DIVISION OF PLANT ENVIRONMENTAL RESPONSES

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Plant organs have the ability to sense various vectorial stimuli such as light, humidity and gravity as well as reorient their growth direction so as to be in a suitable position to survive and acclimate to their environment. These types of responses are referred to as tropisms. Gravitropism is a major determinant in the directing of plant organ growth angles. In gravity sensing cells (statocytes), plastids accumulating starch at a high-density relocate toward the direction of gravity. Amyloplast relocation serves as a physical signal trigger for biochemical signal transduction, which in turn leads to the regulation of polar auxin transport necessary for change in the direction that a given plant is growing. The above points raise the following important questions: 1) How is amyloplast relocation converted into a biochemical signal? 2) How do signals affect directional plant growth?

In keeping with this, we aim to understand the detailed molecular mechanism of gravity signaling by applying genetic and molecular biological approaches that use the model plant *Arabidopsis thaliana*.

I. Molecular mechanisms associated with gravity signaling

1-1 LZYs and their interactors RLDs are involved in gravitropism

*LAZY1 f*amily genes are involved in gravitropism in many plant species. We previously found that *LAZY1-LIKE (LZY) 1*, *LZY2*, and *LZY3,* are required for gravity signal transduction in statocytes following amyloplast relocation, which leads to the determination of the growth angle of roots and shoots in Arabidopsis. LZY2 and LZY3 are major contributors to root gravitropism (Figure 1A). *LAZY1* family genes encode plant specific proteins with no domain in which the function is inferable. To elucidate the molecular function of these LZY proteins, we performed yeast two-hybrid screens and immunoprecipitation coupled with mass spectrometry to identify their interactors. We found that LZY2 and LZY3 interact with RCC1-like domain (RLD) proteins. There are eight *RLD* family genes in *Arabidopsis* genome. *RLD* family genes are conserved among land plants and share a similar domain combination 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. *Promoter:GUS* analysis indicates that four *RLD* genes are expressed in the root caps and vascular tissues of primary roots and lateral roots (Figure 1B). While *lzy* multiple mutants exhibit the disturbed gravity setpoint angle both in primary roots and shoots, *rld1 rld4* double mutants display a similar root phenotype to that found in *lzy* multiple mutants (Figure 1A, C). *rld1 rld4* double mutants fail to establish the asymmetric auxin distribution in roots as observed in *lzy* multiple mutants. These findings suggest that both *RLD*s and *LZY*s play an important role in gravity signaling.

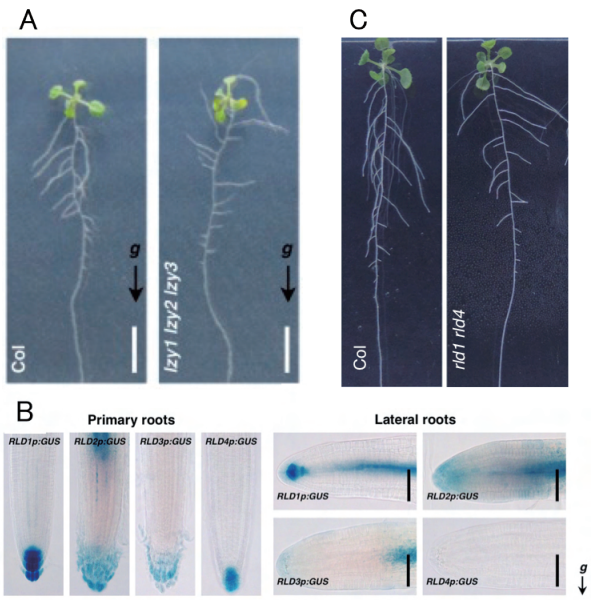

Figure 1. Role of *RLDs* and *LZYs* in gravitropic setpoint angle. **A and C**. Growth angle of lateral roots in *lzy1 lzy2 lzy3* and *rld1 rld4* mutants. Arrows indicate the direction of gravity. **B**. Histochemical assay for *RLD* gene promoter activities. GUS staining of the primary and lateral roots in *RLD1p, RLD2p, RLD3p or RLD4p:GUS* lines are shown.

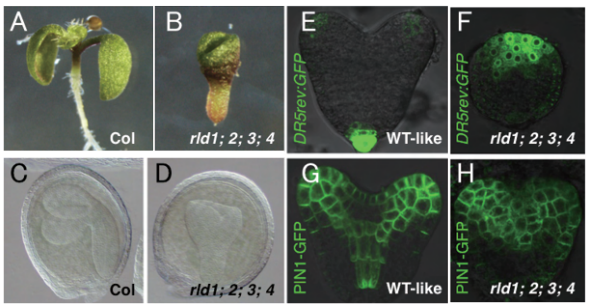

Figure 2. Loss-of-function of *RLD* genes disrupt the patterns of PIN1-GFP expression and auxin distribution. **A and B**. Seven-day-old seedlings of Col wild-type (A) and *rld1 rld2 rld3 rld4* (*rld1; 2; 3; 4*). **C and D**. Embryos of Col (**C**) and *rld1; 2; 3; 4* (**D**). **E-H**. *DR5rev:GFP* (**E, F**) and PIN1-GFP (**G, H**) expression in WT-like (**E, G**) and *rld1; 2; 3; 4* (**F, H**) embryos dissected from ovules of plant homozygous for *rld1*, *rld3*, and *rld4* and heterozygous for *rld2* at the heart stage.

1-2 LZYs recruit RLDs to the plasma membrane

rld1 rld4 mutants exhibited a significant reduction in the level of GFP-tagged PIN3 (PIN3-GFP), which itself is a member of auxin efflux carrier PIN family expressed in root cap columella cells. Moreover, *rld1 rld2 rld3 rld4* quadruple

mutant embryos and seedlings displayed severe morphological defects (Figure 2A-D). In the quadruple mutant embryos, a severe reduction of PIN1-GFP and abnormal auxin distribution pattern were observed (Figure 2E-H). These embryonic phenotypes closely resembled those of *gnom*, a loss-of-function mutant of an ARF-GEF GNOM involved in the trafficking of PIN proteins. 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.

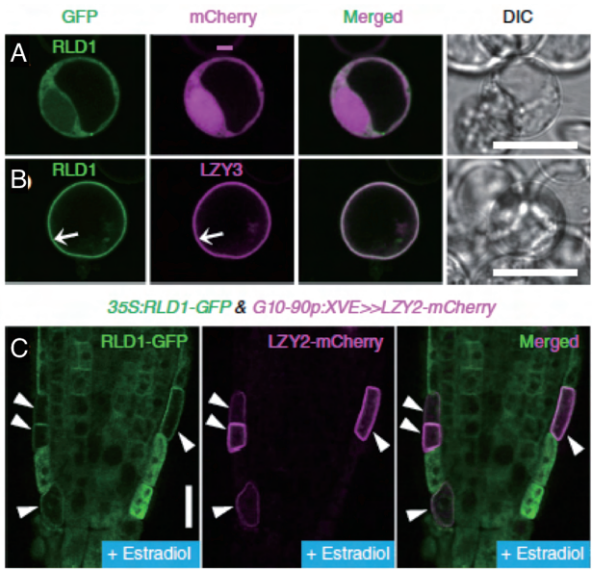

Figure 3. LZYs recruits RLDs to the plasma membrane. **A and B**. Co-expression of RLD1-GFP with mCherry (**A**) and LZY3-mCherry (**B**) in Arabidopsis protoplasts. **C**. Localization of RLD1-GFP (left) and LZY2-mCherry (middle) in the primary root harboring *35Sp:RLD1-GFP* and estradiol-inducible *LZY2-mCherry*. Arrowheads indicate LZY2-mCherry-expressing cells, where RLD1-GFP colocalized with LZY2-mCherry in the plasma membrane.

While RLD-GFPs were observed in the cytoplasm in Arabidopsis protoplast cells, they were localized on the plasma membrane only when LZY2/3-mCherry were co-expressed (Figure 3A, B). Chemical inducible expression of LZY2-mCherry in the root cells constitutively expressing RLD1-GFP led to the localization of RLD1-GFP on the plasma membrane whereas RLD1-GFP was detected in the cytosol in cells not expressing LZY2-mCherry (Figure 3C).

We also demonstrated through yeast two-hybrid experiments and *in vitro* pull-down assays that the CCL domain on the C-terminus of LZY and the BRX domain on the C-terminus of RLD are responsible for the interaction between LZY3 and RLDs. The interaction between the CCL domain and the BRX domain was required for their co-localization of LZY and RLD on the plasma membrane in protoplasts. To elucidate the structural basis of the interaction, we determined the crystal structures of the RLD2 BRX domain bound to the LZY3 CCL peptide at 1.35 Å resolution. The structural analysis reveals the mode of the interaction as an intermolecular β-sheet between the CCL and the BRX domain in addition to the structure of the BRX domain (Figure 4). Based on the structural analyses, we introduced mutations that impaired the interaction into the BRX or the CCL and demonstrated the importance of the interaction for gravitropism. These findings indicate RLDs, possible regulators of PIN trafficking, are recruited from the cytosol to the plasma membrane by LZYs through the interaction between the BRX domain and the CCL domain.

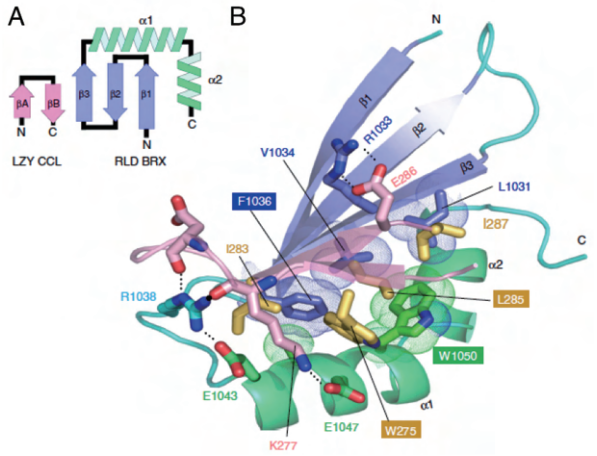

Figure 4. The structure of the CCL-BRX complex. **A**. Topology diagram of RLD2 BRX domain bound to LZY3 CCL. **B**. Ribbon representation of the crystal structure of the BRX-CCL complex. Color codes are as in **A**. The RLD2 BRX domain comprises a three-stranded antiparallel β-sheet (blue) and two α-helices (green). The LZY3 CCL peptide adopts a β-hairpin structure (pink). Dashed lines represent inter-molecular hydrogen bonds. The colors of corresponding positions in the CCL-BRX complex at which mutations were introduced in RLD1-GFP and LZY3-mCherry are reversed.

1-3 LZY3 controls RLD1 localization in columella cells of lateral roots

PIN3 is involved in the asymmetric auxin distribution in roots, and has been reported to polarly localize on the plasma membrane in the columella cells. Interestingly, we have found that LZY3-mCherry is also polarly localized on the basal side of the plasma membrane in the columella cells of lateral roots when plants are vertically grown. Intriguingly, 180˚ rotation of plants expressing LZY3-mCherry reduces the signal of LZY3-mCherry on the basal side of the plasma membrane and increases the signal on the apical side of plasma membrane where the latest gravity direction is at least 30 minutes after the rotation. This observation indicates that LZY3 is polarly localized on the plasma membrane in response to gravistimulation (Figure 5A). RLD1-GFP exhibits the same behavior as LZY3-mCherry in the columella cells whereas the asymmetric distribution of PIN3-GFP is observed 300 minutes after the rotation. Taken together, we have proposed a model in which gravity stimuli leads to polarization of LZYs and the polarized LZYs recruit RLDs on the plasma membrane in order to establish the polar auxin transport (Figure 5B). Little is known about how the amyloplast sedimentation information is transduced in the form of localization of LZYs and the molecular function of RLDs in gravity signaling. Accordingly, we are now exclusively focusing on revealing those mechanisms.

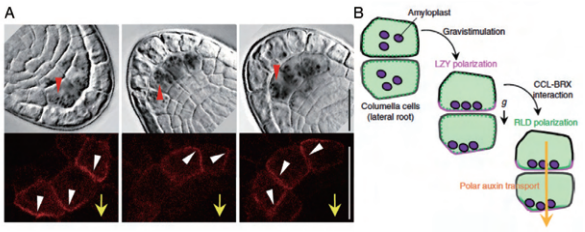

Figure 5. Asymmetric localization of LZY3-mCherry toward the direction of gravity and the model on gravity signaling. **A**. The localization of LZY3-mCherry in the lateral root tips of *lzy1 lzy2 lzy3* seedlings harboring *LZY3p:LZY3-mCherry* at the stage 2 before rotation (left) and at 5 min (middle), 30 min (right) after 180˚ rotation. White and red arrowheads indicate polarized LZY3-mCherry localization and amyloplasts, respectively. The yellow arrows indicate the direction of gravity estimated from the growth orientation of lateral root tips. **B**. Schematic diagrams of LZY-RLD-mediated gravity signaling in columella cells of lateral roots.

II. Determination mechanism of gravitropic setpoint angle

Plant posture is controlled by various environmental cues, such as light, temperature, and gravity. Their overall architecture is determined by the growth angles of lateral organs, such as roots and branches. The branch growth angle affected by gravity is known as the gravitropic setpoint angle (GSA). Many gravitropic mutants show abnormal GSA in lateral branches; meaning they produce wider growth angle phenotypes likely due to reduced gravitropism. Lateral branches of Arabidopsis *shoot gravitropism* (*sgr*) mutants lacking the endodermal cells, which themselves are shoot statocytes, grow horizontally as well as those possessed by wild type plants grown under microgravity condition.

The primary shoots of *lzy1 lzy2 lzy3* triple mutants exhibited non-responsiveness to gravistimulation, so it was expected that the lateral branches of the triple mutant would grow horizontally like *sgr* mutants do. However, they unexpectedly grew downward and showed positive gravitropism upon gravistimulation. Moreover, primary and lateral roots of *lzy2 lzy3 lzy4* triple mutant showed negative gravitropism. We accordingly refer to the phenotype of reversed growth direction observed in primary roots and lateral branches of *lzy* triple mutants as “anti-gravitropic”.

It has been proposed that GSA is determined by balancing two opposing growth components: gravitropism and anti-gravitropic offset (AGO). We assumed that the balance between gravitropism and AGO was disrupted in *lzy* triple mutants, and hypothesized that AGO would be manifested as an “anti-gravitropic” phenotype. The molecular mechanisms underlying gravitropism have been studied extensively, but little is known about the nature of AGO. To investigate the mechanism of GSA control, we are currently focusing on understanding the nature of AGO by analyzing “anti-gravitropic” phenotypes of *lzy* triple mutants.

Publication List:

〔Original papers〕

- Furutani, M., Hirano, Y., Nishimura, T., Nakamura, M., Taniguchi, M., Suzuki, K., Oshida, R., Kondo, C., Sun, S., Kato, K., Fukao, Y., Hakoshima, T., and Morita, M.T. (2020). Polar recruitment of RLD by LAZY1-like protein during gravity signaling in root branch angle control. Nat. Commun. *11*, 76. doi: 10.1038/s41467-019-13729-7
- Kimata, Y., Kato, T., Higaki, T., Kurihara, D., Yamada, T., Segami, S., Morita, M.T., Maeshima, M., Hasezawa, S., Higashiyama, T., Tasaka, M., and Ueda, M. (2019). Polar vacuolar distribution is essential for accurate asymmetric division of *Arabidopsis* zygotes. Proc. Natl. Acad. Sci. USA *116*, 2338-2343. doi: 10.1073/pnas.1814160116
- Mergner, J., Frejno, M., List, M., Papacek, M., Chen, X., Chaudhary, A., Samaras, P., Richter, S., Shikata, H., Messerer, M., Lang, D., Altmann, S., Cyprys, P., Zolg, D.P., Mathieson, T., Bantscheff, M., Hazarika, R.R., Schmidt, T., Dawid, C., Dunkel, A., Hofmann, T., Sprunck, S., Falter-Braun, P., Johannes, F., Mayer, K.F.X., Jürgens, G., Wilhelm, M., Baumbach, J., Grill, E., Schneitz, K., Schwechheimer, C., and Kuster, B. (2020). Mass-spectrometry-based draft of the *Arabidopsis* proteome. Nature *579*, 409-414. doi: 10.1038/s41586-020-2094-2
- Ohbayashi, I., Huang, S., Fukaki, H., Song, X., Sun, S., Morita, M.T., Tasaka, M., Millar, A.H., and Furutani, M. (2019). Mitochondrial pyruvate dehydrogenase contributes to auxin-regulated organ development. Plant Physiol. *180*, 896-909. doi: 10.1104/pp.18.01460

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〔Review articles〕

- Nakamura, M., Nishimura, T., and Morita, M.T. (2019). Bridging the gap between amyloplasts and directional auxin transport in plant gravitropism. Curr. Opin. Plant Biol. *52*, 54-60. doi: 10.1016/j.pbi.2019.07.005
- Nakamura, M., Nishimura, T., and Morita, M.T. (2019). Gravity sensing and signal conversion in plant gravitropism. J. Exp. Bot. *70*, 3495-3506. doi: 10.1093/jxb/erz158