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# Leaf Development

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**Leaves are the most important organs for plants. Without leaves, plants cannot capture light energy or synthesize organic compounds via photosynthesis. Without leaves, plants would be unable perceive diverse environmental conditions, particularly those relating to light quality/quantity. Without leaves, plants would not be able to flower because all floral organs are modified leaves. *Arabidopsis thaliana* is a good model system for analyzing mechanisms of eudicotyledonous, simple-leaf development. The first section of this review provides a brief history of studies on development in Arabidopsis leaves. This history largely coincides with a general history of advancement in understanding of the genetic mechanisms operating during simple-leaf development in angiosperms. In the second section, I outline events in Arabidopsis leaf development, with emphasis on genetic controls. Current knowledge of six important components in these developmental events is summarized in detail, followed by concluding remarks and perspectives.**

## INTRODUCTION

The shoot system is a fundamental unit in the body plan of angiosperms and seed plants in general. It is composed of a leaf, a stem, and a lateral bud that differentiates into a lateral shoot. The most specialized organ in angiosperms, the flower, can be considered to be a derived shoot system since floral organs, such as the sepal, petal, stamen, and carpel, are all modified leaves (see Weberling 1981; Cronk 2009). Scales, bracts, and certain kinds of needle, such those on cacti, are also derived from leaves (see Bell 1991; Cronk 2009). Thus, an understanding of leaf development is critical to a more general understanding of shoot development. Moreover, leaves play important roles in photosynthesis, respiration, and photoperception. Therefore, improved understanding of leaf development contributes directly to a more comprehensive concept of angiosperm biology.

The details of leaf development remain unclear, even though there has been extensive recent progress in understanding mechanisms in the model plant *Arabidopsis thaliana* (L.) Henyn. (hereafter, Arabidopsis). Although leaf shape seems very simple, processes of development are not so simple, as demonstrated in classic studies on the ways in which division and elongation of cells occur in the leaf primordium of eudicot species (Maksymowych 1963; Poethig and Sussex 1985).

Genetic studies of Arabidopsis have provided a powerful tool for understanding the mechanisms of the complex processes of leaf development. This plant has become a model for studying eudicotyledonous, simple leaves. Various details of the mechanisms that control leaf development have emerged in recent developmental and molecular genetic studies of Arabidopsis. Developmental mechanisms of complex leaves, such as those on the tomato (*Solanum lycopersicum* L.) *Cardamine hirsuta* L. and the garden pea (*Pisum sativum* L.), differ from those of the simple leaves in Arabidopsis. This issue was examined by Efroni et al. (2010) and Canales et al. (2010), and Townsley and Sinha (2012) also provided relevant information. Monocotyledonous leaves also develop differently from those of Arabidopsis (Yamaguchi et al. 2004, 2010). My focus here is on current information that is available for various mechanisms of eudicotyledonous, simple-leaf development in Arabidopsis. Vascular patterning is also an important determinant of leaf shape, and this topic has been reviewed by Turner and Sieburth (2003). Readers should consult Moon and Hake (2011) for information on the roles of class I *Knotted-1Homoeobox* (*KNOX*) genes, which have been extensively studied. The terminology used here is schematized in Fig. 1. I begin this review with a brief summary of the history of Arabidopsis leaf development research. The overview of the mechanisms of leaf development begins in the section titled “Outline of events in Arabidopsis leaf development”.

## A BRIEF HISTORY OF STUDIES ON LEAF DEVELOPMENT IN ARABIDOPSIS

Many Arabidopsis mutants with alterations in leaf morphology were isolated more than 50 years ago (*e.g.*, Rédei 1962; Lee-Chen and Steinitz-Sears 1967; Barabas and Rédei 1971), but each was used merely as a positional marker for genetic mapping (*e.g.*, Koornneef et al. 1983). Associated phenotypes were not analyzed ini-

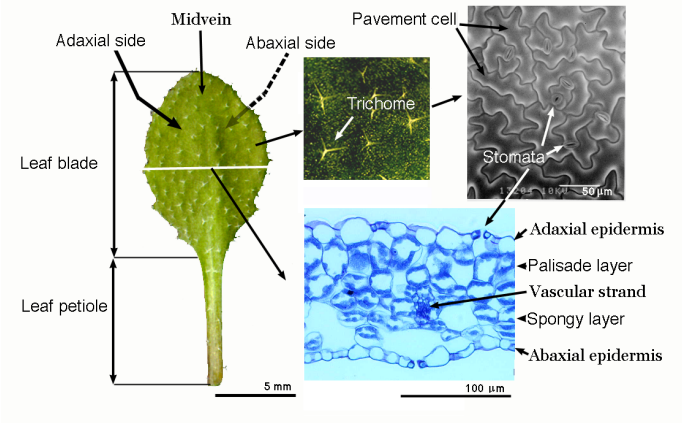


**Figure 1.** Terms used to describe leaf morphology. Left, gross morphology of the fifth rosette leaf of Arabidopsis. Upper right, magnified views of the leaf surface. Lower right, magnified view of cross section of the leaf blade.

tially from a developmental genetics perspective, except in a few cases (Rüffer-Turner and Napp-Zinn 1979). Anatomical analyses of leaf development in several other species with broad leaves, such as tobacco, *Xanthium pennsylvanicum* Gand., and *Phaseolus vulgaris* L., were published in the later decades of the 20th century (for reviews, see Maksymowych 1963; Marx 1983; Dale 1988). Until the mid-1990s, genetic and molecular explorations of leaf development were restricted to functions of the *knotted1* (*kn1*) gene in maize (*Zea mays* L.; Smith and Hake, 1992, 1993). Apart from the descriptions of heterophylly by Röbbelen (1957), studies of leaf development in Arabidopsis were very limited.

Developmental and molecular analyses of leaf development and its controls began a few years after a report by Pyke et al. (1991) on kinetic and anatomical features of development in the first set of foliage leaves on the Landsberg *erecta* strain of Arabidopsis. Arabidopsis leaves are very suited to studies of leaf morphogenesis because they have a simple, stable form and genetic analyses are readily performed. A turning point in studies of leaf development was reached in 1994 when Arabidopsis was recognized as a useful model plant. Tsukaya et al. (1994) performed anatomical analyses of cotyledon development in the Columbia wild-type strain of Arabidopsis and showed that embryonic leaves may be used as a model for studies of leaf morphogenesis. They also demonstrated that the *angustifolia* (*an*) mutant has a defect in polarity-dependent elongation of cotyledon cells. In the same year, Van Lijsebettens et al. (1994) showed that insertion of T-DNA into the gene for the S18 ribosomal protein caused expression of the *pointed first leaves* (*pfl*) phenotype, which has extremely narrow first leaves and pale coloration at low temperatures. Lincoln et al. (1994) isolated and characterized a first homolog of the maize *knotted1* gene from the Arabidopsis genome, i.e., the *KNAT1* (*knotted*-like from *Arabidopsis thaliana1; AT4G08150*) gene. Subsequent reports of new mutations in leaf morphology were published with rapidly increasing frequency. Tsuge et al. (1996), for example, reported anatomical and genetic characterizations of the *an* mutant and newly isolated *rotundifolia* mutants. They also showed that two-dimensional growth of leaf blades is controlled by two independent, polarity-dependent systems. Their study was the

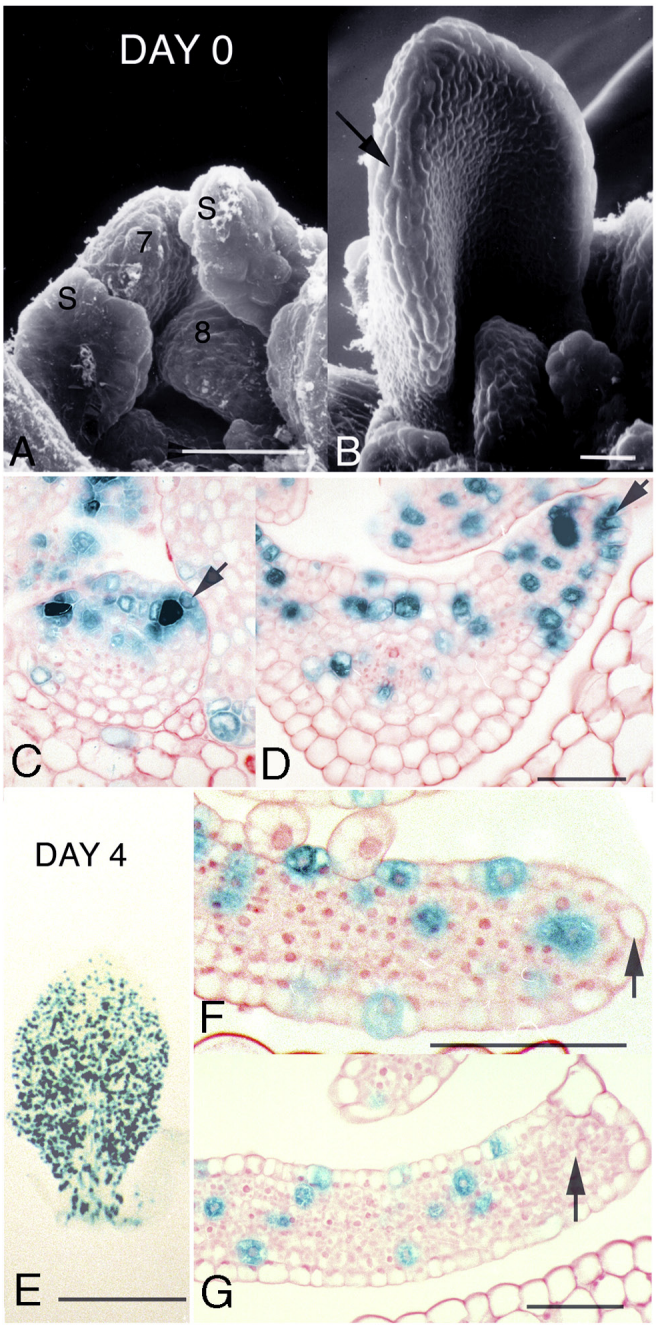


**Figure 2.** Meristematic activity in early leaf primordia of Arabidopsis. Development of primordia of eighth foliage leaves (Leaf 8 primordia) monitored with the *pCYCB1;1::GUS-DB* reporter gene, which acts as a specific marker for the G2/M phase of the cell cycle. Blue color indicates the expression of the reporter gene. Arrows indicate cells at the margins of leaf primordia. (A and B) Scanning electron micrographs of *gl1* leaves. Bar, 50 µm. (A) Leaf 8 primordium (indicated by 8) 50 µm in length. Also shown are Leaf 7 (7) and stipules (S) of older leaves. (B) Leaf 8, 0.4 mm long. Arrow indicates enlarged cells at margin. (C) Cross section of Leaf 8 primordium at stage of Leaf 8 primordium (indicated by 8) 50 µm in length. (D) Cross section of Leaf 8 primordium 0.16 mm in length, sectioned at a distance 25% of total length above the base. Bar, 50 µm. (E) Cleared Leaf 8 1.2 mm in length. Bar, 0.5 mm. (F and G) Cross sections of Leaf 8 primordia 1.5 mm in length. Bar, 50 µm. (F) Sections cut at a distance 25% of total length above the leaf base. (G) Section cut at a distance 50% of total length above the leaf base. Modified from Donnelley et al. (1999; Dev. Biol. 215: 407-419 with permission from the authors.

first developmental genetic analysis of mutations in the shape of Arabidopsis leaves.

Understanding of leaf development in Arabidopsis has progressed significantly since Donnelly et al. (1999) analyzed the expression pattern of a *pCYCLIN B1:1(CYCB1;1)::GUS* fusion gene (with a destruction box inside the *GUS* coding region for β-glucuronidase). Expression of this gene is a specific marker of the G2/M phase of the cell cycle. Donnelly et al. (1999) were able to monitor patterns of division and enlargement of cells in leaf primordia from the first protrusion of leaf primordia to leaf maturity (Fig. 2). This analysis forms a basis for our present understanding of basic behavior in leaf primordial cells of Arabidopsis. In the same year, comprehensive genetic categorizations of mutations in leaf morphology deposited in Stock Centers were first published (Berná et al. 1999; Serrano-Cartagena et al. 1999, 2000). A description of the basic pattern of venation in leaf blades of Arabidopsis also became available (Candela et al. 1999). A series of studies by J. L. Micol and collaborators later advanced knowledge of the many genes that affect leaf organogenesis (see *e.g.*, Mollá-Morales et al. 2011), although some of these loci are not leaf-specific regulators but are implicated rather in the control of whole-plant architecture (*e.g.,* DNA polymerase α: Barrero et al. 2007; genes for histone monoubiquitination enzyme: Fleury et al. 2007; genes for subunits of cellulose synthase complex: Rubio-Díaz et al. 2012). In the same time period, there were extensive genetic analyses of natural variation in leaf architecture by the same research group (Perez-Perez et al. 2002; Alonso-Peral et al. 2006).

As the Arabidopsis genome project progressed, diverse loci for leaf development were isolated, with an accompanying increase in annual numbers of publications on leaf development (reviewed by Tsukaya 2010). The decade following the late 1990s was a fruitful period with a burgeoning literature on mechanisms of leaf development. For example, McConnell et al. (2001) cloned the *PHABULOSA* (*PHB; AT2G34710*) and *PHAVOLUTA* (*PHV; AT1G30490*) genes, which have similar functions in determination of adaxial fate in leaf primordia, by searching for homologous genes shared by two chromosomal regions to which the two genes had been mapped. There were breakthrough studies of leaf development in Arabidopsis, leading to identifications of mutations (and genes) that affect the establishment of dorsiventrality of the leaf primordium (*e.g.*, Bohmert et al. 1998; McConnell and Barton 1998; Lynn et al. 1999; Sawa et al. 1999; Siegfried et al. 1999; Kerstetter et al. 2001; McConnell et al. 2001). This series of studies are greatly indebted to a pioneer study on the role of dorsiventral identity for flat lamina growth in snap dragon (*Antirrhinum majus* L.) (Waites and Hudson 1995). Explorations of such mutations and genes in Arabidopsis suggest that establishment of polarities around the leaf primordium and activity of the shoot apical meristem might be tightly linked (Fig. 3). The mechanisms that determine dorsiventral polarity are very complex, as confirmed by recent investigations. An important advance on this topic was a revelation that the *YABBY* gene family is not a fundamental factor that identifies the abaxial fates of leaves (Sarojam et al. 2010). This view diverges from earlier literature (*e.g.,* Sawa et al. 1999). Vegetative *YABBY* genes operate in the induction of leaf lamina-specific genetic programs and shut-down SAM programs, such as *WUSCHEL* (*WUS; AT2G17950*) in the leaf primordia (Sarojam et al. 2010).

As information on the functions of individual genes in the control of leaf development has accumulated, a developing research focus has been on the roles of genetic networks and interactions among such genes, for example, in the control of dorsiventrality. Certain genes negatively regulate the expression of class I *KNOX* genes in leaf primordia of Arabidopsis (*e.g.*, Byrne et al. 2000; discussed later). Genetic interactions between the SAM and leaf primordia via and/or in parallel with the regulation of class I *KNOX* genes (Fig. 3) are quite complex, and studies on the mechanisms are now in progress.

Cell-cell communication is a component of organogenesis in leaf primordia. Following a proposal by Nath et al. (2003) that there exists a 'cyclic arrest front' (a conceptual front in develop-

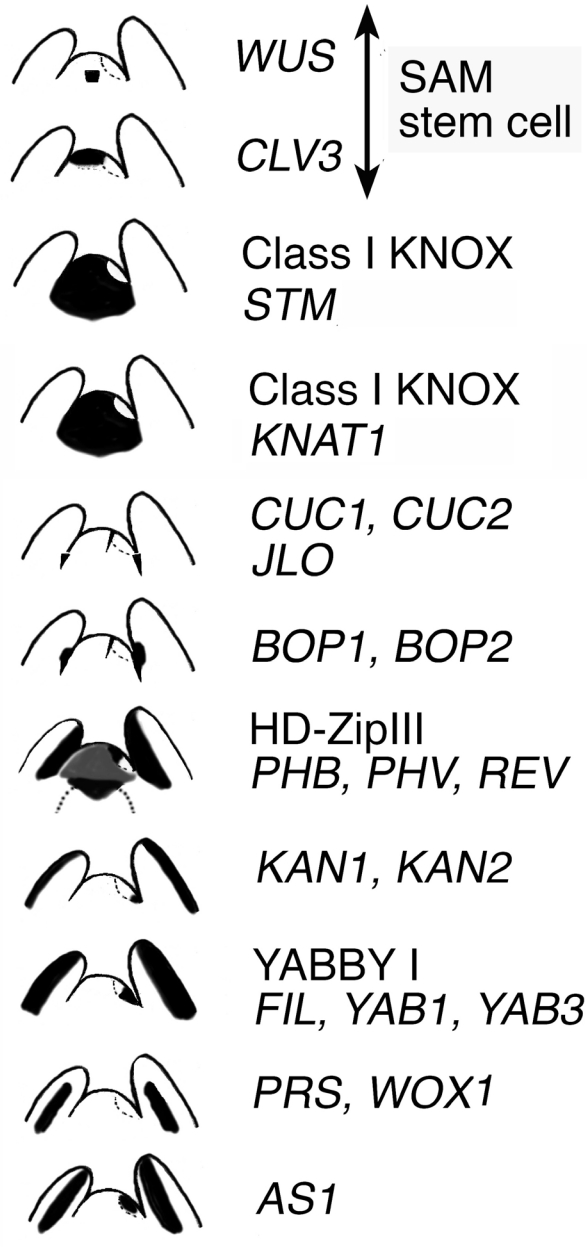


**Figure 3.** Schematic representation of patterns of expression of key genes required for early steps in leaf development in Arabidopsis. Cross sections of a shoot apex with two young leaf primordia and one predicted area of a leaf primordium are shown. Regions in which the indicated genes are expressed are shaded. See text for details.

ing leaf primordia that distinguishes a meristematic or cell-proliferative area from an area where cells exit the mitotic process and begin expansion and differentiation), there have been many explorations of the ways in which this front is regulated. This is a very popular current topic in leaf organogenesis studies. Tsukaya (2002a) also proposed the existence of unknown cell-cell interactions in leaf primordia based on the concept of 'compensation,' which is an abnormal increase in cell volume triggered by defects in cell proliferation in leaf primordia. The mechanism of cell-cell communication in the regulation of leaf organogenesis is a subject of intensive research efforts. Two important findings to date relate to the concepts of the 'cyclic arrest front' and 'compensation': (i) initially, it was believed that the arrest front moves gradually from tips to bases of leaf primordia, but Kazama et al. (2010) showed that this is not the case, and (ii) an unknown cell-cell communication system indeed links the level of cell proliferation with that of cell expansion in the leaf primordium; this was elegantly demonstrated by Kawade et al. (2010).

Studies of leaf morphogenesis are performed not only on single leaves, but also at the whole-plant level. Following Kersteller and Poethig's (1998) finding of Arabidopsis heteroblasty, Poethig and colleagues conducted extensive investigations of heteroblastic mechanisms using Arabidopsis as a model system (*e.g.,* Wu and Poethig 2006; Wu et al. 2009; Li et al. 2011).

Since the early 2000s, studies have also extended to environmental adaptations of leaf shape. Before the work of Tsukaya et al. (2002a), explorations of genetic factors involved in the shade-avoidance syndrome focused largely on those in hypocotyls of Arabidopsis. Tsukaya et al. (2002a) extended analyses to leaves and demonstrated that leaf petioles and blades respond differently to phytochrome signals. Research is progressing toward identifying the roles of phytohormones and the genetic pathways relating to their functions.

Finally, to which direction the Arabidopsis leaf development studies can extend? For example, developments in procedures for the analysis of molecular genetic controls of leaf organogenesis in the past decade have enabled studies on alterations in genetic systems operating in 'unusual' types of leaves (in comparison with those of Arabidopsis) (*e.g.*, Yamaguchi et al. 2010). Such evolutionary developmental biology studies or 'Evo-devo' approaches will progress rapidly in the near future with advances in Arabidopsis leaf studies.

Below, I summarize the present understanding of each of the processes functioning during Arabidopsis leaf development. Phytohormone-dependent controls are not included in this review; readers interested in those controls may wish to consult the latest reviews on the roles for each phytohormone in the *Arabidopsis Book*. Understanding of genetic controls in tissue- or cell-type differentiations such as in leaf venation patterning has also advanced in recent years (*e.g.*, Dengler and Kang 2001; Alonso-Peral et al. 2006; Robles et al. 2010), but these topics will be covered in another review article.

## OUTLINE OF EVENTS IN ARABIDOPSIS LEAF DEVELOPMENT

Early events in leaf development fall into three main processes (Foster 1936; Steeves and Sussex 1989; Smith and Hake 1992): (i) initiation of the leaf primordium, (ii) establishment of dorsiventrality, and (iii) development of a marginal meristem. Recent understanding of genetic regulation in early leaf primordia differs from the classic view. In the following, I summarize a current perspective on leaf development based on that of Efroni et al. (2010), with some modifications based on findings by Kazama et al. (2010), Ichihashi et al. (2011), and Nakata et al. (2012).

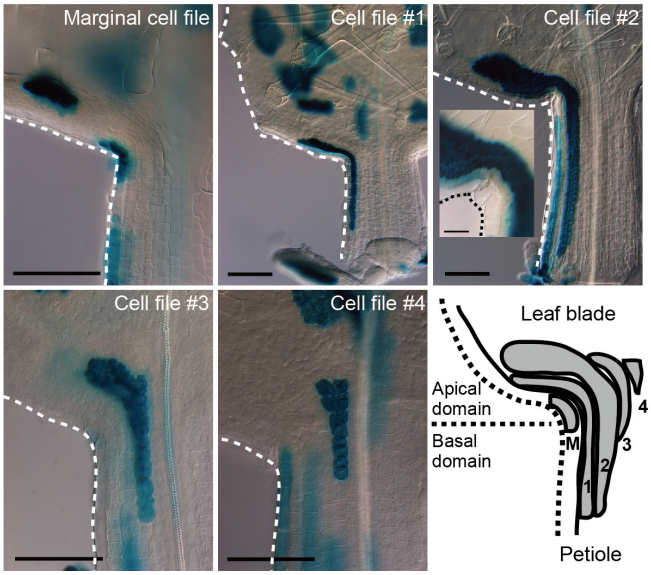


**Figure 4.** Two-directional cell supply from "leaf meristem" region. Clonal cell files are visualized by a heat-shock-inducible homologous recombination system with a GUS reporter gene. Note extension of a cell file in both directions, to the leaf lamina and to the leaf petiole, from a junction region between them. Note also the presence of five distinct cell files. Figures courtesy of Dr. Y. Ichihashi.

Initially, leaf primordia or founder cells for leaves are specified as auxin maxima in a flanking region of the shoot apical meristem (SAM) (Reinhardt et al. 2000) following the well-known rules of phyllotaxy (Jönsson et al. 2006; de Reuille et al. 2006). Because phyllotaxy in Arabidopsis is spiral, auxin distribution is unequal between left and right sides, resulting in asymmetric growth of leaf laminas; in a clockwise phyllotactic spiral pattern, the left side will grow more than the right side (Chitwood et al. 2012). Subsequently, dorsiventrality (= dorsoventrality; here I use the spelling "dorsiventrality," following recommendations of D. Kaplan) or abaxial-adaxial polarity (Ad-Ab polarity) is established with bulging of the primordia. Genetic controls of dorsiventrality are dealt with the "Establishment of dorsiventrality and leaf-lamina identity" section below. While leaf protrusion results from a significant enhancement in cell proliferation levels above those occurring in the shoot apical meristem, initial cell proliferation activity in the bulge of early leaf primordia is somewhat reduced in comparison with that following establishment of the leaf blade/petiole junction region (Ichihashi et al., 2011). The apical part of the leaf primordium is occupied by small, non-polarized cells with longitudinal, transverse, and oblique arrangements of crosswalls relative to the proximal-distal axis. The basal part of the leaf primordium has large, longitudinally polarized cells that are arranged paral-

lel to the proximal-distal axis. After differentiation of apical and basal regions, cell proliferation in the leaf primordium accelerates and the narrowed morphology between the leaf blade/petiole becomes conspicuous. New cells are directionally supplied from the junction region to the tip for construction of the leaf blade or lamina and to the base for construction of the petiole. Chimera analysis has indicated that particular cell populations at the junctions between petioles and blades function as common sources (intercalary meristems or "leaf meristems") for this bi-directional cell supply (Ichihashi et al. 2011: Fig. 4). Cell division is anticlinal in the petiole-forming cell lineage and rather random in the blade-forming cells, especially after a particular developmental stage has been reached (Horiguchi et al. 2011a). Although there is a long history of discussion as to whether or not the marginal meristem is a major contributor to lamina expansion in eudicotyledenous leaves (Boyce 2007), it has been known for some time that while leaf blade development in diverse ferns with marginally-ending dichotomous veins depends on the marginal meristem, development of angiosperm leaves with many higher-order vein reticulations and internally directed, free-ending internal veinlets depends on dispersed, non-marginal growth (Boyce 2007). Observations of Arabidopsis by Donnelley et al. (1999) confirmed that activity of the so-called marginal meristem is very limited and contributes to leaf-blade development or expansion to a very small extent (Fig. 2). Although the *CINCINNATA*-class *TEOSINTE BRANCHED1, CYCLOPEDEA, AND PCF* (TCP) gene was once proposed as a controller of cell proliferation at the leaf margin [operating during progression of the so-called cyclic arrest front from leaf tip to base; Nath et al. (2003)], this concept has been replaced (Efroni et al. 2008: see the "Positive and negative regulation of cell proliferation" section below) by another, which proposes that lamina growth in angiosperms is sustained by active cell proliferation in the plate meristem located along the Ad-Ab polarity junction. Since the work of Waites and Hudson (1995), a theory has developed that flat, two-dimensional growth of leaf blades depends on and occurs along the Ad-Ab polarity junction. The plate meristem (Donnelly et al. 1999) occurs along the Ad-Ab junction (Fig. 2). *PRESSED FLOWER* (*PRS; AT2G28610*) is expressed at the margins of the leaf primordia, resulting in two-dimensional growth of leaf laminas (Matsumoto and Okada 2001), but recent re-examinations have demonstrated that the *PRS*-expressing domain is a subset part of the plate meristem (Nakata et al. 2012: Figs. 3 and 5). On the basis of this finding, Nakata et al. (2012) proposed a modified model in which leaf Ad-Ab polarity establishes three domains, i.e., the adaxial, middle, and abaxial domains. This concept is referred to again in the "Establishment of dorsiventrality and leaf-lamina identity" section below.

Interestingly, areas of leaf primordia expressing the positive regulator of cell proliferation in leaf primordia *ANGUSTIFOLIA3*(*AN3*)*/AtGRF-INTERACTING FACTOR1* (*AtGIF1; AT5G28640*) (Kim and Kende 2004; Horiguchi et al. 2005) mostly overlap with the cell-proliferating zone, suggesting that *AN3/AtGIF1* is important in the control of leaf-lamina formation (Ichihashi et al. 2011). The mRNA expression domain of the *AN3/AtGIF1* is, however, restricted to inner tissue but not in epidermis in the leaf primordia (Kawade et al. 2013). Detailed analyses of the *an3* mutant showed that both the epidermis and inner tissue are under a control of the *AN3/AtGIF1* activity, in terms of number of cells. GFP-tagged approaches revealed that the AN3

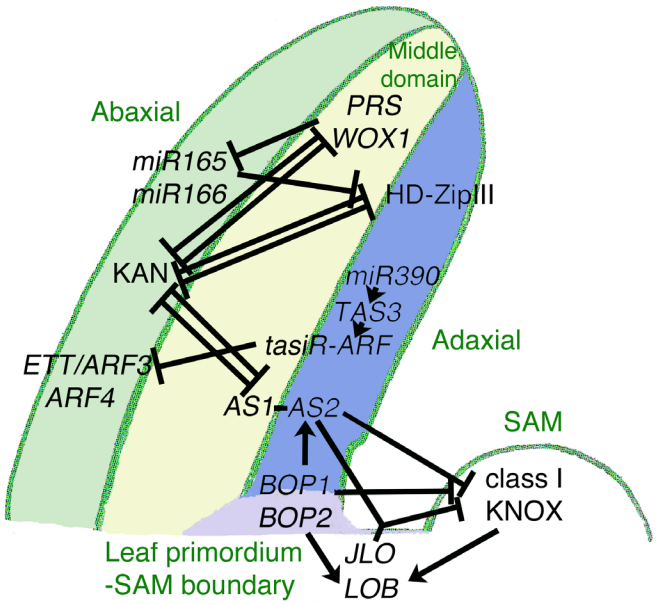


**Figure 5.** Genetic network for adaxial-abaxial polarities of leaf primordia linked to a genetic control system for leaf primordium-SAM boundary formation. See text for details.

protein moves from inner tissue to epidermis thereby coordinating cell proliferation between different tissue layers; interference with AN3 movement results in abnormal leaf size and shape, indicating that AN3 signaling is indispensable for normal leaf development with a coordinated growth between epidermis and inner tissue (Kawade et al. 2013). This is the first report on the importance of the role of mesophyll layers as a signaling source for the coordinated cell proliferation in a leaf primordium.

The regulation mechanisms referred to above appear to be dominant in cell proliferation in the cross-sectional plane. However, in a longitudinal orientation, other forms of regulation are more important. One is the cell-cycle arrest front that borders the cell-proliferating domain or "leaf meristem"; others are areas of cell expansion and differentiation (Fig. 6) recognizable as two fronts: a primary front that determines arrest of general cell proliferation and a secondary arrest front that arrests dispersed meristematic cell proliferation (White 2006). The secondary arrest front is thought to be under control of *PEAPOD* (*PPD*)*1* (*AT4G14713*) and *PPD2* (*AT4G14720*; White 2006). It was previously believed that cessation of mitotic cell division occurs gradually from tip to base in the leaf primordia of Arabidopsis, but Kazama et al. (2010) determined that the cyclic arrest front remains located at an almost constant distance from the leaf blade base over a certain period, and then progresses toward the base, disappearing relatively quickly. Leaf size is determined fundamentally by the duration of the period of cyclic arrest and/or active cell proliferation. A cyclic arrest front also occurs in the leaf petiole zone but has yet to be precisely characterized. Similarly, no details are yet available for the genetic regulators of proximal-distal axis formation in Arabidopsis leaf primordia, although these details are

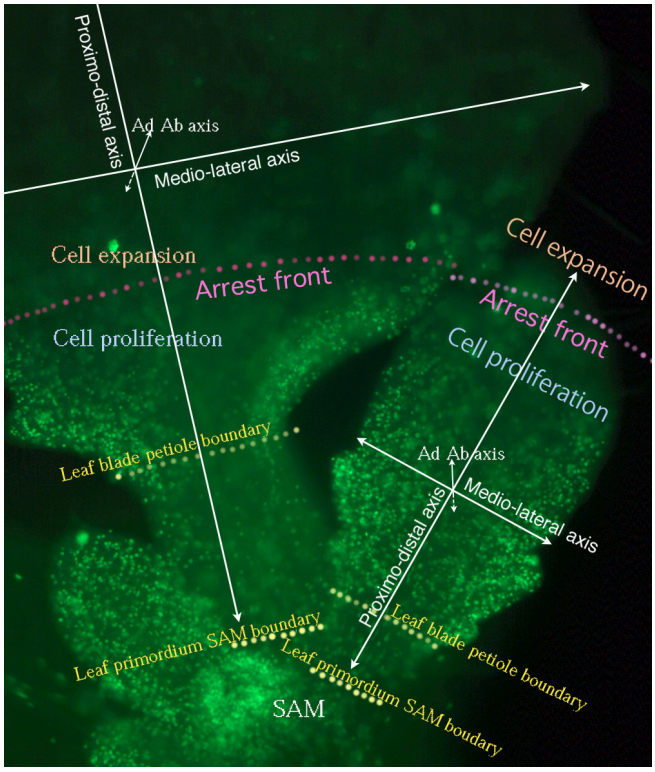


**Figure 6.** Cyclic arrest front in Arabidopsis leaves. S-phase nuclei are visualized by EdU incorporation (shown by green spots) to indicate the active region of cell proliferation in the leaf primordia and the shoot apical meristem (SAM) of a Columbia wild-type plant rosette. The border between the EdU positive and negative regions is the arrest front (shown in pink). Three axes around leaf primordia are also indicated by white arrows. Note that the EdU-positive zone in the leaf primordia has a constant size measured as distance from the base of the lamina, as reported by Kazama et al. (2010) from analysis of *pCYCB1;1::GUS-DB* reporter gene expression (see also Fig. 2). Note also the presence of leaf blade-petiole boundary and leaf primordium-SAM boundary indicated by yellow dots.

available for monocotyledonous leaf primordia (*e.g.,* Ramirez et al. 2009). As indicated in the "Role of the leaf primordium-SAM boundary" section below, the leaf primordium-SAM boundary also appears to function in the regulation leaf organogenesis, particularly in control of the Ad-Ab axis and the "leaf meristem" (Fig. 6). The ROTUNDOFOLIA4 (ROT4; AT2G36985) peptide and similar peptides are encoded by 23 members of the *ROT4* paralogs (*RTFL/DVL:* reviewed by Tsukaya et al. 2013a) that may be involved in longitudinal position cueing in lateral organs of Arabidopsis (Ikeuchi et al. 2011). Detailed analysis of this peptide has yet to be undertaken.

The supply of cells from the meristematic junction region between the blade and petiole to the upper leaf sector is neither uniform nor steady. Along leaf margins, there are periodic spurts and slowdowns in cell proliferation that are reflected in the formation of leaf serrations. This periodic cell supply may be predicted from developmental observations in many plant species; it is linked to formation of auxin maxima controlled by PIN-FORMED1 (PIN1; AT1G73590) activity (Kawamura et al. 2010: Fig. 7) in a manner resembling control of auxin maxima in the SAM. Another key factor in formation of serrations is the *CUP-SHAPED COTYLEDON2* (*CUC2; AT5G53950*) gene (Nikovics et al. 2006), which is a member of the CUC gene family that is generally required for boundary formation between two organs (Fig. 3). Before the work of Kawamura et al. (2010), it was believed that *CUC2* deepens serration sinuses, but their recent study demonstrated that *CUC2* activity enhances growth of the serration tip via stabilization of the auxin maxima in the leaf margin (Fig. 7). Because auxin maxima cannot be stably positioned at a particular site when CUC2 or PIN1 is lost (Fig. 7), the leaf margins become smooth, integrated, or irregular. Simulation analysis revealed that auxin maxima along leaf margins are automatically formed through PIN1 activity along the marginal epidermis of leaf primordia (Bilsborough et al. 2011).

Interestingly, Kawamura et al. (2010) showed that in Arabidopsis leaves the marginal cell file, which has characteristically elongated epidermal cells, does not surround the leaf Ad-Ab junction. The proximal part of this characteristic cell file splits into two in the more distal position and merges into abaxial epidermal cells at the distal end, suggesting spatial and temporal shift of the Ad-Ab border from abaxial to adaxial in the course of leaf primordial development. Growth and development of leaf primordia are more dynamic than previously expected.

Subsequently, cell proliferation ceases, and leaves are mature after cell differentiation and expansion have been completed. The processes of cell expansion are not independent of cell proliferation activity when the cyclic arrest front is maintained in a fixed location, as indicated in the "Positive and negative regulators of cell proliferation in leaf primordia" section below. The numbers of cells and their sizes in a given leaf lamina are also controlled by genetic pathways regulating heteroblasty (Usami et al. 2009), as mentioned in the "Heteroblasty" section below. Importantly, leaf morphogenesis has not terminated at this point, even though leaves have matured. To some extent, leaf shape can be adjusted to environmental factors, particularly light and gravity, via additional cell expansion/elongation (reviewed by Ferjani et al. 2008; as mentioned in the "Environmental control of leaf shape" section below).

The following section presents more details on aspects of the processes mentioned above.

## NEGATIVE REGULATION OF THE CLASS I *KNOX* MEMBERS IN PRIMORDIA

The class I *KNOX* members (*SHOOT APICAL MERISTEMLESS*: *STM: AT1G62360*; *KNAT1*, *KNAT2: AT1G70510*, and *KNAT6: AT1G23380*) are key factors in formation and maintenance of the SAM identity; at the same time, their elimination from early leaf primordia is required (Long et al. 1996: Fig. 3). Class I KNOX works with diverse co-factors in many kinds of functions (reviewed by Hay and Tsiantis 2010). Of particular importance is the direct suppression of gibberellin biosynthesis genes in the SAM and leaf primordia by class I KNOX (Sakamoto et al. 2001). Although re-activation of class I *KNOX* genes in the leaf primordia is required for proper formation of complex leaves in most angiosperms (Bharathan et al. 2002), primordia of simple leaves, such as those in Arabidopsis, have very limited expression. In

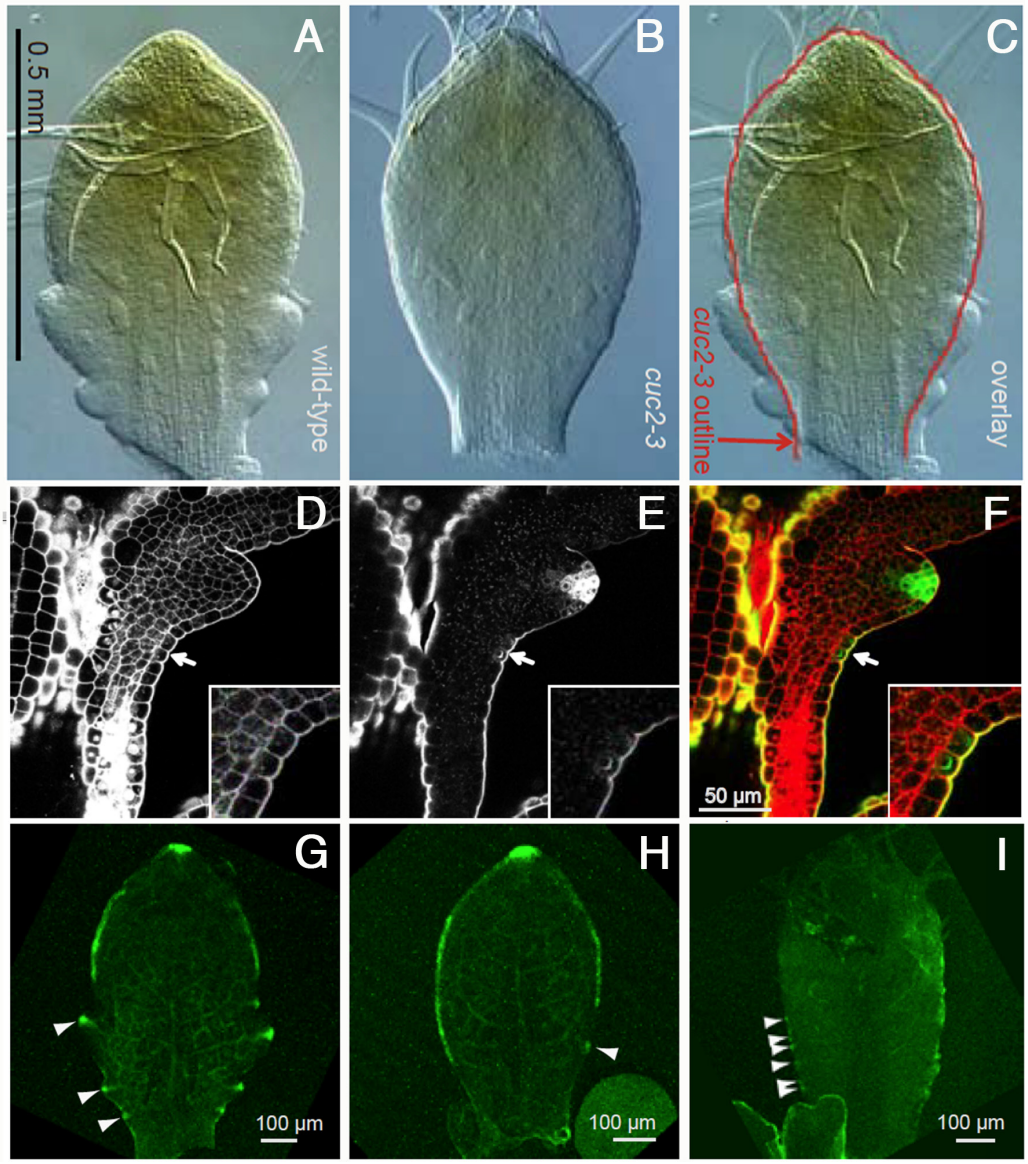


**Figure 7.** Patterning of marginal serration.

**(A,B)**, Wild-type leaf primordia **(A)** compared to a *cuc2-3* mutant with a smooth leaf margin **(B)**.
**(C)**, Overlay image of **(A)** and **(B)** showing that the *cuc2-3* mutant has a defect in serrated margin outgrowth.
**(D-F)** Early development of serration. **D**, Propidium-iodide stained cell image; **(E)** auxin maxima visualized by DR5rev::GFP signal; **(E)**, an overlay of **(D)** and **(E)**. Arrows indicate positions where teeth are predicted to develop.
**(G-I)**, Auxin maxima (arrowheads) along the leaf margin seen in the wild-type **(G)**, *cuc2-3* mutant **(H)**, and *pin1-8* mutant **(I)** leaf primordia expressing DR5rev::GFP. Note that the *cuc2-3* mutant failed to maintain distinct auxin maxima; *pin1-8* has irregularly dense but smaller auxin maxima. Modified from Kawamura et al. (2010).

brief, all leaf primordia initially down-regulate class I *KNOX*. There is subsequent persistent repression in simple-leaf primordia through functioning of the K-box region in the promoter, but there is a class I *KNOX* reactivation in compound leaf primordia (Bharathan et al. 2002; Uchida et al. 2007). When expression is ectopic in the primordia, leaves become deformed, developing irregular lobes or deep serrations due to an abnormally increased potential for organogenesis. Many genetic factors are involved in the suppression of class I *KNOX* genes expression in leaf primordia: *ASYMMETRIC LEAVES1* (*AS1; AT2G37630*), *AS2* (*AT1G65620*), *BLADE-ON-PETIOLE1* (*BOP1; AT3G57130*), *BOP2* (*AT2G41370*), *SAWTOOTH1* (*BEL1-LIKE HOMEODOMAIN* (*BHL*)*2/SAW1; AT4G36870*), *BHL4/SAW2* (*AT2G23760*), *JAGGED* (*JAG; AT1G68480*), *JAGGED LATERAL ORGANS*

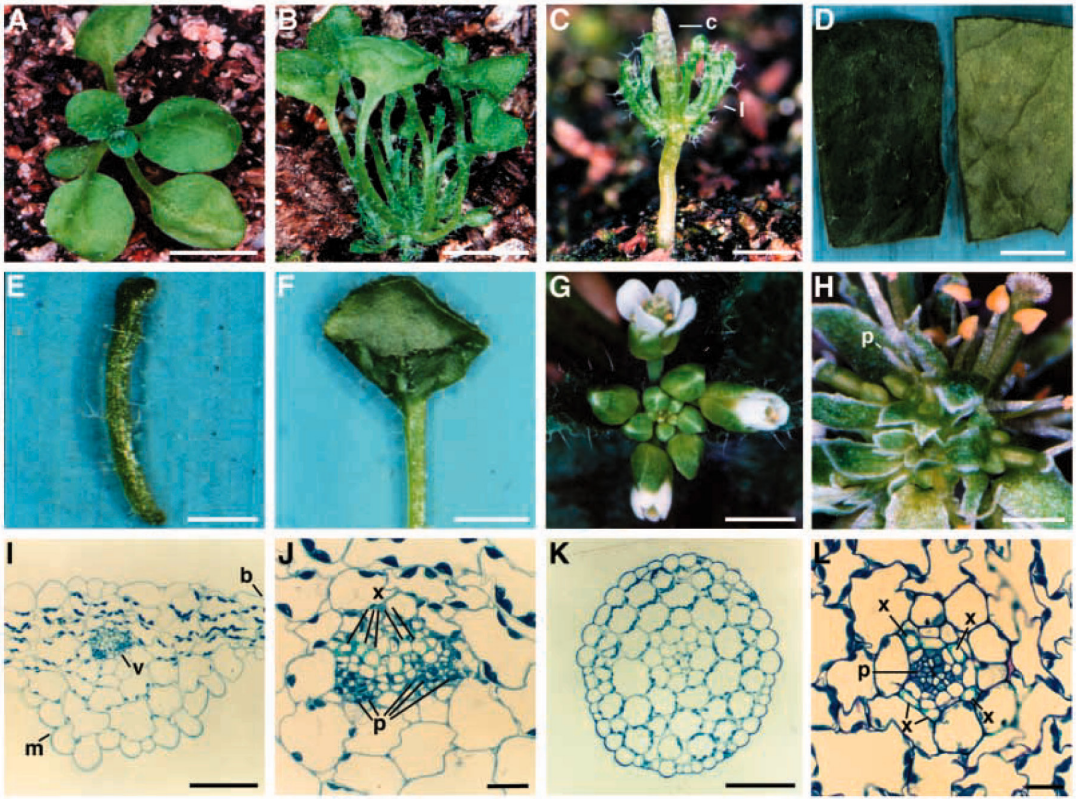


**Figure 8.** Leaves and floral organs of wild-type and *phb-1d* mutant of Arabidopsis.

**(A)** Wild-type rosette. Bar, 5 mm.
**(B)** Rosette of *phb-1d*/+ heterozygote. Note that leaves grow upward, with trumpet-like or rod-like shapes. Bar, 5 mm.
**(C)** Rosette of *phb-1d* /*phb-1d* homozygote. Foliage leaves (l) and cotyledons (c) are extremely radialized and grow vertically. Bar, 1.25 mm.
**(D)** Adaxial (left) and abaxial (right) side of a wild-type foliage leaf. The adaxial surface is glossy and dark green whereas the abaxial surface is matte, dull or pale green. Bar, 1.75 mm.
**(E)** Severely adaxialized *phb-1d* leaf. The glossy, dark-green surface characteristic of the adaxial surface extends around the circumference of the radialized leaf. The petiole is highly reduced. Bar, 1 mm.
**(F)** Less severely adaxialized leaf. This trumpet-shaped leaf exhibits adaxial characters on the outside of the cup. The inside of the cup has abaxial characters. Bar, 1 mm.
**(G)** Wild-type inflorescence. Bar, 2 mm.
**(H)** Inflorescence of *phb-1d*/+ ; sepals fail to enclose the developing flower (p, petal). Bar, 1.25 mm.
**(I)** Cross section of wild-type foliage leaf at midvein; adaxial surface is up (b, leaf blade; m, midrib; v, vascular tissue). Bar, 100 μm.
**(J)** Magnification of wild-type vascular tissue in midrib (x, xylem; p. phloem). Bar, 20 μm.
**(K)** Cross section of extremely radialized leaf of *phb-1d/* + heterozygote. Bar, 100 μm.
**(L)** Magnification of vascular tissue in a moderately radialized *phb-1d/* + leaf. Note that xylem cells surround phloem cells. Bar, 20 μm. Photographs are reproduced from McConnell and Barton (1998; Development 125, 2935-2942) with permission.

(*JLO; AT4G00220*), *TCP2* (AT4G18390), *TCP3* (*AT1G53230*), *TCP10* (*AT2G31070*), among others (Byrne et al. 2000; Semiarti et al. 2001; Iwakawa et al. 2002; Ha et al. 2003; Kumar et al. 2007; Guo et al. 2008; Schiessl et al. 2012). Some of these factors form protein complexes and function in the same genetic pathway (Xu et al. 2003; Li et al. 2012; Rast and Simon 2012). Others, however, are not specific repressors of the class I KNOX pathway; they have other specific roles, *e.g.*, in separate components of protein complexes. Although *AS1* and *AS2*, for example, form a protein complex (Xu et al. 2003), their loss-of-function phenotypes are not the same: *as1* and *as2* are somehow expressed as shrunken leaf blades with a few incomplete leaflets (Tsukaya and Uchiniya 1997); the *as1* phenotype is expressed as stunted leaf blades with very short petioles, whereas *as2* is responsible for an elongated leaf petiole (Semiarti et al. 2001). The *bop1 bop2* double mutant is expressed as a rather normal leaf blade in the upper region but is expressed as abnormally prolonged morphogenesis in the lower part of leaf blades and petioles (Ha et al. 2003), possibly due to misregulation of the meristematic junction region between blade and petiole (Ichihashi et al. 2011). BOP1 protein directly activates expression of *AS2* by binding to the *AS2* promoter (Ha et al. 2007; Jun et al. 2010), but it is still not clear why the *as2* and *bop1 bop2* phenotypes are different. Why does loss-of-function in the *BOP* genes have synergistic effects on *as1* and *as2* mutation

phenotypes? The answer may relate to local regulation at the leaf primordium-SAM boundary (discussed later: Fig. 6). Further study is needed to improve understanding of this very complicated system for suppression of the class I *KNOX* in leaf primordia.

## ESTABLISHMENT OF DORSIVENTRALITY AND LEAF-LAMINA IDENTITY

As indicated in the "Outline of events in Arabidopsis leaf development" section above, establishment of dorsiventrality is required for flat outgrowth of the lamina, and lack of either adaxial or abaxial identity in leaves results in rod-, lotus-leaf-, or trumpet-shaped leaves when the defect is partial (Fig. 8). The first establishment of dorsiventrality in leaf primordia is believed to depend on an unknown factor from the shoot apical meristem ("anlagen factor"). Not only is the identity of the factor obscure, we do not know whether or not it is indeed present (Efroni et al. 2010). Some of the compounds synthesized by catalytic activities of succinic semialdehyde dehydrogenase have been proposed as key elements to promote better understanding of the nature of this factor, since loss-of-function in this enzyme causes instability of the Ad-Ab border in Arabidopsis leaves (Toyokura et al. 2011). The factor may be a γ-aminobutyric acid (GABA) shunt metabolite, or related compounds that await identification.

Genetic mechanisms determining dorsiventrality or DV-axis formation are very complex (*e.g.*, reviewed by Kidner and Timmermans 2010) and appear even more so as studies proceed (*e.g.*, Nakata and Okada 2012: Fig. 5). Before moving on to the three-domain theory, I will first examine the classic two-domains view in which leaf primordia may be divided into adaxial and abaxial domains (Yamaguchi et al. 2012, see also Fig. 5). Briefly, these two domains are controlled by two distinct regulators. The class III Homeodomain-Zinc finger (HD-ZipIII) family [*PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV; AT5G60690*)] identifies the adaxial domain; the KANADI (KAN) family (*KAN1; AT5G16560* and *KAN2; AT1G32240*) (McConnell et al. 2001; Emery et al. 2003) and *ETTIN (ETT)/AUXIN RESPONSE TRANSCRIPTION FACTOR* (*ARF*)*3* (*AT2G33860*) and *ARF4* (*AT5G60450*) identify the abaxial domain (Pekker et al. 2005). The two domains suppress one another. HD-ZipIII is degraded by miR165/166, which is expressed in abaxial sides of leaf primordia (Emery et al. 2003). *ETT/ARF3* and *ARF4* are degraded by tasiR-ARF, which is derived from adaxially expressed *TRANS-ACTING CIS RNA3* (*TAS3; AT3G17185*) via the action of miR390 (*AT2G38325*; Garcia et al. 2006; Fahlgren et al. 2006; reviewed by Kidner 2010). KAN and HD-ZipIII suppress one another. These key factors are also regulated by many other factors, such as the AS1-AS2 protein complex (Iwakawa et al. 2002; Ishibashi et al. 2012). Importantly, *AS2* seems to act as a stabilizer of Ad-Ab polarity in leaf primordia by suppressing class I KNOX, *KAN* genes, and *ETT/ARF3* (Ishibashi et al. 2012; Fig. 5). Negative regulation of class I KNOX (see above) is also linked to the Ad-Ab regulation network via "junction genes" or "boundary genes" that express at organ boundaries. Since boundary genes have many important roles in plant organogenesis, not only in leaves but also in diverse developmental phenomena (*e.g.*, reviewed by Majer and Hochholdinger 2010), I shall briefly discuss the organ boundary issue later.

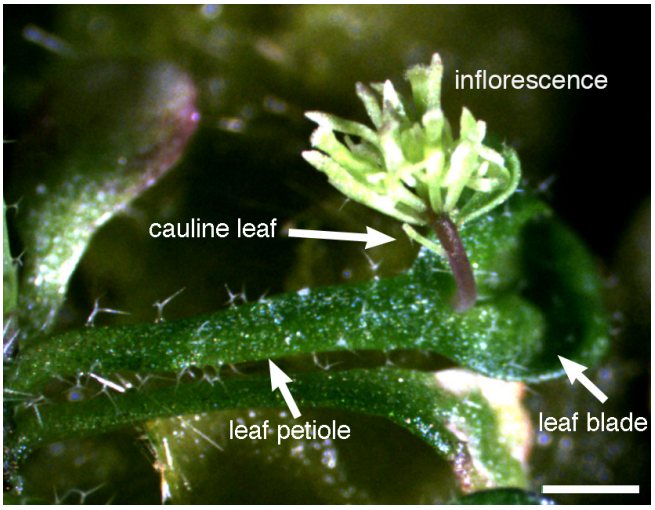


**Figure 9.** An epiphyllous inflorescence on foliage leaves of the Arabidopsis *yab3 fil* double mutant. Loss of activity in the leaf-lamina identifier YABBY gives deformed leaves the ectopic identity of a shoot apical meristem. Seeds were a gift from J. Bowman (Monash University, Australia). Bar, 1 mm.

Until a few years ago, the YABBY gene family was seen as a key component in establishing abaxial identity of leaf primordia, but detailed analyses of four vegetative YABBY genes (YABs: *FILAMENTOUS FLOWER* (*FIL=YAB1; AT2G45190*), *YAB2* (*AT1G08465*), *YAB3* (*AT4G00180*), and *YAB5* (*AT2G26580*)) proved that this is not the case (Sarojam et al. 2010). Before 2004, expression of this gene family in abaxial sides of leaf primordia promoted an early theory of abaxial identity control in leaves (Sawa et al. 1999; Siegfried et al. 1999). However the discovery of adaxially expressed members of *YABBY* in *Amborella trichopoda* Baill. (Yamada et al. 2004) and a member of *YABBY* in rice that specifically expresses in midrib of leaves (Yamaguchi et al. 2004) provided contradictory evidence. Sarojam et al. (2010) determined that even without the activity of the four vegetative *YABs* functions, developed leaves maintained dorsiventrality. Transcriptome analyses showed that the *fil yab3 yab5* triple mutant failed to activate gene expressions specific to the leaf lamina [represented by *CIN-TCP* genes: described later]] and did not repress the SAM-specific program represented by *WUS*. Indeed, when *miR-YAB* is driven by the leaf-primordia-specific promoter of *AINTEGUMENTA* (*ANT; AT4G37750*), "leaf primordia" express *WUS* at their tips. Similarly, double mutants of *fil yab3* ectopically develop inflorescence shoots on their leaves (Siegfried et al. 1999; Fig. 9). Auxin-related regulation is also disturbed in the loss-of-function YABs mutants. Thus, the four vegetative YABs are essential in switching from the SAM program to the leaf-specific program because they translate dorsiventral polarity into activation of leaf-lamina programs. The mechanisms may also be interpreted as conversion of dorsiventral axis information into the process of medio-lateral expansion. Lateral buds always occur in the junction regions between adaxial sides of leaves and the main stems; abaxial sides of leaves do not form epiphyllous buds,

other than in a few exceptional cases (*e.g.*, Okada et al. 1999). These classic observations might be linked to alternative regulation between leaf-lamina and SAM-specific programs.

I return now to the new model, the three-domain theory (Nakata et al. 2012: Fig. 5). Nakata et al. (2012) found that *PRESSED FLOWER* (*PRS*)/*WUSCHEL-RELATED HOMEOBOX* (*WOX*)*3* (*AT2G28610*) and *WOX1* (*AT3G18010*) genes are expressed in mid-sectors of leaf primordia (Figs. 3, 5). This 'middle domain' is a part of the abaxial domain (if we define it as the *FIL*-expressing domain), and *PRS, WOX1*, and *FIL* are expressed there. Since (i) loss-of-function in both *PRS* and *WOX1* causes instability in the establishment of Ad-Ab polarity and (ii) KAN family genes suppress the expression of *PRS* and *WOX1* (Nakata et al. 2012), this domain is important for establishment/maintenance of a previously recognized Ad-Ab axis in leaves. Interestingly, this domain overlaps the plate meristem, which supplies new cells to the leaf blade along the Ad-Ab border that disperses outward from the leaf tip (Donnelly et al. 1999; M. Nakata, personal communication). According to the three-domain theory, leaf primordia may be divided in three domains along the Ad-Ab axis from the top: (i) adaxial domain, which expresses *AS2* and HD-ZipIII (after elimination by miR165/166 in the other domains); (ii) middle domain expressing *PRS*, *WOX1*, and *FIL*; (iii) abaxial domain *sensu stricto*, which expresses KAN and *FIL.* Although the original concept of Nakata et al. (2012) treated the *FIL* as a key abaxial identity regulator, it was already known through the work of Sarojam et al. (2010) that the *FIL* is not the Ad-Ab polar identity gene, but is rather a leaf-lamina identifier. Therefore, it is also possible that the middle domain expressing *FIL* might be the central part of the leaf primordium possessing the leaf-lamina programs and is thus required for proper establishment of two-dimensional growth of leaf laminas and stability of the Ad-Ab axis. More detailed examination of this concept will provide clues that advance understanding of the relationship between dorsiventrality and leaf lamina growth.

In addition to the above factors, against expectations, ribosomal protein genes are involved in Ad-Ab regulation (Pinon et al. 2008; Yao et al. 2008; Horiguchi et al. 2011b). Interestingly, Horiguchi et al. (2011b) found that the amounts by which the numbers of cells per lamina level decrease and the levels of enhancement in the abnormality in Ad-Ab polarity are not always correlated among loss-of-function mutants of ribosomal genes, indicating that Ad-Ab polarity regulation by ribosomal genes is not directly linked to 'housekeeping' functions. The activities of so-called housekeeping genes drastically affect not only by dorsiventrality but also by the other developmental programs (*e.g.*, Nelissen et al. 2005; 2010; Barrero et al. 2007; Fleury et al. 2007; Mollá-Maňus et al. 2011). The ways in which these housekeeping genes are involved in such highly organized developmental programs are intriguing and need to be the subject of further investigation (reviewed by Tsukaya et al. 2013b).

## ROLES OF THE LEAF PRIMORDIUM-SAM BOUNDARY

As indicated above, the leaf primordium-SAM boundary seems to have an important function in the regulation of leaf polarities. Many genes express at the boundary and regulate leaf organogenesis (Figs. 3, 5). Here, I discuss some typical examples from three gene families: (i) the *LATERAL ORGAN BOUNDARY* (LOB) gene family, (ii) *BOP1* and *BOP2*, and (iii) CUC gene family.

*JAGGED LATERAL ORGANS* (*JLO*) is a member of the LOB family (Shuai et al. 2002) and is expressed in the leaf primordium-SAM boundary (Borghi et al. 2007: Figs. 3, 5); it functions in suppression of class I *KNOX* genes, such as *STM* and *KNAT1*, in the basal parts of leaf primordia through formation of the JLO-AS2 heteromer (Rast and Simon 2012). If JLO activity is lost, the SAM becomes inactive; weak alleles result in leaflet-like structures when combined with the *as2* mutation, owing to mis-expression of the *KNOX* genes in leaves (Rast and Simon 2012). Curiously, ectopic expressions of *STM* and *KNAT1* are induced in leaves under the influence of the 35S promoter, resulting in serrated and deformed leaves (Borghi et al. 2007). Why loss-of-function and ectopic overexpression result in the same mis-expression of the *KNOX* genes has yet to be resolved.

*BOP1* and *BOP2* are additional key boundary genes. Loss-of-function in the *bop1 bop2* mutant results in ectopic lamina formation in the place of the petiole (Ha et al. 2003) and in mispositioning of either the leaf blade-leaf petiole boundary or the proliferative zone of the leaf primordia (Ichihashi et al. 2010). *BOP1* and *BOP2* are expressed not only in the boundary but also on the adaxial sides of leaf primordium bases (Norberg et al. 2005; Jun et al. 2010; Figs. 3, 5). *BOP1* and *BOP2* suppress expression of class I *KNOX* genes in leaf primordia (Ha et al. 2004) and induce local expression of *AS2* on the adaxial sides of leaf primordium bases by direct binding of BOP1 to the promoter region of the *AS2* promoter (Jun et al. 2010). Ad-Ab polarity is disturbed in the *bop* mutant (Ha et al. 2007), indicating that the boundary region has some role in stabilization/maintenance of Ad-Ab polarity in leaves. Since *as2* and *bop1bop2* in combination have synergistic effects on leaf shape (Ha et al. 2003, 2007), there must be other components that are targets of *BOP* genes in the regulation of leaf morphogenesis.

The well-known organ boundary gene *CUC3* (*AT1G76420*) is also expressed in the boundary between leaf primordia and the stem, but we do not yet know whether it directly participates in leaf organogenesis or is otherwise involved in lateral bud formation (Aida and Tasaka 2006). To date, the organ boundary in shoot systems has been viewed as a key domain with meristematic activities. Similar concepts may be also applicable to all junctions/boundaries in the leaf primordium, such as the "leaf meristem" at the junction between the leaf blade and leaf petiole (Ichihashi et al. 2010: Figs. 4, 6) and boundaries between serrations (Kawamura et al. 2010; Bilsborough et al. 2011; Hasson et al. 2011: Fig. 7). For example, as mentioned above, outgrowth of serrations depends on the activity of *CUC2* in the leaf margin that stabilizes auxin maxima at the tips of the serrations (Kawamura et al. 2010; Fig. 7). Nevertheless, extrapolation to a generalized viewpoint is perhaps unwarranted given the abstract, conceptual nature of the current knowledge base. As indicated in the discussion of *JLO* and *BOP* above, the boundary appears to have more complex roles that influence not only the boundary itself but also neighboring organs. Further careful studies of the real roles of the "boundary genes" are awaited.

## POSITIVE AND NEGATIVE REGULATORS OF CELL PROLIFERATION IN LEAF PRIMORDIA

All leaf organogenesis depends on cell proliferation. Although the apical meristem is often believed to have the highest meristematic/cell proliferative activity, proliferation in leaf primordia is much greater than in the SAM. Without enhancement of cell proliferation, no lateral organ primordia would protrude from the apical meristem. Thus, importantly, factors that accelerate cell proliferation are indispensable for organogenesis in leaf primordia.

To date, many genes have been postulated as controllers of proliferative activity in leaf primordia. *ANT* (Mizukami and Fischer 2000), the *Arabidopsis thaliana GROWTH REGULATING FACTOR* (*AtGRF*) family (*e.g., AtGRF5* (*AT3G13960*): Kim and Kende 2004; Horiguchi et al. 2005; Lee et al. 2009), which are regulated by miR396 (Rodriguez et al. 2010), *AN3/AtGIF1* (Kim and Kende 2004; Horiguchi et al. 2005), *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*; AT3G59900: Hu et al. 2003), *DA1* (Li et al. 2008), G protein $\gamma$ subunit (AGG3; AT5G20635) (Li et al. 2012), and *STRUWWELPETER* (*SWP*; AT3G04740: Autran et al. 2002) are positive regulators. *SPATULA* (*SPT*; AT4G36930: Ichihashi et al. 2010) and the *ROT4-Like* (*RTFL*)/*DVL* family (Narita et al. 2004; Wen et al. 2004), among others, are negative regulators. Of course, in addition to specific regulators for lateral organs, general factors or 'housekeeping genes' affect the activity of cell proliferation in leaf primordia; these include ribosomal genes (Fujikura et al. 2009) and the H+-pyrophosphatase gene (*AVP1; AT1G15690*: Ferjani et al. 2011). New reports of novel genes regulating cell numbers in the leaf blade are increasing annually.

Beyond advancing understanding of the molecular roles of components listed above, it is essential that we tackle the more important issue of the mechanisms that control meristematic activity in the leaf primordia. The concept of the arrest front is useful for schematic understanding, but the molecular background is confused. As noted above, although it was previously thought that the arrest front "gradually moves from the tip to the base of leaf primordia," Kazama et al. (2010) disproved this. The cyclic arrest front is held at a constant position in the leaf primordium and then suddenly moves to the base of the blade primordium. Movement of CIN-TCPs mRNA expression over time was once speculated to operate in controlling the cyclic arrest front (Nath et al. 2003), but Efroni et al. (2008) elegantly showed that the CIN-TCPs are not directly involved in control of cell proliferation; rather, they are heterochronic regulators, i.e., CIN-TCPs are factors for the change from a phase of lateral and distal expansion via active cell supply to a phase of differentiation in functional leaves [Efroni et al. (2008) used the terms "primary morphogenesis" (PM) phase and "secondary morphogenesis" (SM), respectively, for these two phases, but since those authors stated that "the understanding of SM regulation is even more fragmented," I do not use these terms here]. In 2013, Efroni et al. (2013) reported that the CIN-TCPs reduce sensitivity of leaf primordia to cytokinin via SWI/SNF chromatin remodeling ATPase, BRAMA (BRM; AT2G46020). They showed that TCP4 and BRM bind the promoter of *Arabidopsis thaliana RESPONSE REGULATOR4* (*ARR4*; *AT1G10470*), an inhibitor of cytokinin response, resulting induction of *ARR4.* The reduced sensitivity to cytokinin by expression of the *ARR4* is thought to link to differentiation in leaf primordia (Efroni et al. 2013). However, we still do not know whether CIN-TCPs are the only regulators of the phase transition.

The AN3/AtGIF1-AtGRF system, for example, is also a candidate for phase shift control. The co-activator AN3/AtGIF1 and the transcriptional factor AtGRF5 are expressed in the proximal, cell-proliferating zone of leaf primordia (Horiguchi et al. 2005; Ichihashi et al. 2011), whereas *miR396*, which degrades *AtGRF* mRNA, begins expressing in the distal part of leaf primordia and increases expression level through leaf maturation (Rodriguez et al. 2010). This dipolar system might also be a key regulator in the shift from the cell proliferative phase to the cell expansion and differentiation phase.

Andriankaja et al. (2012) demonstrated that chloroplast differentiation is required to trigger cell expansion in leaves. A retrograde signal from the chloroplast to the nuclei may be involved in this mechanism. Mutational analyses also indicate that mitochondrion- and/or chloroplast-dependent signals play roles in the development of leaf architecture in Arabidopsis (*e.g.,* Wetzel et al. 1994; Hricová et al. 2006; Quesada et al. 2011). But, even in plants treated with norflurazon, the onset of cell expansion in leaves is delayed although not cancelled (Andriankaja et al. 2012), suggesting that other components are also important in the phase shift.

## INTEGRATED REGULATION OF CELL DIVISION AND CELL EXPANSION

Some mutations or transgenics have increased numbers and sizes of cells in the leaf lamina (*e.g.*, ectopic overexpression of *ORGAN SIZE RELATED1: ORS1*; AT2G41230; Feng et al. 2011); most small-leaved mutants have similar decreases in both numbers and sizes of cells (Horiguchi et al. 2006). Among leaves of diverse species, different numbers and sizes of cells produce different leaf sizes, indicating that there are species-specific and organ-specific determination mechanisms for both the numbers and sizes of cells across organs and taxa. Are cell proliferation and cell expansion independently regulated? Is organ size merely the sum of cell size and cell number? In a leaf primordium, very active cell proliferation occurs in a region proximal to the arrest front and cell expansion occurs above the arrest front (as indicated above) giving a first impression that regulation of cell proliferation and cell expansion are independent spatially and temporally. However, this is not the case.

Compensation is a phenomenon that demonstrates the presence of integration systems that link levels of cell proliferation to those of cell expansion in leaves (and floral organs derived from leaves); this is defined as an abnormal cell volume increase triggered by defective cell proliferation in leaf primordia (Tsukaya 2002a). Interestingly, reverse relationships (*e.g.*, increased numbers of cells triggered by decreased cell volume, or decreased cell volume triggered by increased number of cells) do not occur. For example, loss-of-function in *AN3/AtGIF1* results in fewer, larger cells in leaves, whereas overexpression of *AN3/AtGIF1* results in many more cells with normal volumes (Horiguchi et al. 2005: Fig. 10). Although some mutants develop leaves with larger numbers of small cells, cell size and cell number do not have a mutual

causal-result relationship in such cases (Usami et al. 2009; see the "Heteroblasty" section below). Reports of compensation or compensation-like phenotypes are increasing rapidly. The phenomenon is not Arabidopsis-specific, indicating that compensation is a general phenomenon, at least in angiosperm leaves and floral organs (Horiguchi and Tsukaya 2011). The first discovery of the mechanism known as compensation and its general occurrence in Arabidopsis leaves and floral organs (Tsukaya 2002a; Beemster et al. 2003) were misinterpreted as proof of the Organismal Theory (*e.g.,* Hemerly et al. 1995), which proposes that organ size is determined by some unknown genetic pathways acting directly on organs and independently of the behavior of cells (Kaplan and Hagemenn 1991; reviewed in Tsukaya 2002a). It was also wrongly believed that compensation may be explained as an uncoupling of cell division and cell expansion, i.e., a passive result of fewer divisions leading to increased cell volume. Tsukaya (2002a) demonstrated that compensation is not proof of the Organismal Theory, but instead supports a well-known cell theory, which proposes that all organogenesis is based on cells. The cell becomes a unit of morphogenesis when we introduce the concept of cell-cell communication ("Neo-cell Theory": Tsukaya 2002a). Indeed, compensation or compensated cell enlargement occurs in many cases [except in the case of the *KIP-RELATED PROTEIN2* (*KRP2; AT3G50630*) over-expressor (Ferjani et al. 2007)] only after the exit from mitosis, ruling out an interpretation based on a possible uncoupling of cell division and cell expansion. *JAG* is a key factor that links the cell size and the cell cycle during the mitotic phase in floral organ development (Schiessl et al. 2012). Moreover, mutants with mild cell proliferation defects do not trigger compensation, but double mutants that express severe decreases in cell numbers do (Fujikura et al. 2009), suggesting that compensation cannot be triggered unless a severe defect crosses a threshold line. It is unlikely that the total number of cells per lamina can be used as a criterion of severity because compensated cell enlargement occurs in the distal area of a leaf primordium even when active cell proliferation is ongoing in the proximal, meristematic area of the leaf primordium.

How then is a defect in cell proliferation translated into enhanced cell expansion? Interestingly, Kawade et al. (2010) showed that this occurs by cell-cell communication (Fig. 10). When *AN3/AtGIF1* overexpressing cells are introduced into an *an3* mutant leaf in a chimera-like configuration, both *an3* mutant cells and *AN3/AtGIF1* overexpressing cells have levels of compensated cell enlargement closely similar to that in *an3* mutant leaves (Kawade et al. 2010). Curiously, this unknown signal appears unable to pass through the midrib regions of leaves. Moreover, if the same chimera experiment is performed using an over-expressor of *KRP2*, a negative regulator of the cell cycle,

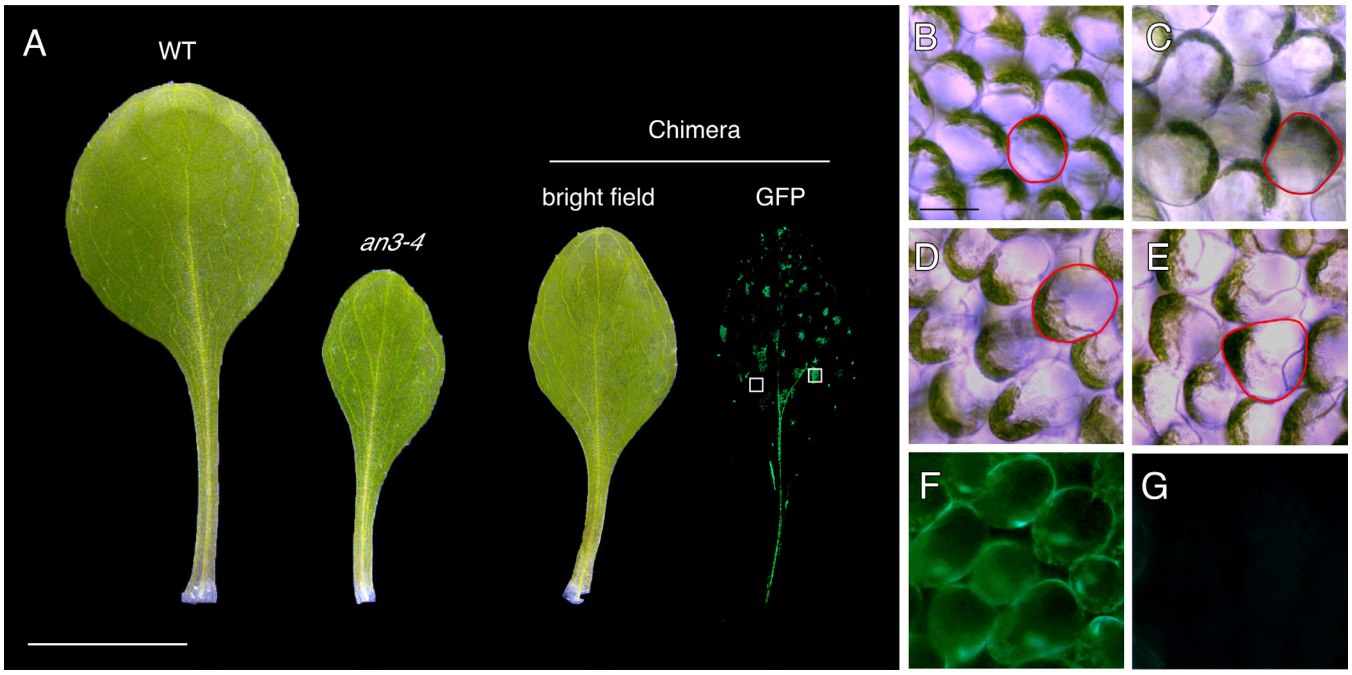


**Figure 10.** Compensation is regulated non-cell autonomously. (A) Leaf morphology and size of a wild-type leaf, an *an3-4* leaf, and a chimera leaf harboring both the *an3-4* mutant and *AN3/AtGIF1*-overexpressing (marked by GFP expression) cells in the configuration of a spotted chimera. Bar, 5 mm. (B-G) Micrographs of subepidermal palisade cells of the wild type (B), *an3-4* (C), and the chimera (D-G). (F and G) Fluorescent microscopy of the cells in (D) and (E), respectively. Bar, 50 μm. The *an3-4* leaves have much larger cells (C) than do wild-type leaves (B), showing a typical compensation. Note that in the chimera leaf, both *an3-4* cells (D, F) and AN3::GFP-overexpressing cells (E, G) expand to the same extent as the cells in the *an3-4* mutant leaf (C), indicating non-cell-autonomous regulation of compensated cell enlargement. In a chimera harboring both the *an3* mutant and *AN3/StGIF1*-overexpressing (marked by GFP expression) cells, both cells have the same level of compensated cell enlargement as cells of *an3* mutant leaves. Figures courtesy of K. Kawade.

a different outcome is obtained. In chimeric leaves, *KRP2*-overexpressing cells have typical compensation, but wild-type cells do not undergo any abnormal enlargement processes, even when neighboring *KRP2* overexpressing cells (Kawade et al. 2010). Thus, once more, it appears that there are several types of compensation in mechanisms that are discernible in comparative kinematic analyses of cell behaviors in leaf primordia among compensation-exhibiting mutants/transgenics (Ferjani et al. 2007). The most important unsolved question is: what is the cell-cell signal that links defective cell proliferation with enhanced cell expansion? It may be physical force, a chemical signal, or something quite different. The answer to this question will aid in elucidating the mechanisms that determine the shapes and sizes of multicellular organs, at least for seed plants.

Is compensated cell enlargement a special cell expansion system differing from normal cell expansion? Fujikura et al. (2007) demonstrated that *an3*-dependent compensated cell enlargement in leaves depends on a subset of normal cell expansion pathways. The next unsolved question is: how and why is only a subset of normal cell expansion pathways enhanced in compensated cell enlargement?

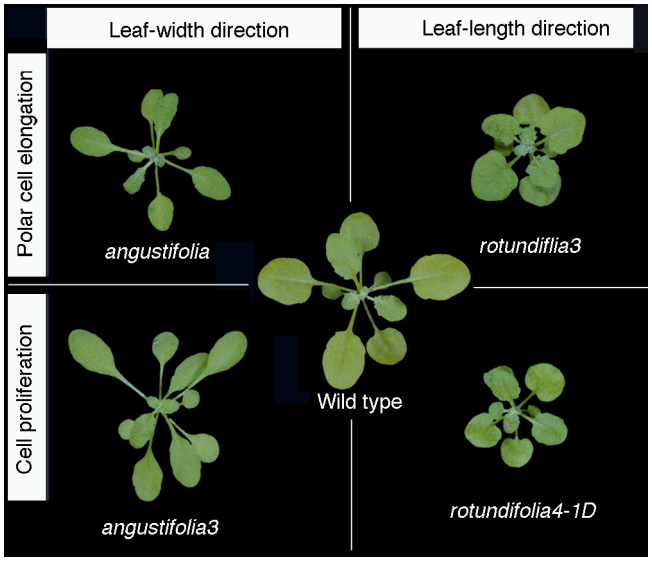


**Figure 11.** Genes that control the leaf index value in Arabidopsis. See text for details. Photograph is reproduced from Tsukaya (2005; Int. J. Dev. Biol. **49:** 547-555) with permission.

## TWO-DIMENSIONAL LEAF-LAMINA GROWTH

Leaf size is largely determined by behaviors of the cyclic arrest front, activities of cell proliferation, and cell enlargement. To date, we have accumulated much knowledge on genetic controls of these processes. But how is leaf-shape controlled? It is affected significantly by dorsiventrality, although most of the natural diversity in leaf shape is independent of alterations in dorsiventral controls except for the case of compound leaves (Kim et al. 2003). The most frequently seen differences in leaf shape between closely related species are in the numbers and depths of serrations and in the leaf index (leaf length / leaf width ratio) (Tsukaya 2002b; 2005). We have already seen (in the section 'Outlines of Developmental Events') how the patterning and depths of leaf serrations are determined in Arabidopsis. Below, the mechanisms of leaf index control are considered.

When Tsuge et al. (1996) reported on independent effects of *AN* and *ROTUNDIFOLIA3* (*ROT3; AT4G36380*) genes on leaf shape, they postulated that leaf index is largely determined by a balance between polar-dependent cell expansion systems. Thus, *AN* regulates lateral expansion of leaf cells whereas *ROT3* regulates longitudinal cell expansion. This concept is closely congruent with extensive information available on the histology of 'rheophyte' ferns in tropical forests; these ferns have narrower leaves than closely related species and can thus grow along banks of rivers where frequent floods disturb growth (reviewed by Tsukaya 2002a, b). But we also now know that rheophyte angiosperms or seed plants have narrower leaves, which result from altered distributions of leaf cells and not from polarity-dependent cell expansion; this mechanism differs from that of rheophyte ferns (Tsukaya et al. 2002b).

Indeed, four genes influence the leaf index in Arabidopsis through effects on cell numbers or cell shape along two axes, the lateral and the longitudinal (Tsukaya 2005, 2006: Fig. 11). Different from *AN* and *ROT3*, the *an3* mutant has narrower leaves owing to a defect in appropriate leaf lamina cell proliferation (Horiguchi et al. 2011a) as indicated above; *ROT4* influences leaf length through a negative effect on the cell proliferation zone in leaf primordia (Narita et al. 2004; Ikeuchi et al. 2011). *LONGIFOLIA*(*LNG*)*1*(*AT5G15580*)*/LNG2*(*AT3G02170*) (Lee et al. 2006) also controls leaf length. Overexpression of *LNG* genes results in longer, more slender leaves and loss-of-function *lon* mutants have shorter leaves due to stunted cell elongation. Among these genes, only the precise molecular function of *ROT3* is known: it encodes cytochrome P450 (CYP90C1), which catalyzes brassinosteroid C23 hydroxylation redundantly with CYP90D1 (AT3G13730), resulting in biosynthesis of active forms of brassinosteroids (Ohnishi et al. 2006). As brassinosteroids control both number and size of leaf cells in general (Nakaya et al. 2002), the specific effect of ROT3 on the longitudinal cell expansion in leaves is of interest. Since *ROT3* is also a component of control in petiole elongation in the shade-avoidance syndrome (see the "Environmental control of leaf shape" section below), some of the intermediate compounds may have hidden functions other than the well-known 'biological activity' among species of brassinosteroids.

As indicated above, the co-activator AN3/AtGIF1 has many roles. Although the *an3* mutant has narrow leaves, it is important to note that AN3 does not directly affect the direction/pattern of the cell division plane (Horiguchi et al. 2011a)). Rather, loss-of-function in *AN3* specifically affects cell proliferation activity in phase II mitosis when longitudinal and oblique divisions increase at the expense of transverse divisions that are dominant in the earlier phase (phase I) (Horiguchi et al. 2011a). AN3 regulates not only coordinated cell proliferation activity among tissue layers of leaf primordia by its intercellular movement (Kawade et al. 2013), but also dorsiventrality of leaves (Horiguchi et al. 2005; 2011a) and the identity of cotyledons through suppression of expansion in the expression domain of *PLETHORA1* (*PLT1; AT3G20840*)

(Kanei et al. 2012). Many genes are directly or indirectly regulated by this gene (Horiguchi et al. 2011a). Further detailed studies are awaited to enable fuller understanding of the roles of AN3.

The molecular roles of genes other than *ROT3* and *AN3* in leaf morphogenesis remain enigmatic. AN is a member of C-terminal Binding Protein (CtBP)/Brefeldin-A-ADP ribosylated substrate (BARS), for which we have only an incomplete understanding (Chinnadurai 2006). This was first postulated to be a co-suppressor, as is the case for animal CtBP members (Folkers et al. 2002; Kim et al. 2002). Comparative analysis of *Drosophila* CtBP and AN demonstrated that they differ in molecular function within *Drosophila* embryos (Stern et al. 2007). Detailed analysis has shown that AN is localized in the trans-Golgi network (TGN) and a nuclear localization signal (NLS) is not necessary in the coding region (Minamisawa et al. 2011). Since the *an* mutant has an abnormal distribution of plant-specific cytoskeletal structure expressed in leaf cortical microtubules (Kim et al. 2002), AN may have acquired plant-specific functions during evolution that differ from those of CtBP and BARS.

*ROT4* is a curious gene that encodes a peptide of 6.2 kDa without a signal sequence for secretion (Narita et al. 2004); it has 23 paralogs (*ROT-FOUR-LIKE* (*RTFL*)*/DEVIL* (*DVL*)) in the Arabidopsis genome (Tsukaya et al. 2013a). When fused with GREEN FLUORESCENT PROTEIN (GFP), regardless of whether the fusion site is in the C-terminal or N-terminal, fusion proteins are localized on the plasma membrane (Narita et al. 2004; Ikeuchi et al. 2011). In wild-type leaf primordia, *ROT4* seems to express at a very limited level in the proximal region; if ectopically overexpressed with Cauliflower Mosaic Virus 35S promoter, it causes stunted leaves, short stems, arrowhead-like deformation of fruits (Narita et al. 2004), and abnormal protrusion of the main stem at the bases of pedicels that appear to be ‘bends’ in the pedicels (Ikeuchi et al. 2011). Since paralogs are believed to share the same function when overexpressed (Wen et al. 2004), loss-of-function mutant phenotypes are as yet unknown. However, phenotypic occurrences of chimeric ROT4 overexpression in leaves, and abnormal protrusions of main stems caused by 35S-driven overexpression (Ikeuchi et al. 2011) suggest that the ROY4 peptide is involved in determination of longitudinal positional cueing in shoots. The RTFL/DVL family occurs in the genome of *Selaginella moellendorffii* Hieron. (Lycopodiophyta) as a single copy (Floyd and Bowman 2007) and is extensively duplicated in dozens of angiosperm genomes. Detailed functional analysis of these genes will promote better understanding of the ways in which genetic mechanisms involved in longitudinal growth of the angiosperm shoot have evolved.

## ENVIRONMENTAL CONTROL OF LEAF SHAPE

Adaptation of leaf shape to environmental factors is a fundamental component of physiological function in plants because leaves are the primary organs of photosynthesis. Thus, final leaf size and shape are adjusted to the intensity and direction of light, and to the direction of gravity. Under weak light, leaf blades are underdeveloped and leaf petiole elongation is promoted (Fig. 12). This is part of the “shade-avoidance” syndrome, which is also expressed in extensive elongation of stems and hypocotyls. The syndrome


**Figure 12.** Gross morphology of rosette and flowering plants of the wild type (left) and the *phyB-9* mutant (right). Plants were cultivated at 22°C under 12 hours of strong light and 12 hours of darkness daily. Note the altered growth of the leaf lamina and leaf petiole between wild type and the *phyB-9* mutant. Bar, 5 mm.

typically occurs in a loss-of-function mutant of the photoreceptor gene *PHYTOCHROMEB* (*PHYB; AT2G18790)* (Tsukaya et al. 2002; Kozuka et al. 2005). In Arabidopsis leaves, both blue and red light are important for controlling shade avoidance. Blue and red light have different effects on the control of leaf shape (Kozuka et al. 2005). Interestingly, opposite reactions of leaf blades and petioles to the same light condition are not attributable to simple contrasting reactions to the same trigger. Transcriptome analysis of leaf petioles and blades treated with end-of-day far-red light (EODFR) demonstrate that nearly half of the genes induced by this treatment in both parts of the leaves are auxin-responsive; BR-responsive genes are also overrepresented among EODFR-induced genes (Kozuka et al. 2010). Contributions of auxin and BR to shade-avoidance-dependent petiole elongation have also been confirmed by genetic studies of auxin- and/or BR-deficient mutants. Interestingly, spotlight irradiation experiments indicated that phytochrome in leaf blades regulates petiole elongation, but phytochrome in petioles does not (Kozuka et al. 2010), a result that is similar to the control of stem elongation by light perceived by leaves (Black and Shuttleworth 1974).

Light information is not used in only in an on/off manner. Leaves developing under high light intensity are thick ("sun leaves"), whereas leaves developing under low light intensity, such as on the forest floor, are thin ("shade leaves"). The thickness of leaf palisade cells is also under the control of light intensity (Björkman 1981); this is an important phenotypic plasticity trait that maximizes the efficiency of photosynthesis activity. Phototropin 2 (PHOT2; AT5G58140) is the major photoreceptor regulating high-light-dependent elongation of palisade cells in the direction of leaf thickening or of the Ad-Ab axis in a tissue-autonomous manner (Kozuka et al. 2011).

Gravity is also an important environmental factor for leaves because it provides key information on the expected direction of illumination. The direction of light (or the sky) is important for the rosette life form of Arabidopsis, which lacks elongated stems in the vegetative stage. On slopes and stone walls, rosette plants are able to grow on surfaces that are not horizontally flat. How do Arabidopsis plants determine leaf position under such circumstances? The positions of Arabidopsis rosette leaves are in fact determined by combined responses to light and gravity (Mano et al. 2006). Intriguingly, the radial positioning of rosette leaves is not affected by the direction of gravity under continuous white light and depends on the orientation of the shoot axis only (Fig. 13). In contrast, when Arabidopsis plants are shifted to darkness, rosette leaves have negative gravitropism and nastic movement (Fig. 13). The positioning of Arabidopsis rosette leaves is determined by sum of these controls.


**Figure 13.** Environmental control of the direction of rosette leaf radial positioning in Arabidopsis. A-C, Position of rosette leaves under continuous light illuminated from all sides. Plants were grown on transparent gel in a normal position (A), after a 90˚ rotation (B), or inversion (C). D, Schematic model of leaf movement in darkness, grown in a normal position (left) or inverted (right). Figures are reproduced and modified from Mano et al. (2006; Plant Cell Physiol. 47: 217-223) with permission.

## HETEROBLASTY

Understanding of molecular mechanisms in heteroblasty has progressed rapidly in recent years (reviewed in Poethig and Scott 2010). Several morphological characters in Arabidopsis are affected by heteroblasty (i.e., age-dependent change in leaf traits; heteroblasty reflects a phase change from juvenile to adult form during the vegetative period). In the juvenile phase, leaves are smaller, rounded, have trichomes only on the adaxial side, infrequent serrations or hydathodes along the margin, and small numbers of large volume cells (Fig. 14). In contrast, adult-

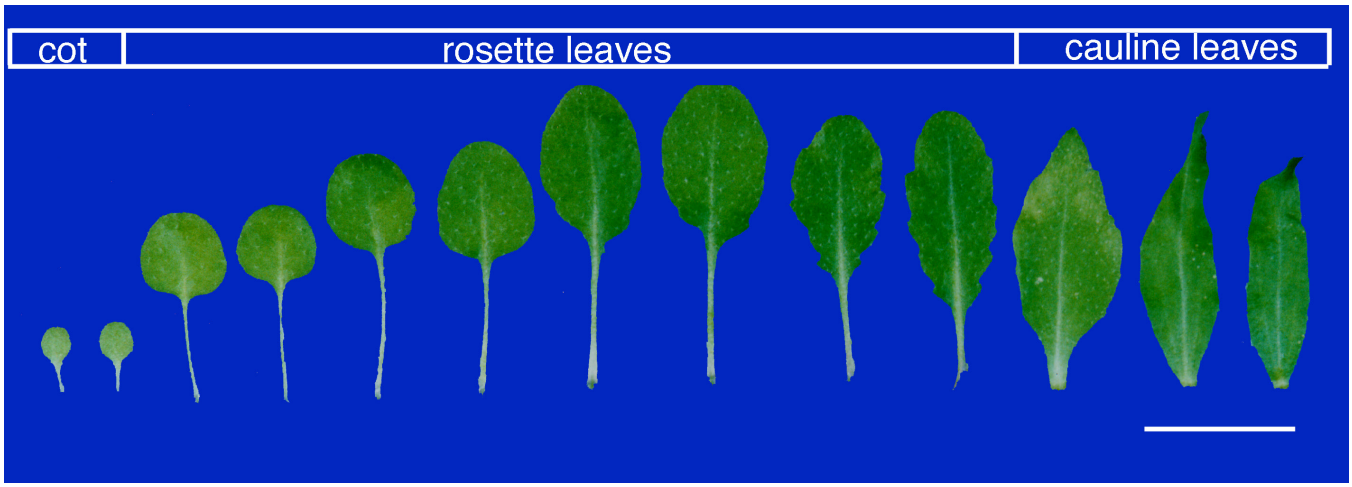


**Figure 14.** Heteroblasty in Arabidopsis (Columbia wild type) under continuous light at 22°C. The image shows gradual changes in the shape of leaves. From left: two cotyledons (cot), eight foliage, rosette leaves (rosette leaves), and three cauline leaves. Foliage leaves and cauline leaves are arranged from left as: first foliage leaf; second foliage leaf; third, fourth ... eighth foliage leaf; first, second, and third cauline leaf. Bar = 5 mm. Reproduced from Tsukaya et al. (2000; Planta 210, 536-542) with permission.

phase leaves are larger, elongated, have trichomes on both adaxial and abaxial surfaces, frequent serrations or hydathodes, and large numbers of small-volume cells (Kersteller and Poethig 1998; Tsukaya et al. 2000; Tsukaya and Uchimiya 1997; Usami et al. 2009). Two distinct genetic pathways are currently known to switch between the juvenile and adult phases of Arabidopsis. One is a pathway of *miR156*-mediated regulation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes and the other is a pathway of *tasiR-ARF*-mediated regulation of *ETT/ARF3* and *ARF4*, which also regulates dorsiventrality of leaves (Wu and Poethig 2006). Only the former pathway regulates cell size and cell number per leaf blade in Arabidopsis (Usami et al. 2009), while both regulate traits of gross morphology, such as the trichome pattern and overall shape. Interestingly, the *miR156-SPL* system also regulates *miR172*, which targets *TARGET OF EARLY ACTIVATION TAGGED* (*TOE1; AT2G28550*) and *TOE2* (*AT5G60120*) that control flowering, that is another phase change in plant development (Wu et al. 2009). Since *miR172* acts downstream from *miR156*, which regulates the expression of *miR172* via *SPL9* and *SPL10* mRNA levels that positively regulate the expression of *miR172b*, there is a negative feedback loop in the control of juvenile-adult phase changes. Importantly, a leaf-derived signal, purportedly a product of photosynthesis, represses expression of *miR156*, which is a key microRNA required for degradation of *SPL* genes that promote expression of the adult phase (Li et al. 2011).

## CONCLUDING REMARKS AND PERSPECTIVES

Most studies on mechanisms of leaf development have focused on particular components of organogenesis. However, many unsolved enigmas remain. The nature of the factor(s) that links cell proliferation to cell expansion in leaf primordia, for example, is still problematic. We do not know what the anlagen factor is, nor do we understand the process by which the cyclic arrest front is regulated. The way in which the leaf petiole and leaf blade differentiate from a common meristematic region between them also remains unexplained. These are not the only important unknowns in plant foliar morphogenesis. Other, untouched themes are important too, but remain unstudied simply because the research community is too small to tackle them at the present time (see Fig. 1 of Tsukaya 2006). For example, the mechanisms determining the shapes of apices or bases of leaf blades (acute, obtuse, emarginated, or aristate for apices; attenuate, cuneate, obtuse, rotund, truncate, reniform or sagittate for the bases) are not at all understood. Factors determining whether leaf blades are decurrent along the petiole remain enigmatic, although there are clues (*e.g.*, ectopic overexpression of *LEAFY PETIOLE* (*LEP; AT5G13910*) causes the leaf lamina to be decurrent, but this is a case of ectopic expression: van der Graaff et al. 2000). Why polyploidy affects leaf size is also unclear (Tsukaya 2008). Although many reports (*e.g.*, Breuer et al. 2007) indicate that endoreduplication is important for proper leaf size gain in Arabidopsis, many other species, such as lettuce and rice, never exhibit endoreduplication in their leaves (Barow and Meister 2003), indicating that endoreduplication is not necessarily linked to development by default. What types of modifications in genetic systems linked the endoreduplication process to a basic program of leaf development during the evolution of Arabidopsis? The processes regulating leaf thickness have been only partially explored. Leaf thickness is under the control of light intensity, temperature, and other environmental variables. Light-intensity-dependent leaf-thickness control is a very important plastic mechanism in plants that might be used to manipulate photosynthetic ability in plants that are useful to humans. Interestingly, at least in *Chenopodium album* L., a

long-distance signal(s) from mature leaves regulates thickness of emerging new leaf primordia (Yano and Terashima 2004). But we do not know the nature of the signals or those of the downstream pathways. Usually we cultivate Arabidopsis under very low intensity of light in the laboratory (*e.g.*, 60 μmol photons/$m^2$/s), but wild Arabidopsis grows under strong light (1000–2000 μmol photons/$m^2$/s); thus at present our knowledge of morphogenesis in this plant is restricted to very unusual conditions. Detailed analyses of leaf morphogenesis under high light are expected to produce important results.

During the writing of this second edition of my "Leaf Development" review chapter for the *Arabidopsis Book*, 10 years after releasing the first edition in 2002, I found that much knowledge has accumulated in this research field and some of the unresolved enigmas have been solved. Studies on leaf development in Arabidopsis have increased in this past decade, as described in the Introduction. I hope many more enigmas will be resolved in the next 10 years.

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