The ROTUNDIFOLIA3 gene of Arabidopsis thaliana encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells

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The polarized processes of cell elongation play a crucial role in morphogenesis of higher plants. We reported previously that the rotundifolia3 (rot3) mutant of Arabidopsis has a defect in the polar elongation of leaf cells. In the present study, we isolated two additional alleles with mutations in the ROT3 gene. The ROT3 gene was cloned by a T-DNA-tagging method and isolation of the gene was confirmed by a molecular analysis of three rot3 mutant alleles obtained from different mutagenesis. The ROT3 gene encodes a cytochrome P-450 (CYP90C1) with domains homologous to regions of steroid hydroxylases of animals and plants. Expression of the ROT3 gene was detected in all major plant organs. Especially, higher expression was detected in the tissues that had high activity of cell division. We confirmed that the ROT3 gene controls polar elongation specifically in leaf cells by an analysis of three rot3 mutants obtained from different mutagenesis experiments. Our results imply that the ROT3 protein is a member of a new class of cytochrome P-450 encoding putative steroid hydroxylases, which is required for the regulated polar elongation of cells in leaves of Arabidopsis.

[Key Words: Arabidopsis thaliana; cytochrome P-450; leaf morphogenesis; polar cell elongation; rotundifolia3 mutant; T-DNA tagging]

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The morphology of multicellular organisms is largely attributable to the shape, size, and number of constituent cells. Cell shape, in plants in particular, is dependent on processes of polar elongation. Phytohormones, such as auxin and gibberellic acids, are involved in elongation of cells along the long axis (Leopold 1955; Koornneef and van der Veen 1980; Cleland 1988; Shibaoka 1994; Estelle 1996; Kende and Zeervaart 1997). Brassinolides have also been shown to be involved in polar elongation of cells in the longitudinal direction (Takahashi et al. 1995; Bishop et al. 1996; Li et al. 1996; Szekeres et al. 1996; Creelman and Mullet 1997). In contrast, cytokinins and ethylene induce elongation of cells along the short axis (Shibaoka 1994; Kieber 1997). Cytoskeletal components (Giddings and Staehelin 1991; Cyr 1994; Shibaoka 1994) and wall-loosening proteins (McCue-Mason et al. 1992; Cosgrove 1997) are thought to be involved in the control of the polar elongation of cells. However, the molecular mechanisms that control the extent and direction of cell elongation have not been characterized.

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sociated with different phenotypes. Detailed phenotypic and molecular analyses of our rot3 mutants were performed. Molecular cloning by T-DNA tagging of the ROT3 gene showed that tagging abolished the synthesis of a protein with homology in various conserved domains to P-450 monooxygenases, which include steroid hydroxylases (Nelson et al. 1993). Our data indicate that the ROT3 gene product, CY90PC1, might be involved in the biosynthesis of steroids, which somehow play an important role in the regulation of the polar elongation of cells during development in Arabidopsis.

**Results**

Genetic and phenotypic re-examination of rot3 mutant alleles

A recessive rot3 mutant allele, rot3-1, was isolated and characterized in a previous study (Tsuge et al. 1996). To characterize the function of the ROT3 gene in greater detail we searched for new rot3 mutant alleles in an analysis of Arabidopsis plants obtained after different types of mutagenesis. We isolated two additional alleles: one (rot3-2) from a screening of ethylmethane sulfonate (EMS)-treated plants and the other (rot3-3) from a screening of plants that had been transformed with T-DNA insertions. The rot3-3 mutant was isolated from a screening of plants from 22,000 seeds (11 pools) of Arabidopsis. We characterized the rot3-3 gene showed that tagging abolished the synthesis of a protein with homology in various conserved domains to P-450 monooxygenases, which include steroid hydroxylases (Nelson et al. 1993). Our data indicate that the ROT3 gene product, CY90PC1, might be involved in the biosynthesis of steroids, which somehow play an important role in the regulation of the polar elongation of cells during development in Arabidopsis.

The lengths of all the true leaves (foliage leaves) of the rot3-1 and rot3-3 mutants were longer than those of the wild type (Fig. 1A–D). The length of adaxial epidermal cells in the petiole of the rot3-2 mutant was 52% of that of wild type at the fully expanded stage (Table 2). Petals, sepals, stamens, and pistils were all of reduced length in the rot3-1 mutant (Tsuge et al. 1996), whereas those of the rot3-2 mutant were all larger than the wild type and the rot3-1 mutant (data not shown). The phenotypes of F1 plants from each cross between rot3-1 and rot3-2, and between rot3-2 and rot3-3 were similar to those of the rot3-2 mutant, which had round leaf blades that were larger than those of the rot3-1 mutant (Fig. 1E–G). The results of our phenotypic analysis demonstrated that the rot3-1 mutant allele was the strongest of the three mutant alleles.

The rot3-2 mutant had enlarged cells in its leaves and stems

As described above, the rot3-2 mutant had a clearly distinct phenotype, having enlarged leaf blades and a thick

**Figure 1.** Morphology of wild-type and rot3 mutant plants. (A–D) Photographs of wild-type (A), rot3-1 (B), rot3-2 (C), and rot3-3 (D) plants are shown. (E–G) The morphology of heterozygotes from the following crosses: rot3-1 × rot3-2 (E), rot3-1 × rot3-3 (F), and rot3-2 × rot3-3 (G). The plants were photographed 4 weeks after sowing of seeds. Bars, 10 mm.
stem (Figs. 1C and 2B,H,J). Therefore, we analyzed this mutant in further detail.

To determine whether the abnormal size of leaf blades and internodes in the rot3-2 mutant was attributable to a change in cell expansion (elongation) or a change in cell number, we performed an anatomical analysis of rot3-2 leaves and internodes, comparing them with the wild type (Tables 2 and 3; Fig. 2C–J).

Although the rot3-1 mutant had a defect in the elongation of leaf palisade cells, specifically in the leaf-length direction (Tsuge et al. 1996), the width, length, and thickness of palisade cells of the rot3-2 mutant were 112%–126% of those of wild type at the fully expanded stage (Table 2; Fig. 2C,D). The numbers of palisade cells in the fifth rosette leaf of the rot3-2 mutant in the leaf-length direction and in the leaf-length direction were not very different from the wild type (Table 3). The epidermal cells of the rot3-1 mutant had smaller numbers of protrusions in the leaf-length direction (Tsuge et al. 1996). However, the mean area of individual epidermal cells of the rot3-2 mutant was elevated on both the adaxial and the abaxial side (Fig. 2E,F). The cell areas on the adaxial and the abaxial side were 134% and 141% of the wild-type areas, respectively. These anatomical results suggested that an increase in cell size directly affected the leaf morphology in the rot3-2 mutant. On the other hand, reduction in the length of petioles of the rot3-2 mutant was similar to those of the wild type at the mature stage (Table 1). However, the internodes were thicker than those of the wild type (Table 1; Fig. 2G–J). The diameter of pith cells of the first internodes of type 2 metamers (Schultz and Haughn 1991) from the rot3-2 mutant was very much greater than that of pith cells in the wild type (Table 1; Fig. 2G,H). However, the number of files of pith cells in the shoots of the rot3-2 mutant did not differ from those of the wild type (Fig. 2G,H). The length of pith cells in the rot3-2 mutant was shorter than that in the wild type. Irregularly arranged cells were also observed in the rot3-2 mutant (Fig. 2I,J). Thus, the rot3-2 mutation also appeared to affect the expansion of cells in the stem.

Molecular cloning of the ROT3 gene

To isolate the ROT3 gene, we examined linkage between the mutant phenotype and T-DNA insertions in the rot3-3 mutant. From F2 families of the rot3-3 mutant with the mutant phenotype, we tested 79 mutants for linkage between the T-DNA insertion and the rot3 mutation. Linkage was assessed by examining patterns of Southern blot analysis of the F2 population with the right-border fragment of the T-DNA as probe (Fig. 3A). Two T-DNA insertions were identified in the rot3-3 line. One of the T-DNAs was inserted in the genomes of all rot3-3 plants examined (data not shown), indicating a tight linkage between the T-DNA insertion and the rot3 mutation (0.0 ± 0.5 cM from the ROT3 locus).

A genomic DNA fragment of 1 kb adjacent to the T-DNA in rot3-3 was isolated by plasmid rescue in Escherichia coli, and it was used for Southern hybridization analysis and isolation of cDNA clones. Southern hybridization experiments, with the Dral–EcoRI fragment of the rescued clone (Fig. 3A, probe D) as probe, revealed that the isolated region had been deleted in the rot3-1 mutant (Fig. 3B), which had been isolated from a population of fast neutron-mutagenized seeds. This result provided preliminary evidence that the isolated region might include the ROT3 gene.

Table 1. Various dimensions of wild-type and rot3-2 mutant plants

| Organ | Wild type (Col) | rot3-2 |
|-------|----------------|--------|
| Cotyledon width | 2.36 ± 0.28 (n = 10) | 2.90 ± 0.21 (n = 10) |
| Cotyledon length | 2.64 ± 0.19 (n = 10) | 2.96 ± 0.35 (n = 10) |
| Hypocotyl length$^b$ | 1.90 ± 0.37 (n = 25) | 1.96 ± 0.28 (n = 38) |
| Petiole length | 15.61 ± 1.47 (n = 18) | 6.38 ± 1.18 (n = 17) |
| Leaf blade width | 6.9 ± 0.1 (n = 2) | 11.3 ± 0.2 (n = 6) |
| Lead blade length | 10.8 ± 0.8 (n = 3) | 13.5 ± 1.9 (n = 6) |
| Internode diameter | 12.1 ± 0.4 (n = 6) | 18.3 ± 1.9 (n = 4) |
| Internode length type 2 metamer (1st) | 42.0 ± 2.3 (n = 6) | 47.5 ± 6.0 (n = 8) |
| type 2 metamer (2nd) | 95.8 ± 15.3 (n = 6) | 97.3 ± 13.2 (n = 5) |
| total length$^c$ | 352.5 ± 27.5 (n = 6) | 333.0 ± 125.3 (n = 5) |

(n) Number of plants examined.

$^a$Cotyledons, petiole of fifth leaves, and fifth rosette leaves were measured at the fully expanded stage, and hypocotyls and internodes were measured at the mature stage.

$^b$Plants were grown on agar plates and then hypocotyls were measured.

$^c$Type 2 metamer + type 3 metamer.
To identify whether or not the isolated genomic fragment included the coding region, we performed Northern hybridization analysis with the isolated genomic fragment as probe. With 1.5-µg of poly(A)+ RNA extracted from rosette leaves, a band that represented a transcribed RNA of ∼1.8 kb was detected with the DraI–HindIII fragment as probe (Fig. 3C, probe C). This Northern hybridization analysis indicated that the level of expression of the detected mRNA was very low (see Materials and Methods). A search among Arabidopsis sequences in the GenBank database revealed that the partial sequence of an EST clone (491 bp; GenBank accession no. N96214) was the same as that of the isolated genomic fragment. We analyzed the DNA sequence of the entire EST clone and found that it encoded a cDNA of 1.8 kb and overlapped the putative first exon sequence of the isolated genomic fragment (Fig. 3A).

The cDNA clone was used as a probe for Southern blot analysis of genomic DNA. Under high-stringency conditions, from one to three genomic fragments were detected; under low-stringency conditions, additional genomic fragments were observed (Fig. 4; arrowhead). Thus, there appeared to be at least one additional sequence that was related to the gene represented by the cDNA clone.

Sequence analysis and studies of the expression of the ROT3 gene

In addition to sequencing of cDNA clones, we determined the corresponding sequence of the genomic DNA and examined homologies in the database. The 1-kb genomic sequence flanking T-DNA was isolated and cDNA sequences were 100% homologous to putative coding sequence of Arabidopsis DNA chromosome 4, ESSA I AP2 fragment no. 2, 177481–181644 bp (GenBank accession no. Z99708; EU Arabidopsis sequencing project ESSA). The ROT3 gene spans ∼6 kb of genomic DNA and consists of nine exons (see Fig. 3A) with consensus splice sites at the exon–intron boundaries. The predicted ROT3 protein has 524 amino acids, with a calculated molecular mass of 59.4 kDa (Fig. 5B). The deduced amino acid sequence of this protein exhibited homology to the conserved amino-terminal membrane-anchoring, proline-rich, oxygen- and heme-binding domains of microsomal cytochrome P-450 (Fig. 5B; 40–90% homology to conserved domains of P-450, as defined by Nebert and Gonzalez 1987). The ROT3 protein appeared, therefore, to include all of the functionally important domains of a P-450 monoxygenase (Pan et al. 1995). This ROT3 protein was designated CYP90C1 by the P-450 Nomenclature Committee (Nebert and Gonzalez 1987). CYP90C1 exhibited the strongest homology to CYP90A1 (36%; cathasterone C23-hydroxylase; Szekeres et al. 1996) from Arabidopsis, strong homology to the DWARF protein of tomato, known also as CYP85 (27%; Bishop et al. 1996), and to maize CYP88 (25%; GA12 to GA 53 gibberellin 13-hydroxylase; Winkler and Helentjaris 1995), as well as to zebrafish P-450-RAI (23%; retinoic acid 4-hydroxylase) (Fig. 5). CYP90C1 differs in several domains from other plant forms of P-450, such as CYP73A of Jerusalem artichoke (11% identity; cinnamate 4-hydroxylase; GenBank accession no. Z17369). CYP90C1 does not have the consensus sequence PFGG(ASV)GRRC(PAV)G around the heme-binding cysteine (Fig. 5B; positions 448–452) that is conserved in members of class A P-450s that catalyze plant-specific reactions, an indication that CYP90C1 (non-class A) is more similar to animal, lower eukaryotic, and bacterial proteins (Fig. 5B; Durst and Nelson 1995). The comparison of sequence also revealed homology between CYP90C1 and specific domains of
Table 2. Dimensions of leaf cells in wild-type and rot3-2 mutant plants

| Direction                | Wild type (Col) | rot3-2          |
|--------------------------|----------------|-----------------|
| Leaf blade\(a\)          |                |                 |
| leaf-width direction     | 40.6 ± 3.3 \((n = 5)\) | 45.3 ± 5.7 \((n = 7)\) |
| leaf-length direction    | 42.6 ± 2.2 \((n = 4)\) | 47.6 ± 4.5 \((n = 6)\) |
| leaf-thickness direction | 32.0 ± 7.6 \((n = 5)\) | 40.4 ± 4.3 \((n = 6)\) |
| Leaf petiole\(b\)        | 470.8 ± 43.9 \((n = 5)\) | 244.8 ± 37.8 \((n = 5)\) |

\((n)\) Number of plants examined.

\(a\) Palisade cells of fifth leaf blade were measured. Standard deviations are shown for results from >150 cells per leaf. Measurements were made on sections.

\(b\) Adaxial epidermal cells of petiole of fifth leaf were measured. Standard deviations are shown for results from >15 cells per petiole.

steroid hydroxylases [at substrate recognition sites (SRSs); Gotoh 1992] such as CYP90A1 (22–70% homology), which encodes cathasterone C23-hydroxylase, an enzyme involved in brassinolide biosynthesis, CYP88 (21–30% homology), which encodes a gibberellin 13-hydroxylase that is involved in gibberellin biosynthesis, and P-450-RAI (20–50% homology), which encodes retinoic acid 4-hydroxylase (Fig. 5B). CYP90C1 also exhibits sequence homology to all conserved domains of animal, fish, and yeast P-450s, such as CYP2A1 of rat (Matsunaga et al. 1990; GenBank accession no. M33312), CYP3A3 of human (GenBank accession no. P05184), and CYP51 of Candida (GenBank accession no. S02713). In addition, CYP90C1 exhibits homology to members of the CYC2 family in several regions (8–33% identity; Fig. 5B), which have the specific SRSs (Gotoh 1992) such as CYP90A1 (22–70% homology).

Molecular analysis of the rot3 mutant alleles

To define the molecular lesions that affect the function of the ROT3 gene, we determined the genomic DNA sequence and the sequence of the mRNA transcript for the three rot3 mutant alleles. One mutant allele (rot3-1), which had been generated by fast neutron irradiation, was found to have a deletion of at least 1 kb at the ROT3 locus by Southern hybridization analysis (see Fig. 3B). The rot3-2 allele, derived from EMS-mutagenized seeds, was found to contain a single base-pair substitution that led to the replacement (marked by an inverted black triangle in Fig. 5B) of Gly-80 by Glu. The rot3-3 allele was derived from insertion of T-DNA, and the T-DNA was localized in the promoter region (see Fig. 3A).

We studied the expression of the three mutant alleles to examine the effects of these lesions on the accumulation of ROT3 mRNA. No ROT3 transcript and a dramatic reduction in the level of the ROT3 transcript were observed in leaves of plants that were homozygous for the rot3-1 and rot3-3 alleles, respectively (Fig. 6A). This result was consistent with the molecular lesions found in these alleles (a deletion and the insertion of T-DNA), which would be expected to disrupt transcription or to alter stability of the transcript. Relatively normal accumulation of ROT3 mRNA was observed in leaves of the

Table 3. Anatomical analysis of the fifth rosette leaves of wild-type and rot3-2 mutant plants

| Number of leaf cells       | Wild type (Col) | rot3-2          |
|----------------------------|----------------|-----------------|
| Aligned in the leaf-width direction |                |                 |
| palisade cells             | 217 ± 30.8 \((n = 5)\) | 212 ± 21.7 \((n = 7)\) |
| parenchymatous cells\(a\) | 721 ± 96.8 \((n = 3)\) | 733 ± 73.7 \((n = 7)\) |
| Aligned in the leaf-length direction |                |                 |
| palisade cells             | 261 ± 21.3 \((n = 4)\) | 247 ± 22.9 \((n = 6)\) |
| parenchymatous cells\(a\) | 808 ± 168 \((n = 3)\) | 860 ± 46.4 \((n = 7)\) |

\((n)\) Number of plants examined.

\(a\) Total numbers of cells in sections in each direction, excluding the cells in the epidermal layer, the xylem, and the phloem.
rot3-2 mutant, a result that suggests that the lesion (a missense mutation) altered the function of the ROT3 protein rather than the synthesis or stability of the ROT3 transcript.

**Discussion**

Role of the product of the ROT3 gene in the polar elongation of cells

In this study we identified a new member of the family of cytochrome P-450s, encoded by the ROT3 gene, that is required for the regulated polar elongation of cells in *A. thaliana*. The rot3 mutants that we studied had very short petioles, leaves with a defect in polar expansion, and abnormal stems (in the case of the rot3-2 allele), but their roots and hypocotyls resembled those of wild-type plants. Light microscopy examination revealed that the decreased or increased dimensions of the leaves of rot3 mutants were a consequence of a severe reduction (Tsuge et al. 1996) or increase in cell length in the leaf-length direction rather than to alterations in cell number (Tables 2 and 3). From the findings presented here it appears that the ROT3 gene is normally required for the proper direction and extent of the elongation of leaf cells, that is, for the appropriately polarized elongation of leaf cells during plant development.

In view of the organ specificity of the mutant phenotype, the ROT3 gene seems to be necessary at various stages during the development of leaves in *A. thaliana* but not during the development of roots and stems. This organ specificity does not correspond exactly to the localization of ROT3 mRNA. The ROT3 transcript was detected not only in leaves, but also in roots and stems throughout plant development. The normal phenotype of roots and stems of loss-of-function type rot3 mutants (rot3-1 and rot3-3) suggests that different pathways might exist for the cell expansion process in roots, stems, and foliages leaves. The expression of ROT3 mRNA was detected at high level in cell-dividing tissues (Fig. 6B) and less in cotyledons that have a low rate of cell division (Tsukaya et al. 1994). Dividing cells in leaves might synthesize some factors by the ROT3 protein CYP90C1 to communicate with elongating cells for regulating the direction of cell elongation.

The rot3-2 mutant had a distinct phenotype; cells were enlarged in the diameter direction in stems and cell lengths were reduced perpendicular to the axes in leaves and stems. In contrast, the null allele rot3-1 was associated with a leaf-specific defect in cell elongation parallel to the longitudinal axis. Our molecular analysis of rot3 mutants provides some insight into some of the amino acid residues that are critical for cell elongation. The rot3-2 allele has a missense mutation that alters a proline-rich domain (position 80; Fig. 5B) that is conserved in all P-450s. One explanation for the rot3-2 phenotype is that this glycine residue is crucial for the protein folding that is necessary for proper function of the protein in cell...
elongation, and the amino acid substitution leads to instability of the protein or a change in substrate specificity. Recently, the experiment of site-directed mutagenesis of the conserved proline-rich region of cytochrome P-450 2C2 (Chen and Kemper 1996; Chen et al. 1998) suggested that a highly conserved proline-rich region with the sequence PPGPTPFP, relative to the sequence flanking it on the carboxy-terminal side, had a very important role in substrate binding. Mutations of this region also may affect the conformational integrity of the proteins (Chen et al. 1998). The \textit{rot3-2} phenotype might represent an altered function caused by mutation in the \textit{CYP90C1} protein.

The \textit{ROT3} gene encodes a new member of the family of cytochrome P-450s with a steroid substrate recognition site.

The molecular cloning of the \textit{ROT3} gene was confirmed by genetic analysis and by analysis of sequences of three mutant \textit{rot3} alleles. The nucleotide sequence of the \textit{ROT3} gene encodes a putative 59.4-kD protein with considerable homology to the conserved domains of cytochrome P-450s. The conservation of exon–intron boundaries between the \textit{ROT3} gene and the gene for \textit{CYP90A1}, which is involved in brassinosteroid biosynthesis (Szekeres et al. 1996), suggest that the \textit{ROT3} gene might encode a cytochrome P-450 steroid hydroxylase. In addition, the absence in \textit{CYP90C1} of the consensus sequence PFGG(ASV)GRRC(PAV)G around the heme-binding cysteine (Durst and Nelson 1995) suggests that \textit{CYP90C1} is more similar to animal, lower eukaryotic, and bacterial proteins. \textit{ROT3} appears to be a gene for a non-class A P-450 that might be involved in steroid biosynthesis. What is the possible role of the product of the \textit{ROT3} gene in cell elongation during plant development? The putative steroid-binding domains in the \textit{ROT3} protein suggest that the protein might be involved in the biosyn-

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**Figure 5.** Phylogenetic tree (A) and alignment of amino acid sequences of \textit{CYP90C1} and other cytochrome P-450s from plants and animals (B). \textit{CYP90C1} exhibits strong homology to \textit{CYP90A1} (36%; cathasterone C23 hydroxylase of \textit{A. thaliana}), \textit{CYP85} (27%; DWARF protein of tomato), \textit{CYP88} (25%; \textit{GA17}-\textit{GA32} gibberelin 13-hydroxylase of maize), and P-450-RA1 (23%; retinoic acid 4-hydroxylase of zebrafish). \textit{CYP2A1} (rat hepatic steroid hydroxylase) is also homologous to \textit{CYP90C1} in the SRSs (SRS-1–SRS-6), indicated above the aligned sequences. The mutation site of \textit{ROT3-2} (Gly-80 to Glu) is marked by an inverted black triangle in the conserved proline-rich region (pro dom.). The locations of the SRSs of microsomal P-450s, including the membrane anchor region, the proline-rich domain, and the O2-, steroid-, and heme-binding domains, are indicated above the aligned sequences. Identical amino acids are indicated as white letters in black boxes. Bar in A indicates percentage of homology to \textit{CYP90C1}.

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Arabidopsis \textit{ROT3} gene encodes \textit{CYP90C1}
brassinosteroids or in related signaling pathways, such as all reported mutants with defects in the synthesis of exogenous brassinosteroid (data not shown). Because was the wild-type phenotype induced by the application of brassinosteroids, as brassinosteroids are the only steroids that have been shown to influence plant growth factors, either GA 3 (Sigma, St. Louis, MO) or brassinolide (kindly provided by Dr. M. Hayashi, Institute of Physical and Chemical Research, Wako, Japan), were applied at 10 μM in M50 medium (Tsukaya et al. 1991). The morphology of leaves and petioles was observed 10 days after germination.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col) was the wild type used for backcrossing and for comparisons with mutant plants. The rot3-1 allele was isolated from fast neutron-irradiated seeds of Arabidopsis (Col ecotype), as described previously (Tsuge et al. 1996). The rot3-2 allele, which was kindly provided by Dr. Y. Komeda (Hokkaido University, Sapporo, Japan), was identified in a population after mutagenesis with EMS (Landsberg erecta ecotype). The rot3-3 allele was isolated from a population of Arabidopsis (Wassilewskija ecotype) with T-DNA insertions (Feldmann 1991) that had been generated by the DuPont Corporation (Wilmington, DE). The mutant strains had mutations at the same locus, as demonstrated by an allelism test that involved crosses between the various strains. To avoid any background effect, the rot3-2 mutant strain was backcrossed three times to the Col ecotype before detailed analysis. Allelism tests were performed using lines with a similar phenotype from the EMS-mutagenized line and the T-DNA-tagged line.

Plants were grown in small plastic pots with rockwool or vermiculite, or both, that had been moistened with MGRL medium (Tsukaya et al. 1991) at 22°C under continuous illumination from fluorescent lamps, as described previously (Tsuge et al. 1996). Fully extended shoots at each position (type 2 metamers; Schultz and Haughn 1991) were measured after growth under the conditions described above. For analysis of the growth of roots and hypocotyls, plants were cultured on plates. For cultivation of plants on agar-solidified medium (Okada and Shimura 1992), seeds were surface sterilized as described previously (Tsukaya et al. 1991) and were washed extensively with sterilized water. The plates were incubated vertically, for observation of the growth of roots and hypocotyls, under the conditions described above. Fully extended shoots at each position (type 2 metamers; Schultz and Haughn 1991) were measured after growth under the conditions described above. For analysis of the growth of roots and hypocotyls, plants were cultured on plates. For cultivation of plants on agar-solidified medium (Okada and Shimura 1992), seeds were surface sterilized as described previously (Tsukaya et al. 1991) and were washed extensively with sterilized water. The plates were incubated vertically, for observation of the growth of roots and hypocotyls, under the conditions described above. Fully extended shoots at each position (type 2 metamers; Schultz and Haughn 1991) were measured after growth under the conditions described above. Fully extended shoots at each position (type 2 metamers; Schultz and Haughn 1991) were measured after growth under the conditions described above.

Genetic analysis

After outcrossing of the rot3-3 mutant with the wild type, F2 families included 221 wild-type and 79 mutant plants (2.80:1), fitting the expected ratio of 3:1 for monogenic segregation of the recessive rot3-3 mutation (χ² = 0.284; P = 0.60). From among the F2 progeny plants with a mutant phenotype, 79 mutants were tested for linkage between the T-DNA insertion and the rot3-3 mutation. Linkage was assessed by Southern blot analysis with the right border fragment of the T-DNA as probe. In the course of these studies, two T-DNAs were identified in the rot3-3 line. One of the T-DNAs was inserted in the genomes of all lines examined that were homozygous for the rot3-3 mutation, indi-
cating a tight linkage between the insertion of the T-DNA and the rot3-3 mutation (±0.5 cm at the ROT3 locus).

Anatomy of leaves
For anatomical analysis, samples were embedded in Technovit 7100 resin (Kulzer & Co. GmbH, Wehrheim, Germany) and examined as described previously (Tsukaya et al. 1993). For transverse sections, samples were cut at the center of the fifth rosette leaf blade. Longitudinal sections were prepared by cutting samples along the midrib of the leaf blade (Tsuge et al. 1996). For observations of epidermal cells, samples of petals and leaves were fixed in FAA solution [5% (vol/vol) acetic acid, 45% (vol/vol) ethanol, and 5% (vol/vol) formaldehyde] under a vacuum and rendered transparent by incubation overnight in a solution of chloral hydrate (200 grams of chloral hydrate, 20 grams of glycerol, 50 ml of H2O) as described by Tsuge et al. (1996). Then samples of leaves were stained with a 0.1% (wt/vol) solution of toluidine blue in 0.1 M sodium phosphate buffer (pH 7.0) for 2 min. Samples were photographed under bright-field illumination to obtain paradermal images of the layers of leaf cells. The average area of an epidermal cell was determined by measuring the total area of epidermal cells on a photograph and then dividing this area by the number of epidermal cells in the photograph.

Molecular biological analysis
Routine procedures, including isolation of DNA, Southern blot analysis, and sequencing, were performed essentially as described elsewhere (Sambrook et al. 1989). For standard-stringency (high-stringency) Southern blot analysis, hybridization was carried out in 5× SSC, 0.5% SDS, 5× Denhardt’s solution, and 100 μg/ml denatured salmon sperm DNA at 65°C, with final washing in 1× SSC and 0.1% SDS at 65°C twice for 10 min. For reduced-stringency Southern blot analysis, hybridization was performed in the same solution as described above at 50°C, with final washing in 1× SSC and 0.1% SDS at 50°C twice for 15 min.

For the plasmid rescue experiment, genomic DNA was isolated from the rot3-3 line, digested to completion with either EcoRI or SalI, self-ligated by T4 DNA ligase (Toyobo, Tokyo, Japan), and used to transform E. coli DH5α cells as described previously (Feldmann 1991). After selection for ampicillin-resistant colonies, plasmid DNA was prepared and analyzed by restriction mapping and Southern hybridization to identify rescued plasmids that contained Arabidopsis genomic DNA. The fragment that included the T-DNA—plant DNA junction (flanked by BamHI and EcoRI sites in Fig. 3A) was subcloned into pBluescript SK− (Stratagene, La Jolla, CA) and sequenced.

The complete genomic DNA sequence of the ROT3 gene (Col ecotype) contig has been registered in GenBank (ESSA 1 AP2 contig fragment no. 2, accession no. Z99708) as part of the Arabidopsis Genome Project. Simultaneously, the ROT3 gene was identified in an ap2 contig from chromosome IV. The deduced amino acid sequence encoded by ROT3 in the Col ecotype was identical to that in the WS ecotype except for a Lys(55) to Phe missense alteration in the
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