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Review

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Molecular Biodesign of Plant Leaves and Flowers

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Abstract

The morphology of the leaves and the flowers of angiosperms exhibit remarkable diversity. One of the factors showing the greatest variability of leaf organs is the leaf index, namely, the ratio of leaf length to leaf width. In some cases, different varieties of a single species or closely related species can be distinguished by differences in leaf index. To some extent, the leaf index reflects the morphological adaptation of leaves to a particular environment. In addition, the growth of leaf organs is dependent on the extent of the expansion of leaf cells and on cell proliferation in the cellular level. The rates of the division and enlargement of leaf cells at each stage contribute to the final shape of the leaf, and play important roles throughout leaf development. Thus, the control of leaf shape is related to the control of the shape of cells and the size of cells within the leaf. The shape of flower also reflects the shape of leaf, since floral organs are thought to be a derivative of leaf organs. No good tools have been available for studies of the mechanisms that underlie such biodiversity. However, we have recently obtained some information about molecular mechanisms of leaf morphogenesis as a result of studies of leaves of the model plant, *Arabidopsis thaliana*. For example, the *ANGUSTIFOLIA (AN)* gene, a homolog of animal *CtBP* genes, controls leaf width. AN appears to regulate the polar elongation of leaf cells via control of the arrangement of cortical microtubules. By contrast, the *ROTUNDIFOLIA3 (ROT3)* gene controls leaf length via the biosynthesis of steroid(s). We provide here an overview of the biodiversity exhibited by the leaf index of angiosperms. Taken together, we can discuss on the possibility of the control of the shapes and size of plant organs by transgenic approaches with the results from basic researches. For example, transgenic plants that overexpressed a wild-type *ROT3* gene had longer leaves than parent plants, without any changes in leaf width. Thus, The genes for leaf growth and development, such as *ROT3* gene, should be useful tools for the biodesign of plant organs.

Key words: *Arabidopsis*, biodesign, cell division, cell elongation, leaf expansion, leaf index, leaf morphogenesis

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Introduction

The leaf is a major component of the shoot and it is the organ that is the key to a full understanding of not only plant morphogenesis, but also plant biodiversity. The processes of leaf expansion play important roles in the establishment of leaf morphology, as well as in natural productivity as a consequence of the capacity of the leaf for photosynthesis. The specific pattern and morphology of multicellular organs are attributable, in part, to mechanisms that regulate the shape, size, and number of cells in those organs (Steeves and Sussex 1989). Leaf morphogenesis has long been a focus of studies of the correlation between leaf shape and patterns of cell division and elongation (Ashby 1948; Arkebuer and Norman 1995). The rates of division and elongation of cells at each stage contribute to the final shape of the leaf (Maksymowych 1963; Poethig and Sussex 1985) and play important roles throughout leaf development. Nonetheless, the mechanisms controlling these basic aspects of leaf development are poorly understood, since the pattern of growth within the lamina is surprisingly complex (Steeves and Sussex 1989). For example, the roles of cell division in leaf development remain unclear. Organismal theory claims that cell division is just a general factor during plant growth, but that it does not influence plant growth or morphology (Kaplan and Hagemann 1991). Recently, several molecular genetic studies, including a study of transgenic tobacco plants expressing the dominant negative *cdc2a* mutant gene and a genetic study of the *tangled*-1

mutant, have lent support to this organismal theory (Hemerly et al. 1995; Smith et al. 1996). Hemerly et al. (1995) suggested that reduced CDK activity affects cell division, but does not affect leaf shape directly. Smith et al. (1996) reported that the *tangled*-1 mutation of maize altered the orientation of leaf cell division without altering leaf shape.

On the other hand, several molecular genetic studies support the idea that cell division can directly affect leaf morphogenesis (Riou-Khamlichi et al. 1999; Wang et al. 2000). Recently, *ICK1* genes, which are negative cell-cycle-regulating CDK inhibitors, were identified and characterized in *Arabidopsis* (Wang et al. 1997). Leaves of transgenic plants overexpressing *ICK1* were smaller and serrated (Wang et al. 2000). CDK activity in 35S-*ICK1* plants was reduced, resulting in alteration of the length /width ratio and shape of leaves (Wang et al. 2000). Interestingly, the leaf cells in 35S-*ICK1* plants were two or three times larger, while the number of leaf cells was significantly reduced (Wang et al. 2000). This suggests that cell division affects leaf shape. This idea was supported by a study of transgenic plants ectopically expressing *Arabidopsis CycB1 (Cyc1At)*, a mitotic cyclin gene, via the cdc2 promoter (Doerner et al. 1996). A study of transgenic plants overexpressing *CycD3*, a G1 cyclin, indicated that cell-cycle regulators affected leaf shape directly (Riou-Khamlichi et al. 1999).

In the past seven years, studies of the developmental genetics of leaf morphogenesis in *Arabidopsis thaliana* (L.) Heynh. have begun to dissect this complicated process (Tsukaya 1995). Many mutations that cause defects in initiation, primary morphogenesis, and secondary morphogenesis have been identified and characterized. This review focuses on the genetic regulation of leaf expansion in *Arabidopsis*, a model of dicotyledonous plants, from the perspective of the spatial and temporal balance between cell division and cell enlargement, with special emphasis on our own studies. Other recent reviews of leaf morphogenesis cover leaf initiation and primary morphogenesis (Dale 1988; Smith and Hake 1992; Tsukaya 1995; Dengler 1999; Dengler and Tsukaya 2001).

Spatial and temporal patterns of cell division and cell enlargement

Cell elongation in leaves continues throughout leaf expansion, while cell division can stop before the leaf is mature (Dale 1976). Our previous genetic and histological study of the curly leaf (*clf*) mutation is a good way to examine the balance between cell division and cell enlargement in leaf expansion (Kim et al. 1998a).

The leaves of the *clf* mutant are narrow, short, and curl upwards (Figure 1A), while the average length of the hypocotyl and primary root of the *clf* mutant are not significantly different

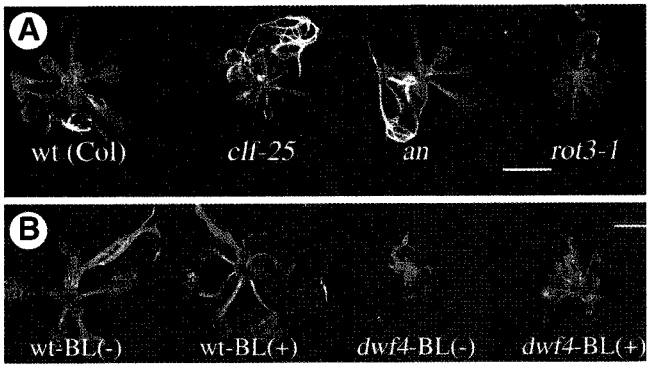

Figure 1. Morphology of wild-type (wt) and mutant plants. (A) Photographs of, from the left, wt, *clf*-25, an, and *rot*3-1 plants are shown. (B) The morphology of wt and *dwf*4 plant after treated (-) or untreated (+) 0.1 mM brassinolide (BL) for 3 days. Bars, 10 mm.

from those of the wild type (Goodrich et al. 1997; Kim et al. 1998a). The width and length of leaf blades of the *clf*-25 mutant are significantly smaller than those of the wild type at the same stage (Table 1). The reduced size and number of leaf cells (Table 1) results in the narrow short leaves of the *clf* mutant. The leaf of the *clf*-25 mutant grows more slowly than that of the wild type throughout its development (Kim et al. 1998a). Palisade cell division begins to stop in both the wild type and *clf*-25 mutant four days after the appearance of the primordium. The rate of cell production is lower in the *clf*-25 mutant than in the wild type throughout the period of cell division (Kim et al. 1998a). During leaf cell elongation, two phases were recognized. While cells were dividing, the size of cells in the *clf*-25 mutant did not differ from that in the wild type (Kim et al. 1998a). After completion of the cell-division phase (8 d after the appearance of the leaf primordium), in the observed area of the leaf blade, expansion continued in both the wild type and the *clf*-25 mutant. The rate of cell elongation at this stage was lower in the *clf*-25 mutant than in the wild type (Kim et al. 1998a). These data show that the spatial and temporal balance between cell proliferation and cell enlargement in leaves can affect leaf morphology. The proper balance of leaf cell proliferation and enlargement in wild-type plants, both spatially and temporally, was revealed in a recent study of *Arabidopsis* using transgenic plants harboring a G2/M-specific marker gene, *Cyc1At* promoter-*GUS* (Donnelly et al. 1999). GUS-positive cells in the leaves of transgenic plants were distributed uniformly at the early stage of leaf primordia. GUS activity was still high in the leaf blade, but its activity was reduced to a limited area of the petiole, basal portion of the mid-vein, and margin of the leaf blade 4 days after the appearance of the leaf primordium (Donnelly et al. 1999). The distribution of GUS-positive cells began to show a longitudinal gradient along the leaf blade, with the most in the basal part of the leaf blade at 8

Table 1. Dimensions of wild-type (wt), *clf-25, an, rot3-1* and *an clf-25* mutant plants[a]

	wt (Col)	*clf-25*	*an*	*rot3-1*	*an clf-25*
Leaf width[b]	6.9± 0.1	2.4±0.1	5.5± 0.5	-	1.4± 0.2
Leaf lenght[b]	10.8± 0.8	4.0±0.3	13.7± 1.1	-	4.3± 1.5
Cell size in leaf[c]					
- width	40.6± 3.3	24.1±1.3	33.8± 1.6	44.0± 9.6	17.8± 3.3
- length	42.6± 2.2	25.2±2.1	41.8± 2.7	37.1± 8.3	18.6± 4.2
- thickness	32.0± 7.6	20.8±6.8	54.5± 9.2	41.2± 7.3	37.0± 4.9
Number of palisade cells along lamina					
- width[d]	216.0±30.8	119.0±4.2	156.0±12.2	252.0±27.0	66.0± 4.4
- lenght	216.0±21.3	174.0±9.5	272.0± 9.1	270.0±37.2	142.0±23.1

[a]Data are means±SD for more than 4 plants.
[b]Fifth rosette leaves were measured at the fully expanded stage. All measurements are in mm.
[c]Measurements were made on sections. All measurements are in μm.
[d]Cell numbers were measured at the portion with maximum lamina-width.
-,not measured.

days. GUS-positive cells were limited to the basal third of the leaf blade at 12 days (Donnelly et al. 1999). On the other hand, palisade cell enlargement continued after cell division decreased, until the leaf was fully expanded (Donnelly et al. 1999). These results support the cell size control of cycling model proposed by Francis (1998), in which a minimum size is required for commitment to cell division in a tissue-specific manner.

Polar elongation of leaf cells in leaf morphogenesis

As shown in our study of the leaves of the *clf* mutant, the process of cell enlargement can be divided into two distinct phases (Kim et al. 1998a). The earlier phase of cell enlargement has a tight relationship with the process of cell proliferation, as described above. The later phase of cell enlargement involves a polar-dependent process of leaf cell elongation. A previous genetic study of *angustifolia (an)* and *rotundifolia3 (rot3)* mutations revealed that genetic regulation of the polar elongation of cells controls the two-dimensional growth of the leaf blade (Tsukaya et al. 1994; Tsuge et al. 1996).

How the two phases of cell elongation are related was examined using a double mutant combining the *an* and *clf* mutations. The *AN* gene regulates the width of leaf cells in a polarity-dependent manner and functions independently of *ROT3* (Tsuge et al. 1996). The *an* mutant has narrower leaves of normal length (Figure 1A). The *an clf*-25 double mutant had fewer cells in the leaf-width and leaf-length directions than either single mutant (Table 1). The cells themselves were also smaller in both directions than in either single mutant (Table1). In the leaf-thickness direction, cell length was intermediate between those of the two single mutants (Table 1 and Kim et al. 1998a). Thus, the *an* and *clf* mutations have an additive effect, which results in an altered pattern of polar elongation of leaf cells during the late stages (stages III and IV), as described previously (Tsuge et al. 1996; Kim et al. 1998a). This suggests that the two phases are regulated independently, at least in terms of the functions of the *AN* and *CLF* genes. Moreover, the results show that the *AN* and *CLF* genes act independently in the later-phase of leaf-cell elongation.

The *AN* gene was identified by positional cloning (Kim et al. 2002) and was found to encode a member of the C-terminal binding protein (CtBP) family, whose members are known to act as transcriptional co-repressors in animals (Turner and Crossley 2001). The *AN* gene is the first gene for a member of the CtBP family to be isolated from plants. Cytological analysis of the cortical microtubules (MTs), which are considered to be important in the regulation of the polar elongation of cells, in the an mutant revealed an altered pattern of cortical MTs that can fully explain the morphological phenotype of the leaf cells (Kim et al. 2002). Thus, *AN* might function in the regulation of the arrangement of cortical MTs in leaf cells. It is noteworthy, moreover, that the *AN* protein can interact with the ZWICHEL protein, a kinesin-like protein that might interact with tubulin, in a yeast two-hybrid system (Folkers et al. 2002).

By contrast to the *an* mutant, *rot3* mutants have short leaves and petioles with normal leaf width (Figure 1A), suggesting that *ROT3* controls leaf length specifically. To examine this hypothesis from a genetic perspective, the *ROT3* gene was cloned using a T-DNA tagging strategy (Kim et al. 1998b). A molecular genetic study of the *ROT3* gene suggested that it encoded a novel cytochrome P450, CYP90C1 (Kim et al. 1998b). A study of transgenic plants overexpressing the *ROT3* gene

revealed that it regulated polar elongation in leaf cells, but it did not affect the number of leaf cells throughout leaf expansion (Table 1; Kim et al. 1999). In addition, the leaves of transgenic plants were longer than those of wild-type plants, while they had the same width (Table 1; Kim et al. 1999). Thus, the *ROT3* gene might specifically regulate leaf elongation.

On the other hand, genes that have high homology to the *ROT3* gene, such as *CPD (=CYP90A1)* and *DWARF4 (=CYP90B1)*, are involved in brassinosteroid biosynthesis (Kim et al. 1998b). Brassinosteroid is a general plant growth factor that regulates both the division and elongation of cells in all plant organs (Szekeres et al. 1996; Azpiroz et al. 1998). A defect in brassinosteroid biosynthesis results in severe dwarfism, with a non-polar defect in leaf expansion, which can be restored by the exogenous supply of brassinosteroid (Figure 1B). Thus, all members of the CYP90 gene family, except *ROT3*, are involved in the general regulation of the proliferation and enlargement of plant cells. The *ROT3* gene appears to have evolved from a family of genes that are general regulators of plant growth.

Molecular biodesign of leaf and flower shapes

Transgenic *Arabidopsis* plants that overexpress the *ROT3* gene have leaves with elongated petioles and leaf blades (Kim et al. 1999; Figure 2A), demonstrating that *ROT3* stimulates the elongation of leaves in the leaf-length direction exclusively without any effect on the expansion of leaf blades in the leaf-width direction. Such a gain-of-function type of mutation might plausibly have contributed to the evolution of narrow leaves with large leaf indices. This hypothesis is strengthened by the fact that morphological phenotypes of rheophytes are known to mostly be dominant or semi-dominant in terms of heredity. In our studies of *ROT3*, we identified one allele of the *rot3* mutation, *rot3-2*, that encoded an amino-acid substitution in the gene product ($rot3^{G80E}$). The *rot3-2* plants had a slightly different phenotype from null-type mutants, having larger leaf blades and thicker stems (Kim et al. 1998b). Although the leaf blades were larger in the *rot3-2* mutant, the lengths of leaf blades were proportionally shorter than the widths of leaf blades, as is the case for leaves of plants with the other two rot3 alleles. Plants that overexpressed the *rot3-2* mutant gene, on a background of the *rot3-1* null mutation, had leaves that were very similar to those of the original *rot3-2* mutant (Kim et al. 1999; Figure 2A). Diversity of leaf form, in terms of the relative sizes of petiole and leaf blade, might be due, in part, to such mutation. Ectopic overexpression of *ROT3* also enhanced the longitudinal elongation of floral organs (Figure 2B-D), whereas petals, sepals, stamens, and pistils were all of

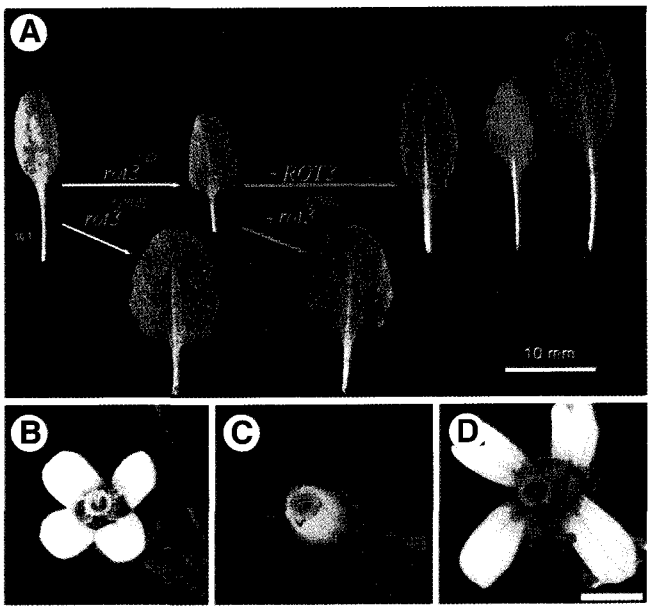

Figure 2. Alterations in leaf (A) and flower (B-D) shapes in *Arabidopsis* as a result of different alleles of the *ROT3* gene. (A) Morphology of the fifth foliage leaves. White arrows indicate mutations in the *ROT3* gene and pink arrows indicate a wild-type *ROT3* transgene or a mutated *ROT3* transgene. The petiole and blade of Columbia wild-type leaves (far left) are shortened by a null mutation in the *ROT3* gene (*rot3-1*; Upper left, $rot3^{null}$). This effect is reversed by overexpression of the wild-type *ROT3* gene (+*ROT3*, upper right; leaves of three independent transgenic lines are shown). A mutation causing an amino acid substitution in the product of the *ROT3* gene ($rot3^{G80E}$) results in short petioles and broad leaf blades (lower left), similar to those induced by expression of the mutated gene on a background of the null allele *rot3*-1 (+ $rot3^{G80E}$; Lower right). (B-D) Flowers of a wt plant (B), a *rot3*-1 mutant plant (C), and a transgenic plant of overexpression of *ROT3* gene (D). (Bar=10 mm.)

reduced length in the null-type *rot3-1* mutant (Figure 2B-D). Elongation of internodes and hypocotyls was unaffected by induction of the transgene, supporting the hypothesis that the *ROT3* gene is involved specifically in the elongation of leaves and leaf-based organs. These results suggest that we could control the organ shapes by ectopically overexpressing or downregulating the morphology-related genes and biodesign the novel shape of plants within near future.

Conclusions

The mechanism joining cell division, cell elongation, and leaf shape is still controversial. There are major unresolved questions about perturbations in the course of cell division and cell enlargement, including how the patterns of cell division are controlled with such precision and how cells are regulated spatially and temporally during organ morphogenesis. The answers to these questions have eluded researchers for decades.

Several studies introduced in this review have contributed partial answers to some of these questions. In addition, the examples presented here show that it will be possible to engineer horticultural plants and crops with diverse phenotype by ectopically overexpressing or downregulating the genes that control the morphology. The advent of new techniques of genetic and molecular analysis will offer considerable promise for horticultural plant improvement.

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