ROTUNDIFOLIA4 Regulates Cell Proliferation Along the Body Axis in Arabidopsis Shoot

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Molecular genetics has been successful in identifying leaf-size regulators such as transcription factors, phytohormones, and signal molecules. Among them, a ROTUNDIFOLIA4-LIKE/DEVIL (RTFL/DVL) family of Arabidopsis, genes encoding peptides with no secretion-signal sequence, is unique in that their overexpressors have a reduced number of leaf cells specifically along the proximodistal axis. However, because the RTFL/DVL lack any obvious homology with functionally identified domains, and because of genetic redundancy among RTFL/DVL, their molecular and developmental roles are unclear. In this study we focused on one member in the family, ROTUNDIFOLIA4 (ROT4), and identified the core functional region within it and we found no proteolytic processing in planta. Developmental analysis of leaf primordia revealed that ROT4 overexpression reduces the meristematic zone size within the leaf blade. Moreover, induced local overexpression demonstrated that ROT4 acts as a regulator of the leaf shape via a change in positional cue along the longitudinal axis. Similarly, ROT4 overexpression results in a protrusion of the main inflorescence stem, again indicating a change in positional cue along the longitudinal axis. These results suggest that ROT4 affects the positional cue and cell proliferation along the body axis.

Keywords: Arabidopsis thaliana • Cell proliferation • Organ size and shape • Polarity • ROTUNDIFOLIA4 • Stem protrusion.

Abbreviations: AF, arrest front; AZ, abscission zone; CaMV35S, Cauliflower mosaic virus 35S promoter; DAI, days after imbibition; GFP, green fluorescent protein; o/x, overexpressor.

Introduction

In contrast to animals that can deal with harsh conditions by moving, plants are sessile, and thus the physical characteristics of a plant body are especially important for its survival. The size of lateral organs such as leaves and petals is of particular interest because it partially determines the efficiency of energy capture or pollinator attraction. In leaf primordia, the cell population having a high proliferation activity (the meristematic zone) resides in the proximal part of the leaf blade (Donnelly et al. 1999), and the transition from the meristematic phase to the differentiating phase is postulated to be under the control of a hypothetical, proximally progressing “arrest front” (Nath et al. 2003, White 2006). We recently found that the progression of the arrest front is not gradual: for a certain period during the development of a leaf primordium, the distance of the arrest front from the base of the leaf blade is constant and then suddenly decreases at the last moment (Kazama et al. 2010).

A powerful strategy for identifying novel factors that regulate lateral organ size is the isolation and characterization of mutants of Arabidopsis thaliana (hereafter, Arabidopsis) that exhibit polarity-dependent phenotypes, that is, mutants that exhibit phenotype (such as size reduction) along a specific body axis (Tsukaya 2005, 2006, 2008, Horiguchi et al. 2006). This is because polarity-dependent phenotypes likely result from the perturbation of developmental systems that specifically control organ size, whereas polarity-independent phenotypes are more likely perturbed in more general biological aspects, such as basic metabolism (Fujikura et al. 2009) or cell division (Wang et al. 1998, Verkest et al. 2005).
Several mutants with short leaves have supplied good clues for understanding the roles of a particular phytohormone or organogenetic signaling pathway on leaf size regulation. For example, Ikezaki et al. (2010) revealed that gibberellic acid deficiency is responsible for the short-leaved phenotype through the analysis of asymmetric leaves1 (as1) and asymmetric leaves2 (as2), and Kim et al. (1998, 2005) identified that mutation of brassinosteroid biosynthetic enzyme CYP90C1 is responsible for *rotundifolia*3. The gene responsible for *rotunda*1 encodes an enzyme hydrolyzing inositol triphosphate (IP3) (Robles et al. 2010), which in turn is possibly involved in important morphogenetic events through the regulation of PIN-FORMED1 (PIN1) intercellular localization (Carland and Nelson 2009, Naramoto et al. 2009).

*rotundifolia*4-1D (rot4-1D) was isolated through activation tagging screening of phenotypes with short leaves and a reduced number of cells in the proximodistal axis (Narita et al. 2004). The ROT4/DVL16 putatively encodes a small protein of 6.2 kDa without a signal sequence for secretion (Narita et al. 2004). A database search identified 22 other putative homologs of ROT4 encoded in the Arabidopsis genome, and they are called the RTFL/DVL family (Narita et al. 2004, Wen et al. 2004). Overexpression experiments suggested that at least six members of the RTFL/DVL share the same function (Wen et al. 2004). Neither genome-wide gene annotation nor large-scale loss-of-function mutant screening had ever discovered this gene family, the former due to the small size of the open reading frame (ORF), and the latter possibly because of functional redundancy among family members. The RTFL/DVL genes are widely conserved among land plants (Floyd and Bowman 2007), indicating their essential role(s) in plant life. Since the RTFL/DVL share no sequence similarity with well-characterized motifs or domains, virtually no clues about their function have been obtained from their sequence data.

Wen and Walker (2006) thought the functional region of RTFL/DVL might be processed to a shorter biologically active form, based on the following three premises. First, they experimentally demonstrated that one or two conserved basic amino acid(s) (K or R) at the N-terminus of the functional region was necessary for function (Wen et al. 2004). They speculated that these conserved residues might function as a possible recognition site of some endopeptidase(s), since the residues are near the border of a domain that is conserved throughout the RTFL/DVL family. Second, the N-terminal region of RTFL/DVL family members is highly divergent in length and sequence, suggesting that this region might be cleaved from the conserved domain. Third, they reported that the DVL1/RTFL18 with green fluorescent protein (GFP) fused to the N-terminus (GFP-DVL1) was fully functional, whereas DVL1 with GFP fused to the C-terminus (DVL1-GFP) was nonfunctional. The DVL1 is 6.2 kDa and GFP is 27 kDa, thus they speculated that GFP fusion possibly interfered with the function of the DVL1. If GFP is fused at the N-terminus, and if the N-terminus-GFP adduct is cleaved from the functional domain, then the processed DVL1 would be functional. Although these indirect facts were consistent with their hypothesis, direct evidence was lacking. In contrast, Narita et al. (2004) reported that ROT4-GFP was functional. The inconsistency of this result in the case of DVL1-GFP suggests that a reexamination is warranted.

The RTFL/DVL-dependent phenotypes are also enigmatic. Although RTFL/DVL overexpresor (o/x) was first characterized by its short leaves with a decreased number of cells along the proximodistal axis (Narita et al. 2004, Wen et al. 2004), pleiotropic phenotypes, such as a bent pedicel or a protrusion of the trichome base, have also been reported (Wen and Walker 2006). Recently a legume homolog of the RTFL/DVLs was implicated in nodulation. MtDVL1 of *Medicago truncatula* was expressed in an early stage of nodule formation, and its overexpression aborted nodule development (Combier et al. 2008).

To better understand these issues, we focused on ROT4/DVL16, a member of the RTFL/DVL gene family. We determined the core functional domain in the ROT4 protein, examined the possibility of posttranslational processing, and characterized the pleiotropic phenotypes caused by overexpression of ROT4. These data are essential for understanding the native role of ROT4.

### Results

#### Identification of the core functional region within the ROT4 peptide

Narita et al. (2004) roughly showed that N- or C-terminal domains are not required for the ROT4 function, but the precise functional region was not determined. Thus we generated transgenic plants overexpressing various truncated portions of ROT4 using the 35S promoter of the cauliflower mosaic virus (CaMV35S) and determined the precise domain. Fruits of full-length ROT4 o/x are shorter and wider than those of the wild type, in agreement with the rosette compactness originally described as the morphological phenotype of ROT4 o/x (Narita et al. 2004). Because fruit size and morphology are simple to evaluate and are relatively insensitive to plant age and growth conditions, they are used here for the quantitative analysis. Then we performed cluster analysis on the fruits data to judge if each transgenic line exhibits phenotype (Supplementary Fig. S1; see Materials and Methods for details). The effect of the introduced transgene was evaluated with the ratio of transgenic lines with phenotypes per total number of transgenic lines obtained.

Experiments were performed in two steps. First, either the N- or C-terminus was truncated by an interval of three residues (Fig. 1A). The constructs with N-terminal truncation are called the dN series, and those with C-terminal truncation are called the dC series. dN1 lacks two residues at the N-terminus and dC1 lacks seven residues at the C-terminus, and as the designation number increases, the construct becomes shorter by three residues. Thus dN2 lacks 5 residues at the N-terminus, dN3 lacks 8 residues, and so forth, while dC2 lacks 10 residues at the C-terminus, dC3 lacks 13 residues, and so on. As a result,
the ratios of phenotype-positive lines per total lines of dC1 and dN1–dN4 were comparable to that of full length o/x, while those of dC2, dC3, and dN5–9 were comparable to that of wild type (Fig. 1B, C). These results suggest that the region from K13 to Q46 is important for ROT4 function. The boundaries of the functional region apparently reside in the 30–34 residues beginning at K13–F15 on the N-terminal side and extending to H44–Q46 on the C-terminal side (Fig. 1A).

Second, to identify the functional region more precisely, various constructs of this region were truncated at both the N- and C-termini by an interval of one amino acid (Fig. 1D). After overexpression of these constructs, some transgenic lines of dN4-dC1, dN4-dC4, and dN10-dC4 exhibited phenotype, while none exhibited phenotype in dN4-dC5, dN11-dC4, and dN5-dC4. The ratio of transgenic lines that exhibit phenotype was much lower in dN4-dC1, dN4-dC4, and dN10-dC4 than in full-length o/x (Fig. 1E, F). These results suggest that the 32 residues-long region from T14 and D45 is the core region for the molecular function of ROT4 (Fig. 1D), while other regions are also required for the full function.

**ROT4 is not subject to proteolytic processing**

To test the possibility of proteolytic processing of ROT4, we generated GFP-ROT4 o/x and ROT4-GFP o/x and compared the morphological phenotypes with the degree of GFP expression in the transgenic lines. In both GFP-ROT4 o/x and ROT4-GFP o/x, the reduction in leaf length was roughly correlated with the fluorescence intensity (arising from the presence of GFP), indicating that the reduction in leaf length was correlated with the degree of gene expression. When transgenics of similar
fluorescence intensity were compared, the GFP-ROT4 o/x consistently had shorter leaves than the ROT4-GFP o/x (Fig. 2A–H), but all of the transgenics had shorter leaves than the wild type. Average petiole length in the GFP-ROT4 o/x was 79% that of the wild type, and the average blade length was 60% (P < 0.001 for both blade and petiole, Tukey’s pairwise comparisons). In the ROT4-GFP o/x, average petiole length was 85% that of the wild type, and the average blade length was 81% (P < 0.001 for both blade and petiole). This result suggests that ROT4-GFP has a significant effect, although the effect is significantly smaller than GFP-ROT4 (relative value of ROT4-GFP to GFP-ROT4 was 107% for blade: P = 0.26; relative value of ROT4-GFP to GFP-ROT4 was 135% for petiole: P < 0.01). We also found that GFP-ROT4 localized primarily on cell periphery, and also on limited numbers of small dots in cytoplasm (Fig. 2I–K). The localization pattern was quite similar to ROT4-GFP (Narita et al. 2004; also confirmed in Fig. 2J). Narita et al. (2004) concluded that the signal is on plasma membrane based on plasmolysis, suggesting that GFP-ROT4 also localizes on plasma membrane.

Next, we performed immunoblotting analysis on the plant lysate using an anti-GFP antibody. Only the fusion protein was detected; no free GFP was observed, as would have been the case had processing occurred at either the N- or C-terminal domain of the ROT4 peptide (Fig. 2L). The mobility of GFP-ROT4 was slightly but reproducibly smaller than that of ROT4-GFP, even though the two protein sizes are calculated to be approximately the same (33.6–33.7 kDa). Although size is not as accurate, the estimated protein sizes from their mobility in the gel for ROT4-GFP and GFP-ROT4 were both slightly larger than expected.

**The rate of cell proliferation is reduced along the proximodistal axis in ROT4 o/x leaves**

To characterize the developmental basis of polarity-dependent phenotypes of leaf, we first carried out a time-course analysis of
leaf development. In particular, one must distinguish whether the reduction in cell number in a mutant leaf originates from a reduction in the rate of cell production or from a shortening of the cell proliferative growth. As reviewed in Anastasiou and Lenhard (2007), most mutants with a reduced cell number in leaves, such as *aintegumenta*, *argos*, *nubbin*, and *jagged*, are of the latter type. Some, such as *fugu2*, *fugu5*, *erecta* (Ferjani et al. 2007), and *spatula* (Ichihashi et al. 2010), are of the former type.

The time course of leaf development in *rot4-1D* indicated a lower rate of cell production compared to the wild type, without any reduction in the period of cell proliferative growth (Fig. 3A). This suggests that ROT4 acts as a negative regulator of cell proliferation rather than as a regulator of the transition from cell proliferative growth to cell expansion.

The visualization of proliferating cells by pCYCB1;1:CYCB1;1-GUS and an estimation of the position of the arrest front by numerical analysis (Kazama et al. 2010; Fig. 3B) revealed that it was significantly closer to the proximal end of the leaf blade in *rot4-1D* (0.18 ± 0.04 mm, P < 0.01, Student’s t-test) than in the wild type (0.23 ± 0.02 mm). This result indicates a reduction in the size of the meristematic zone along the proximodistal axis in *rot4-1D* leaf primordia. This observation suggests that the polarity-dependent leaf shape in ROT4 o/x is attributable to the polarity-dependent reduction in the size of the meristematic zone in leaf primordia.

**ROT4 acts locally in the regulation of leaf shape**

To obtain further insights on the effect of ROT4 on leaf morphogenesis, we adopted a local expression system using the heat shock promoter Cre/Lox recombination (Ichihashi et al. submitted) to GFP-ROT4. In this system, Cre recombinase is expressed when the transgenic plant experiences heat shock, leading to site-specific recombination at Lox sites. When recombination occurs, GFP-ROT4 is expressed under the control of the CaMV35S promoter. Recombination stochastically occurs at the single-cell level, thus an appropriate heat treatment can generate a progenitor-cell population that overexpresses GFP-ROT4 (Fig. 4A). Since the rate of cell proliferation is only slightly reduced in the ROT4 overexpressor, the effect of GFP-ROT4 expression is detectable only when the cell population is large enough. Therefore chimeric leaves with large sectors were selected for the following analyses. While overexpression of 3xGFP did not affect leaf shape (Fig. 4B, C), when the GFP-ROT4-expressing sector appeared at the medial part of the leaf along the midrib, the leaf blades were shorter (4.99 ± 1.49 mm, P < 0.01, Student’s t-test) than those of the wild type (7.33 ± 0.83 mm) (Fig. 4D, E). Two alternative hypotheses can be advanced to explain this observation: locally expressed GFP-ROT4 can affect the whole leaf by some long-distance signaling, or the medial portion of the leaf locally affected by GFP-ROT4 determines the entire leaf length. On the other hand,
when the transgene-expressing sector appeared at the leaf margin, the petiole on the sector side was significantly shorter (4.44 ± 1.62 mm, P < 0.01) than the wild type (7.94 ± 0.87 mm), while the petiole on the opposite side showed no significant alteration (7.15 ± 1.53 mm, P = 0.08) (Fig. 4F, G). This result strongly suggests that GFP-ROT4 acts locally in the regulation of leaf shape.

**ROT4 o/x phenotypes exhibiting an altered trichome base or inflorescence share a common protrusion event**

Besides the short-leaf phenotype, a protrusion of the trichome base, a turn or bending in the pedicel or secondary inflorescence stems have been reported in DVL9 o/x (Wen and Walker 2006); however, they have not been characterized in detail. We also found these phenotypes in some of the transgenic lines of ROT4 o/x (Fig. 5). The phenotype in the inflorescence structure is prominent and unique, and have never been reported for other Arabidopsis mutants or transgenics as far as we know. We analyzed it in a overexpressor constructed with a truncated version of ROT4 that exhibited the most conspicuous and stable expression of the phenotype. We also found these phenotypes in some of the full-length overexpressors of ROT4 (data not shown).

To elucidate the developmental basis of the abnormal turn in pedicels, a time-course observation of pedicel growth was carried out. At an early stage of development, when the floral...
bud was approximately 0.5 mm in length, the pedicel was straight up in both the wild type and \textit{ROT4dN4C2\#1} (Fig. 6A, E). Later, when the floral bud was approximately 1 mm in length, the turning point appeared at the base of pedicels of \textit{ROT4dN4C2\#1}, but not in wild-type pedicels (Fig. 6B, F). The direction of growth of the part of the pedicel proximal to the turning point changed to downward at around the stage of anthesis (Fig. 6C, G).

Next, to elucidate the organ identity of the part proximal to the turning point in pedicels and secondary inflorescence stems, longitudinal sections of mature inflorescence stems were examined. In the wild type, anatomically distinct cell files called vestigial abscission zones (AZs), characterized by cytoplasmically dense small cells, are observed at the adaxial side of the basal-most part of pedicels and secondary inflorescences (Stenvik et al. 2006; Fig. 6J, O). The vestigial AZs mark the organ boundaries between pedicels or secondary inflorescence stems and the main inflorescence stem. In \textit{ROT4dN4C2\#1}, vestigial AZs were observed at the turning point of pedicels (Fig. 6M), but not at the base (Fig. 6L). Similarly, vestigial AZs were seen in the secondary inflorescences at the turning point (Fig. 6R), but not at the base (Fig. 6Q). These observations suggest that
the part proximal to the turning point is a protrusion of the main stem rather than a part of the pedicel or secondary inflorescence.

To further confirm these interpretation, we used *puchi-1* as a morphogenetic marker and *pLOB:GUS* as a gene expression marker for the organ boundary. *puchi-1* is reported to develop ectopic “stipules” (a type of glandular hair) at the base of pedicels (Hirota et al. 2007, Karim et al. 2009; Fig. 7A). Similarly, *LOB* expresses specifically between pedicels or secondary inflorescences and the main stem (Shuai et al. 2002; Fig. 7C, D). In *puchi1 ROT4dN4C2*#, stipules were observed only at the turning points (Fig. 7B). Similarly, in *pLOB:GUS ROT4dN4C2*#, GUS staining was detected only at the turning points (Fig. 7E, F). These data support the interpretation that the ROT4 o/x phenotype exhibiting an altered inflorescence is caused by an abnormal protrusion of the main stem, which appears to be analogous to the protrusion of the trichome base.

### Discussion

#### Molecular nature of ROT4

Previously Wen and Walker (2006) proposed a hypothesis that the functional region of RTFL/DVL might be processed to a shorter biologically active form. However, our data suggest that no processing of ROT4 occurred in planta. Moreover, from analysis of phenotypes of the GFP-ROT4 local overexpressor, we concluded that GFP-ROT4 locally regulates leaf shape, suggesting that GFP-ROT4 is not processed into a small, long-distance signal molecule. These data are inconsistent with the processing hypothesis. To explain the milder interference of the ROT4 function by the GFP tag when the tag was at the N-terminus compared to the C-terminus, we suggest the following possible interpretations. Application of a computational prediction (Akiyama et al. 1998) to the ROT4 sequence predicted that the N-terminal sequence outside of the functional region may form a coil structure, thus serving as a flexible structural buffer that minimizes the interference effect of GFP on the fused ROT4 function. An alternative, but not mutually exclusive possibility is that the functional region needs to reside near the C-terminus of the protein. In fact, among the RTFL/DVL family, the number of residues outside the conserved functional region on the C-terminal side is less than 11, while on the N-terminal side, the length of the non-conserved region varies from 18 to 60 residues. Consistent with this idea, we observed that a small tag, such as FLAG (eight amino acids), at the C-terminus of ROT4 did not interfere with the molecular function (data not shown).

Since ROT4 was primarily localized on the plasma membrane, and since no transmembrane domain exists in ROT4, the protein presumably interacts with some membrane component(s). The importance of the intracellular localization of RTFL/DVL was experimentally tested in a previous study (Wen and Walker 2006), in which two types of DVL1 o/x were generated, with altered intracellular distribution. One, sGFP-DVL1, had a secretion-signal sequence attached at the N-terminus. The other, eGFP-DVL1, had both a secretion-signal sequence at the N-terminus and an endoplasmic reticulum (ER)-retention-signal sequence at the C-terminus for localization in the ER. Overexpressors of these modified genes exhibited no phenotype, while overexpressors of the authentic GFP-DVL1 genes showed phenotypes similar to...
that of unmodified DVL1. Therefore localization on the plasma membrane appears to be essential for the molecular function of RTFL/DVL. Because the core functional region of ROT4 is only 32 amino acids in length, it is unlikely to have any signal-receptor domain or output domain. Therefore ROT4 may exert its biological effect via an interaction with some component of a signal transduction pathway on the plasma membrane.

Developmental role of ROT4

As reported by Narita et al. (2004), rosette leaves of rot4-1D have a reduced number of cells, specifically along the proximodistal axis. From this observation, ROT4 was implicated in the control of polarity-dependent cell proliferation. The time course of leaf development in ROT4 o/x in this study revealed that the period of cell proliferation is not altered in the leaf primordia, but the size of the meristematic zone is reduced along the proximodistal axis. Interestingly, the results of local overexpression experiments raised the possibility that some positional cue during leaf morphogenesis along the proximodistal axis was perturbed in ROT4 o/x, because in a chimeric leaf, the leaf blade–petiole boundary on the side of the leaf that had ROT4 being expressed in the leaf margin shifted proximally. This phenotype is distinct from that of chimeras for other growth-regulating factors. For example, in the case of the hobbit mutant, which exhibits smaller leaves, hobbit sectors located at the leaf margin grow in cooperation with the remaining wild-type parts of the leaf, without a change in the leaf blade–petiole boundary (Serralbo et al. 2006). Thus these results suggest that ROT4 could be involved in the determination of organ boundary and positional cues of leaf primordia along the proximodistal axis.

If we simplify the effect of ROT4 expression in leaf primordia as the perturbation of boundary or positional cues along the proximodistal axis, then the same interpretation is possible for the perturbation of boundary or positional cues along the proximodistal axis. Interestingly, the results of local overexpression experiments suggested the possibility that some positional cue during leaf morphogenesis along the proximodistal axis, then the same interpretation is possible for the perturbation of boundary or positional cues along the proximodistal axis. From this observation, ROT4 was implicated in the control of polarity-dependent cell proliferation. The time course of leaf development in ROT4 o/x in this study revealed that the period of cell proliferation is not altered in the leaf primordia, but the size of the meristematic zone is reduced along the proximodistal axis. Interestingly, the results of local overexpression experiments raised the possibility that some positional cue during leaf morphogenesis along the proximodistal axis was perturbed in ROT4 o/x, because in a chimeric leaf, the leaf blade–petiole boundary on the side of the leaf that had ROT4 being expressed in the leaf margin shifted proximally. This phenotype is distinct from that of chimeras for other growth-regulating factors. For example, in the case of the hobbit mutant, which exhibits smaller leaves, hobbit sectors located at the leaf margin grow in cooperation with the remaining wild-type parts of the leaf, without a change in the leaf blade–petiole boundary (Serralbo et al. 2006). Thus these results suggest that ROT4 could be involved in the determination of organ boundary and positional cues of leaf primordia along the proximodistal axis.

Materials and Methods

Plant materials and growth conditions

The Arabidopsis accession Columbia (Col) was used as the wild type. Plants were grown at 23°C under continuous light. We used rot4-1D (Narita et al. 2004), puchi-1 (Hirota et al. 2007) and pLOB:GUS (Shuai et al. 2002). A single line of p35S:ROT4-GFP, previously reported by Narita et al. (2004), was used in this study and represented as p35S:ROT4-GFP1. To confirm the results of this study, novel transgenic lines of p35S:ROT4-GFP were generated and represented as p35S:ROT4-GFP2. The intervening sequence between the GFP tag and ROT4 was different between the two; both versions of transgenic lines were used for the biochemical analysis.

Vector construction and transformation

For constitutive overexpression of truncated versions of ROT4, polymerase chain reaction (PCR) was used to amplify the coding regions of ROT4 from Arabidopsis genomic DNA using the following primers:

- Full length-Fw, 5′-CACCATGGCCACCGAGGAGAATAGGC-3′; dN1-Fw, 5′-CACCATGCGGGAAATGGCGACCTGT-3′; dN2-Fw, 5′-CACCATGAAAGCCACTGATGACCTGC-3′; dN3-Fw, 5′-CACCATGGAGCCGTGCAAGACTTTTGG-3′; dN4-Fw, 5′-CACCATGAAAGTTTGGCGCAAGAATGCG-3′; dN5-Fw, 5′-CACCATGGGCAAAAGTGCAGCGCACTGATC-3′; dN6-Fw, 5′-CACCCAAAAATGCAAGCCATGCTCGT-3′; dN7-Fw, 5′-CACCCATGTCGTAAGAAGGACGAAGG-3′; dN8-Fw, 5′-CACAAGGAGGCCAAGTTTAC-3′; dN9-Fw, 5′-CACCGGGCAGTTTACG-3′; dN10-Fw, 5′-CACCATGACTTTTGGCGCAAGAATGCG-3′; dN11-Fw, 5′-CACGACTTTGGAAGAAGGAGGACGAAGG-3′; full length-Rv, 5′-TCAGATGCTCTGTTTGCGG-3′; dC2-Rv for the first-round experiment, 5′-TCAGTTGCTGTCCAGCACAAAATACAC-3′; dC2-Rv for the second-round experiment, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′; dC3-Rv, 5′-TCAGATGCTCTGTTTGCG-3′; dC4-Rv, 5′-TCAGATGCTCTGTTTGCG-3′; dC5-Rv, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′; dC6-Rv, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′.

The PCR products were first cloned into the pENTR D-TOPO vector (Invitrogen, Carlsbad, CA, USA), then introduced downstream of the 35S promoter in a destination vector (pH35G). For construction of p35S:GFP-ROT4, PCR was used to amplify RTFL/DVL. Because the core functional region of ROT4 is 32 amino acids in length, it is unlikely to have any signal-receptor domain or output domain. Therefore ROT4 may exert its biological effect via an interaction with some component of a signal transduction pathway on the plasma membrane.

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If we simplify the effect of ROT4 expression in leaf primordia as the perturbation of boundary or positional cues along the proximodistal axis, then the same interpretation is possible for the other pleiotropic phenotypes of the overexpressor, namely, protrusions in inflorescences and the trichome base. In both phenotypes, overexpressors exhibited an abnormal shift of the boundary (inflorescence stem/pedicle; primary inflorescence stem/secondary inflorescence stem; and trichome/socket). Although all examined overexpressors were expected to express ROT4 ubiquitously and uniformly, perturbation of some positional cues may have occurred, if ROT4 was involved in the determination of the cues by some gradient. The possibility also exists that ROT4 is involved in the determination of positional cues during shoot development. Further studies of the role of ROT4 may lead to a deeper understanding regarding the molecular regulation of polar growth in plants.

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Vector construction and transformation

For constitutive overexpression of truncated versions of ROT4, polymerase chain reaction (PCR) was used to amplify the coding regions of ROT4 from Arabidopsis genomic DNA using the following primers:

- Full length-Fw, 5′-CACCATGGCCACCGAGGAGAATAGGC-3′; dN1-Fw, 5′-CACCATGCGGGAAATGGCGACCTGT-3′; dN2-Fw, 5′-CACCATGAAAGCCACTGATGACCTGC-3′; dN3-Fw, 5′-CACCATGGAGCCGTGCAAGACTTTTGG-3′; dN4-Fw, 5′-CACCATGAAAGTTTGGCGCAAGAATGCG-3′; dN5-Fw, 5′-CACCATGGGCAAAAGTGCAGCGCACTGATC-3′; dN6-Fw, 5′-CACCCAAAAATGCAAGCCATGCTCGT-3′; dN7-Fw, 5′-CACCCATGTCGTAAGAAGGACGAAGG-3′; dN8-Fw, 5′-CACAAGGAGGCCAAGTTTAC-3′; dN9-Fw, 5′-CACCGGGCAGTTTACG-3′; dN10-Fw, 5′-CACCATGACTTTTGGCGCAAGAATGCG-3′; dN11-Fw, 5′-CACGACTTTGGAAGAAGGAGGACGAAGG-3′; full length-Rv, 5′-TCAGATGCTCTGTTTGCGG-3′; dC2-Rv for the first-round experiment, 5′-TCAGTTGCTGTCCAGCACAAAATACAC-3′; dC2-Rv for the second-round experiment, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′; dC3-Rv, 5′-TCAGATGCTCTGTTTGCG-3′; dC4-Rv, 5′-TCAGATGCTCTGTTTGCG-3′; dC5-Rv, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′; dC6-Rv, 5′-TTAGTGCCAGCACAAAATACACACACAC-3′.
and by replacing the GUS gene by GFP::ROT4. The vector was cut by XbaI and SalI and ligated with pH35G vector cut by XbaI and Xhol.

Binary vectors were transformed into agrobacterium (Rhizobium radiobacter) Cs8 C1Rif and in turn used to transform Arabidopsis by the floral dip method (Clough and Bent 1998). Wild-type plants were used as hosts in most experiments, but pHS:Cre222.2 and pHS:Cre222.3 (provided by Dr. Taku Takahashi, Okayama University) were used for the chimeric expression of GFP-ROT4.

**Protein immunoblotting**

For biochemical analysis of ROT4 in Arabidopsis, total soluble protein was extracted from the aerial part of plants 12 days after imbibition (DAI). The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 14% (w/v) polyacrylamide gel. Immunoblotting was performed by the standard method using horse-radish peroxidase-coupled secondary antibody and ECL-plus (Amersham Biosciences, Uppsala, Sweden).

**Leaf anatomy**

Leaves were fixed in a solution containing formalin, acetic acid, and alcohol (FAA) and then cleared in hydrated chloride, as previously described (Tsuge et al. 1996). The leaves were inspected under a microscope (Leitz DMRXE; Leica Microsystems, Wetzlar, Germany) equipped with a cooled charged-coupled device (CCD) camera (DFC300FX; Leica Microsystems).

**Stem anatomy**

For Technovit sectioning, plant samples were first peeled off manually for better infiltration. They were then vacuum-infiltrated for 30 min in FAA, dehydrated through a graded ethanol series, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Sections 7–8 mm thick were prepared with Microm HM 360 (GMI, Ramsey, MN, USA), stained by toluidine blue and observed under a stereomicroscope or a microscope equipped with a CCD camera.

**Intracellular localization of ROT4**

For observation of the intracellular localization of ROT4, GFP fluorescence from plants 6 DAI excited by an Ar+ laser at 488 nm was imaged on a fluorescence microscope (BX51; Olympus, Tokyo, Japan) equipped with a confocal scanner unit (CSU10; Yokogawa Electric, Tokyo, Japan) and a cooled CCD camera (ORCA-AG; Hamamatsu Photonics, Shizuoka, Japan). Images were processed with IPLab software (BD Biosciences, Franklin Lakes, NJ, USA).

**Estimation of the cell proliferation zone in leaf**

To visualize G2/M cells in leaf primordia, the transgenic line pCYCB1;1:CYCB1;1-GUS (Donnelly et al. 1999) was introgressed into rot4-1D and used for further analysis. GUS staining was detected as previously described (Donnelly et al. 1999). Numerical analysis was performed as previously described (Kazama et al. 2010).

**Statistical analysis**

For cluster analysis, we performed both hierarchical and model-based analysis using R software (R Foundation for Statistical Computing, 2009). In hierarchical clustering, we tried several different methods for calculation. Of those, single-linkage clustering did not work well in clustering obtained data into two groups, while other methods (complete linkage clustering, average linkage clustering, centroid linkage clustering, median linkage clustering, Ward’s criterion and McQuitty’s criterion) gave similar results. Moreover, a model-based clustering method called expectation maximization clustering gave similar results to the hierarchical clustering, thus we adopted the results obtained from expectation maximization clustering.

**Induction of a chimera**

pHS:Cre:Lox-GFP-ROT4 at the T$_2$ generation (homozygous for the pHS:Cre transgene, and homozygous or heterozygous for Lox-GFP-ROT4) was used as the starting plant material. Sixteen hours after imbibition, the seeds were sown on Murashige and Skoog media and kept at 4°C in the dark for 72 h. After 6 h acclimation at room temperature, a heat shock was given by keeping the plantlets at 37°C for 1.5 h. After the heat shock, plates were incubated in the growth chamber. After 10 days of growth on Murashige and Skoog plates, plants exhibiting fluorescence due to GFP-ROT4 in sectors were grown on rock wool for 4–5 days. The leaves were then observed under a stereomicroscope equipped with a CCD camera.

**Supplementary data**

Supplementary data are available at PCP online.

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