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RESEARCH ARTICLE

The Arabidopsis LAZY1 Family Plays a Key Role in Gravity Signaling within Statocytes and in Branch Angle Control of Roots and Shoots

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Short title: Genes for gravitropism and branch angle control

One-sentence summary: The LAZY1 family expressed in statocytes is likely to regulate the polar auxin transport in response to gravistimulation in gravitropism of roots and shoots, and in GSA control of lateral roots.

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ABSTRACT

During gravitropism, the directional signal of gravity is perceived by gravity-sensing cells called statocytes, leading to asymmetric distribution of auxin in the responding organs. To identify the genes involved in gravity signaling in statocytes, we performed transcriptome analyses of statocyte-deficient *Arabidopsis thaliana* mutants and found two candidates from the LAZY1 family, *AtLAZY1*/*LAZY1-LIKE1* (*LZY1*) and *AtDRO3*/*AtNGR1*/*LZY2*. We showed that *LZY1*, *LZY2*, and a paralog *AtDRO1/AtNGR2/LZY3* are redundantly involved in gravitropism of the inflorescence stem, hypocotyl, and root. Mutations of *LZY* genes affected early processes in gravity signal transduction without affecting amyloplast sedimentation. Statocyte-specific expression of *LZY* genes rescued the mutant phenotype, suggesting that *LZY* genes mediate gravity signaling in statocytes downstream of amyloplast displacement, leading to the generation of asymmetric auxin distribution in gravity-responding organs. We also found that *lzy* mutations reversed the growth angle of lateral branches and roots. Moreover, expression of the conserved C-terminal region of LZY proteins also reversed the growth direction of primary roots in the *lzy* mutant background. In lateral root tips of *lzy* multiple mutants, asymmetric distribution of PIN3 and auxin response were reversed, suggesting that *LZY* genes regulate the direction of polar auxin transport in response to gravity through the control of asymmetric PIN3 expression in the root cap columella.

INTRODUCTION

Plants are able to sense the direction of gravity and alter the orientation of their growth accordingly. In general, primary shoots and roots of vascular plants exhibit negative and positive gravitropism, respectively (Knight, 1806). As described by the starch-statolith hypothesis (Haberlandt, 1965; Sack, 1991), the direction of gravity is perceived mainly by gravity-sensing cells, called statocytes, which detect the sedimentation of starch-accumulating high-density amyloplasts. The resulting gravitropic signal is converted to a biochemical signal within the statocytes (gravity signaling) and then transmitted to responding tissues (auxin transport), where the signal induces the differential growth of the lower and upper surfaces of gravity-responsive organs through the asymmetrical distribution of auxin, as described by the Cholodny-Went theory (Went, 1974; Hart, 1990). Recent molecular genetic studies have provided evidence that strongly supports these hypotheses. Starch accumulation in amyloplasts has been shown to be important for mobility in response to gravistimulation, and amyloplast sedimentation is influenced by large central vacuoles and actin

75 cytoskeletons (Fitzelle and Kiss, 2001; Hashiguchi et al., 2013; Blancaflor et
76 al., 2013). Moreover, many aspects of the molecular mechanisms for auxin
77 transport, auxin signaling, and auxin response, which are crucial not only
78 for tropisms but also for plant development and growth, have recently been
79 characterized (Sato et al., 2015; Rakusová et al., 2015; Žádníková et al.,
80 2015).

81 However, the molecular mechanism underlying the change in polar
82 auxin transport following amyloplast sedimentation in statocytes, which
83 bridges the gap between the two long-standing hypotheses, remains
84 unknown (Morita, 2010). In *Arabidopsis thaliana*, endodermal cells sense
85 gravity in the shoots, whereas columella cells sense gravity in the roots
86 (Fukaki et al., 1998; Blancaflor et al., 1998), and the auxin efflux facilitator
87 PIN3 and the membrane-associated DnaJ domain proteins ALTERED
88 RESPONSE TO GRAVITY1 (ARG1) and ARG1-LIKE2 (ARL2) are
89 reportedly involved in the gravity signaling in statocytes. More specifically,
90 PIN3, which is expressed in both endodermal and columella cells, is thought
91 to relocate to the lower side of statocytes upon reorientation, resulting in a
92 subsequent redistribution of auxin in the responding organs (Harrison and
93 Masson, 2008; Kleine-Vehn et al., 2010; Rakusová et al., 2011). This polar
94 localization of PIN3 has been observed in the endodermal cells of hypocotyls
95 and in the columella cells of roots but has not been reported in the
96 endodermal cells of inflorescence stems. Meanwhile, ARG1 and ARL2, which
97 are both expressed in statocytes, are involved in the gravitropism of
98 hypocotyls and roots (Sedbrook et al., 1999; Boonsirichai et al., 2003),
99 although ARG1 is not involved in gravitropism in inflorescence stems
100 (Fukaki et al., 1997). ARG1 contributes to the polarization of PIN3
101 distribution in the columella cells of roots (Harrison and Masson, 2008),
102 indicating that ARG1 is involved in gravity signaling by root statocytes.

103 In the present study, we aimed to identify genes involved in the
104 gravity signaling process of inflorescence stems, by performing

transcriptome analyses of the endodermis-defective mutants *shoot gravitropism1* [*sgr1*; also known as *scarecrow* (*scr*)] (Fukaki et al., 1996) and *endodermal amyloplast less 1* (*eal1*) (Fujihira et al., 2000). The *sgr1/scr* mutant possesses inflorescence stems that lack an endodermis and exhibit agravitropism (Fukaki et al., 1998), whereas *eal1*, a hypomorphic allele of *SGR7/SHORT-ROOT* (*SHR*), retains the ability to form an endodermis-like cell layer but is also agravitropic, indicating that the endodermis-like cells of *eal1/shr* were not functional statocytes (Morita et al., 2007). Since both SGR1/SCR and SGR7/SHR are transcription factors (Helariutta et al., 2000), we hypothesized that any genes downregulated in both *sgr1/scr* and *eal1/shr* would include genes that are expressed in the endodermis and involved in the gravitropism of stems. Among the differentially expressed genes, we focused on two members of the *LAZY1* family in Arabidopsis (Yoshihara et al., 2013), namely *AtLAZY1*/*LAZY1-LIKE1* (*LZY1*) and *AtDRO3*/*AtNGR1*/*LZY2*. The *LAZY1* family contains plant-specific genes with unknown molecular functions that are involved in gravitropism in rice, Arabidopsis, maize, and *Medicago truncatula* (Table 1; Yoshihara and Iino, 2007; Li et al., 2007; Yoshihara et al., 2013; Dong et al., 2013; Uga et al., 2013; Ge and Chen, 2016). Here, we show that *LZY1*, *LZY2*, and their paralog *AtDRO1/AtNGR2/LZY3* are redundantly involved in both shoot and root gravitropism and that their corresponding proteins play a key role in controlling lateral auxin flow after the reorientation of statocytes. Our findings also demonstrate that the regulation of auxin flow by the three LZY proteins in statocytes influences plant architecture by controlling the growth angle of lateral shoots and lateral roots.

RESULTS

Role of *LZY1, LZY2,* and *LZY3* in shoot gravitropism

To identify genes involved in regulating gravity signaling in shoot statocytes in Arabidopsis, we performed DNA microarray analyses on inflorescence stems of wild-type, *sgr1/scr*, and *eal1/shr* mutant plants. Gene expression profiles were then compared among the wild type and mutants. The genes downregulated by more than 5- and 3-fold in the *eal1/shr* and *sgr1/scr* mutants were identified (Table 2). Among these genes were two members of Arabidopsis *LAZY1* family: *AtLAZY1*, which is known to be involved in shoot gravitropism, and *At1g17400* (Yoshihara and Iino, 2007; Li et al., 2007; Yoshihara et al., 2013). Here, we refer to *AtLAZY1* as *LAZY1-LIKE1* (*LZY1*) and to *At1g17400* as *LZY2* (Table 1) and focused our subsequent analyses on them and on a paralog *LZY3/At1g72490* that shares 66% amino acid sequence identity with LZY2. *LZY2* and *LZY3* have recently been reported as *AtDRO3/AtNGR1* and *AtDRO1/AtNGR2*, respectively, and investigated for their roles in root gravitropism (Table 1; Ge and Chen, 2016; Guseman et al., 2017). We confirmed that the expression levels of *LZY1*, *LZY2,* and *LZY3* were significantly reduced in both *sgr1/scr* and *eal1/shr* stems by qRT-PCR analyses (Figure 1A). We also evaluated the promoter activities of these *LZY* genes using β-glucuronidase (GUS) activity assays in wild-type plants harboring *LZY1p:GUS, LZY2p:GUS,* or *LZY3p:GUS* constructs (Figures 1B and Supplemental Figure 1). We found that *LZY1, LZY2*, and *LZY3* exhibited different expression patterns that partially overlapped and that all three genes were active mainly in the endodermis of both inflorescence stems and hypocotyls.

To ascertain the role of the *LZY* genes in shoot gravitropism of inflorescence stems and hypocotyls, we obtained T-DNA insertion lines (*lzy1*; GABI_591A12, *lzy2*; FLAG_199G07, *lzy3*; SAIL_723_H12) and crossed these to generate multiple mutant lines (Supplemental Figure 2). Although the inflorescence stems of *lzy2*, *lzy3*, and *lzy2 lzy3* mutant plants did not show

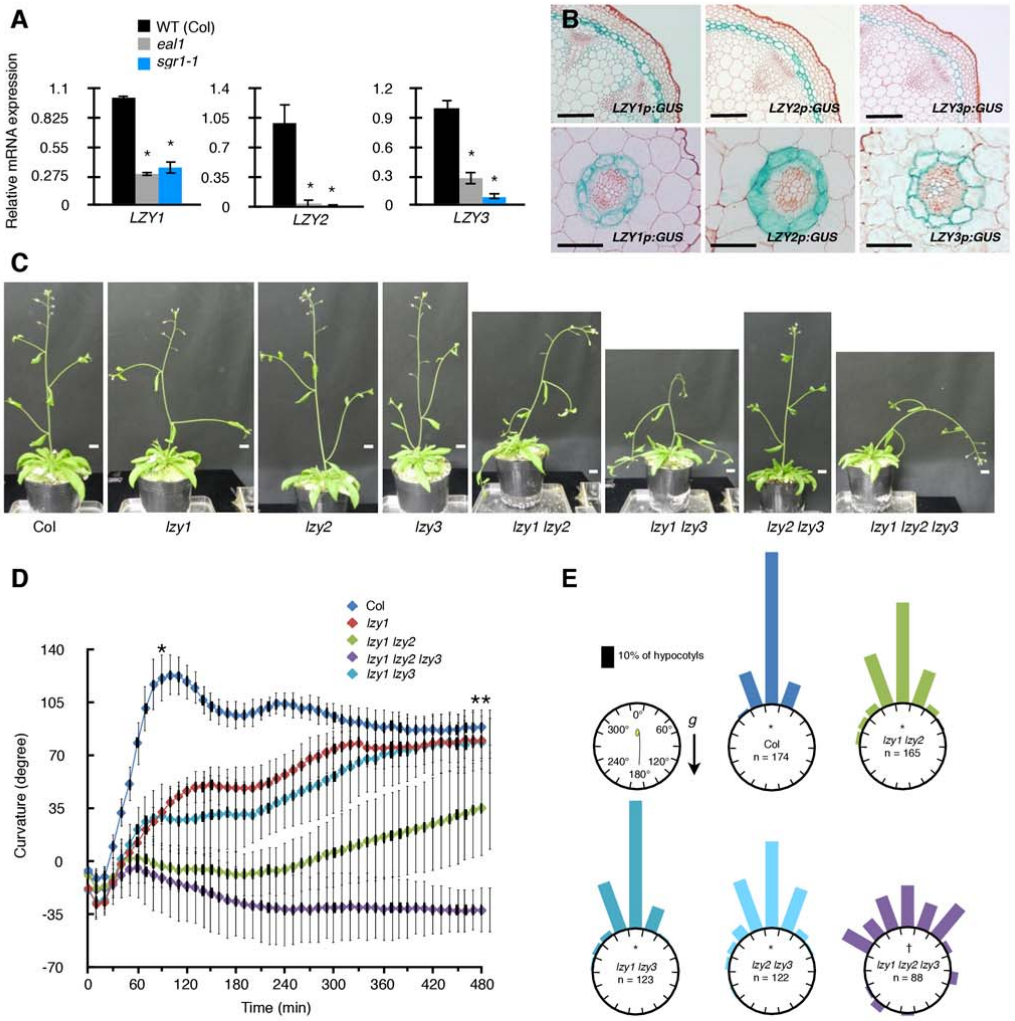

Figure 1. Arabidopsis *LZY* genes function in shoot gravitropism.

(A) Transcript levels of *LZY1* (left), *LZY2* (middle), and *LZY3* (right) in inflorescence stems of wild-type Col (black), *eal1* (gray), and *sgr1* (blue). Data represent relative values where the mRNA level of each gene in Col was set as 1. Data show mean ± SD of three technical replicates for three independent samples with SD. Asterisks indicate significant differences by Student's *t*-test compared to wild type (**P* value < 0.01).(B) Expression pattern of the *LZY* genes. GUS staining of plants expressing *LZY1p:GUS* (left), *LZY2p:GUS* (middle), and *LZY3p:GUS* (right) in inflorescence stems (top) and etiolated hypocotyls (bottom). (C) Aerial parts of 5-week-old plants. (D) Time course of the gravitropic response (mean ± SD) of inflorescence stems after being placed horizontally (n ≥ 12 for each genotype). For statistical analysis, the Tukey-Kramer method was used to compare curvature measurements after 90 (*) and 480 minutes (**) of horizontal placement. There were significant differences in *lzy1*, *lzy1 lzy2*, *lzy1 lzy3*, and *lzy1 lzy2 lzy3* after 90 min compared to Col (*P* value < 0.05), in *lzy1 lzy2* and *lzy1 lzy2 lzy3* after 480 min compared to Col (*P* value < 0.05), between *lzy1* and *lzy1 lzy2*, *lzy1* and *lzy1 lzy2 lzy3*, *lzy1 lzy2* and *lzy1 lzy3*, and *lzy1 lzy3* and *lzy1 lzy2 lzy3* after 90 min (*P* value < 0.05) and between *lzy1* and *lzy1 lzy2*, *lzy1* and *lzy1 lzy2 lzy3*, *lzy1 lzy2* and *lzy1 lzy3*, *lzy1 lzy2* and *lzy1 lzy2 lzy3*, and *lzy1 lzy3* and *lzy1 lzy2 lzy3* after 480 min (*P* value < 0.05). (E) Growth direction of 3-day-old etiolated hypocotyls, at intervals of 20°. The number of individuals examined for each line is shown within the corresponding circles. Arrow marked with *g* represents the direction of gravity. For statistical analysis, means of the absolute value of the angle between growth direction and horizontal axis were compared. Means not sharing the same symbol (*, †) are significantly different (Tukey-Kramer, *P* < 0.05). Scale bars = 100 μm (B) and 1 cm

significant phenotypic changes in gravitropic responses, *lzy1* mutant plants displayed a reduced gravitropic response, and their lateral branches tended to grow in a horizontal direction (Figures 1C and 1D; Yoshihara, 2013). The *lzy2* mutation enhanced the *lzy1* phenotype, but *lzy3* did not (Figures 1C,

1D, and Supplemental Figure 3A). In addition, *lzy1 lzy2 lzy3* triple mutant plants displayed more severe defects in gravitropism than did *lzy1 lzy2*. Remarkably, the primary shoots of *lzy1 lzy2 lzy3* triple mutant plants grew along the ground and showed almost complete loss of the gravitropic capacity for reorientation. In dark-grown hypocotyls, single and double mutants showed slight phenotypic changes in growth directions, indicating retention of gravitropic capability. However, triple mutants exhibited significantly reduced gravitropism (Figure 1E and Supplemental Figure 3B). The phenotypes of the single and multiple *lzy* mutants demonstrated that the *LZY* genes have redundant functions and different levels of contribution to shoot gravitropism (*LZY1* > *LZY2* > *LZY3* in the stem; *LZY1* ≈ *LZY2* ≈ *LZY3* in dark-grown hypocotyls). The gravitropic phenotype of the *lzy1 lzy2 lzy3* triple mutant was rescued by introducing genomic fragments of *LZY2* or *LZY3*, thus confirming their role in gravitropism (Supplemental Figure 4).

Since organ elongation is essential for tropic responses, we also measured the elongation of inflorescence stems, finding that even the *lzy1 lzy2 lzy3* triple mutants elongated normally (Supplemental Figure 5A and 5B). In addition, *lzy1 lzy2 lzy3* stems and hypocotyls exhibited positive phototropic responses to unilateral blue light, indicating that *lzy1 lzy2 lzy3* shoots retained the capacity for asymmetric organ growth in response to a directional light stimulus (Supplemental Figures 5C and 5D). These observations suggest that these *LZY* genes are involved in processes that occur before organ elongation during tropic responses.

LZY1, LZY2, and *LZY3* genes function in the endodermis in shoot gravitropism

Our above analysis using the GUS reporter system to investigate the locations of *LZY1, LZY2,* and *LZY3* expression suggested that the genes function in the shoot endodermis (Figures 1B and Supplemental Figure 1).

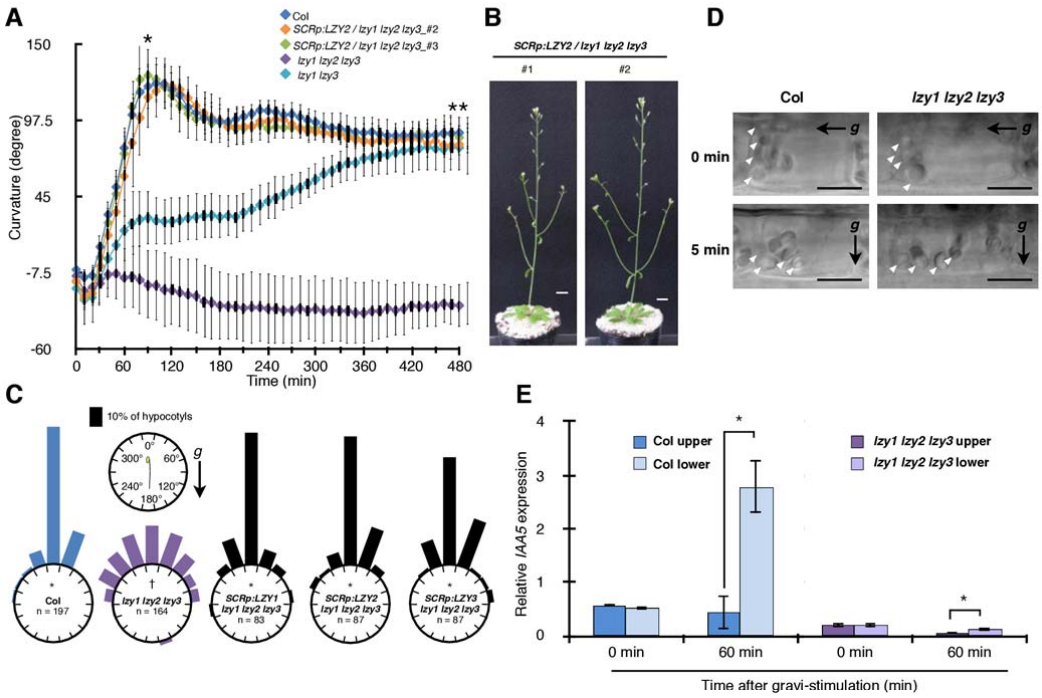

Figure 2. *LZY* genes regulate shoot gravitropism in statocytes.

(A) Time course of the gravitropic response (mean ± SD) of inflorescence stems of the two independent transgenic plants expressing *SCRp:LZY2* in *lzy1 lzy2 lzy3* background. For statistical analysis, the Tukey method was used to compare curvature measurements after 90 (*) and 480 minutes (**) of horizontal placement. There were significant differences in *SCRp:LZY2* expressing *lzy1 lzy2 lzy3_#2* and *SCRp:LZY2* expressing *lzy1 lzy2 lzy3_#3* compared to *lzy1 lzy2 lzy3* (P value < 0.05), but no significant differences compared to Col (P value < 0.05). (B) Aerial parts of 5-week-old plants expressing *SCRp:LZY2* in *lzy1 lzy2 lzy3* background. (C) Growth direction of 3-day-old etiolated hypocotyls expressing *SCRp:LZY1, SCRp:LZY2,* and *SCRp:LZY3* in *lzy1 lzy2 lzy3* background at intervals of 20°. For statistical analysis, means of the absolute value of the angle between growth direction and horizontal axis were compared. Means not sharing the same symbol (*, †) are significantly different (Tukey-Kramer, $P < 0.05$).(D) Amyloplasts in the endodermal cells of wild-type and *lzy1 lzy2 lzy3* inflorescence stems before (0 min) and after reorientation (5 min). Arrowheads indicate amyloplasts.(E) Relative expression of *IAA5* in the upper and lower flanks of wild-type (blue left panel) and *lzy1 lzy2 lzy3* (red right panel) inflorescence stems after reorientation. Expression levels were normalized against expression of the actin gene *ACT8*. Values and bars represent means ± SD of three biological replicates, and differences between the means were assessed for statistical significance using a Student *t* test (*$P < 0.01$). Arrows marked with *g* represent the direction of gravity. Scale bars = 1 cm (B) and 10 μm (D).

196 To investigate whether endodermis-specific *LZY* expression could rescue the
197 *lzy1 lzy2 lzy3* phenotype, the *LZY* genes were expressed in *lzy1 lzy2 lzy3*
198 plants under the control of the *SGR1/SCR* promoter, which was previously
199 reported to drive expression in the endodermis of both shoots and roots

(Wysocka-Diller et al., 2000). We found that the gravitropic phenotype of *lzy1 lzy2 lzy3* stems was fully rescued by *SCRp:LZY2* and *SCRp:LZY3* constructs (Figures 2A, 2B, and Supplemental Figure 6A) but was only partially rescued by the *SCRp:LZY1* construct (Supplemental Figure 6B), possibly owing to non-optimal expression of *LZY1* when under control of the *SCR* promoter. Each *LZY* driven by the *SGR1/SCR* promoter was also able to rescue the gravitropic phenotype of *lzy1 lzy2 lzy3* hypocotyls (Figure 2C). These results indicate that *LZY1*, *LZY2*, and *LZY3* all function in the statocytes during shoot gravitropism, which suggests that the three *LZY* genes share redundant molecular functions.

To investigate the role of the *LZY* genes in statocytes, we first determined whether they influence statocyte development. This analysis showed that the endodermis on the inflorescence stems of *lzy1 lzy2 lzy3* plants formed normally and that accumulation of starch in the hypocotyl endodermis was similar to that in the wild type (Supplemental Figure 7). In addition, we observed the relocation of amyloplasts upon reorientation in endodermal cells of the stems of *lzy1 lzy2 lzy3* (Nakamura et al., 2015), and found that amyloplasts were sedimented in the direction of gravity at 5 min after reorientation in endodermal cells of both mutant and wild-type plants (Figure 2D). Quantitative comparison of amyloplast sedimentation during the first 5 min after reorientation showed that wild-type and *lzy1 lzy2 lzy3* plants behaved similarly (Supplemental Figure 8A). This finding suggests that the *LZY* genes are likely to be involved in downstream processes of amyloplast-mediated gravity perception in shoot statocytes.

Accordingly, we investigated whether an asymmetric auxin signal could be generated in the inflorescence stems of *lzy1 lzy2 lzy3* (Figure 2E), by monitoring *IAA5* transcript levels (Taniguchi et al., 2014). A slight difference in *IAA5* transcript levels was detected between the upper and lower sides of *lzy1 lzy2 lzy3* inflorescence stems at 60 min after reorientation, whereas a >4-fold difference was observed in wild-type stems.

Thus, *LZY* genes play a key role in the statocytes to generate asymmetric auxin distribution in inflorescence stems.

LZY1, LZY2, and *LZY3* genes have the same molecular function in root gravitropism

By analyzing *LZY* promoter activity, we found that *LZY2* and *LZY3* were expressed in columella cells, i.e., root statocytes, of both primary and lateral roots, whereas *LZY1* was not (Figure 3A and Supplemental Figures 1G to 1L). Plants carrying the double mutation *lzy2 lzy3* showed defective root gravitropism, whereas plants with a single or other double combination of *lzy* mutations showed normal gravitropism (Figure 3B, and Supplemental Figure 9A). In addition, there was no significant difference in root gravitropism between *lzy2 lzy3* and *lzy1 lzy2 lzy3*. Thus, *LZY1* appears to have little or no role in root gravitropism; this conclusion is consistent with its lack of expression in root columella cells (Figure 3A). The gravitropic phenotype of *lzy1 lzy2 lzy3* was rescued by introducing genomic fragments of *LZY2* or *LZY3*, thus confirming their role in root gravitropism (Supplemental Figures 9B and 9C). These results suggest that *LZY2* and *LZY3* redundantly contribute to root gravitropism.

The *LZY2* promoter was active mainly in the columella cells, while *LZY3* was active in the stele above the elongation zone as well as in the columella cells in roots. To investigate whether the *LZY* genes function in root statocytes, the genes were expressed in *lzy1 lzy2 lzy3* plants under the control of *ACTIN DEPOLYMERIZING FACTOR9* (*ADF9*), *SCR*, or *SHR* promoters. In roots, the promoter activity of *ADF9,* whose expression was severely decreased in *eal1/shr* and *sgr1/scr* (Table 2), was found in statocytes, shoot endodermis, and columella cells of both primary and lateral roots, without detectable expression in other tissues (Supplemental Figure 10). The *SCR* promoter has previously been shown to promote expression in the endodermis and the quiescent center, while the *SHR*

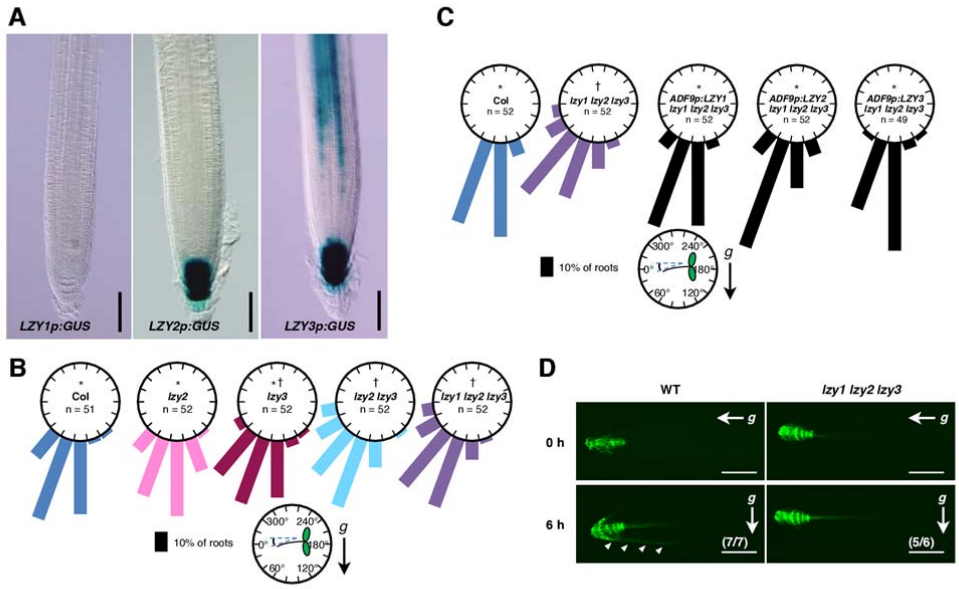

Figure 3. *LZY* genes function in root gravitropism.

(A) Expression patterns of the *LZY* gene family in roots. GUS staining of roots expressing *LZY1p:GUS* (left), *LZY2p:GUS* (middle), and *LZY3p:GUS* (right). (B) Root gravitropism of 5-day-old seedlings. Root angles were measured at 12 h after a 90° reorientation. The number of individuals examined for each Arabidopsis line is shown within the corresponding circles. Means not sharing the same symbol (*, †) are significantly different (Tukey-Kramer, $P < 0.05$). (C) Root gravitropism of 5-day-old seedlings expressing *ADF9p:LZY1*, *ADF9p:LZY2*, and *ADF9p:LZY3* in *lzy1 lzy2 lzy3* background. Root angles were measured at 12 h after a 90° reorientation. Means not sharing the same symbol (*, †) are significantly different (Tukey-Kramer, $P < 0.05$). (D) *DR5rev:GFP* expression in wild-type and *lzy1 lzy2 lzy3* roots before and after 6 h of reorientation. Arrowheads indicate the asymmetric GFP signals. Asymmetric GFP expression was found in wild type (7 out of 7), whereas symmetric expression was detected in *lzy1 lzy2 lzy3* (5 out of 6). Two independent replicates were carried out. Arrows marked with *g* represent the direction of gravity. Scale bars = 100 μm.

promoter has been reported to drive expression in the stele of roots; neither promoter is active in the columella cells (Wysocka-Diller et al., 2000; Nakajima et al., 2001). *LZY* genes driven by the *ADF9* promoter were able to rescue the gravitropic phenotype of *lzy1 lzy2 lzy3* in roots (Figure 3C,

Supplemental Figures 11A to 11C). By contrast, *LZY2* and *LZY3* driven by the *SCR* or the *SHR* promoter failed to rescue the gravitropic phenotype of *lzy1 lzy2 lzy3* roots (Supplemental Figures 11D to 11G). These results demonstrate that *LZY2* and *LZY3* function in the statocytes of roots during gravitropism and that *LZY1* has the same molecular function as *LZY2* and *LZY3* in root statocytes, although *LZY1* is not expressed in root statocytes under natural conditions.

We investigated whether the *LZY* genes influenced the development of statocytes and found that the morphology and starch accumulation behavior of the root caps of both primary and lateral roots were indistinguishable between wild-type and *lzy1 lzy2 lzy3* plants (Supplemental Figure 12A to 12H). In addition, amyloplast sedimentation in *lzy1 lzy2 lzy3* plants was normal in both root cap columella cells and shoot endodermal cells (Supplemental Figure 12I), suggesting that the *LZY* genes act downstream of amyloplast-mediated gravity perception in root statocytes as in the shoots. Moreover, asymmetric expression of the auxin responsive marker *DR5rev:GFP* (Ottenschläger et al., 2003) did not occur in *lzy1 lzy2 lzy3* roots at 6 h after reorientation, whereas GFP fluorescence was observed in the lower flank of wild-type roots (Figure 3D). Taken together, these results demonstrate that *LZY1, LZY2,* and *LZY3* share redundant and ubiquitous molecular functions in statocytes of both roots and shoots despite their relatively low sequence similarity; the genes also play a key role in the production of asymmetric auxin distribution in roots and shoots.

We examined whether *lzy* mutations affected the behavior of PIN3 in the columella cells. In wild-type root columella cells, PIN3 is uniformly distributed but becomes polarized upon reorientation (Harrison and Masson, 2008; Kleine-Vehn et al, 2010). We observed a low level of polarization of PIN3-GFP after reorientation in wild-type columella cells, and we did not detect any significant difference in PIN3-GFP polarization between wild-type and *lzy1 lzy2 lzy3* plants (Supplemental Figure 13).

Role of the LZY C-terminal region in gravity signaling

The lack of recognized functional domains or motifs in LZY family proteins makes it difficult to assign potential molecular functions to these proteins. Here, we investigated their potential functions by analysis of the well-conserved domain between LAZY1 family proteins including OsLA1, ZmLA1, and Arabidopsis LZY proteins. Because the C-terminal 14-amino acid sequence is well conserved among all LAZY1 family members despite relatively low sequence similarity of the overall proteins, we designated the domain as Conserved C-terminus in LAZY1 family proteins (CCL) (Figure 4A and Supplemental Figure 14). In addition, three Arabidopsis proteins (AtNGR3/At1g19115, At3g24750, and At3g27025) that have CCL domains at the C-terminus were designated as LZY4, LZY5, and LZY6, respectively (Table 1). To investigate the role of the CCL in LZY2 and LZY3, genomic fragments of *LZY2* and *LZY3* lacking the CCL domain were individually expressed in the *lzy1 lzy2 lzy3* triple mutant background. We found that the truncated LZY3 protein, for which transcript was detected, did not rescue the gravitropic phenotype of *lzy1 lzy2 lzy3* roots, and nor did the truncated LZY2 (Figure 4B and Supplemental Figures 15 and 16A). This observation indicates that the CCL domain is important for the molecular function of both LZY2 and LZY3 in roots.

Next, we investigated the subcellular localization of the LZY proteins. We first established transgenic lines of *lzy1 lzy2 lzy3* harboring *LZY2p:LZY2-mCherry* or *LZY3p:LZY3-mCherry*, which partially and fully rescued the *lzy1 lzy2 lzy3* phenotype, respectively (Supplemental Figure 16B and 16C) as expected of the respective levels of contribution of LZY2 or LZY3 to root gravitropism (Figure 3B). Our observations indicate that LZY2-mCherry and LZY3-mCherry fused proteins are functional *in planta.* However, mCherry fluorescence was not detectable in root columella cells, possibly because of low abundance or high turnover rate of proteins. Upon

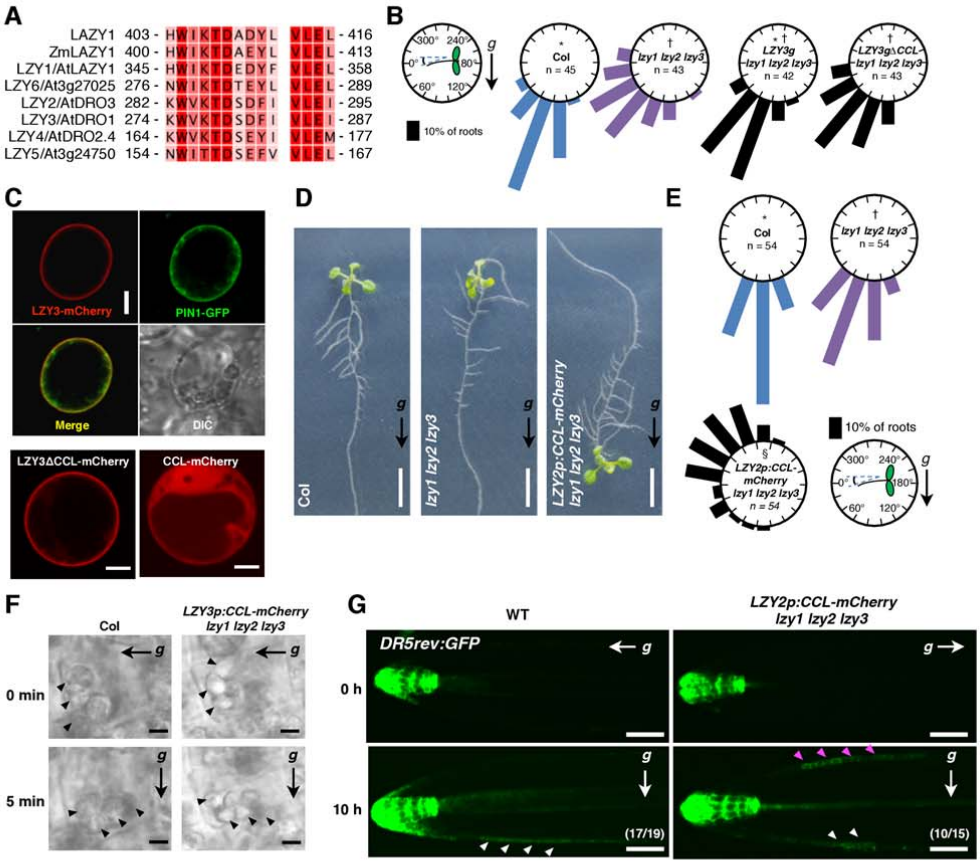

Figure 4. Important role of the C-terminal region of LZY proteins in gravity signaling.

(A) The alignment of C-teminal 14-amino acid sequences of LAZY1, ZmLAZY1, and Arabidopsis LAZY1 family. (B) Root gravitropism of 5-day-old seedlings. Root angles were measured at 12 h after a 90° reorientation. Means not sharing the same symbol (*, †) are significantly different (Tukey-Kramer, $P < 0.05$). (C) Intracellular localization of coexpressed LZY3-mCherry and PIN1-GFP, transiently-coexpressed LZY3ΔCCL-mCherry, and CCL-mCherry in the protoplast cells. (D) Effect of the CCL-mCherry driven by the *LZY2* promoter on the direction of root gravitropism in *lzy1 lzy2 lzy3*. 12-day-old seedlings were grown vertically on MS plates. (E) Root gravitropism of 5-day-old seedlings. Root angles were measured at 12 h after a 90° reorientation. Means not sharing the same symbol (*, †, §) are significantly different (Tukey-Kramer, $P < 0.05$). (F) Amyloplasts in the columella cells of Col (left) and *LZY3p:CCL-mCherry* in *lzy1 lzy2 lzy3* (right) before (0 min) and after reorientation (5 min). Arrowheads indicate amyloplasts. (G) *DR5rev:GFP* expression in wild-type and *LZY2p:CCL-mCherry* expressing *lzy1 lzy2 lzy3* roots before and after 10 h of reorientation. White and magenta arrowheads indicate GFP signals in the lower side and upper side of lateral root cap, respectively. WT primary roots displayed asymmetric GFP expression (17 out of 19), whereas *lay* mutant primary roots showed additional GFP expression in the upper side (10 out of 15). Three independent biological replicates were carried out. Each replicate includes data from more than three seedlings. Arrows marked with *g* represent the direction of gravity. Scale bars = 5 μm (C, F), 1 cm (D), and 100 μm (G).

transient overexpression of LZY tagged with GFP in Arabidopsis protoplasts, we detected fluorescence mainly at the cell periphery (Supplemental Figure 17). The LZY1-GFP signal was also observed in the nucleus, as reported previously (Yoshihara et al., 2013). LZY3-mCherry

signals colocalized with that of a plasma membrane protein, PIN1-GFP, at the cell periphery, indicating that LZY proteins are localized at the plasma membrane, and that only LZY1 is present in the nucleus (Figure 4C). To examine whether the CCL domain, which has relatively high hydrophobicity, plays a role in the subcellular localization of LZY3, we transformed protoplasts with LZY3ΔCCL-mCherry and examined expression of the transgene (Figure 4C). We found that LZY3ΔCCL-mCherry was localized at the plasma membrane, in a similar manner as LZY3-mCherry. By contrast, when CCL-mCherry was expressed in protoplasts and *in planta* (*LZY2p:CCL-mCherry/lzy1 lzy2 lzy3*), fluorescence was observed in the cytoplasm of protoplasts and columella cells, indicating that the CCL domain did not contribute to plasma membrane localization (Figure 4C and Supplemental Figure 18A). Overall, our observations indicate that the CCL domain is important for LZY function but not for LZY localization.

When CCL-mCherry was expressed in the *lzy1 lzy2 lzy3* background, both primary and lateral roots of the transgenic seedlings grew upward (Figure 4D and Supplemental Figures 18B and 18C). Since the amino acid sequence of LZY2-CCL is identical to that of LZY3-CCL, the effect of CCL-mCherry can be considered to be equivalent between LZY2 and LZY3. When the seedlings were gravistimulated *via* horizontal orientation, their primary roots grew upward (Figure 4E, and Supplemental Figure 18D). The perturbation effect of CCL-mCherry on root gravitropism was also observed in the wild-type background, although it was milder in effect than in the *lzy1 lzy2 lzy3* background, suggesting that the effect of CCL-mCherry was influenced by the level of endogenous *LZY* gene expression (Supplemental Figure 18D). We also observed that amyloplasts were relocated in *lzy1 lzy2 lzy3* columella cells expressing *LZY3p:CCL-mCherry*, and found that amyloplasts sedimented normally in the direction of gravity (Figure 4F and Supplemental Figure 8B). These results confirm that mutations of the *LZY*

genes did not affect amyloplast sedimentation, and suggest that *lzy1 lzy2 lzy3* roots expressing *LZY3p:CCL-mCherry* recognized the directional signal of gravity, but that the signal was perturbed and resulted in negative gravitropism. These findings also support our conclusion that *LZY* genes are involved in downstream processes in amyloplast-mediated gravity perception in root statocytes.

We tested whether the perturbed signal causes asymmetric auxin signaling derived from *DR5rev:GFP* in primary root tips of *LZY2p:CCL-mCherry* expressing *lzy1 lzy2 lzy3* before and after reorientation. Since roots of *LZY2p:CCL-mCherry / lzy1 lzy2 lzy3* grew upward, we investigated GFP fluorescence distribution before reorientation. The patterns of GFP signals were indistinguishable between wild-type and transgenic plants (Figure 4G). After reorientation, GFP fluorescence was observed asymmetrically at the lower flank in wild-type roots, whereas additional GFP fluorescence was observed at the upper flank of *lzy1 lzy2 lzy3* roots expressing *LZY2p:CCL-mCherry* (Figure 4G). Given that *LZY2p* seems to impart columella-specific expression in roots (Figure 3A), it is likely that CCL-mCherry perturbed the gravity signaling in columella cells by interfering with the function of the LZY proteins, thus leading to the disorientation of auxin flow at the root tip.

Effect of *lzy* mutations on the growth angles of lateral shoots and roots

In addition to abnormal gravitropism in primary shoots and primary roots, we also observed that plants carrying *lzy* mutations exhibited abnormal growth angles in both lateral shoots and roots. The lateral shoots of *lzy1* plants showed a larger growth angle (in almost a horizontal direction) as previously reported (Yoshihara et al., 2013), whereas those of the *lzy2*, *lzy3*, and *lzy2 lzy3* mutants were similar to wild-type plants (Figure 1C); both *lzy2* and *lzy3* appeared to enhance the *lzy1* phenotype, as found above for gravitropic response of inflorescence stems. We also found that the lateral

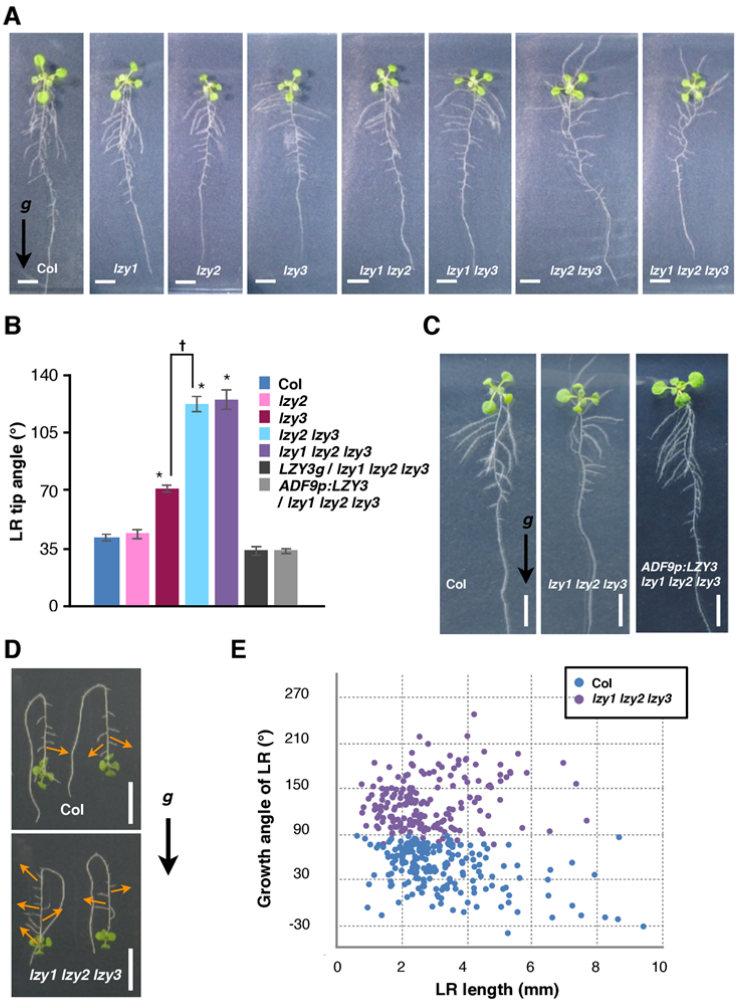

Figure 5. *LZY* genes control the growth angle of lateral roots.

(A) Two-week-old seedlings grown vertically on MS plates. (B) Lateral root tip angle of 12-day-old seedlings of each line to the direction of gravity. Statistical analysis was performed compared to Col (* *P* value < 0.01), and between *lzy3* and *lzy2 lzy3* († *P* value < 0.01) by the Tukey-Kramer method. (C) 12-day-old seedlings of Col, *lzy1 lzy2 lzy3*, and *ADF9p:LZY3* expressing *lzy1 lzy2 lzy3*. (D) 9-day-old Col and *lzy1 lzy2 lzy3* seedlings, rotated 180° at 4 days after germination. Orange arrows indicate the growth direction of lateral roots. (E) Scatter plots of length and growth angle of lateral roots of 9-day-old plants rotated 180° at 4 days after germination. The angle between the direction of gravity and growth direction of lateral root tip was measured. There was a significant difference in the correlation coefficient between Col and *lzy1 lzy2 lzy3* (r_{Col} = -0.420, r_{lzy} = 0.200, P < 0.01). Black arrows marked with *g* represent the direction of gravity. Scale bars = 1 cm.

roots of *lzy3* plants exhibited larger growth angles than those of wild-type plants (Figure 5A). Quantitative analyses confirmed these observations and demonstrated that *lzy2* enhanced the *lzy3* phenotype leading to upward growth of lateral roots, although *lzy2* plants were indistinguishable from

wild type (Figure 5B). The growth angle was not affected in *lzy1* plants; this observation was consistent with our earlier observation that *LZY1* was not expressed in roots (Figure 3A, Supplemental Figures 1G and 1H). More importantly, we also found that the growth angle phenotype of *lzy1 lzy2 lzy3* lateral shoots and roots could be rescued by *LZY* genes expressed in the statocytes. Lateral branches of *lzy1 lzy2 lzy3* plants grew downward (Figure 1C); *SCRp:LZY2* almost completely rescued the growth angle phenotype in these plants (Figure 2B). With regard to lateral roots, the growth angle phenotype of *lzy1 lzy2 lzy3* plants was rescued by columella-specific expression of *LZY3* under the control of the *ADF9* promoter (Figure 5B and 5C). These results suggest that *LZY* genes expressed in statocytes play an important role in controlling the growth angle of lateral shoots and roots.

Decrease of *LZY* activity reverses auxin flow in lateral root statocytes

Interestingly, the lateral roots of *lzy2 lzy3* and *lzy1 lzy2 lzy3* plants grew slightly upward (Figure 5B). To investigate this phenotype in detail, we measured the growth angles of lateral roots over 2 mm in length at various growth stages (Supplemental Figure 19A). The growth angles of wild-type lateral roots gradually decreased as the roots grew, and finally they grew almost vertically (Mullen and Hangarter, 2003). By contrast, *lzy1 lzy2 lzy3* lateral roots scarcely grew below the horizontal level at any growth stage. To test whether the lateral roots of the triple mutant were capable of responding to reorientation, we measured the growth angles of lateral roots that emerged after turning young seedlings upside down (Figures 5D and 5E). Lateral roots from inverted *lzy1 lzy2 lzy3* and wild-type seedlings grew upward and downward, respectively, indicating that the lateral roots of the triple mutant recognized the direction of gravity and then grew in the opposite direction.

We subsequently analyzed whether asymmetric auxin signaling was generated in the lateral roots of *lzy1 lzy2 lzy3* growing the opposite

direction. It has been reported that asymmetric distribution of auxin signaling by *DR5rev:GFP* is mostly observed in stage II lateral roots of wild-type plants (Rosquete et al., 2013). We examined whether the growth angle phenotype was present in young lateral roots of sub-millimeter lengths. Most of the lateral roots of *lzy1 lzy2 lzy3* and wild-type plants grew above and below the horizontal level, respectively (Supplemental Figure 19B). Prior to the enlargement of the central columella cells (stage 1), the majority of wild-type and *lzy1 lzy2 lzy3* lateral roots displayed symmetric *DR5rev:GFP* expression (Figures 6A and 6D). During and after the enlargement of the central S2 columella cells (stage 2 and 3), expression of *DR5rev:GFP* was observed in the lower side of wild-type lateral root caps as reported previously, and the GFP signal was decreased in the upper side of the columella cells (Figures 6B and 6C, and Supplemental Figure 20; Rosquete et al., 2013). Interestingly, most *lzy1 lzy2 lzy3* lateral roots exhibited significant *DR5rev:GFP* expression in the upper side of the lateral root cap cells, and less GFP expression in the lower side of the columella cells (Figures 6E and 6F). It has been reported that PIN3, which is expressed in columella cells before PIN4 and PIN7 (Rosquete et al., 2013), is involved in the growth angle control. We analyzed the effects of *lzy1 lzy2 lzy3* mutations on PIN3 localization in columella cells of lateral roots at early developmental stages. At stage 1, both wild-type and *lzy1 lzy2 lzy3* plants showed symmetric PIN3-GFP distribution in columella cells of lateral roots (Figures 6G and 6J). At later stages (stage 2 and 3), PIN3-GFP signal intensity in the lower lateral columella cells in wild-type lateral roots was much higher than in the upper columella cells, although we were unable to observe polarized localization of PIN3-GFP within the columella cells (Figures 6H and 6I, and Supplemental Figure 21). By contrast, the signal intensity in the upper lateral columella cells was higher than that of the lower cells in *lzy1 lzy2 lzy3* lateral roots (Figures 6K and 6L, and Supplemental Figure 21). These asymmetric distributions of PIN3-GFP

were in agreement with the asymmetric patterns of *DR5rev:GFP* and the growth directions of lateral roots. These results demonstrate that *LZY* genes are required for the flow of auxin toward the direction of gravity in lateral root columella cells. Furthermore, our data also indicate that the decrease in LZY activity causes reversal of the auxin flow in the lateral root columella with recognition of the direction of gravity, and that LZY activity affects PIN3 expression pattern in columella cells.

DISCUSSION

Previous studies have reported that *LAZY1* and its orthologs are involved in shoot gravitropism of rice (Yoshihara and Iino, 2007; Li et al., 2007), *Arabidopsis* (Yoshihara et al., 2013), and maize (Dong et al., 2013), and that other *LAZY1* gene family members are involved in root gravitropism of rice (*DRO1*; Uga et al., 2013), *Medicago truncatula* (*MtNGR*), and *Arabidopsis* (*AtLAZY1/LZY1*, *AtNGR1/LZY2, AtDRO1/AtNGR2/LZY3, AtNGR3/LZY4*; Ge and Chen, 2016; Table 1). In spite of their apparently well-conserved physiological function in gravitropism in various species, the similarities among the entire sequences of LAZY1 family proteins are low (e.g., sequence similarity between LZY1 and LZY2 or LZY3 was around 30%); the proteins share five short conserved regions though have no domain with an identified function (Yoshihara et al., 2013). In the present study, we demonstrated that three *LZY* genes are redundant and share the same molecular function with regard to gravitropism in both shoots and roots (Figures 1 to 3).

In rice coleoptiles, LAZY1 is required for the formation of auxin gradients after reorientation, although it has been unclear in which cells LAZY1 is necessary (Yoshihara and Iino, 2007). We demonstrated in the present study that three *LZY* genes function in the gravity signaling process inside the statocytes of both shoots and roots in *Arabidopsis thaliana* and that they function to positively regulate lateral auxin flow to the direction of gravity upon reorientation (Figures 2 and 3). The statocytes of roots, hypocotyls, and inflorescence stems have distinct developmental origins, and their cellular functions and morphologies are almost completely different, except for their shared role in gravity sensing. However, since amyloplast displacement in the statocytes is used in the gravity perception systems of all organs (Kiss et al., 1989; Weise and Kiss, 1999), it is reasonable that the statocytes of all gravity-responding organs share a common molecular mechanism for gravity signaling. Genes involved in the initial process of gravitropism across all organs have not been reported,

except for *PHOSPHOGLUCOMUTASE* (*PGM*), which is required for starch accumulation in amyloplasts (Kiss, 2000). In contrast to *PGM*, *LZY* genes did not affect amyloplast sedimentation (Supplemental Figure 8). Our results demonstrate that *LZY1, LZY2,* and *LZY3* genes are involved in early gravity signaling processes following amyloplast sedimentation in the statocytes of all organs, and more specifically, that the *LZY* genes induce auxin flow toward the direction of gravity. Thus, they likely function in processes bridging the gap between those described by the starch-statolith hypothesis and those in the Cholodny-Went theory.

The suggested function of the *LZY* genes in mediating auxin flow in statocytes in response to reorientation implies the involvement of these genes in the regulation of auxin transporters such as PIN3, which is expressed in statocytes of all organs and is involved in gravitropism in roots and hypocotyls (Friml et al., 2002). In columella cells of primary roots, uniformly distributed PIN3 becomes polarized upon reorientation (Harrison and Masson 2008, Kleine-Vehn et al., 2010). In addition, polarized localization of PIN3 has been observed in columella cells of young lateral roots (Rosquete et al., 2013). In this study, we did not detect any significant difference in PIN3 polarization between wild-type and *lzy1 lzy2 lzy3* primary roots (Supplemental Figure 13). This finding might explain the mild root gravitropism phenotype of *lzy1 lzy2 lzy3* plants. As *AtNGR3/LZY4* activity is thought to remain in *lzy1 lzy2 lzy3* primary roots (Ge and Chen, 2016; Table 1), additional analyses using *lzy1 lzy2 lzy3 lzy4* quadruple mutants will be necessary. On the other hand, polarized localization of PIN3-GFP was not detected in columella cells of wild-type and mutant lateral roots in the present study. Rather, asymmetry of PIN3-GFP expression in the columella of lateral root was observed (Figure 6). The opposite asymmetric pattern of PIN3 distribution as a consequence of a decrease in *LZY* gene activity might be an underlying mechanism of the anti-gravitropic capability (see below). Further investigations are required

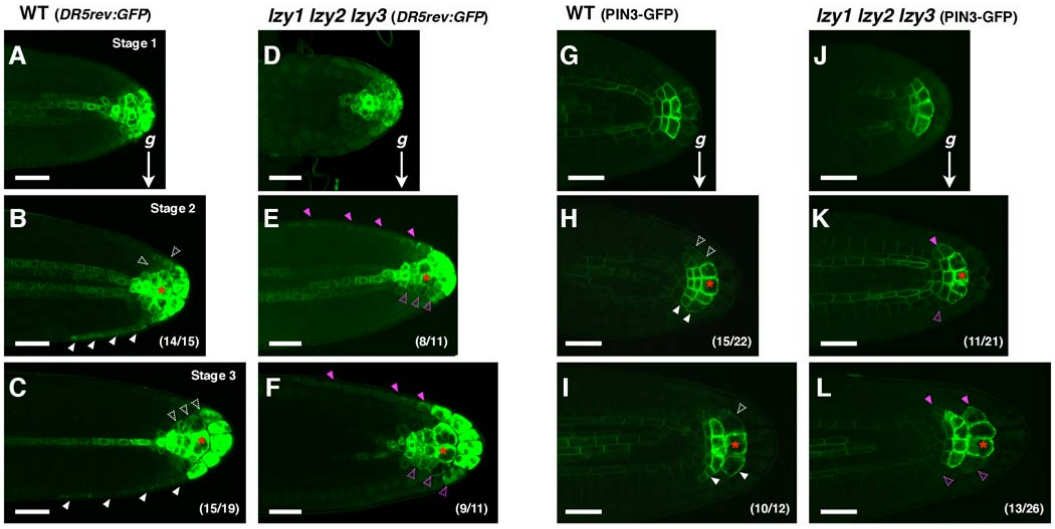

Figure 6. *LZY* genes control the direction of auxin transport from lateral root tips.
(A-F) *DR5rev:GFP* expression in lateral root tips of Col (A-C) and *lzy1 lzy2 lzy3* (D-F) at the stages 1 (A and D), 2 (B and E), and 3 (C and F) of lateral root development. Stages of lateral root growth were defined as described in the Materials and Methods. White and magenta filled arrowheads indicate the GFP signals in the lower and upper sides of the lateral root cap, respectively. Arrowheads enclosed by white and magenta dotted-lines indicate reduced GFP signals in the upper and lower sides of columella cells, respectively. Asymmetric GFP expression was found in wild-type lateral roots at the stage 2 (14 out of 15) and at the stage 3 (15 out of 19), and in *lzy* lateral roots at the stage 2 (8 out of 11) and at the stage 3 (9 out of 11). Three independent biological replicates were carried out. Each replicate includes the data from more than two seedlings. (G-L) PIN3-GFP expression in lateral root tips of wild type (G-I) and *lzy1 lzy2 lzy3* (J-L) at the stage 1 (G and J), 2 (H and K), and 3 (I and L) of lateral root development. White and magenta filled arrowheads indicate the asymmetric PIN3 localization in the lower and upper sides of columella cells, respectively. Arrowheads enclosed by white and magenta dotted-lines indicate reduced signals in the upper and lower sides of columella cells, respectively. Asymmetric GFP expression was found in wild-type lateral roots at the stage 2 (15 out of 22) and at the stage 3 (10 out of 12) and in *lzy* lateral roots at the stage 2 (11 out of 21) and at the stage 3 (13 out of 26). Three independent biological replicates were carried out. Each replicate includes the data from more than three seedlings. Arrows marked with *g* represent the direction of gravity. Red asterisks present central S2 columella cells. Scale bars = 20 μm. The classification of lateral root development was based on development of columella cells (see Materials and Methods).

521 to elucidate how *LZY* genes regulate auxin flow in statocytes in response to
522 reorientation and the regulatory mechanisms for asymmetric distribution of
523 PIN3 in the lateral root columella.

524 The CCL-mCherry construct was shown here to perturb root

gravitropism (Figure 4). This effect might have resulted from an excess of the CCL moiety, compared to a low level of endogenous LZY family proteins; this explanation is supported by the observation that the effect of CCL-mCherry was consistently reduced in the presence of endogenous *LZY* genes (Supplemental Figure 16D). It is also possible that LZY proteins interact with other proteins in gravity signaling *via* the CCL domain and that CCL-mCherry may interfere with these protein–protein interactions in a competitive manner. Therefore, we speculate that a complete loss of function of the *LZY* gene family could cause root phenotypes similar to that of CCL-mCherry in the *lzy1 lzy2 lzy3* background. A recent study demonstrated that primary roots of the triple mutant *atngr1 atngr2 atngr3* (*lzy2 lzy3 lzy4* in our nomenclature) grow upward in a similar fashion to that of *lzy1 lzy2 lzy3* plants expressing *CCL-mCherry* (Ge and Chen, 2016), although it is unclear whether *AtDRO2/AtNGR2/LZY4* is expressed in columella cells. Further investigations will be necessary to understand the perturbation mechanism of gravitropism by CCL-mCherry in both roots and shoots and will provide valuable information regarding the gravity signaling process in statocytes.

The findings of the present study also demonstrate that LZY-mediated gravity signaling in statocytes regulates the growth angles of both lateral roots and shoots, which influence the architecture of the whole plant (Figures 1C, 2B, and 5). The growth angle of organs is maintained at specific angles with respect to gravity (gravitropic set-point angle; GSA) according to developmental control and environmental factors, a concept that provides a unifying explanation for ortho-, plagio-, and diagravitropism (Digby and Firn, 1995). Recent investigations of growth angle control in the lateral roots and shoots of Arabidopsis suggested that an antagonistic interaction between two balancing auxin-dependent growth components, gravitropism and antigravitropic offset, underlies the mechanism of GSA control (Roychoudhry et al., 2013; Roychoudhry and Kepinski, 2015). Based

555 on the concept of GSA control, the growth angle phenotype of *lzy1 lzy2 lzy3*
556 lateral roots and shoots might be the result of an imbalance between
557 gravitropism and antigravitropic offset. The present study demonstrated
558 that *LZY* genes are positive regulators of auxin flow toward the direction of
559 gravity according to amyloplast relocation. Simply thinking, the phenotype
560 caused by the loss of function of such *LZY* genes would be expected to
561 include loss of responsiveness to reorientation, as in *pgm*, *pin2*, or *aux1*
562 roots (Kiss et al., 1989; Müller et al., 1998; Chen et al., 1998; Luschnig et
563 al., 1998; Bennett et al., 1996). However, decreased *LZY* gene activity
564 caused a reversal of the auxin flow in the lateral root, with recognition of
565 the direction of gravity (Figure 6). Downward growth of lateral shoots of
566 *lzy1 lzy2 lzy3* plants (Figure 1C) and upward growth of lateral roots of *CCL-*
567 *mCherry* expressing *lzy1 lzy2 lzy3* plants (Figures 4D and 4E) could be
568 similarly caused by a decrease in *LZY* activity. Our observations suggest
569 that lateral roots, and possibly lateral shoots, of wild-type plants contain an
570 anti-gravitropic capability as well as LZY-dependent gravitropism. The anti-
571 gravitropic capability became obvious following the loss of function of the
572 *LZY* genes. This conclusion is partly consistent with a previous proposal
573 (Roychoudhry et al., 2013). Primary roots of *atngr1 atngr2 atngr3* (*lzy2 lzy3*
574 *lzy4*) and of *CCL-mCherry* expressing *lzy1 lzy2 lzy3* plants also exhibited
575 reverse gravitropism (Ge and Chang, 2016; Figure 4E and Table 1). It is
576 possible that both lateral roots and primary roots have a balance between
577 LZY family-dependent gravitropism and LZY family-independent anti-
578 gravitropic capability. Thus, the LZY family proteins could play a key role in
579 elucidating a unified mechanism for ortho-gravitropism of primary organs
580 and GSA control of lateral branches via regulation of auxin flow according to
581 amyloplast relocation in statocytes.
582
583 **MATERIALS AND METHODS**
584 **Plant materials and growth conditions**