GNOM-Mediated Vesicular Trafficking Plays an Essential Role in Hydrotropism of Arabidopsis Roots[1][W][OA]

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Roots respond not only to gravity but also to moisture gradient by displaying gravitropism and hydrotropism, respectively, to control their growth orientation, which helps plants obtain water and become established in the terrestrial environment. As gravitropism often interferes with hydrotropism, however, the mechanisms of how roots display hydrotropism and differentiate it from gravitropism are not understood. We previously reported *MIZU-KUSSEI1* (*MIZ1*) as a gene required for hydrotropism but not for gravitropism, although the function of its protein was not known. Here, we found that a mutation of *GNOM* encoding guanine-nucleotide exchange factor for ADP-ribosylation factor-type G proteins was responsible for the ahydrotropism of Arabidopsis (*Arabidopsis thaliana*), *miz2*. Unlike other *gnom* alleles, *miz2* showed no apparent morphological defects or reduced gravitropism. Instead, brefeldin A (BFA) treatment inhibited both hydrotropism and gravitropism in Arabidopsis roots. In addition, a BFA-resistant GNOM variant, GN^{M696L}, showed normal hydrotropic response in the presence of BFA. Furthermore, a weak *gnom* allele, $gnom^{B/E}$, showed defect in hydrotropic response. These results indicate that GNOM-mediated vesicular trafficking plays an essential role in hydrotropism of seedling roots.

Stationary growth is a distinct feature of plants and distinguishes them from other organisms. Plants have evolved a variety of mechanisms for responding to environmental cues, which enables them to survive in the presence of limited resources or environmental stresses. One of the most important growth adaptations plants have acquired is tropism, growth response that involves bending or curving of plant organs toward or away from a stimulus. For example, roots display tropisms in response to environmental cues such as gravity, light, touch, and moisture (Darwin and Darwin, 1880; Takahashi, 1997; Correll and Kiss, 2002; Monshausen et al., 2008). Gravitropism has been the subject of intense study, while other tropic responses of roots have been less well characterized. There is some evidence of hydrotropism in roots, but this response has proven difficult to differentiate from gravitropism, as the latter always interferes with hydrotropism (Jaffe et al., 1985; Takahashi, 1994; Takahashi, 1997). The demonstration of true hydrotropism in roots has facilitated the identification of some of the physiological aspects of hydrotropism and its existence in a wide range of plant species. However, the underlying mechanisms that regulate hydrotropism remain unknown. The limited supply of water and precipitation in many parts of the world greatly affects agriculture and ecosystems. Elucidating the molecular mechanism of hydrotropism in roots is therefore important not only for understanding how terrestrial plants adapt to changes in moisture, but also for improving crop yields and biomass production.

The isolation and analysis of hydrotropism-deficient mutants using the model plant species Arabidopsis (*Arabidopsis thaliana*) represents a potent tool for dissecting the molecular mechanism of hydrotropism. Previously, we isolated an ahydrotropic mutant of Arabidopsis, *mizu-kussei1* (*miz1*), and showed that *MIZ1* encodes a protein of unknown function (Kobayashi et al., 2007). In light of both the physiological features of hydrotropism, as well as what we have learned from genetic studies of other tropisms, it is unlikely that *miz1* alone governs the hydrotropic response. In support of this, we have identified a second ahydrotropic mutant, *miz2*, a unique allele of *gnom* that confers ahydrotropic but not agravitropic growth, which implies distinct roots of vesicular trafficking between hydrotropism and gravitropism in roots.

RESULTS AND DISCUSSION

A hydrotropic mutant of Arabidopsis, *miz2*, was isolated by an experimental procedure previously

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described (Kobayashi et al., 2007). Initial analysis showed that *miz2* appeared to be totally lacking root hydrotropism (Fig. 1, A and B). We next analyzed the kinetics of the hydrotropic response of *miz2* roots. In the presence of a moisture gradient, vertically positioned wild-type roots gradually bent toward the source of moisture in 1% (w/v) agar and had grown to contact the agar by 12 h post-hydrostimulation. In contrast, *miz2* roots did not display hydrotropic bending. Instead, they tended to bend away from the agar surface (Fig. 1C). This wrong-way curvature has been often observed during ahydrotropic response in several species and is likely due to a loss of turgidity on the dry side of the root (Takahashi, 1994; Kobayashi et al., 2007). There were no significant differences in root elongation rate between wild-type and *miz2* roots until 8 h, and the differences became apparent 8 to 12 h after hydrostimulation (Fig. 1D). This was most likely due to the enhanced elongation of wild-type roots as the tips came into contact with the source of moisture, because there were no obvious differences in root elongation when seedlings were grown under conventional conditions (data not shown). The growth rate of *miz2* roots did not differ from that of wild-type roots under humidity-saturated conditions.

The *miz2* mutation was mapped to a site on chromosome 1 (At1g13980), between the simple sequence-length polymorphism markers CER432406 and 4894kb. This genomic locus was different from that of *miz1*, which suggested that *miz2* was not allelic to *miz1* (Fig. 2A). Complementation analysis using a genomic fragment of wild-type At1g13980 confirmed that *miz2* is a new recessive mutant allele of *gnom* (Fig. 2B). The mutation in *miz2* consisted of a single base change of G to A, which resulted in a single amino acid substitution in GNOM, Gly951Glu.

GNOM (also called *EMB30* or *VAN7*) encodes a guanine-nucleotide exchange factor for ADP-ribosylation factor-type G proteins (ARF-GEF). Loss-of-function alleles of Arabidopsis *GNOM* lead to severe defects in apical-basal pattern formation in the embryo (Mayer et al., 1993; Steinmann et al., 1999). Nearly all of the defects associated with *gnom* alleles involve altered auxin transport, and proper localization of the auxin efflux carrier has been shown to require GNOM function (Steinmann et al., 1999; Geldner et al., 2003). The missense mutation we identified in *miz2* was downstream of the central catalytic Sec7 domain of GNOM. Weak *gnom* alleles carrying mutations in this C-terminal region have defects in postembryonic development (Geldner et al., 2004). Moreover, the *no hydrotropic response1* (*nhr1*) mutant exhibits altered root elongation and root cap development (Eapen et al., 2003); thus, while the *nhr1*gene has not been identified, it is reasonable to predict that the ahydrotropic response of *miz2* plants is due to defects in root development. We therefore examined whether *miz2* mutants had defects in root morphology or not. In contrast to

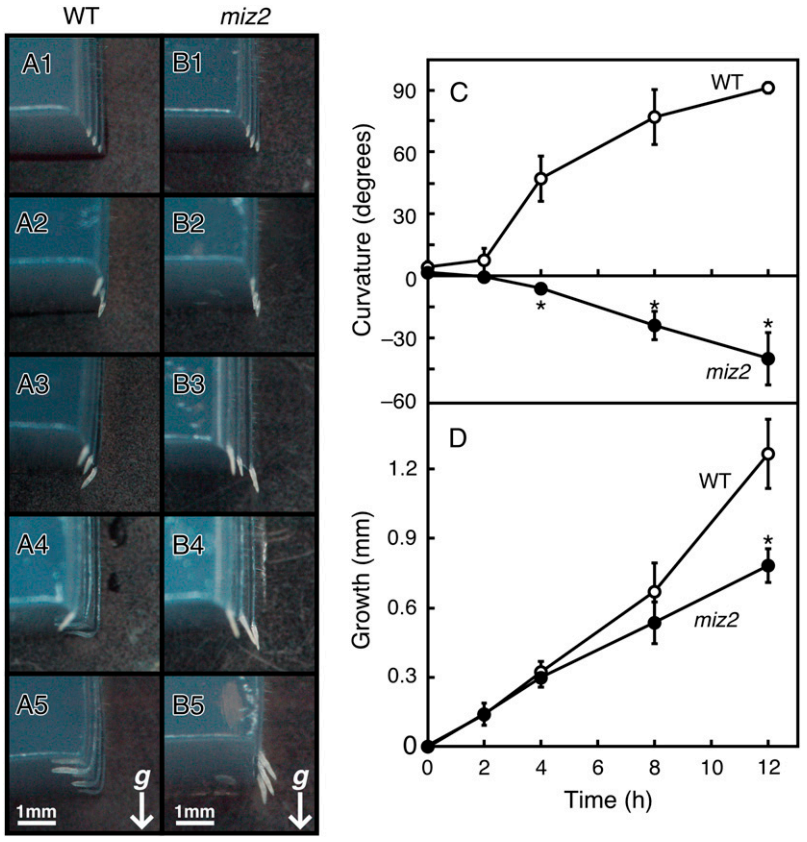

Figure 1. Hydrotropism and elongation of *miz2* and wild-type (WT) roots. A and B, Hydrotropic curvatures of WT (A1–A5) and *miz2* (B1–B5) roots at 0 h (A1 and B1), 2 h (A2 and B2), 4 h (A3 and B3), 8 h (A4 and B4), and 12 h (A5 and B5) after the start of hydrotropic stimulation. The arrow (*g*) indicates the direction of the gravitational force. Scale bars = 1 mm. C and D, Time course of hydrotropic curvature (C) and elongation (D) of *miz2* and WT roots. White circles, WT roots; black circles, *miz2* roots. Data represents the means ± SD of three independent experiments ($n = 20$ for each experiment). Asterisks indicate statistically significant differences, as determined by the Student's two-tailed *t* test ($P < 0.01$).

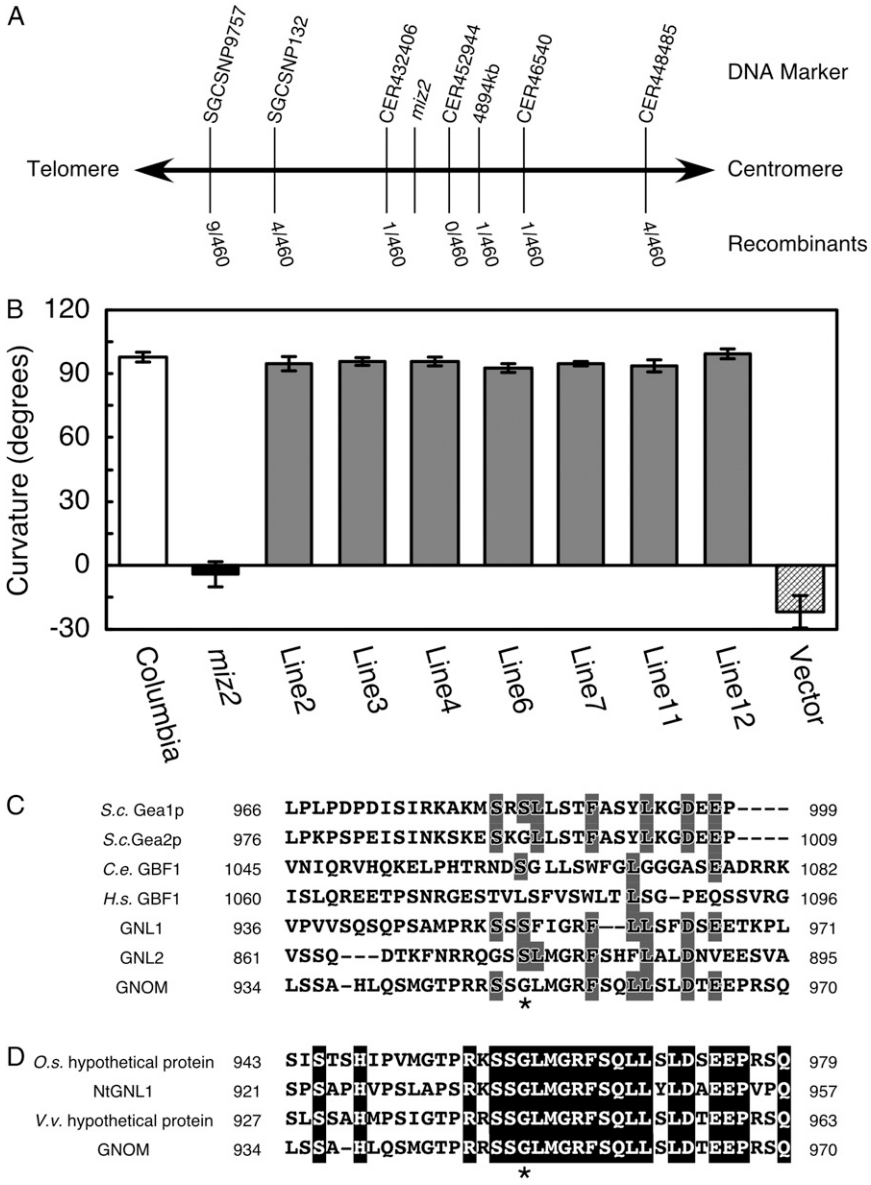

Figure 2. Identification of the *miz2* gene. A, Map-based cloning of the *miz2* locus. DNA markers and the number of recombinants observed for the corresponding marker, as a fraction of the total number of chromosomes, are shown. B, Complementation test for hydrotropism in *miz2* plants. Hydrotropic curvature 12 h after hydrostimulation is presented. White bar, wild type; black bar, *miz2*; gray bars, independently generated transgenic lines; hatched bar, vector control. Data represents the means ± SD of at least 17 individuals. C, Amino acid sequence alignment of the *miz2* mutation and the flanking residues for the following Gea/GNOM/GBF family members: budding yeast Gea1p and Gea2p (*S.c.* Gea1p and *S.c.* Gea2p, respectively), *Caenorhabditis elegans* GBF1 (*C.e.* GBF1), human GBF1 (*H.s.* GBF1), and Arabidopsis *GNL1* (At5g39500) and *GNL2* (At5g19610) gene products. Sequences were aligned with GNOM using ClustalW (Thompson et al., 1994). Identical residues are shaded. D, Amino acid sequence alignment of the *miz2* mutation and the neighboring amino acids for plant GNOM homologs. Putative proteins of rice (*O.s.* hypothetical protein; EAY91294), grape (*V.v.* hypothetical protein; AM461845), and tobacco GNOM-like1 (NtGNL1; EF520731) proteins were aligned with GNOM using ClustalW (Thompson et al., 1994). Identical residues are highlighted. In both C and D, asterisks indicate the mutated residue in *miz2*.

other weak alleles of *gnom*, there were no obvious differences in the morphological features of either the roots or shoots of wild-type and *miz2* plants, including root tip morphology (Fig. 3, A, C–E, G, and H; Supplemental Fig. S1). In addition, no obvious phenotypes in vascular development, which is often observed in weak alleles of *gnom*, were observed (Fig. 3, B and F). These results demonstrate that the *miz2* mutation unlikely affects the functions of GNOM required for proper developmental patterning.

As mentioned above, mutations in *GNOM* generally disrupt the polarity of auxin transport and thereby cause defects in gravitropism (Geldner et al., 2004). As gravitropism has been shown to interfere with hydrotropism (Jaffe et al., 1985; Takahashi, 1994, 1997; Kobayashi et al., 2007), we were interested in whether the defect in hydrotropism of *miz2* mutants was due to altered gravitropism. To test whether the *miz2* mutation altered gravitropism, we compared the kinetics of gravitropic growth of wild-type and *miz2* plants. Unexpectedly, there were no significant differences in gravitropic curvature or growth between wild-type and *miz2* plants (Fig. 3, I and J). This was in a striking contrast to the phenotype of $gnom^{B/E}$, a weak allele of *gnom*, which displays a significant defect in root gravitropism (Geldner et al., 2004).

Previously, it was reported that GNOM-mediated vesicular trafficking is inhibited by brefeldin A (BFA) in Arabidopsis root cells (Geldner et al., 2003). To examine the involvement of vesicular trafficking in root hydrotropism, we treated wild-type seedlings with BFA and monitored the effect on hydrotropism. As shown in Figure 4A, treatment with BFA inhibited the development of root hydrotropic curvature in a dose-dependent manner. Thus, the hydrotropic response of *miz2* was phenocopied by treatment of

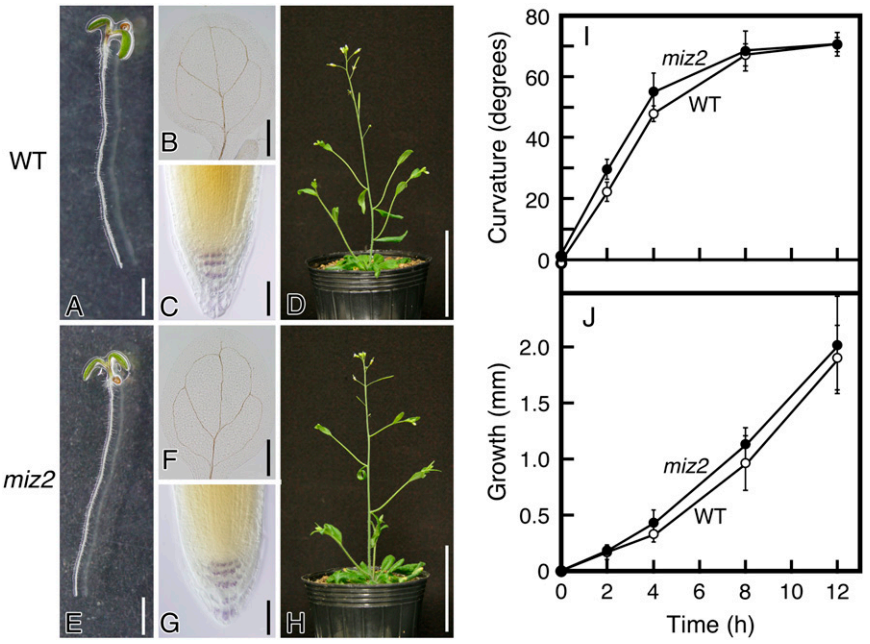

Figure 3. Morphological features and gravitropism of *miz2* and wild-type (WT) plants. WT (A) and *miz2* (E) seedlings; vascular patterns in the cotyledons of WT (B) and *miz2* (F) seedlings; iodine-stained columella cells of WT (C) and *miz2* (G) plants; and inflorescence of 4-week-old WT (D) and *miz2* (H) seedlings are shown. Scale bars = 2 mm (A and E), 500 μm (B and F), 50 μm (C and G), and 5 cm (D and H). I and J, Gravitropism of WT and *miz2* seedling roots. Curvature (I) and growth (J) were measured 0, 2, 4, 8, and 12 h after gravitropic stimulation. White circles, WT; black circles, *miz2*. Data represents the means ± SD of three independent experiments (n = 20 for each experiment).

wild-type plants with BFA at a concentration of 10^{-5} M, which demonstrated that vesicle trafficking was involved in root hydrotropism. In contrast to *miz2* mutants, BFA treatment to wild-type seedlings also decreased root gravitropic response (Fig. 4, C and D), which probably is attributable to the inhibition of proper auxin transport. On the other hand, our recent demonstration showed that polar auxin transport is not essential for root hydrotropism in Arabidopsis. Namely, Arabidopsis roots treated with inhibitors of auxin efflux, TIBA or NPA, showed normal hydrotropic response, while their gravitropic response was substantially reduced (Kaneyasu et al., 2007). Also, hydrotropic response of *pin2/wav6-52* mutant roots in which polar auxin transport and gravitropism were altered, did not differ from that of wild-type roots (Takahashi et al., 2002). We further confirmed the apparent normality of auxin transport in *miz2* by monitoring *DR5*::*GUS* expression, which is widely used to visualize auxin gradients in roots (Geldner et al., 2004). We found no obvious alteration in auxin response gradients between wild type and *miz2* (Supplemental Fig. S2). In addition, localization of PIN1 protein in the roots of *miz2* did not differ from that of the wild-type roots (data not shown). Thus, it is likely that *miz2* affects hydrotropism by a mechanism apart from the role of GNOM that regulates polar auxin transport.

Because phenotypes of *miz2* differed from those of other known *gnom* alleles, we confirmed the involvement of GNOM in root hydrotropism using Arabidopsis seedlings that express the BFA-resistant GNOM variant, GN^{M696L} (Geldner et al., 2003). Root hydrotropic response was completely inhibited by BFA treatment in wild-type plants, whereas Arabidopsis seedlings expressing GN^{M696L} displayed normal hydrotropic response irrespective of the BFA treatment (Fig. 5, A and B). This result virtually confirmed that *miz2* is a novel allele of *gnom* and that GNOM function is essential for root hydrotropic response. In addition, we investigated the hydrotropic response of $gnom^{B/E}$, a weak allele of *gnom*, to

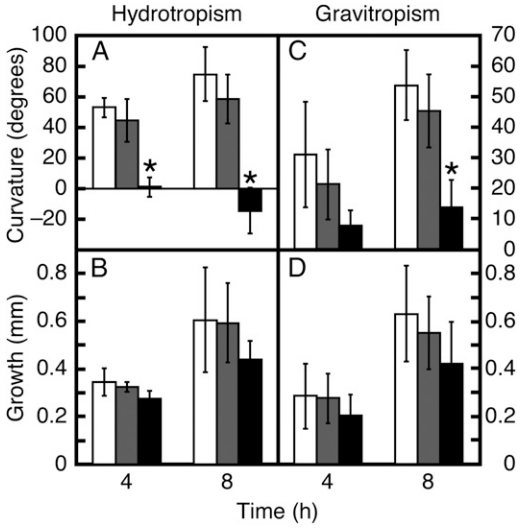

Figure 4. BFA inhibits both hydrotropism and gravitropism. Wild-type seedlings were treated with BFA at concentrations of 10^{-6} M (gray bars) and 10^{-5} M (black bars), and hydrotropism (A and B) and gravitropism (C and D) were compared to mock-treated seedlings (white bars). Curvature (A and C) and growth (B and D) were collected 4 and 8 h after hydrotropic and gravitropic stimulation. Data represents the means ± SD of three independent experiments (at least 15 individuals for each hydrotropism assay and 10 individuals for each gravitropism assay). Asterisks indicate statistically significant differences, as determined by the Student's two-tailed t test ($P < 0.01$).

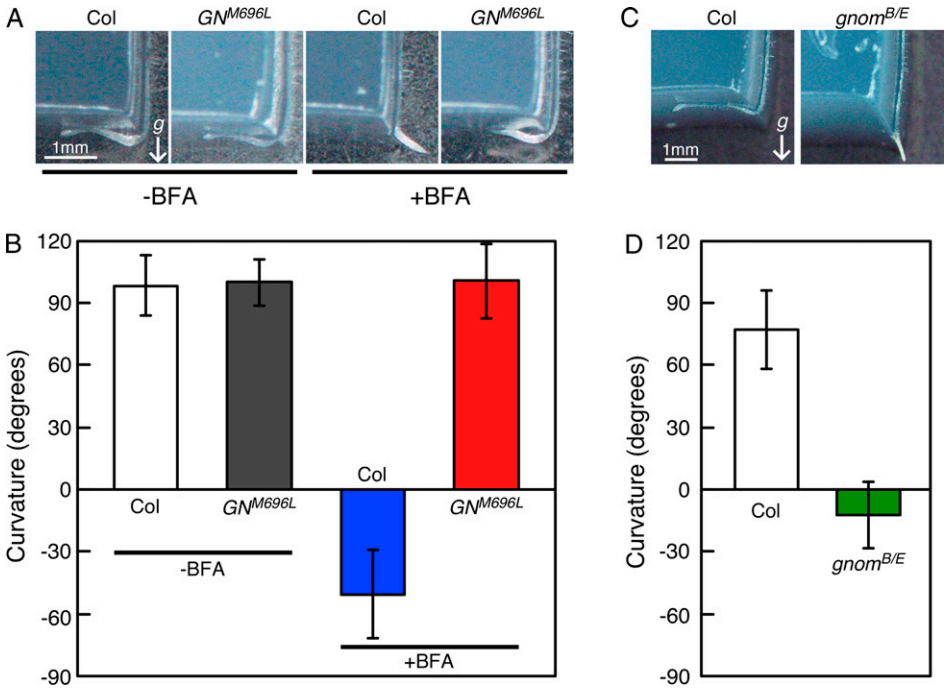

Figure 5. Hydrotropic response of *gnom* variants. A and B, Effect of BFA on root hydrotropic response of a GNOM BFA-resistant line. Seedlings expressing BFA-resistant GNOM (GN^{M696L}) were subjected to hydrotropic assay together with those of wild type (Col). Seedlings were treated with BFA at a concentration of 10^{-5} M, and hydrotropism was compared to mock-treated seedlings. Photomicrographs (A) and curvature (B) were collected 12 h after hydrotropic stimulation. Data in B represent the means ± SD of 16 individuals. White bar, Control roots of wild type; gray bar, control roots of GN^{M696L}; blue bar, BFA-treated roots of wild type; red bar, BFA-treated roots of GN^{M696L}. C and D, Hydrotropic response of a weak *gnom* allele, $gnom^{B/E}$. Seedlings of $gnom^{B/E}$ were subjected to hydrotropic assay together with those of wild type (Col). Photomicrographs (C) and curvature (D) were collected as described above. Data in D represent the means ± SD of 28 and 23 for Col and $gnom^{B/E}$ individuals, respectively. White bar, wild type; green bar, $gnom^{B/E}$. In A and C, the arrow (*g*) indicates the direction of the gravitational force.

reveal the specificity of *miz2* mutation on root hydrotropism. Seedlings of $gnom^{B/E}$ showed no hydrotropic response as *miz2* did (Fig. 5, C and D), suggesting a stronger requirement of GNOM for hydrotropism relative to gravitropism.

GNOM is a member of the Gea/GBF/GNOM subfamily of ARF-GEF proteins (Jackson and Casanova, 2000). Members of this subfamily contain a significant level of similarity in the regions of the Sec7 domain as well as upstream and downstream of the Sec7 domain. While the Sec7 domain of ARF-GEFs is well characterized, relatively little is known about these conserved flanking regions. The only information that has been shown is that the N-terminal DCB-domain of GNOM is required for the direct interaction between DCB, HUS, and SEC7 domains of GNOM molecules (Anders et al., 2008). The amino acid substitution of *miz2* was located in the C-terminal region of GNOM, which includes a 300-amino acid region located downstream of the Sec7 domain that is shared specifically by members of the Gea/GBF/GNOM family (Jackson and Casanova, 2000). The overall C-terminal region of GNOM is believed to be required for full auxin canalization, but no functions other than regulating auxin transport polarity have been shown. While the amino acid residue affected by the *miz2* mutation was not conserved among Gea/GBF/GNOM family members, it was conserved among GNOM homologs of other plant species such as grape (*Vitis vinifera*), tobacco (*Nicotiana tabacum*), and rice (*Oryza sativa*; Fig. 2, C and D). In budding yeast (*Saccharomyces cerevisiae*), the corresponding C-terminal region of Gea1p and Gea2p, which are homologues of GNOM, is required for their ability to interact with the Golgi protein Gmh1p (Chantalat et al., 2003). While the precise role of Gmh1p is unclear, it is probable that such C-terminal binding protein of GNOM confers its full activity necessary for specific plant phenomena.

In conclusion, we have shown that GNOM is involved in the regulation of an important step in hydrotropism in addition to its role in different aspects of plant development, including the gravitropic response. Our results clearly suggest that vesicular trafficking plays crucial roles in both hydrotropism and gravitropism, but their regulatory mechanisms differ from one another in the two tropisms. Further analysis of *miz2* mutants will shed a new light on the importance of membrane trafficking in some of the unsolved issues of plant biology such as hydrotropism in roots.

MATERIALS AND METHODS

Plant growth conditions, the procedure for the hydrotropism and gravitropism assays, and the screening of *miz* mutants have been described previously (Kobayashi et al., 2007). A *miz2* homozygous mutant (ecotype Columbia) was crossed with Landsberg *erecta* (L*er*) wild-type plants to generate a mapping population. In the F_2 population, *miz2* mutant seedlings were selected based on an impaired hydrotropic response 12 h after exposure to a moisture gradient, and their genomic DNA was isolated from the leaf tissues. Polymorphisms between Columbia and L*er* ecotypes were analyzed using a combination of cleaved amplified polymorphic sequence analysis and simple sequence length polymorphism markers, and data obtained from The Arabidopsis Information Resource and the Monsanto Arabidopsis Polymorphism and L*er* Sequence collection (Jander et al., 2002). For complementation analysis, a 11.4-kb genomic fragment of the *GNOM* gene, containing approximately 6.2 kb upstream of the ATG start codon and approximately 0.4 kb downstream of the stop codon, was subcloned into pPCR-Script Amp SK+ (Stratagene) according to the manufacturer's instructions. After sequencing, the 11.4-kb GNOM fragment was excised by restriction digestion with *Kpn*I and *Sac*I and inserted into the *Kpn*I-*Sac*I sites of the binary plant transformation vector pPZP211 (Hajdukiewicz et al., 1994). Further complementation analyses were performed as described previously (Kobayashi et al., 2007). Treatment with BFA was performed as previously described (Kaneyasu et al., 2007), with the exception that the stock solution of BFA (Sigma Chemicals) was prepared at a concentration of 1,000× in dimethyl sulfoxide. As a control, an equivalent volume of dimethyl sulfoxide was added to the culture medium. We observed root tip morphology of propidium iodide stained wild-type and *miz2* roots under FV-1000 confocal laser scanning microscopy (Olympus). GUS staining and microscopic observations were done as described previously (Kobayashi et al., 2007).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NC_001142 (S.c. Gea1), NC_001137 (S.c. Gea2), NM_067121 (C.e. GBF1), NM_004193 (H.s. GBF1), NM_123312 (AtGNL1), NM_121966 (AtGNL2), U36432 (AtGNOM), AP005778 (O.s. hypothetical protein), EF520731 (NtGNL1), and AM461845 (V.v. hypothetical protein).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Primary root meristem of *miz2* mutant.

Supplemental Figure S2. Auxin response gradients in *miz2* root tips upon exogenous auxin treatment.

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