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Changes in the shapes of leaves and flowers upon overexpression of cytochrome P450 in *Arabidopsis*

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ABSTRACT In *Arabidopsis*, the two-dimensional expansion of leaves is regulated via the polarized elongation of cells. The ROTUNDIFOLIA3 (ROT3) protein, a member of the family of cytochromes P450, is involved in this process and regulates leaf length. Transgenic plants that overexpressed a wild-type *ROT3* gene had longer leaves than parent plants, without any changes in leaf width. The shapes of floral organs were also altered, but elongation of the stem, roots, and hypocotyls was unaffected. To our knowledge, no similar specific regulation of leaf length has been reported previously. Transgenic plants overexpressing the *rot3-2* gene had enlarged leaf blades but leaf petioles of normal length. Morphological alterations in such transgenic plants were associated with changes in shape of leaf cells. The *ROT3* gene seems to play an important role in the polar elongation of leafy organs and should be a useful tool for the biodesign of plant organs.

Leaves are important for the production of plant biomass. They are also morphologically diverse (1) and are the basis for floral organs, such as petals. Appropriate molecular tools for the biodesign of shapes of leaves and/or floral organs are necessary for improvement of crops and other economically important plants. For the biodesign of leaf shape, we need to understand the genetic mechanisms that control leaf morphogenesis. Because floral organs are derived from leaves, an understanding of the genetic control of leaf shape should also be useful for the biodesign of floral organs. However, leaf morphogenesis is still poorly understood, particularly in dicotyledonous plants, because the development of leaves is a complex phenomenon (2, 3). Studies of the developmental genetics of leaf morphogenesis have recently identified several important steps in leaf morphogenesis (4–8). We showed previously that the two-dimensional expansion of leaves is regulated via the polarized elongation of cells in *Arabidopsis thaliana* (4–6).

The *rot3-1* null mutant of *Arabidopsis thaliana* (L.) Heynh. has been identified as a mutant with a specific defect in the polar elongation of leaf cells, without any abnormalities in internodes and roots (5). The length of the *rot3-1* leaves is shorter than those of wild type, whereas the width of the *rot3-1* leaves is normal (5). Molecular cloning of the *ROT3* gene indicated that it encodes a cytochrome P450, CYP90C1, which might be involved in steroid biosynthesis (6). Although the *ROT3* transcript is ubiquitous, the function of the gene seems to be specific to the polar elongation of leaf cells (6), and it seems likely that the *ROT3* gene might be a key factor in leaf growth and development. To test this hypothesis, we constructed transgenic *Arabidopsis* by introducing a wild-type version or a *rot3-2* allele (6) of the *ROT3* gene into *Arabidopsis* with the null mutation *rot3-1*. The phenotypes of transgenic plants showed clearly that the *ROT3* gene plays an important role in the polar elongation of leafy organs and is a good tool for the biodesign of plant organs. Moreover, we should be able to manipulate leaf shape at will by using the wild-type and/or *rot3-2* allele of the *ROT3* gene in *Arabidopsis.*

MATERIALS AND METHODS

Construction and Culture of Transgenic *Arabidopsis*. We amplified a 1.9-kbp fragment of a sequence that started 8 bp upstream of the ATG triplet of the *ROT3* gene (6) by PCR with the oligonucleotides ROT3PRO-1 (5′- CCCAAGCTTGGGCTCCAATCACATGTCGTAGG-3′) and ROT3PRO-2 (5′-CCCAAGCTTGGGCAGTTAAACCGGTTTCTTGGG-3′), which introduced a *Hin*dIII restriction site at each end. PCR was performed with an Extra Long PCR kit (PE Applied Biosystems, Norwalk, CT) with genomic DNA from the Columbia ecotype of *Arabidopsis* as a template. Amplified fragments were sequenced and ligated into the *Hin*dIII site of the promoterless β-glucuronidase (GUS) expression vector pHTS18, which was derived from pBI101.2 (H.T., unpublished work). Thus, we constructed a *ROT3P*::*GUS* chimeric gene. Lines of transgenic plants (ecotype Columbia) harboring this construct were established by *Agrobacterium*-mediated transformation, which was performed by a simplified *in planta* infiltration method without application of a vacuum (9). Transgenic lines were selected in the presence of 10 mg/liter hygromycin. Histochemical analysis was performed on 2-week-old T2 transgenic *Arabidopsis* plants that had been grown on MS medium (10) as described (11).

The coding sequence of *ROT3* or of a *rot3-2* allele ($ROT3^{G80E}$; ref. 6) in pBluescript SK(−) (Stratagene) was introduced into the vector pTA7002 (12), such that transcription was controlled by a cis-activating element that responded to glucocorticoids. These constructs were introduced into *Arabidopsis* (ecotype Columbia) plants that were homozygous for the *rot3-1* null mutation (5) by a modified version of a previously published method for the *Agrobacterium*-mediated transformation of roots (13). Transgenic lines were selected on MS medium that contained hygromycin and made homozygous for the transgene. Heterozygous *InducibleP*::$ROT3^{G80E}$ transgenic plants were generated by back-crossing a line that was homozygous for the transgene with wild-type Columbia.

Plants were grown in controlled-environment rooms with continuous light as described (5, 11). Dexamethasone (Sigma), as a 10 μM solution, was applied either by spraying every second day or by addition to the growth medium. Leaves were measured 24 days after the start of treatment with dexamethasone.

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Abbreviation: RT-PCR, reverse transcription–PCR.

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Morphological and Molecular Analysis. For observations of cell shape, leaves were cleared and viewed with Nomarski optics as described (5).

Fully extended shoots at each position (type 2 metamers) were also measured (6). For cultivation of plants on agar-solidified medium (14), seeds were surface-sterilized as described (11) and incubated on vertical plates for observation of the growth of roots and hypocotyls, under the conditions described above (5, 11).

For reverse transcription–PCR (RT-PCR) analysis, total RNAs from wild-type and transgenic leaves of 2-week-old seedlings were purified. The following oligonucleotides were used as the primers for RT-PCR: sense 2 (5′-TCTCGGCCTGTTACCTTC-3′) and antisense 2 (5′-GTCTTCCTCAGCCGTCCA-3′). The conditions for direct amplification by RT-PCR have been described (6).

RESULTS

To examine the tissue specificity of expression of the *ROT3* transcript, we analyzed transgenic *Arabidopsis* that carried the chimeric gene *ROT3P*::*GUS* histochemically. Preliminary observation on more than 20 independent transgenic lines showed a similar pattern of GUS expression. Thus, we chose four independent lines for further analysis. As shown in Fig. 1 *A–C*, we observed the non-organ-specific expression of the GUS reporter gene in leaves, stems, floral organs, and roots of all four transgenic lines examined. This result was consistent with those of previous studies of the quantitative analysis of levels of transcripts (6). Furthermore, in leaves, histochemical analysis indicated that the *ROT3* promoter was expressed more strongly in distal, mature regions than in proximal, immature regions (Fig. 1 *B* and *D*). Moreover, as shown in Fig. 1*E*, expression of the fusion gene was ubiquitous in all cell layers, including epidermis, palisade tissue, and the spongy layer.

To regulate the level of the *ROT3* transcript, we constructed a gene from *ROT3* cDNA that was driven by a glucocorticoid-inducible promoter (12), and we generated transgenic plants. As shown in Fig. 2*A* and Table 1, five independently raised lines had dramatic changes in leaf shape. The level of expression of the wild-type *ROT3* transgene before induction was nearly the same with the native expression level of the *ROT3* gene (Fig. 3) and sufficient for morphological transformation (Table 1). Induction of the wild-type *ROT3* transgene by dexamethasone caused even more dramatic changes in leaf shape (Table 1). Treatment with dexamethasone had no effect on wild-type and *rot3-1* mutant plants. We confirmed a correlation between the level of expression of transgenes by RT-PCR (Fig. 3) and alterations in leaf shape (Fig. 2). Transgenic *rot3-1* plants (expressing the wild-type *ROT3* gene) had longer leaves than the *rot3-1* mutant. The increases in length of leaf blades and leaf petioles ranged from 1.2- to 1.6-fold and 2.2- to 3-fold, respectively (Table 1). Ectopic overexpression of the *ROT3* gene affected polar elongation of leaf petioles more significantly than that of leaf blades (Table 1). There were no significant changes in leaf width in transgenic plants (Table 1), supporting the hypothesis that the *ROT3* gene controls elongation of leaves in the leaf-length direction specifically. Effects of the *ROT3* gene were also evident at the level of cell morphology (Fig. 2 *B–D*). Palisade cells in leaves of transgenic plants were fully elongated, with specific polarity and decreased airspace (Fig. 2*D*). Thus, the cell-elongation processes regulated by *ROT3* might be different in leaf blades and leaf petioles.

Ectopic overexpression of *ROT3* also enhanced the longitudinal elongation of floral organs (Fig. 2*G*), whereas petals, sepals, stamens, and pistils were all of reduced length in the null-type *rot3-1* mutant (Fig. 2*F*; ref. 5). Elongation of internodes and hypocotyls was unaffected by induction of the transgene (Table 2), supporting the hypothesis that the *ROT3* gene is involved specifically in the elongation of leaves and leaf-based organs.

The *rot3-2* allele causes enlarged leaf blades with short petioles and thick internodes (6). Results of a previous study

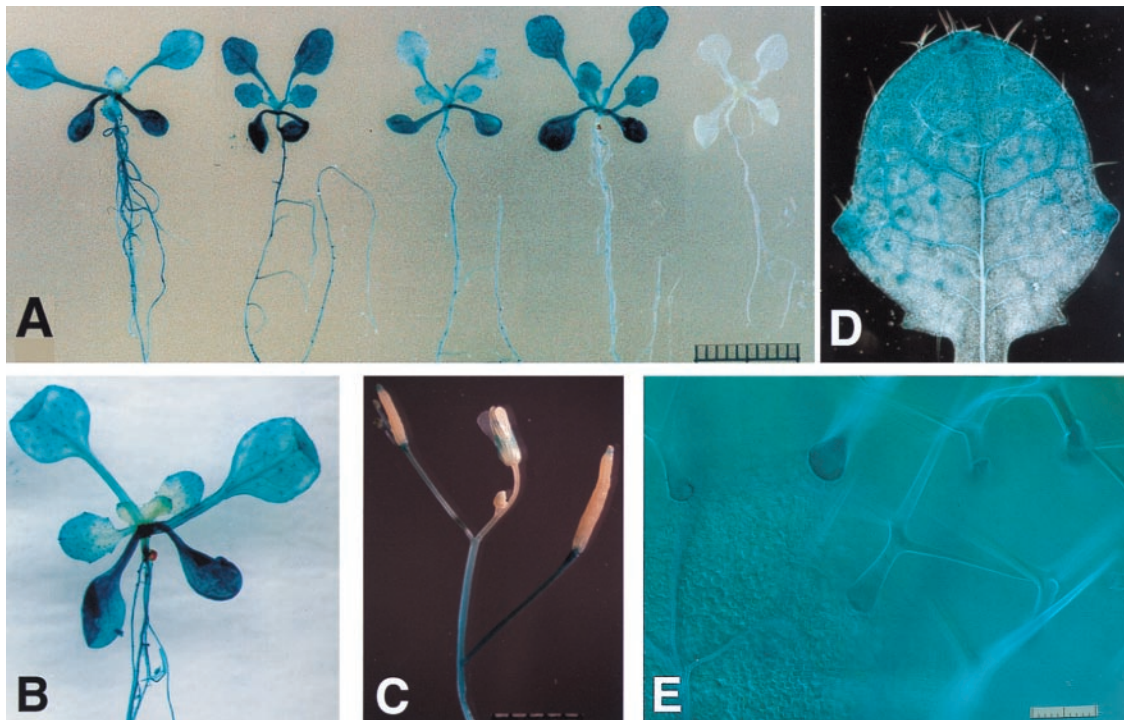

FIG. 1. Expression pattern of GUS encoded by the *ROT3P*::*GUS* fusion gene. (*A*) Histochemical staining for GUS activity in four independent transgenic plants (left four) and a nontransgenic plant (right). (Bar = 1 cm.) (*B–D*) Magnified views of parts of a transgenic plant that carried the *ROT3P*::*GUS* fusion gene. A young rosette (*B*), inflorescence (*C*), and young leaf (*D*) are shown. (*E*) A mature leaf stained for GUS activity shows ubiquitous expression of the *ROT3P*::*GUS* fusion gene (Nomarski optics). (Bar = 100 μm.)

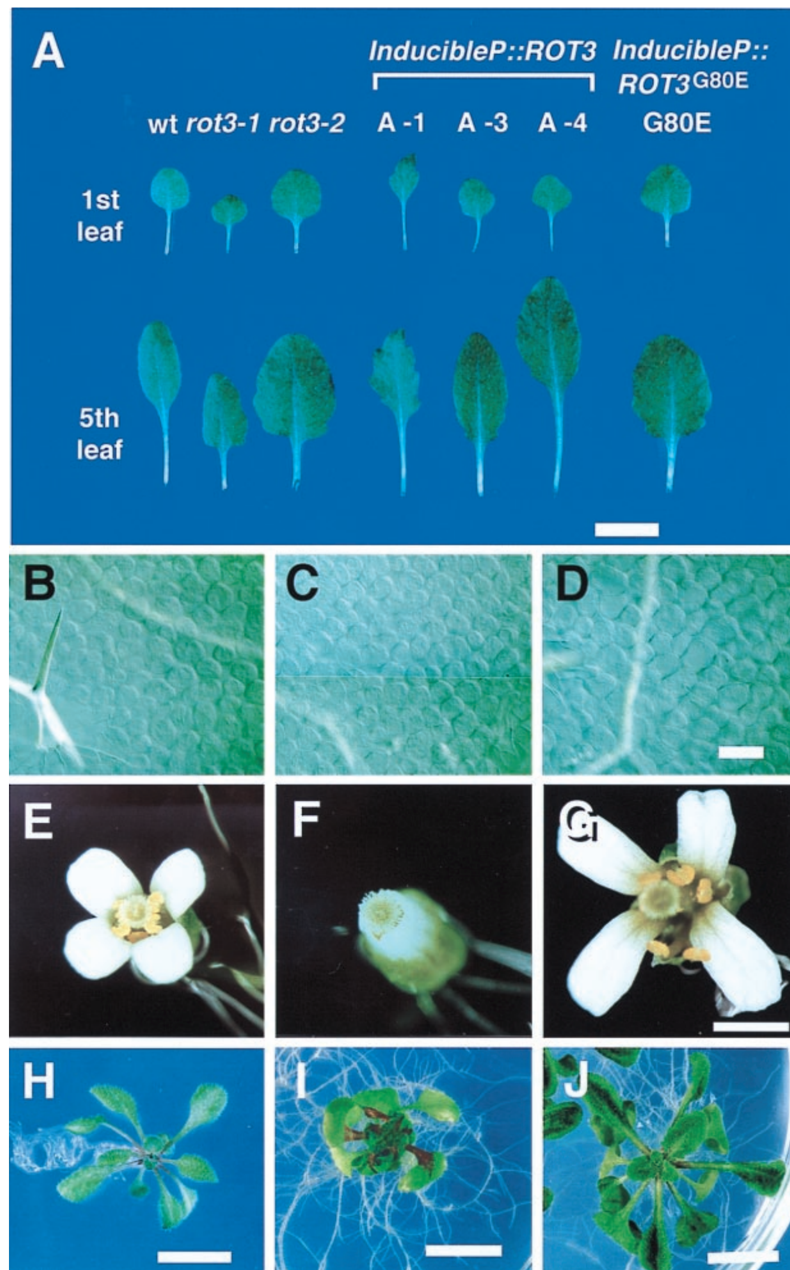

FIG. 2. Phenotypes of wild-type (wt) plants, plants with one of two *rot3* alleles, and transgenic *rot3-1* plants that overexpressed *ROT3*. (*A*) The leaves in each row were taken from the following plants: (from left) wt, *rot3-1*, *rot3-2*, transgenic plants that overexpressed the wild-type *ROT3* gene (A-1, A-3, and A-4), and a plant that expressed the *ROT3*^{G80E} gene (G80E). Upper and lower rows show the first leaves and the fifth leaves, respectively. (Bar = 10 mm.) Paradermal images of palisade cells in the fifth leaves of a wt plant (*B*), a *rot3-1* mutant plant (*C*), and an A-3 transgenic plant (*D*) at the fully expanded stage. (Bar = 100 μm.) Flowers of a wt plant (*E*), a *rot3-1* mutant plant (*F*), and an A-1 transgenic plant (*G*). (Bar = 1 mm.) Gross morphology of plants heterozygous for the transgene (*InducibleP*::*ROT3*^{G80E}). Heterozygous 17-day-old plants (*H*) were transferred to medium plus 10 μM dexamethasone (*I*) or to control medium (*J*) and photographed 8 days later (*I* and *J*). (Bars = 10 mm.)

suggested that the *rot3-2* gene (with a point mutation that results in replacement of Gly-80 by Glu in a proline-rich domain) might function differently from the *ROT3* gene in cell morphogenesis (6). When we induced the expression of a *rot3-2* allele, *ROT3*^{G80E}, into a null-type *rot3-1* parent, plants had slightly enlarged leaf blades without longer leaf petioles (Table 1). The leaves from two of three independent lines showed the same phenotype and were indistinguishable from those of *rot3-2* plants (Fig. 2*A*). Moreover, transgenic plants had 1.5-fold thicker internodes that were of normal length, as compared with the wild-type and *rot3-1* plants. In this respect, they resembled *rot3-2* plants (Table 2). Elongation of internodes, primary roots (not shown), and hypocotyls of transgenic plants was unaffected (Table 2). Without induction of the transgene, plants that were heterozygous for the transgene developed wild-type leaves (Fig. 2 *H* and *J*), suggesting that low-level expression of the ROT3^{G80E} function was masked by wild-type ROT3. Dexamethasone induced the accumulation of anthocyanin in leaf petioles and enlarged leaf blades, without elongation of young leaf petioles, in plants heterozygous for the transgene (Fig. 2*I*). Thus, the function of the ROT3^{G80E} protein in plants that were heterozygous for the transgene seemed to be regulated by a balance between the expression of *ROT3* and that of *ROT3*^{G80E}.

DISCUSSION

Cytochromes P450 are involved in several important biochemical pathways, such as the biosynthesis of brassinosteroids,

Table 1. Dimensions of the fifth leaves of wild-type *Arabidopsis*, of *rot3-1* plants, and of transgenic plants that overexpressed the *ROT3* gene

Plant line	Width of leaf blade, mm	Relative width, %	Length of leaf blade, mm	Relative length, %	Length of leaf petiole, mm	Relative length, %	No. of plants examined
wt (Col) (−)	5.6 ± 0.8	87.6	9.2 ± 1.3	135.3	11.4 ± 1.4	314.0	15
wt (Col) (+)	6.2 ± 1.1	97.5	9.9 ± 1.6	144.4	11.4 ± 1.9	313.2	10
rot3-1 (−)	6.4 ± 0.7	100	6.8 ± 0.9	100	3.6 ± 0.5	100	14
rot3-1 (+)	5.6 ± 0.7	88.7	6.6 ± 0.6	96.6	3.5 ± 0.7	96.2	11
A-1 (−)	6.3 ± 0.8	99.5	10.9 ± 1.7	159.4	11.0 ± 1.5	302.2	15
A-1 (+)	5.9 ± 0.9	92.0	11.4 ± 1.4	167.2	10.8 ± 1.8	295.3	10
A-3 (−)	6.6 ± 0.5	103.8	8.4 ± 0.8	123.2	8.6 ± 1.0	236.3	15
A-3 (+)	7.8 ± 1.4	122.6	12.2 ± 2.8	178.9	9.0 ± 2.9	247.3	10
A-4 (−)	7.2 ± 1.3	112.7	9.0 ± 1.4	132.6	8.0 ± 1.6	219.8	13
A-4 (+)	7.6 ± 1.1	119.5	11.0 ± 1.6	160.6	8.8 ± 1.6	240.4	10
G80E (−)	6.7 ± 1.3	104.9	9.0 ± 2.4	131.5	5.4 ± 1.1	147.5	15
G80E (+)	6.8 ± 0.8	106.8	8.1 ± 1.0	118.3	5.2 ± 1.0	143.1	7

Fifth rosette leaves were measured 24 days after sowing. Plants were grown on medium plus 10 μM dexamethasone (+) or on control medium (−). G80E (+) represents transgenic plants that overexpressed the $ROT3^{G80E}$ gene. %, percentage of the value for the fifth leaf of *rot3-1* mutant plants grown without dexamethasone; wt, wild type; Col, Columbia.

flavonoids, and lignin (15).** We found previously that a null-type mutation in the *ROT3* gene, which encodes a cytochrome P450, caused specific defects in leaf length and in the length of floral organs derived from leaves (5, 6). In the present study of transgenic plants that carried an *InducibleP*::*ROT3* chimeric gene, we found that overexpression of the *ROT3* gene in whole plant organs accelerated elongation exclusively of leaves and of floral organs derived from leaves. In addition, our results confirmed that the *ROT3* gene regulates only the length of leaves without affecting their width. Thus, in spite of the apparently ubiquitous pattern of gene expression (Fig. 1), the *ROT3* gene seems to regulate specifically the lengths of leaves and floral organs (6). Judging from the expression pattern of GUS activity, the *ROT3* mRNA seems to express more strongly in the distal region of leaves than in the basal region of leaves. This cline of expression along the longitudinal axis of leaves might be responsible for the *ROT3* control of polar leaf expansion to the longitudinal direction of leaves.

Several factors that control polar elongation are known to affect plant morphogenesis, such as cytoskeletal components (16, 17), wall-loosening proteins (18, 19), and phytohormones (20–23). Recently, genes for biosynthesis or perception of brassinosteroids have been shown to be involved in the elongation of stems and leaves (24, 25). The *ROT3* gene has strong homology to some genes for the biosynthesis of brassinosteroids (6). However, in earlier studies, no adequate evidence has been provided to explain the involvement of such genes in the polarized elongation of cells, particularly in specific organs.

This report describes a method for the control of leaf shape and flower shape by ectopic expression of a wild-type or mutant *ROT3* gene. We confirmed that expression of the wild-type *ROT3* gene accelerated polar elongation of leaves and floral organs specifically in our transgenic system. We also found that the *rot3-2* allele $ROT3^{G80E}$ induced enlarged leaf blades and thicker stems, acting to counterbalance the effect of the wild-type ROT3 protein. Our transgenic system should prove to be a useful tool, not only for investigation of factors that regulate leaf morphogenesis, but also for molecular breeding of leaf shape.

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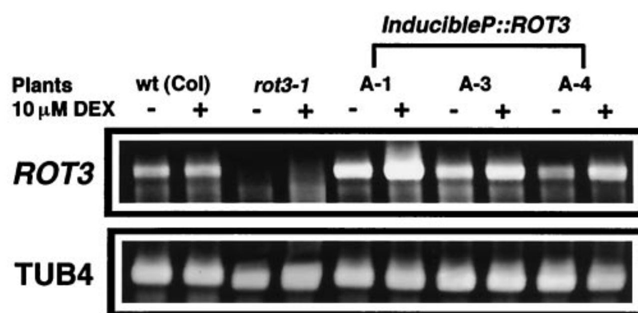

FIG. 3. Amplification by RT-PCR of *ROT3* mRNA. (From left) Nontransgenic wild type (wt), nontransgenic *rot3-1* null mutant (*rot3-1*), and three independent transgenic lines that carry the *InducibleP*::*ROT3* chimeric gene are shown. Plants were cultivated with (+) or without (−) treatment with 10 μM dexamethasone (DEX). Total RNA (1 μg) was amplified for each strain. (*Upper*) The result of amplification by RT-PCR of the *ROT3* cDNA. (*Lower*) The result for amplification by RT-PCR of β-tubulin 4 (TUB4) fragments is shown for standard controls.

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Table 2. Various dimensions of wild-type *Arabidopsis* plants, *rot3* plants, and transgenic plants that overexpressed the *ROT3* gene

	Dimension (mm)		
Plant line	Hypocotyl length, mm (no.)	Internode length, mm (no.)	Internode diameter, mm (no.)
wt (Col) (−)	1.2 ± 0.3 (16)	98.2 ± 16.6 (9)	0.9 ± 0.1 (5)
wt (Col) (+)	1.2 ± 0.4 (13)	107.0 ± 14.1 (15)	0.8 ± 0.1 (9)
rot3-1 (−)	1.2 ± 0.4 (9)	89.7 ± 19.5 (9)	0.9 ± 0.1 (5)
rot3-1 (+)	1.3 ± 0.5 (9)	85.5 ± 16.9 (4)	0.9 ± 0.1 (6)
rot3-2 (+)	—	—	1.2 ± 0.1 (4)
A-1 (−)	1.3 ± 0.4 (13)	90.6 ± 10.5 (5)	0.9 ± 0.0 (9)
A-1 (+)	1.4 ± 0.4 (14)	88.2 ± 10.2 (6)	0.9 ± 0.1 (4)
A-3 (−)	1.3 ± 0.4 (13)	99.2 ± 15.0 (6)	1.0 ± 0.1 (5)
A-3 (+)	1.3 ± 0.6 (7)	94.8 ± 12.6 (8)	1.0 ± 0.0 (6)
A-4 (−)	1.3 ± 0.4 (13)	99.2 ± 15.0 (6)	1.0 ± 0.1 (5)
A-4 (+)	1.3 ± 0.6 (7)	94.8 ± 12.6 (8)	0.9 ± 0.1 (4)
G80E-1 (−)	1.1 ± 0.4 (12)	96.6 ± 6.8 (5)	—
G80E-1 (+)	1.3 ± 0.5 (10)	79.3 ± 11.6 (6)	1.2 ± 0.1 (7)

Plants were grown on medium plus 10 μM dexamethasone (+) or on control medium (−). Hypocotyls and internodes were measured at the mature stage. Total lengths of type 2 metamers of fully extended shoots and diameters of middle parts of first internodes of type 2 metamers were measured. Plants were incubated on vertical plates for observation of the growth of roots and hypocotyls. A-1 (+) to A-4 (+) represent transgenic plants that overexpressed the *ROT3* wild-type gene. G80E (+) represents transgenic plants that overexpressed the $ROT3^{G80E}$ gene. —, not measured.

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