3 CCL peptide is folded into a β-hairpin structure and docks into the hydrophobic groove between the β3-strand and the α1-helix of the RLD2 BRX domain by forming an antiparallel intermolecular β-β association between the ββ-strand of the CCL β-hairpin and the β3-strand of the BRX domain. This results in structural extension from the three-stranded β-sheet of the BRX domain to a five-stranded antiparallel β-sheet in the complex, which creates an additional hydrophobic core comprising nonpolar residues (Trp275, Ile283, and Leu285) from the CCL β-hairpin and the surface nonpolar residues (Leu1031, Val1034, Phe1036, and Trp1050) from the BRX domain (Fig. 5a and Supplementary Figs. 13 and 14). The complex is also stabilized by polar interactions and the electrostatic surface potential of the BRX domain is complementary to charged residues of the CCL hairpin (Supplementary Fig. 14a). At the interface, salt bridges between charged side chains from the CCL hairpin and the BRX domain are formed (Glu286-Arg1033 and Lys277-Glu1047), and the CCL hairpin loop is stabilized by Arg1038 from the BRX domain (Supplementary Fig. 14b). Our mutational analysis suggests a dominant contribution of hydrophobic interactions to the complex formation and the importance of Arg1038-mediated stabilization of the hairpin loop (Fig. 5b and Supplementary Fig. 14c, d). Using transient expression in protoplast cells, mutations at corresponding Phe1052 and Trp1066 of the RLD1 BRX domain or at Trp275 and Leu285 of LZY3 CCL were found to prevent the recruitment of RLD1 to the PM by LZY3 (Fig. 5c, d and Supplementary Figs. 14e, f, 15). These results demonstrate that the CCL-BRX interaction is sufficient for LZY-dependent RLD recruitment to the PM. In addition, to evaluate the importance of the CCL-BRX interaction in GSA control of LRs, the same construct caused negative root gravitropism in the lzy1 lzy2 lzy3 triple mutants with wider GSA of LRs under the control of own promoter. As expected, mutated LZY3-mCherry failed to complement the triple mutant phenotypes (Fig. 5e, f), demonstrating the significance of the CCL-BRX interaction in GSA control of LRs. Statocyte-specific expression of the RLD2 BRX domain caused wider LR growth angles not only in the wild type but also in the rld1 rld4 mutant (Supplementary Fig. 16a–f). Moreover, the same construct caused negative root gravitropism in the lzy1 lzy2
lzy3 background (Supplementary Fig. 16g–k), which was similar to the effect of CCL on lzy1 lzy2 lzy3 roots13. These phenotypes could result from a reduction of the activity of residual LZY and RLD in columella cells by blocking LZY-RLD interaction. These results also support the role of direct CCL-BRX binding in gravity signaling in root statocytes.

Polar recruitment of RLD by LZY in the direction of gravity.

Next, we analyzed LZY3 localization in root statocytes using the complemented transgenic line LYL3p:LZY3-mCherry13 (Supplementary Fig. 17c, d). Although fluorescence of LZY3-mCherry was not detectable in living roots as previously reported13 (Supplementary Fig. 18a, b), the signal became observable in over 80% of LRs (48 out of 58 LR tips in stages 2 and 3) by fixation and clearing with ClearSee solution32 (Fig. 6a and Supplementary Fig. 18c–h). In the stage 2 LRs, LZY3-mCherry was detected in the second-outmost layer of the root tips and mainly localized to the PM. Interestingly, LZY3-mCherry was polarly localized in the lower side of the PM (Fig. 6a and Supplementary Fig. 19). To test whether the polarity was determined based on the apical–basal axis of plant body or the direction of gravity, we analyzed LZY3 localization in response to gravistimulation by 180° reorientation experiments. In columella cells of the stage 2 LRs, the mCherry signal was still on the basal side of PM just 5 min after reorientation, while it was detectable in the apical side after 30 min (Fig. 6b–f). The polarity of LZY3-mCherry in the direction of gravity was clearer at 60 min after reorientation than after 30 min (Fig. 6e). In central columella cells, LZY3-mCherry was fully repolarized in the PM at 30 min after reorientation (Fig. 6f). These results indicate that the polarity of LZY3-mCherry localization is determined based on the direction of gravity. Given that the directional change of gravity is thought to be perceived as relocation of amyloplasts in the statocytes, amyloplasts were analyzed by staining starch granules. In the stage 2 LRs, starch granules were visible at the lower side (gravity direction) of the columella cells (Fig. 6g). After the 180° rotation of seedlings, their relocation to the direction of gravity was observed after 30 min of reorientation (Fig. 6h–j), consistent with the timing of repolarization of LZY3-mCherry. These results show a strong correlation between LZY3 polarization and amyloplast sedimentation in columella cells. Next, to test whether RLDs are also polarly localized to the PM in columella cells, intracellular localization of RLD1-GFP in the stage 2 LRs harboring RLD1p:RLD1-GFP was observed. GFP fluorescence was predominantly found in the cytoplasm and slightly higher signals were occasionally observed at the cell periphery, but without distinct polarity under normal conditions (Fig. 6k). Interestingly, the localization of RLD1-GFP in the PM was polarized toward the direction of gravity after 60 min of reorientation (Fig. 6l–n), the time when repolarization of LZY3-mCherry localization became obvious (Fig. 6d). Asymmetric localization of PIN3-GFP toward the direction of gravity was previously reported in columella cells of LRs13,25. Next, to investigate the gravity-responsive localization of PIN3 and the function of RLDs in the PIN3 response, a localization analysis of PIN3-GFP was performed in the stage 2 wild type and rld1 rld4 LRs (Fig. 6o–q and Supplementary Fig. 20). Since the polarity of PIN3-GFP localization was scarcely detectable in the PM of the columella cells in the LRs, we measured PIN3-GFP fluorescence intensity at the outer lateral PM domains of the lateral columella cell adjacent to the central columella cells and determined the asymmetry of PIN3-GFP as the ratio of the intensity at the basal flank to that at the apical flank (Fig. 6r). In the columella cells of stage 2 wild-type LRs in the second-outmost layer, asymmetry of PIN3-GFP toward the direction of gravity was observed (Fig. 6o, s). The change in the asymmetry of PIN3-GFP was detected at 300 min after reorientation, but not at 60 min (Fig. 6p, q, s), which was delayed from the timing of amyloplast relocation, LZY3-mCherry, and RLD1-GFP. In contrast, asymmetry of PIN3-GFP localization in the stage 3 rld1 rld4 LRs was reversed. 

**Fig. 4 The structure of the CCL-BRX complex.** a Binding of the RLD2 BRX domain to the LZY3 CCL peptide. The obtained ITC profile showed formation of a 1:1 complex with a small K0 value (9.7 ± 2.2 nM). ΔH, TΔS, and N are −11.3 ± 0.09 kcal/mol, −1.68 kcal/mol, and 0.73 ± 0.002, respectively. b Topology diagram of the RLD2 BRX domain bound to LZY3 CCL. c Ribbon representation of the crystal structure of the BRX-CCL complex. Color codes are as in Supplementary Fig. 16a–c.
b). In addition, this localization pattern of the two proteins all over the PM, displaying no polarity (Supplementary Fig. 22a, c). columella cells, LZY3-mCherry and RLD1-GFP were localized mutations in CCL driven by control of the LZY3-mCherry. When measured when the polarity of RLD localization was disrupted in PM in columella cells. Next, to investigate the signistrate that LZYs recruit RLD1 to the PM on the lower side of the opposite direction of gravity (Fig.7a).

localization of RLD1-GFP was sometimes polarized in the protoplast cells.
e, f Complementation test. Ten-day-old seedlings of lzy1 lzy2 lzy3 (e) and lzy1 lzy2 lzy3 uncomplemented by LZY3-mCherry carrying mutations in CCL driven by control of the LZY3 promoter (f). Scale bars, 10 μm (c, d) and 1 cm (e, f).

These results indicate that RLDs are involved in the shift of asymmetry of PIN3-GFP to the direction of gravity following amyloplast sedimentation and polarization of LZY3 and RLD1 localization.

In order to investigate the relationship between LZY3 and RLD1 in the statocytes, the localization of LZY3-mCherry and RLD1-GFP was analyzed in the rld1 rld4 LRs at stages 2 and 3, whereas no asymmetry of PIN3-GFP was detectable in either wild-type or rld1 rld4 stage 1 LRs (Supplementary Fig. 20 h–j). These results indicate that RLDs are involved in the shift of asymmetry of PIN3-GFP to the direction of gravity following amyloplast sedimentation and polarization of LZY3 and RLD1 localization.

Fig. 5 The disruptive mutations of the CCL-BRX interaction interfered with LZY3 function. a Sequence alignment of LZYs CCL with the secondary structures are shown at the top with β-strands (arrowhead). The conserved sequences are filled in pink. Residues whose side chain atoms form intermolecular hydrogen bonds and residues making intermolecular van der Waals contacts are colored in cyan and red, respectively, while residues whose main chain form inter-molecular hydrogen bonds are indicated with open circle. In the consensus sequence, Ψ represents a hydrophobic residue, while x represents any residue. b Pull-down binding assay. RLD2 BRX domain was pulled down with GST-LZY3 CCL (WT and mutants) and analyzed by SDS-PAGE. Arrows indicate the reduced abundance of RLD2 BRX. c, d The importance of CCL-BRX interaction in LZY3-dependent RLD recruitment to the plasma membrane. Co-expression of RLD1-GFP with wild-type LZY3-mCherry (c) and LZY3-mCherry carrying mutations in CCL (d) in Arabidopsis protoplast cells. e, f Complementation test. Ten-day-old seedlings of lzy1 lzy2 lzy3 and lzy1 lzy2 lzy3 uncomplemented by LZY3-mCherry carrying mutations in CCL driven by control of the LZY3 promoter (f). Scale bars, 10 μm (c, d) and 1 cm (e, f).

reduced compared with that in wild-type LRs (Supplementary Fig. 20j). After 180° rotation of the seedlings, the shift of asymmetry of PIN3-GFP to the direction of gravity was decreased in rld1 rld4 LRs at stages 2 and 3, whereas no asymmetry of PIN3-GFP was detectable in either wild-type or rld1 rld4 stage 1 LRs (Supplementary Fig. 20 h–j). These results indicate that RLDs are involved in the shift of asymmetry of PIN3-GFP to the direction of gravity following amyloplast sedimentation and polarization of LZY3 and RLD1 localization.

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with the BFA-sensitive phenotype of rld1 rld4 double-mutant roots (Fig. 1 and Supplementary Fig. 7). Considering the expression patterns of RLDs and the pleiotropic phenotype of rld multiple mutants, RLD1 to 4 redundantly modulate auxin transport and possibly other cellular processes through the regulation of membrane trafficking in various tissues and developmental stages. Particularly in the statocytes, modulation of auxin transport by RLD1 and RLD4 is likely to be linked to gravity signaling by the LZY function. We also demonstrated that the CCL domain of LZY and the BRX domain of RLD form a complex with high affinity in vitro and that they are necessary and sufficient for the recruitment of RLD from the cytosol to the PM (Fig. 3). Both the CCL domain and the BRX domain specifically expressed in the statocytes enhanced the GSA phenotype both in the wild type and the lzy1 lzy2 lzy3 background (Supplementary Fig. 16), possibly due to the interference of LZY-RLD interaction. These results suggest that the interaction between LZY and RLD is a key process in gravity signaling in root statocytes.

The BRX domain was initially identified as a conserved domain that often occurs as a tandem repeat in BRX and BREVIS RADIX-LIKE (BRXL) proteins. Here, we have provided the first structural basis for the BRX domain as a protein–protein interaction domain that forms a 1:1 complex with LZY CCLs. The BRX domain is folded into a compact globular structure, although it is composed of only ~60 residues (Fig. 4). When the results of this structure are compared with known structures in PDB by the DALI server, a module similar to the BRX domain (β-β-α-α-fold) often occurs as part of a larger fold in which extra α-helices or β-strands pack to support the folding of the BRX domain-like module, while they share low sequence similarity with the BRX domain (<10%). Therefore, the BRX domain is a novel domain with a specific structure and function. Our gel filtration analysis showed that the isolated BRX domains of RLDs were eluted later than the complexes with LZY CCLs, suggesting that the BRX domains of RLDs exist as monomers. However, they tend to aggregate and precipitate at higher concentrations (>500 μM), presumably due to the non-specific interactions mediated by the hydrophobic surface between the β3-strand and the α1-helix responsible for the interaction with LZYs. This is consistent with the notion that the binding to LZY3 CCL that covers the hydrophobic surface significantly improves the solubility of the RLD2 BRX domain (>10-fold increase). In contrast to the RLDs, the BRX domain of BRX is suggested to serve as a protein–protein interaction domain mediated by homotypic and heterotypic interactions. This difference might reflect the different properties

Fig. 6 Asymmetric localization of LZY3-mCherry, RLD1-GFP, and PIN3-GFP to the direction of gravity. a–d The localization of LZY3-mCherry in the LR tips of 8-day-old lzy1 lzy2 lzy3 seedlings harboring LZY3p:LZY3-mCherry at the stage 2 before rotation (a) and at 5 min (b), 30 min (c), and 60 min (d) after 180° rotation. White and yellow arrowheads indicate polarized LZY3-mCherry localization and the direction of gravity estimated from the growth orientation of LR tips, respectively. Double-headed arrows show the apical–basal axis of the plant body. Asterisks indicate central columella cells. e, f Comparison of asymmetric LZY3-mCherry localization in columella cells (e) and central columella cells (f) in LR tips at stage 2 before and after 180° rotation. Median and quartile values are provided by the central line and box boundaries. Whiskers show min to max values. g, j The position of amyloplasts in columella cells of LRs of 8-day-old lzy1 lzy2 lzy3 seedlings harboring LZY3p:LZY3-mCherry before and after 180° rotation. Brown arrowhead indicates a biased distribution of amyloplasts in columella cells. k–n The localization of RLD1-GFP in LR tips of 8-day-old seedlings harboring RLD1p:RLD1-GFP before and after 180° rotation. o–q The localization of PIN3-GFP in the LR tips of 8-day-old seedlings harboring PIN3p:PIN3-GFP before and after 180° rotation. Arrowheads indicate lateral columella cells, adjacent to central columella cells (red, apical flank; white, basal flank). r Measurement of PIN3-GFP fluorescence intensity at lateral plasma membrane domains of columella cells adjacent to central columella cells at the basal flanks (white) compared with those at the apical flanks (red) in LR tips of 8-day-old seedlings harboring PIN3p:PIN3-GFP. s Comparison of asymmetric localization of PIN3-GFP in the LR tips of 8-day-old seedlings harboring PIN3p:PIN3-GFP before and after 180° rotation. n, sample number of three biologically independent experiments (e, f, s). Different letters in e, f, s indicate statistical differences (Tukey-Kramer, P < 0.05). Source data for e, f, and s are provided as a Source Data file. Scale bars, 20 μm.
of the hydrophobic groove in the β3-strand and the α1-helix, otherwise a tandem repeat might be required for the oligomerization of the BRX domain. Notably, the sequence alignment of the BRX domains based on structure shows high conservation of the residues interacting with LZYs; among 13 residues responsible for LZY binding, 9 and 12 residues are conserved in the N-terminal and C-terminal of the tandem BRX domains in BRX/BRXL, respectively.

Polar auxin transport is strictly controlled and maintained during development such as axis formation and organogenesis, while it changes rapidly in response to the environmental cue during gravitropism. Here, we demonstrated the polar localization of LZY3, which facilitates asymmetric auxin distribution toward the lower flank of the root, to the PM of the lower side of the columella cell in stage 2 LRs. Our results strongly suggest that the PM is the site of action of the LZY proteins. On the other hand, the mode of binding of LZY to the PM is still unknown. LZY might bind to the PM by associating with other membrane proteins or by direct interaction with membrane lipids. There are proteins that bind reversibly to membrane lipids through electrostatic interaction. Proteins harboring a cluster of positively charged amino acids can bind to negatively charged anionic phospholipids. Interestingly, there are K/R-rich regions in LZY proteins that possibly contribute to membrane binding. Further investigations on the mode of membrane binding are important for understanding the regulatory mechanism of LZY polarization upon gravistimulation. Amyloplast relocation and repolarization of LZY3 occurred 30 min after gravistimulation in the stage 2 LRs (Fig. 6). Following the repolarization of LZY3, a relatively higher fluorescent signal of RLD1-GFP became obvious and was polarized in the columella cell toward the direction of gravity. Although the amount of LZY3 protein might be quite low in the cell, LZY3 could capture RLD1 around the PM due to the high affinity between the CCL and BRX domains. It has been reported that PIN3 localization is polarized in the PM of the columella cells in response to gravistimulation. Although we did not observe polar localization of PIN3-GFP in a columella cell, a shift in the asymmetry of PIN3-GFP toward the direction of gravity in lateral columella cells was observed 300 min after gravistimulation (Fig. 6). Our results prompted us to propose a model in which LZY3 recruits RLD1 to the PM of the columella cell in a polarized manner according to the direction of gravity following amyloplast sedimentation. Subsequently, RLD might regulate PIN3 trafficking as discussed above, leading to asymmetric auxin flow (Fig. 7J). Consistently, PIN1- and PIN3-GFP signal intensities are decreased in the embryo or in columella cells of rld multiple mutants, respectively, and a decrease in PIN3-GFP asymmetry is also observed in rld1 rld4. It has also been reported that polar targeting to the PM and the efflux activity of PIN proteins are regulated by phosphorylation mediated by members of the Arabidopsis AGCVIII Ser/Thr protein kinase family. A member of the D6 PROTEIN KINASES, PROTEIN KINASE ASSOCIATED WITH BRX (PAX), is involved in protophloem sieve element differentiation together with BRX with double BRX domains. Considering that neither PAX nor BRX affect the PIN1 abundance or localization, the mode of action of BRX in regulating auxin transport is likely to be distinct from that of RLD, implying that each BRX domain might interact with a distinct partner to execute its function. Our structural data on the BRX domain of RLD could provide valuable information to help us understand the function of the BRX family proteins.

**Methods**

**Plant materials and growing conditions.** In the present study, A. thaliana accession Columbia-0 (Col) was used as the wild-type line. The following mutant alleles and marker lines were used: lzy1 lzy2 lzy3, DR5rev:GFP, PIN2-GFP, ADF9p:GUS, and LZY3p:LZY3-mCherry. rld2-1 (SALK_138887C), rld2-2 (SALK_067605C), rld2-1 (SALK_042345C), rld3-1 (SALK_059345), rld4-2 (SAIL_503H11), and rld4-2 (SAIL_072865C) were obtained from the Arabidopsis Biological Resource Center, whereas rld2-2 (GABI_2225801) and rld4-1 (GABI_2225801) were obtained from GABI-Kat. All the T-DNA lines were backcrossed with Col at least three times prior to the construction of multiple mutants and phenotype analysis. Surface-sterilized seeds were sown on MS plates at 23 °C in a growth chamber under continuous light for 10–14 days, transplanted to soil, and grown under continuous light.
Yeast two-hybrid screening and interaction assay. Matchmaker™ Gold Yeast Two-Hybrid System Kit (Clontech) was used for library screening and interaction assay. The coding region for LZY3 was fused to the C terminus of GAL4 DNA-binding domain (DBD) of pGBK7 or GAL4 activation domain (AD), and then the plasmids were transformed into Y2HGold yeast strain (Clontech) using Fast™, Yeast Transformation Kit (G-Biosciences). Library screening was performed with Match&Plate™ Librarium-Universal Arabidopsis (Normalized) (Clontech) according to the manufacturer’s instructions with 2 × 10⁶ cfu cells. The colonies and blue color on -Leu/-Trp/-His/-Ade media by incubating for 2 days at 30 °C. After washing the colonies and sequenced. The same genes included in multiple independent yeast colonies were selected as candidate genes for LZY3-interacting proteins (Supplementary Table 1).

For interaction assay, full length or portion of LZY or RLD was fused to the C terminus of GAL4 DBD of pGBK7 or GAL4 activation domain (AD), and then the plasmids were transformed into Y2HGold or Y187, respectively. It was confirmed as follows: set off the threshold at peptide tolerance at ±10 p.p.m. and mass spectrometer (LCITQ-Orbitrap XL (Thermo Fisher Scientific) was used to sequence and transformed into Escherichia coli strain BL21Star (DE3) (Invitrogen) cells for protein expression. RLD2 (residues 1006–1066) and LZY3 (residues 274–287) were cloned into PET47b vector and PETG vector, respectively. The RLD2 was expressed in Luria-Bertani medium at 37 °C and the supernatants were collected into a tube. The extracts were dried in a vacuum concentrator. The yielded peptide powders were stored at −80 °C. For crystallization of the RLD2-LZY3 protein complex, RLD2 (residues 1006–1066) and LZY3 (274–287) were co-expressed at 20 °C in Luria–Bertani medium containing 0.1 mM IPTG for 16–24 h. Harvested cells were suspended in buffer A containing 20 mM HEPES, 200 mM NaCl, 10 mM MgCl₂, 1% (w/v) α-BSA and 5% (v/v) glycerol for re-crystallization. The best crystals of the complex between RLD2 and LZY3 was obtained using the Mosquito crystallization robot (TTP Labtech) with the conditions 274 μM LZY3, 374 μM RLD2, 2.4 μl of M9 medium containing 0.1% (v/v) glycerol, 20 μl of M9 medium containing 0.1% (v/v) glycerol with 2 μl of 0.1 M sodium phosphate (pH 8.0), 100 μM EDTA, 10 μM ferricyanide, 10 mM ferricyanide, 0.1% Triton X-100, and 2 mM 3-bromo-4-chloro-3-indolyl-β-D-glucuronic acid at 30 °C. The crystal was flash-cooled in liquid nitrogen containing 10% ethylene glycol for RLD2-LZY3 crystals and flash cooled at 100 K. X-ray diffraction data were collected at a wavelength of 1.00 Å (for native crystal) or 0.968 Å (for Se-Met crystal) on BL44XU, and BL44XU beamlines at SPring-8 or BL1A beamline at the Photon Factory. All data were processed and scaled using HKL-200053. The crystal data are summarized in Supplementary Table 3.
Structure determination and refinement. Phases of the Se-Met labeled RLD2 V1057M-LZY3 complex crystal were calculated by a single-wavelength anomalous dispersion method using the data collected at 1.93 Å resolution. The Se-Met positions were located using the program SOLVE/RESOLVE54. Two RLD2 V1057M-LZY3 complexes were present in the asymmetric unit of the crystal. The built model was refined through alternating cycles using the Coe35 and PHENIX36 programs. The structure of the binary complex RLD2-LZY3 was determined by molecular replacement using the structure of RLD2 V1057M-LZY3 as a starting model. Molecular replacement was performed with Phaser37. Model building and refinement were performed as well as those for the RLD2 V1057M-LZY3 complex structure. The refinement statistics are summarized in Supplementary Table 3. Binding interfaces and structure comparisons have been deposited in the Protein Data Bank under accession codes 6D6W (the Se-Met labeled RLD2 V1057M-LZY3 complex) and 6D6V (the RLD2-LZY3 complex).

Structure and sequence comparison. Multiple sequence alignments of the BRX domains and LZY family proteins were performed using CLUSTALW58. Pairwise comparisons were performed using Cα atom positions by the PDBeFold server59 and structure figures were prepared using the PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC. Electrostatic potentials were calculated with APBS60 and are displayed in PyMol.

Binding study by ITC analysis. ITC was conducted using a calorimeter (ITC200, GE Healthcare) at 20 °C. Purified protein samples were dialyzed overnight in buffer containing 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl. We performed data fitting using the ORIGIN software program supplied with the instrument. The ITC profile for binding of the RLD2 BRX domain to the LZY3 CCL was obtained by injections of 1 μl of 200 μM LZY3 (residues 274–287) into the RLD2 BRX domain solution (20 μM) at 20 °C. Raw data for 40 sequential injections and the plot of the heat evolved (kcal) per mole of LZY3 CCL added, corrected for the heat of LZY3 CCL by dilution, to the molar ratio of LZY3 to RLD2 BRX domain.

Pull-down binding assay. All mutations were produced by site-directed mutagenesis. For in vitro pull-down binding assays, the purified protein and GST-fusion protein were mixed with a slurry of glutathione sepharose 4B and incubated at 4 °C. After washing with incubation buffer, collected eluates were subjected to SDS-PAGE. The relative amount of the proteins pulled down was measured with error bars representing standard deviation from three independent measurements.

Gravity stimulation analysis. The seedlings were vertically grown on MS plates for 8 days, and then rotated 180° and incubated for additional 5, 30, and 60 min, respectively. The LR sample was cut out with gellan gum block and placed into a 1.5 ml tube, keeping the sample in vertical orientation. Samples were fixed in 4% (v/v) paraformaldehyde in MTSB (15.1 g/l PIPES, 1.23 g/l MgSO4·7H2O, and 1.9 g/l EGTA; pH 7.0) at room temperature for more than 30 min, keeping the samples in vertical orientation. After fixation, samples were washed twice with MTSB buffer for 1 min and cleared with ClearSee solution at room temperature for more than 4 days32. For observation of LZY3-mCherry, samples were mounted in ClearSee solution, and a confocal image of RLD1-GFP/mCherry was obtained using a TCS SP8 DLS (Leica) equipped with a 10× objective. To construct GUS or PIN3-GFP was obtained using a TCS SP8 DLS (Leica) equipped with a 10× objective. To construct GUS or PIN3-GFP was obtained using a TCS SP8 DLS (Leica) equipped with a 10× objective.

Quantitative and semi-quantitative RT-PCR. For real-time quantitative reverse transcription PCR (qRT-PCR), the seedlings, vertically grown on MS medium for 7 days, transferred to new MS medium with or without 1 μM estradiol (10 μM stock in EO), incubated vertically at 23 °C under continuous light for additional 3 days, were used. Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from 0.5 μg of total RNA treated with 1000 IU of RNase-free TURBO DNAse (Ambion) for 20 min at 37 °C and then heated at 80 °C for 5 min to inactivate the enzyme. CDFs were diluted 1:20 into water prior to use. A LightCycler 96 Real-Time PCR System was used. The results of the three technical repeats for three biological replicates, messenger RNA relative expression levels were calculated and expressed as fold changes relative to the reference sample. The derived data were analyzed using a two-way ANOVA with Tukey’s post hoc test.

Plasmid construction. We used the Gateway Cloning System (Invitrogen) to construct RLD1p:GUS, RLD2p:GUS, RLD3p:GUS, RLD4p:GUS, RLD1p:RLD1-GFP, ADF9p:RLD1, ATHB8p:RLD1-mCherry, and 35S:RLD1-GFP. The 2866-, 2178-, 1016-, and 2419-bp upstream from the start codon of RLDR2, RLDR3, RLDR4, respectively, were used as the promoter regions and fused with DNA fragments containing GUS gene and NOS terminator on the pENTR vector. Subsequently, they were introduced into pFAST-B0252. The 3122- and 1700-bp fragments upstream from the start codon of ADF9 and ATHB8 were used as ADF9 and ATHB8 promoters53,54, respectively. The promoter regions of RLDR1, ADF9, and ATHB8 were combined with cloning sites and NOS terminator in pENTR vector. Full-length cDNA of RLDR1, RLDR3-GFP/mCherry fusion gene, BRX of RLDR2 (3′ end of RLDR2 CDS (219 bp) was cloned between the promoter and NOS terminator, following by introducing into pGWBS0154 (RLD1p:GUS, RLD2p:GUS, RLD3p:GUS, RLD4p:GUS, ADF9p:RLD1, ATHB8p:RLD1-mCherry, ADF9p: BRXΔ (RLD2-GRF)), Full-length cDNA of RLDR1 was cloned into pENTR vector, following by introducing into pFAST-B0252. 35S:RLD1-GFP. To introduce mutations into LZY3-LTI6b and LZY3-LTI6b terminator in the multi-cloning site of the pE8R vector65,66. Full PCR amplification and standard primers containing respective mutation was performed and then mutated LZY3 CDS was replaced with wild-type CDS on LZY3p:LZY3-mCherry pENTR vector, followed by introducing into pGWBS01 (LZY3p:LZY3 (K275A2L285A-mCherry)). To construct GUS or PIN3-GFP promoter regions were replaced with wild-type CDS and downstream region of the domain promoter by RT-PCR from the Col wild type, and their fragments were fused with DNA fragments containing GUS gene and NOS terminator (GFP-NOS) on the pUC19 vector under the cauliflower mosaic virus 35S promoter (35S:RLD1-GFP, 35S: RLDR1-APH-GFP, 35S:RLD1ABRX-GFP, 35S:RLD2-GFP, 35S:RLD3-GFP, 35S:RLD4-GFP). To construct 35S:RLD1ARCC1-GFP and 35S:RLD1ATFYVE-GFP, upstream region of respective domains in RLDR1 CDS and downstream region of the domain were amplified by RT-PCR from the Col wild type, and their fragments were fused with DNA fragments containing GUS gene and NOS terminator (mCherry-NOS) on the pUC19 vector under the cauliflower mosaic virus 35S promoter (35S:RLD1-GFP) and NOS terminator (mCherry-NOS). To introduce mutations into LZY3-LTI6b and LZY3-LTI6b terminator in the multi-cloning site of the pE8R vector65,66. Full PCR amplification and standard primers containing respective mutation was performed and then mutated LZY3 CDS was replaced with wild-type CDS on LZY3p:LZY3-mCherry pENTR vector (35S:LZY3 (K275A2L285A-mCherry)).
Supplementary Figs. 7g via the PRIDE67 partner repository with the dataset identifier PXD016219. The authors declare that the data supporting the findings of this study are available within the manuscript and its supplementary files or are available from the corresponding author upon reasonable request. Raw data for underlying Figs. 1d–e, 6e, f, s, and 7i and Supplementary Figs. 7g–j, 12, 14c, 20g–j, and 22c are provided in the Source Data File.

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22c are provided in the Source Data File.
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Author contributions

M.F., Y.H., T.N., T.H. and M.T.M. designed the studies; M.F., T.N., M.N., M.T., K.S., R.O., S.S. and C.K. performed the molecular biological experiments; Y.H. performed structural and biophysical analyses; M.T. and Y.F. identified LZY interactors; M.T., K.S. and M.T.M. conducted initial preliminary studies; and M.F., Y.H., T.N., T.H., K.K. and M.T.M. analyzed the data and wrote the manuscript with input from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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