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]

Received April 13, 1998; revised version accepted June 2, 1998.

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 Zeevaart 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 (McQueen-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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The morphology of leaves of *Arabidopsis thaliana* (L.) Heynh. is regulated by the extent and orientation of the division and elongation of cells (Pyke et al. 1991; Tsukaya et al. 1994; Tsukaya 1995, 1998). Mutations have been identified that affect the development of leaves of *A. thaliana*. These mutations define genes that influence the polar elongation of cells [e.g., *ANGUSTIFOLIA* (*AN*) and *ROT3;* Tsuge et al. 1996], genes that affect both the division and elongation of cells [e.g., *CURLY LEAF* (*CLF*); Kim et al. 1998], and genes that affect the proper arrangement of cells [e.g., *ASYMMETRIC LEAVES* (*AS1*); Tsukaya and Uchimiya 1997]. Mutations in the *ROTUNDIFOLIA3* (*ROT3*) gene are unique because they alter the polarized elongation of leaf cells. Anatomical analysis of a *rot3* mutant showed that the size of leaf cells was reduced specifically in the leaf-length direction (Tsuge et al. 1996). Therefore, it was suggested that the *ROT3* product might be involved in polarized processes of leaf cell elongation.

In this study, in an effort to define molecular mechanisms that control the polar elongation of cells, we performed molecular genetic analysis of the *ROT3* gene and characterized its role in plant development. We isolated two additional *rot3* alleles with mutations that were as-

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, CYP90C1, 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 *A. thaliana* lines that harbored T-DNA insertions as a result of *Agrobacterium tumefaciens*-mediated transformation of seeds (Feldmann 1991).

Each mutant was recognized initially as a putative *rot3* mutant because it exhibited two characteristic features of the *rot3* phenotype: short petioles and round leaves (Fig. 1A–D). The analysis of F_1 and F_2 progeny derived from crosses of these mutants with wild-type plants demonstrated that the defect in each line was inherited as a recessive mutation (data not shown). For tests of allelism, we used the kanamycin resistance of the *rot3-3* allele as a genetic marker. Each pairwise combination of the three *rot3* mutant alleles failed to generate F_1 plants with petioles of normal length and normal leaf blades, demonstrating that each had a *rot3* allele (Fig. 1E–G). We designated the newly isolated mutant alleles as *rot3-2* and *rot3-3*, respectively.

We characterized the *rot3* mutants in terms of the morphology of leaves, stems, hypocotyls, and roots. The *rot3-2* mutant differed from the others in terms of morphology. The average length of the hypocotyl and primary root of the *rot3-2* mutant 9 days after sowing did not differ from those of the wild type (Table 1; Fig. 2K), as was true also for the *rot3-1* mutant (Tsuge et al. 1996). However, cotyledons of the *rot3-2* mutant were slightly larger than those of the wild type (Table 1), whereas the *rot3-1* mutant had normal cotyledons (Tsuge et al. 1996). The lengths of all the true leaves (foliage leaves) of the *rot3-1* and *rot3-3* mutants were shorter than those of the wild type (Fig. 1A,B,D). However, leaves of the *rot3-2* mutant were longer than those of the wild type (Fig. 2A,B). The width of the true leaves of the *rot3-1* and *rot3-3* mutants were similar to those of the wild type. However, leaves of the *rot3-2* mutant were wider than those of the wild type, the *rot3-1*, and *rot3-3* mutants (Table 1; Fig. 1A–D). Petioles of all *rot3* mutant plants were shorter than those of the wild type (Table 1; 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 F_1 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

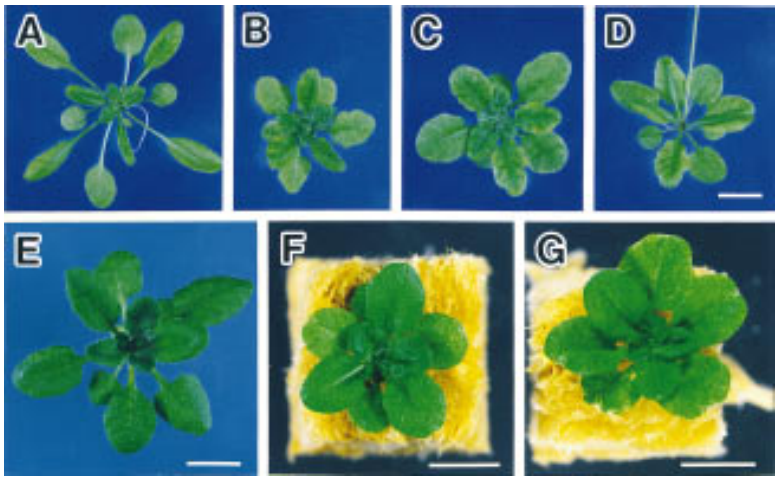

Figure 1. Morphology of wild-type and *rot3* mutant plants. (*A–D*) Photographs of wild-type (*A*), *rot3-1* (*B*), *rot3-2* (*C*), and *rot3-3* (*D*) plants are shown. (*E–G*) The morphology of heterozygotes from the following crosses: *rot3-1* × *rot3-2* (*E*), *rot3-1* × *rot3-3* (*F*), and *rot3-2*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

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

Organ[a]	Wild type (Col) (mm)	*rot3-2* (mm)
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.

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-width 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 abaxial and the adaxial 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 (see Table 1) was attributed to a defect in cell elongation (see Table 2). Thus, the phenotype of the *rot3-2* mutant was similar to that of the *rot3-1* mutant as far as the mutated phenotypes were attributable to changes in cell elongation only. However, the *rot3-2* mutant showed evidence of the accelerated elongation of cells, suggesting that the *rot3-2* mutation might represent a loss of proper *ROT3* function.

Similar results were obtained for floral stems of the *rot3-2* mutant. The length of internodes of floral stems 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 F_2 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 F_2 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 *rot3-3* 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 *Dra*I–*Eco*RI 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.

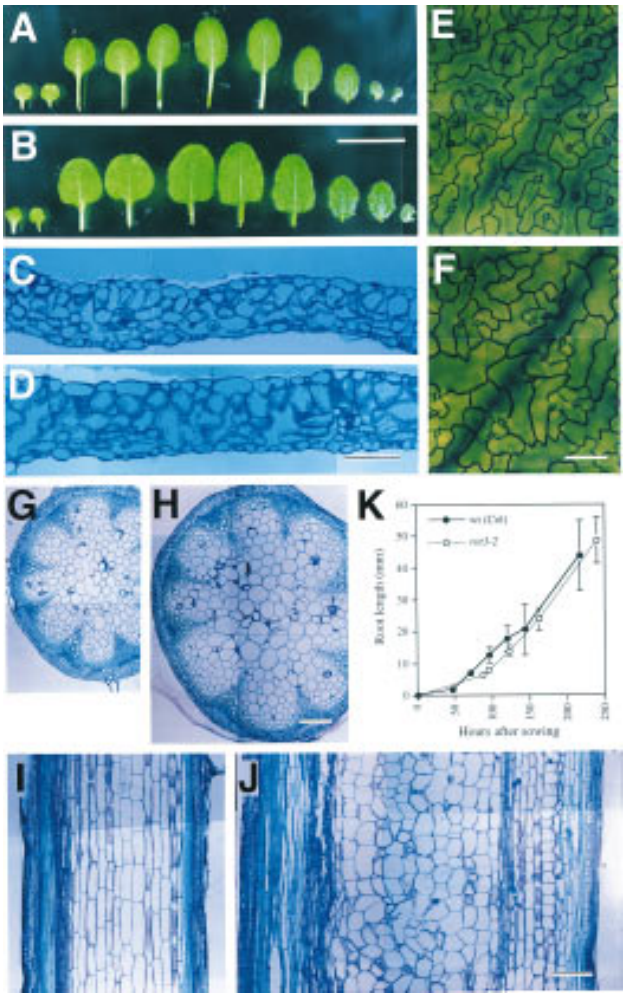

Figure 2. Phenotypes of wild-type and *rot3-2* mutant plants. (*A*,*B*) Leaves of the wild type (*A*) and the *rot3-2* mutant (*B*). The leaves in each row are, from the *left*, the two cotyledons and the rosette leaves from 3-week-old plants. (*C*,*D*) Transverse sections of the fifth rosette leaves of wild-type (*B*) and *rot3-2* (*C*) plants at the stage when leaves were fully expanded. (*E*,*F*) Epidermal cells on the adaxial surface of a wild-type (*E*) and a *rot3-2* (*F*) leaf that was collected when fully expanded. Bar, 100 µm. (*G–J*) Transverse sections of internodes of wild-type (*G*) and *rot3-2* (*H*) plants, and longitudinal sections of internodes of wild-type (*I*) and *rot3-2* (*J*) plants. The sections were prepared from mature plants. Bars, 200 µm. (*K*) Growth of roots of wild-type and *rot3-2* plants. (See text for details.)

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 *Dra*I–*Hin*dIII 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 kD (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 monooxygenase (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%; GA_{12} 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*

	Cell length (μm)	
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 brade 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).

To examine the organ specificity of the expression of *ROT3,* we performed RT–PCR with total RNA from various tissues (suspension culture of cells, roots, 7-day-old seedlings, cotyledons, rosette leaves, stems, and floral buds) of wild-type *Arabidopsis* plants (ecotype Columbia). Two *ROT3*-specific primers for PCR, pROT3-1 and pROT3-2, were designed to amplify a fragment of *ROT3* cDNA. To quantify the *ROT3* transcript, we performed amplification by PCR with increasing numbers of cycles (see Materials and Methods) and then transferred the products to a nylon membrane for Southern hybridization analysis after gel electrophoresis. A single RNA of 0.28 kb was detected in all the tissues examined (Fig. 6B). High level expression was detected in suspension culture of cells, roots, 7-day-old seedlings, and floral buds. Amplification of β-tubulin4 (TUB4; Marks et al. 1987) of transcribed 5′ noncoding sequence (0.35 kb) with two primers, TUB4-sense and TUB4-antisense, was performed as a positive control of RT–PCR (Fig. 6B). This result suggests that *ROT3* is expressed in all major organs, especially in tissues that have high activity of cell division, and that the gene product might play a general role in cell elongation throughout the plant.

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.

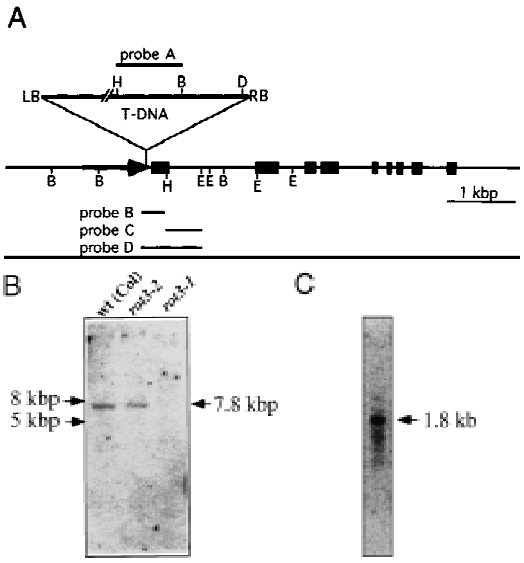

Figure 3. Physical map of the *ROT3* locus and transcription of the wild-type gene. (*A*) Schematic map of the T-DNA-tagged *rot3* gene. An arrow indicates the promoter sequence of the *ROT3* gene. Recognition sites for restriction endonucleases *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H) are shown on the map. (*B*) Southern genomic DNA hybridization analysis of wild-type (wt) and *rot3* alleles. Genomic DNA was digested with *Eco*RI and fragments after electrophoresis were allowed to hybridize with probe D shown in *A*. The hybridization pattern of *rot3-1* indicates a deletion in the promoter and the first exon–intron region of the *ROT3* locus. (*C*) RNA blot hybridization analysis of the wild-type plant 4 weeks after sowing. Hybridization of 1.5 μg of poly(A)$^+$ RNA with probe B shown in *A*. The result indicated that the transcribed RNA was 1.8 kb long. The membrane was exposed to an imaging plate for 1 week, and the band was visualized by an image analyzer (BAS1000; Fujix, Tokyo, Japan).

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 foliage 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

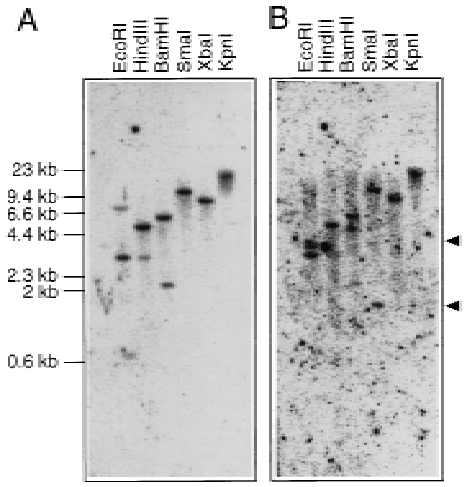

Figure 4. Southern genomic DNA hybridization analysis of the *ROT3* gene. (*A*,*B*) Two blots with similar sets of restriction fragments of genomic DNA were allowed to hybridize with ^{32}P-labeled *ROT3* cDNA with high-stringency conditions (*A*) and with low-stringency conditions (*B*) for hybridization and washing (see Materials and Methods for details). Note the presence of additional bands (*B*, arrowhead) after hybridization and washing under low-stringency conditions.

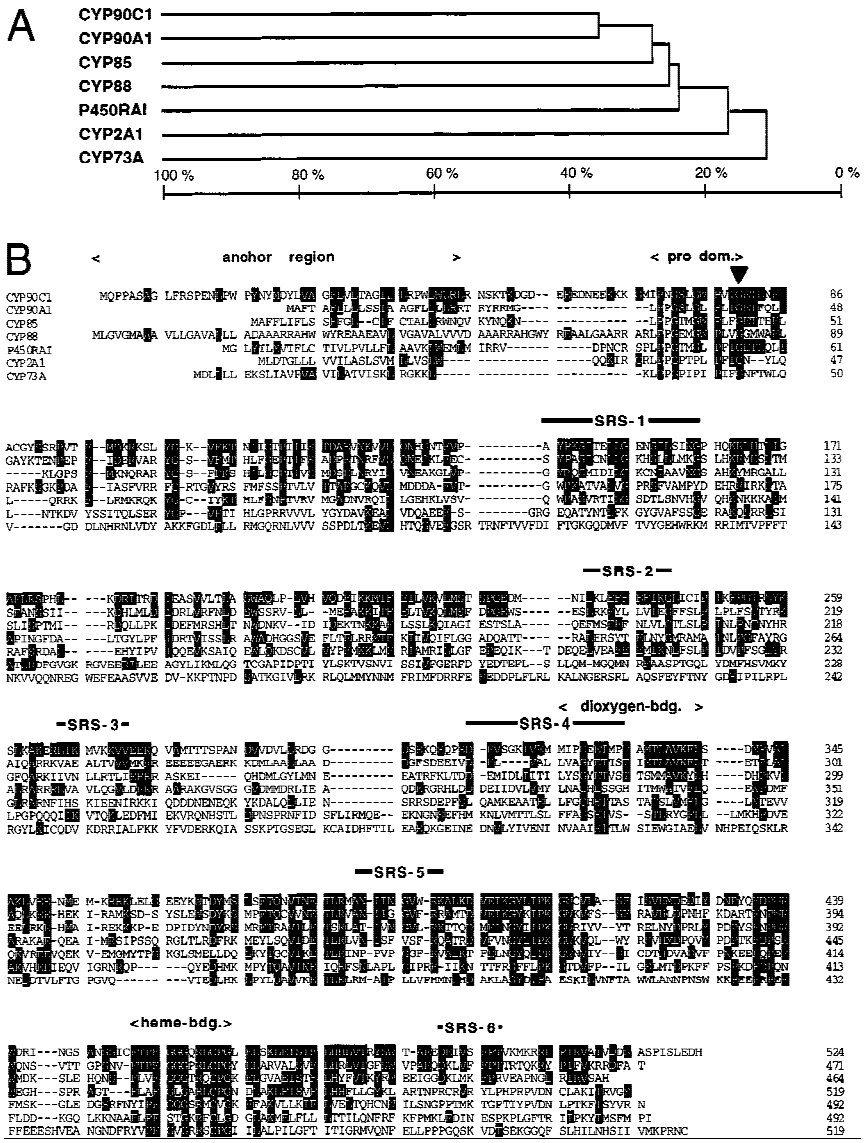

Figure 5. Phylogenetic tree (*A*) and alignment of amino acid sequences of CYP90C1 and other cytochrome P-450s from plants and animals (*B*). CYP90C1 exhibits strong homology to CYP90A1 (36%; cathasterone C23 hydroxylase of *A. thaliana*), CYP85 (27%; DWARF protein of tomato), CYP88 (25%; GA_{12}-GA_{53} gibberellin 13-hydroxylase of maize), and P-450-RAI (23%; retinoic acid 4-hydroxlase of zebrafish). CYP2A1 (rat hepatic steroid hydroxylase) is also homologous to CYP90C1 in the SRSs (SRS-1–SRS-6), indicated above the aligned sequences. The mutation site of ROT3-2 (Gly-80 to Glu) is marked by an inverted black triangle in the conserved proline-rich region (pro dom.). The locations of conserved domains of microsomal P-450s, including the membrane anchor region, the proline-rich domain, and the O_2-, 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 CYP90C1.

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 *rot3-2* phenotype might represent an altered function caused by mutation in the CYP90C1 protein.

The ROT3 *gene encodes a new member of the family of cytochrome P-450s with a steroid substrate recognition site*

The molecular cloning of the *ROT3* gene was confirmed by genetic analysis and by analysis of sequences of three mutant *rot3* alleles. The nucleotide sequence of the *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 *ROT3* gene and the gene for CYP90A1, which is involved in brassinosteroid biosynthesis (Szekeres et al. 1996), suggest that the *ROT3* gene might encode a cytochrome P-450 steroid hydroxylase. In addition, the absence in CYP90C1 of the consensus sequence PFGG(ASV)GRRC(PAV)G around the heme-binding cysteine (Durst and Nelson 1995) suggests that CYP90C1 is more similar to animal, lower eukaryotic, and bacterial proteins. *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 *ROT3* gene in cell elongation during plant development? The putative steroid-binding domains in the ROT3 protein suggest that the protein might be involved in the biosyn-

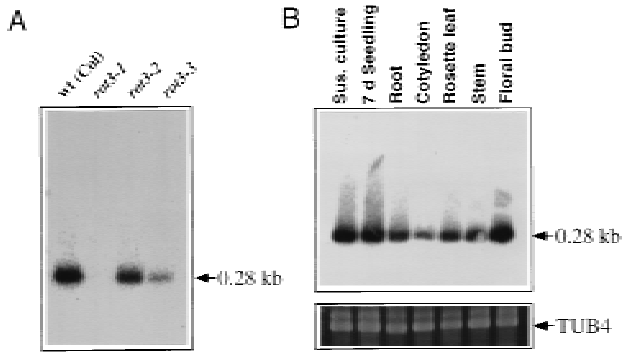

Figure 6. Amplification by RT–PCR of *ROT3* mRNA with primers pROT3-1 and pROT3-2. Total RNA was used for RT–PCR. Amplified DNAs were allowed to hybridizae with ^{32}P-labeled *ROT3* cDNA with high-stringency conditions for hybridization and washing. Arrows indicate 0.28-kb products of PCR. (*A*) Expression of wild-type and *rot3* alleles. Total RNA was isolated from 4-week-old leaves. One microgram of total RNA was used in each case. (*B*) Expression of the mRNA in various tissues of *Arabidopsis*. Total RNA was isolated from suspension cultured cells, 7-day-old seedling, root, cotyledon, rosette leaf, stem, and floral bud. Two micrograms of total RNA was used in each case. Result for amplification by RT–PCR of β-tubulin4 (TUB4) fragments was shown for standard controls (*B; bottom*).

thesis of brassinosteroids, as brassinosteroids are the only steroids that have been shown to influence plant development. However, it is also important to note that the hypocotyls of dark-grown *rot3* seedlings elongate and the cotyledons do not open (data not shown). It suggests that dark-grown *rot3* seedlings show normal skotomorphogenesis. Moreover, none of the *rot3* mutants exhibited dwarfism (Table 1; Tsuge et al. 1996) and in none was the wild-type phenotype induced by the application of exogenous brassinosteroid (data not shown). Because all reported mutants with defects in the synthesis of brassinosteroids or in related signaling pathways, such as *dim*, *det*, and *cpd* mutants (Chory et al. 1991; Takahashi et al. 1995; Kauschmann et al. 1996; Li et al. 1996; Szekeres et al. 1996), do not show skotomorphogenesis under dark conditions and exhibit dwarfism under light conditions, it seems unlikely that the *ROT3* gene is involved in the biosynthesis of brassinosteroids.

The expansion of plant cells is known to be influenced by the action of other plant hormones. Mutants with altered sensitivity to auxin also display altered phenotypes with respect to cell elongation (Maher and Martindale 1980; Estelle and Somerville 1987; Timpte et al. 1992). Abscisic acid (ABA) and ethylene also alter the direction of plant cell elongation. It is unlikely, however, that the signaling pathways for ABA or ethylene are altered in the *rot3* mutants because the mutants lack features expected of such ABA- or ethylene-related mutants. Moreover, treatment of *rot3* mutants with gibberellic acids or auxin did not result in a normal wild-type phenotype (data not shown). Thus, the product of the *ROT3* gene might be involved in processes independent of known hormones in the plant. The product of the *ROT3* gene, expressed in dividing cells, might catalyze synthesis of some factors with a novel effect on cell elongation in leaves.

Further genetic and biochemical studies of the *rot3* mutants and functional analysis of the CYP90C1 protein should resolve some of these issues and provide further insight into basic aspects of cell elongation and its regulation during plant development.

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 all 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.

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 MS0 medium (Tsukaya et al. 1991). The morphology of leaves and petioles was observed 10 days after germination.

Genetic analysis

After outcrossing of the *rot3-3* mutant with the wild type, F_2 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 ($\chi^2 = 0.284$; $P = 0.05$). From among the F_2 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 petioles 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 H_2O) 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 0.1× 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 *Eco*RI or *Sal*I, 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 *Bam*HI and *Eco*RI sites in Fig. 3A) was subcloned into pBluscript SK^- (Stratagene, La Jolla, CA) and sequenced.

DNA sequence analyses and database searches were performed at the Japan National Genetic Institute for Genome Information with the BLAST program (Altschul and Lipman 1990) and multiple sequence alignments were made with the MacDNAsis software (Hitachi, Tokyo, Japan). The *Arabidopsis* EST clone N96214 (Newman et al. 1994) was obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH), and it was fully sequenced using primers derived from the previously determined DNA sequence. The complete genomic DNA sequence of the *ROT3* gene (Col ecotype) contig has been registered in GenBank (ESSA I 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^{45} to Phe missense alteration in the WS ecotype. The cDNA sequence of *ROT3* has been registered in DDBJ (accession no. AB008097).

From 1 to 2 mg of total RNA were treated with DNase I at 37°C for 60 min before use for RT–PCR to avoid amplification of possible contaminating genomic DNA. The conditions for direct amplification by RT–PCR were 1 cycle at 50°C for 30 min and 94°C for 2 min; and then 10, 15, 20, 25, or 30 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min 30 sec with a One Step RT–PCR kit (TaKaRa, Tokyo, Japan). The following oligonucleotides were used as the primers for RT–PCR: pROT3-1, 5′-CGGCAAGCGCAGGACTTTTC-3′; pROT3-2, 5′-GAAGGTAACAGGCCGAGAAG-3′; Sense1, 5′-CGCGGATCCAGTTATTCCCAAGAAACCGG-3′; and Antisense1, 5′-CGCGGATCCCTTCTCTGCTTCCTCTTTGC-3′. Amplification of β-TUB4 of transcribed 5′ noncoding sequence (Marks et al. 1987) with two primers: TUB4-sense, 5′-AGAGGTTGACGAGCAGATGA-3′, and TUB4-antisense, 5′-CCTCTTCTTCCTCCTCGTAC-3′, was performed and the product was used as a positive control of RT–PCR. To test whether the RT–PCR method would allow us to detect *ROT3* mRNA, we isolated total RNA from the rosette leaves plus stems of the *rot3* mutants and the wild type. After DNase I treatment, this RNA was subjected to RT–PCR. A product of 0.28 kb was only detected in the case of the wild type, whereas no product was detected in samples from the *rot3-1* deletion allele. This result confirmed that RT–PCR allowed detection of the rare *ROT3* transcript. The analysis of levels of expression in seven different tissues, namely suspension culture cells, roots, 7-day-old seedlings, cotyledons, rosette leaves, stems, and floral buds from the Col wild type, was performed by RT–PCR. Then, Southern blot analysis (with *ROT3* cDNA as probe) was performed under the conditions described above. Sequence analysis of the *rot3-2* allele was performed by RT–PCR with Sense1 and Antisense1 primers as described above and sequencing of three independent clones.

Acknowledgments

We thank Dr. Y. Komeda (Hokkaido University, Sapporo, Japan) for supplying the mutant seeds from which the *rot3-2* mutant was derived; Dr. T. Casper (DuPont Corporation, Wilmington, DE) for supplying the population of seeds of *A. thaliana* with T-DNA insertions; all workers, as well as the research community, at Arabidopsis Biological Resource Center (ABRC; Columbus, OH) for providing cDNAs; Dr. T. Ueda (University of Tokyo, Japan) for providing total RNA of various tissues; and Dr. M. Hayashi (Institute of Physical and Chemical Research, Wako, Japan) for providing brassinolide. We also thank Drs. T. Tsuge and M. Umeda (University of Tokyo, Japan) for their helpful comments. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, and Culture, Japan (to H.T.). G.-T.K. was supported by a fellowship for Japanese junior scientists from the Japan Society for the Promotion of Science.

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