RESEARCH ARTICLES

The Arabidopsis Auxin-Inducible Gene *ARGOS* Controls Lateral Organ Size

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During plant development, the final size of an organ is regulated and determined by various developmental signals; however, the molecular mechanisms by which these signals are transduced and the mediators involved are largely unknown. Here, we show that *ARGOS*, a novel Arabidopsis gene that is highly induced by auxin, is involved in organ size control. Transgenic plants expressing sense or antisense *ARGOS* cDNA display enlarged or reduced aerial organs, respectively. The alteration in organ size is attributable mainly to changes in cell number and the duration of organ growth. Ectopic expression of *ARGOS* prolongs the expression of *AINTEGUMENTA* (*ANT*) and *CycD3;1* as well as the neoplastic activity of leaf cells. Moreover, organ enlargement in plants overexpressing *ARGOS* can be blocked by the loss of function of ANT, implying that ARGOS functions upstream of ANT to affect the meristematic competence of organ cells. The induction of *ARGOS* by auxin is attenuated or abolished in *auxin-resistant1* (*axr1*), and overexpression of *ARGOS* partially restores *axr1* organ development. These results suggest that ARGOS may transduce auxin signals downstream of AXR1 to regulate cell proliferation and organ growth through ANT during organogenesis.

INTRODUCTION

Developmental control of plant morphogenesis entails the coordination of cell growth, cell division, and cell differentiation; undoubtedly, organ size is one of the most obvious reflections of this coordination (Mizukami, 2001). Organ size is determined by both cell size and cell number. Genetic studies have shown that differences in cell size or cell polar elongation apparently contribute to the size difference of plant organs (Kim et al., 1998, 2002; Kondorosi et al., 2000). On the other hand, larger organs tend to contain more cells than smaller organs, implying that cell division plays a fundamental role in organ size determination during organogenesis. Indeed, some mutants with altered organ size, such as *struwwelpeter* and *phantastica* (Waites et al., 1998; Autran et al., 2002), show a decreased or increased cell number in their organs. However, there are cases in which the alteration of cell proliferation is not correlated with changes in organ size. For example, expression of a dominant-negative Arabidopsis CDKA in transgenic tobacco results in almost normal-sized leaves with fewer but larger cells (Hemerly et al., 1995). In Arabidopsis, overexpression of *CycD3;1*, a G1 cyclin gene, does not increase organ size but leads to a disturbed organogenesis, with numerous small, incompletely differentiated cells (Riou-Khamlichi et al., 1999; Dewitte et al., 2003). Similar data were obtained from overexpression of E2Fa and DPa, two transcriptional factors that play a role in activating cell division genes. Coexpression of E2Fa and DPa in Arabidopsis causes extra cell division, but plant growth

was arrested early (De Veylder et al., 2002). These observations suggest the existence of an intrinsic mechanism to coordinate cell proliferation and growth by which organ development is strictly controlled (Beemster et al., 2003).

The aerial organs of plants are derived from the primordia initiated from apical and lateral meristems. Significant changes in the morphology and size of organs occur when the specification or growth of these meristems or primordia is disorganized or interrupted. A number of genes involved in this developmental process have been identified and characterized, such as *WUSCHEL*, *CLAVATA*, and *SHOOT MERISTEMLESS* (Meyerowitz, 1997; Golz and Hudson, 2002). Nevertheless, lateral organ growth appears to rely on the interactive and durable division of cells within organ or organ meristems (Mizukami, 2001). Although a plant cell can be maintained in a meristematic state (Weigel and Jürgens, 2002), the determinate organ destines these cells to stop dividing as an organ develops. Thus, cell meristematic competence appears to be critical to cell proliferation within an organ and thereby controls organ size (Mizukami, 2001).

Recent studies of Arabidopsis *AINTEGUMENTA* (*ANT*) apparently strengthen this view. ANT seems to function as a coordinator of cell proliferation and lateral organ development. Loss of function of ANT reduces the size of leaf and floral organs (Elliott et al., 1996; Klucher et al., 1996; Mizukami and Fischer, 2000), whereas the ectopic expression of *ANT* increases the sizes of leaf, inflorescence stem, and floral organs. These alterations result mainly from changes in total cell number (Krizek, 1999; Mizukami and Fischer, 2000). Further examination reveals that ANT does not affect the growth rate but regulates the extent of organ growth by maintaining the meristematic competence of organ cells, thereby increasing the intrinsic organ size (Mizukami and Fischer, 2000). To date, little is known about the molecular nature of meristematic compe-

tence (Weigel and Jürgens, 2002). In addition, given the sessile nature of plants and their light-dependent growth, organ size in plants also is influenced greatly by environmental and developmental signals, including light, nutrients, and especially plant hormones. Nevertheless, how these signals are transduced to affect organ development is poorly understood.

The plant hormone auxin plays an essential role in a wide variety of plant growth and developmental processes, such as shoot and lateral root formation, apical dominance, tropism, and senescence (Davies, 1995). Recent genetic and biochemical analyses have suggested that ubiquitin-related proteolysis is central to several aspects of auxin response (Gray et al., 1999, 2001; Dharmasiri and Estelle, 2002; Kepinski and Leyser, 2002; Leyser, 2002). As a model system, some advances in how auxin promotes lateral root formation have been reported recently (Xie et al., 2000; Casimiro et al., 2001; Xie et al., 2002). However, little is known about how auxin regulates the development of the aerial parts of plants. At the cellular level, auxin acts as a signal for cell division, expansion, and differentiation (Leyser, 2001), and some lines of evidence at the whole-plant level indicate that auxin plays a role in organ cell proliferation as well as the determination of organ size (Lincoln et al., 1990; Ecker, 1995). For example, mutation of Arabidopsis *REVOLUTA/INTERFASCICULAR FIBERLESS1* (*REV/IFL1*) prolongs growth and cell proliferation, resulting in larger leaves and flowers and thicker inflorescence stems (Talbert et al., 1995; Zhong and Ye, 1999). The *REV/IFL1* gene product is involved in auxin polar transport, shoot secondary meristem formation, and the differentiation of the interfascicular fiber cell (Zhong and Ye, 2001), suggesting that polar auxin flow also may influence organ development. By contrast, the *auxin-resistant1* (*axr1*) mutant has obviously smaller leaves, inflorescence stems, and floral organs, and anatomic examination shows that the reduced size of its leaf and stem is caused by a decrease in cell number rather than cell size (Lincoln et al., 1990). Although these observations suggest that AXR1 might be involved in auxin-dependent cell proliferation during development (Tsukaya, 2002), there is no molecular data to support this claim.

Here, we describe the characterization of *ARGOS*, a novel auxin-inducible gene that is involved in organ size control. Overexpression or reduced expression of *ARGOS* in Arabidopsis alters the extent of cell proliferation and organ growth, resulting in larger or smaller organs, respectively. Our results also suggest that ARGOS acts downstream of AXR1 to mediate cell proliferation through ANT during organogenesis.

RESULTS

ARGOS Is a Novel Gene That Is Highly Induced by Auxin

Previous studies showed that Arabidopsis NAC1 and SINAT5 mediate auxin signals to promote lateral root development (Xie et al., 2000, 2002). To increase our understanding of the molecular events surrounding auxin-regulated lateral root formation, cDNA microarray analysis was performed by Incyte Genomics (St. Louis, MO) to identify genes responsive to naphthylacetic acid (NAA) treatment in roots of 7-day-old seedlings (our unpublished data). One gene, which is identical to the putative gene At3g59900 in the Arabidopsis database, was found to be highly induced
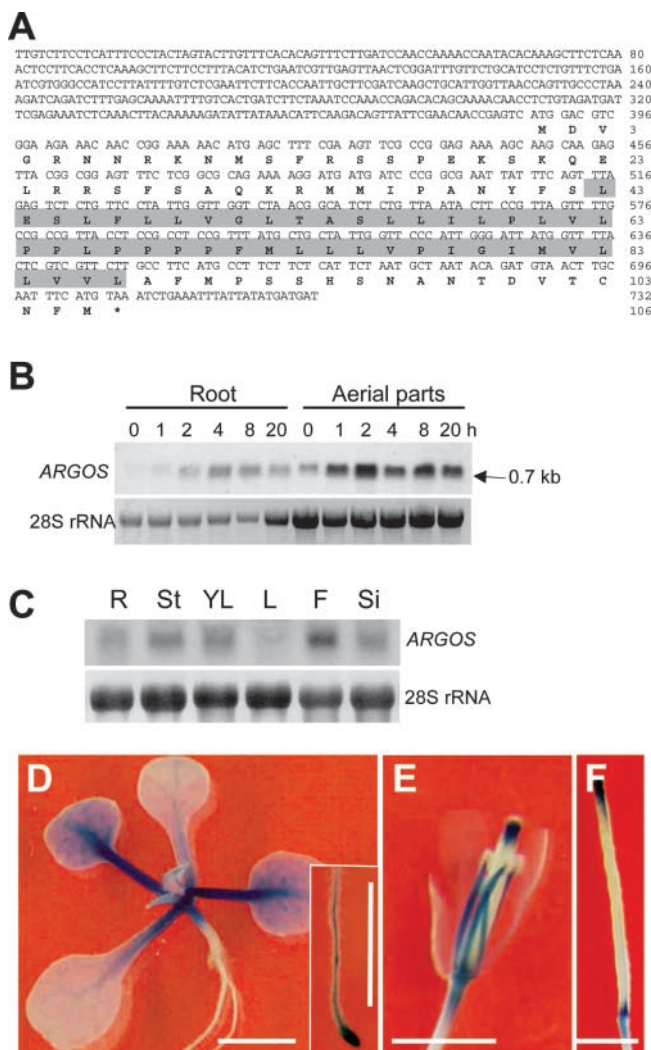

Figure 1. Sequences and Expression of *ARGOS*.

(A) Nucleotide and predicted amino acid sequences of *ARGOS*. The Leu-rich region is shaded.
(B) Induction of *ARGOS* by auxin. Ten-day-old seedlings of Arabidopsis ecotype Columbia grown vertically on MS medium were sprayed with 5 μM NAA. Roots and aerial parts were harvested at the times indicated and processed for RNA gel blot analysis.
(C) Organ-specific expression of *ARGOS*. Inflorescence stems (St), leaves (L), flowers (F), and siliques (Si) were taken from 6-week-old plants grown in a growth chamber. Roots (R) and young rosette leaves (YL) were taken from 2-week-old seedlings grown vertically on MS medium.
(D) to **(F)** *ARGOS-GUS* expression in a 12-day-old seedling **(D)**, flower **(E)**, and young silique **(F)**. The inset in **(D)** shows the GUS expression pattern in the root pericycle and root tip. Bars = 5 mm.

by NAA (data not shown). We designated this gene *ARGOS* (for Auxin-Regulated Gene involved in Organ Size; see below). RNA gel blot analysis confirmed the auxin induction of *ARGOS* in both roots and aerial parts (Figure 1B).

The cloned *ARGOS* cDNA contains 732 bp (Figure 1A), which is almost full length as determined by RNA gel blot analysis. Although the longest open reading frame in *ARGOS* cDNA has

the potential to encode 130 amino acids, protein gel blot analysis of transgenic plants expressing ARGOS cDNA or different mutant cDNA constructs showed that mutation or elimination of the first ATG did not change the size of the protein product (data not shown). This result suggests that ARGOS translation initiates at the second ATG to yield a protein of 106 amino acids, which is the same as predicted in At3g59900. The ARGOS protein contains a Leu-rich domain at its C terminus (Figure 1A). A Basic Local Alignment Search Tool (BLAST) search in GenBank identified only a putative homolog in the rice genome, indicating that ARGOS is a novel protein and possibly plant specific. The Arabidopsis genome contains another putative gene that encodes a protein with ~50% identity to ARGOS, but its organ-specific expression pattern and response to auxin are different from those of *ARGOS* (our unpublished data). Moreover, high-stringency DNA gel blot analysis using a DNA probe prepared from the *ARGOS* open reading frame indicated that *ARGOS* is a single-copy gene (data not shown).

Expression and Cellular Localization of ARGOS

To determine the expression pattern of *ARGOS*, RNAs from dissected organs of mature plants as well as rosette leaves of 2-week-old seedlings were analyzed. *ARGOS* was expressed at low levels in roots, inflorescence stems, flowers, young rosette leaves, and siliques but was almost undetectable in mature leaves (Figure 1C). A detailed examination was performed with transgenic plants expressing an *ARGOS–β-glucuronidase* (*GUS*) fusion gene. In 12-day-old seedlings, GUS staining was observed clearly in leaf primordia as well as juvenile leaf blades and petioles, and moderate GUS expression was detected in cotyledon vascular bundles, root pericycle, and root tips (Figure 1D). In flowers, high expression levels were seen in stamen filaments as well as in the apices and bases of juvenile and elongating siliques (Figures 1E and 1F).

Computer programs predicted the C-terminal Leu-rich domain of ARGOS to be a putative transmembrane domain. Therefore, we investigated the cellular localization of ARGOS in onion epidermal cells using *35S-ARGOS-GFP* and *35S-GFP-ARGOS* fusion genes. GFP florescence was observed in the nucleus, cytoplasm, and cytoplasmic membrane of cells, similar to that obtained with *35S-GFP* alone (data not shown). We tentatively conclude that ARGOS is distributed in the nucleus as well as the cytosol.

Alteration of *ARGOS* Expression Affects Lateral Organ Size

Next, we generated transgenic *35S-ARGOS* and *35S-anti-ARGOS* plants to investigate the function of ARGOS during plant growth and development. Among the 20 lines analyzed for each construct, 12 sense lines and 5 antisense lines showed an enlarged or reduced leaf size, respectively, compared with empty vector control lines (Figure 2A). RNA gel blot analyses performed on two independent lines with a single T-DNA insertion confirmed the overexpression of *ARGOS* RNA in the sense plants and the reduced mRNA level of endogenous *ARGOS* in the antisense plants (Figure 2B).

ARGOS sense and antisense plants differed significantly in their leaf size. Compared with vector control plants, the fresh weight of the corresponding leaf in *ARGOS* sense lines increased by 50 to 120%, whereas that in the *ARGOS* antisense plants de-

creased by 20 to 60% (Figure 2C). The fifth rosette leaf was chosen for further phenotypic analyses, because it was found to be most representative in Arabidopsis (Tsuge et al., 1996). Detailed examination showed that the blade width and length and the petiole length of the fifth leaf were greatly increased or decreased in *ARGOS* sense or antisense plants, respectively (Figure 2D). Similar changes also were observed in floral organs, inflorescence stems, and siliques of *ARGOS* transgenic plants (Figure 2E, Table 1). A corresponding increased or decreased plant height also was found in *ARGOS* transgenic plants (Table 1). However, little difference was observed in the size of cotyledons (Table 1) or in the length of seedling roots grown vertically on Murashige and Skoog (1962) (MS) medium (data not shown).

Transgenic plants overexpressing or underexpressing *ARGOS* also displayed other notable alterations during development. Compared with vector control plants, longer and shorter hypocotyls were found in deetiolated seedlings of sense and antisense lines, respectively (Table 1). Moreover, flowering in transgenic plants overexpressing *ARGOS* was delayed by ~1 week, and these plants produced 20% more seeds in each silique compared with vector control plants. By contrast, *ARGOS* antisense plants flowered earlier, and their siliques contained fewer seeds at maturity (Table 1). These observations indicate that the alteration of *ARGOS* expression in plants affects lateral organ growth and development.

Changes of Organ Size in *ARGOS* Transgenic Plants Are Attributable Mainly to Changes in Cell Number

To assess the contributions of cell division and cell expansion to the phenotypes of *ARGOS* transgenic plants, adaxial epidermal pavement cells of fully expanded fifth leaves were visualized by scanning electron microscopy. Although in Arabidopsis leaf the epidermal pavement cells are jigsaw puzzle like and variable in size (Van Lijsebettens and Clarke, 1998), comparison of pavement cells between vector control and *ARGOS* transgenic plants revealed little size difference between vector control and *ARGOS* sense plants; even pavement cell size was slightly larger in antisense plants (Figure 3A). This finding suggests that cell size is not responsible for the altered leaf size.

We then analyzed transverse and longitudinal sections of leaf blades from vector control, *35S-ARGOS*, and *35S-anti-ARGOS* plants. Leaf blades from these three groups of plants appeared to contain the same number of cell layers (Figure 3B). For further characterization of leaf cells, we defined the leaf width, length, and thickness directions as the X, Y, and Z axes, respectively. As shown in Figures 3B and 3C, the dimensions of palisade cells from these three groups of plants varied slightly in the three axes, and a decreased dimension of palisade cells in the Z axis was observed in *ARGOS*-overexpressing leaves. However, the number of palisade cells and the total number of mesophyll cells in both the X and Y axes differed significantly. The fifth leaf blades of *35S-ARGOS* plants contained ~30% more cells than those of vector control plants, whereas those of *35S-anti-ARGOS* plants contained ~20% fewer cells (Figure 3D). These results suggest that cell number, not cell size, contributes to the enlarged or reduced organ size in *ARGOS* transgenic plants.

To further confirm this observation, we examined inflorescence stems in thin sections and floral organs by scanning
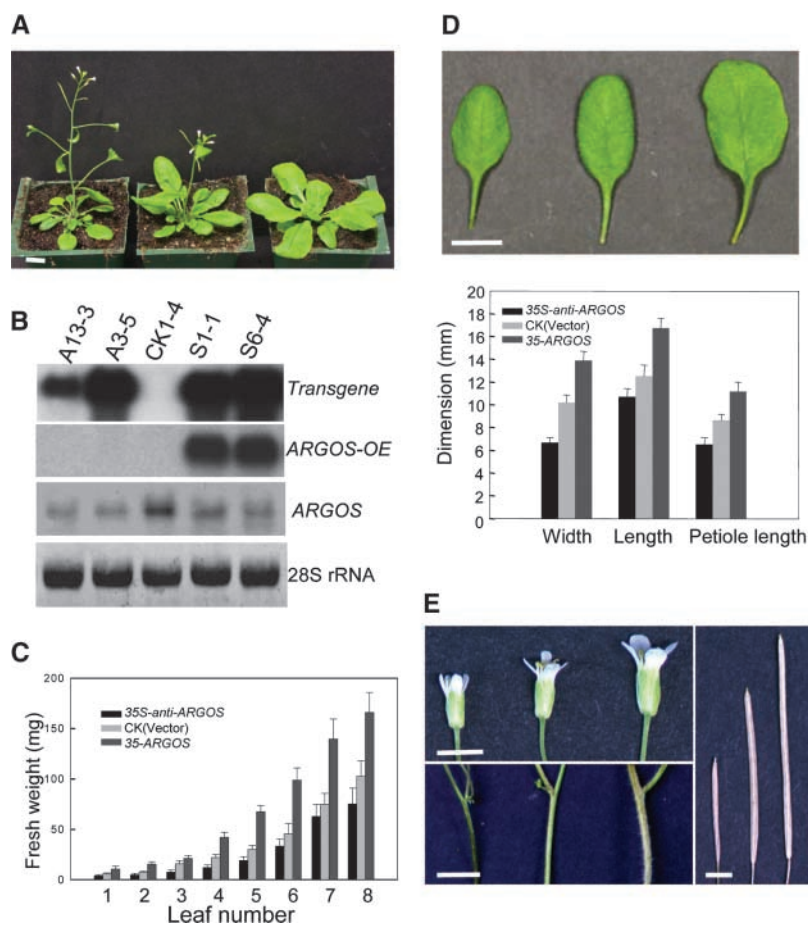
Figure 2. Phenotypic and Molecular Characterization of *ARGOS* Transgenic Plants.

(A) Thirty-day-old transgenic plants carrying *35S-anti-ARGOS* (left), vector control (middle), and *35S-ARGOS* (right) grown in a growth chamber at 23°C under a 16-h-light/8-h-dark photoperiod. Bar = 1 cm.

(B) Expression analyses of *ARGOS* in transgenic plants. A vector control line (CK1-4) and two independent lines of *35S-anti-ARGOS* (A3-5 and A13-3) and *35S-ARGOS* (S1-1 and S6-4) were used to analyze transgene and endogenous gene expression. The RNA gel blot was probed with the *ARGOS* coding region to monitor transgene expression, with an *anti-ARGOS* RNA to detect the overexpression of *ARGOS* (*ARGOS-OE*), and with a 5′ nontranslated region of *ARGOS* for endogenous *ARGOS* expression (*ARGOS*).

(C) Leaf fresh weight of 6-week-old plants. At least 10 plants from two independent lines were measured in vector control, *35S-anti-ARGOS*, and *35S-ARGOS* plants (*n* = 10).

(D) Morphology (top) and dimensions (bottom) of 5-week-old fifth leaves. For the top panel, bar = 5 mm; for the bottom panel, *n* = 10.

(E) Phenotypes of flowers, inflorescence stems, and siliques of *35S-anti-ARGOS*, vector control, and *35S-ARGOS* plants (from left to right). Bars = 5 mm.

electron microscopy. No striking differences in cell size were observed among these three lines, except that the size of petal epidermal cells in *35S-ARGOS* sense plants appeared to be larger than those in vector control and antisense lines (data not shown). However, the slightly larger cell size could not account for the enlarged petal size. Therefore, we conclude that the altered organ size in *ARGOS* transgenic plants results mainly from changes in cell number.

Expression of *ARGOS* Affects the Duration of Plant Growth and Cell Proliferation

At 7 days after germination, seedlings of vector control and transgenic lines were almost indistinguishable. Visible differences in

the first rosette leaf appeared ∼10 days after germination. Eight-week-old vector control plants stopped flowering in most of the inflorescences, whereas *35S-ARGOS* plants of the same age continued to flower in almost all inflorescences (Figure 4A). Visual inspection revealed that *35S-ARGOS* plants may display delayed senescence compared with vector control plants, suggesting that the ectopic expression of *ARGOS* extends the period of growth. To confirm this possibility, we further investigated the growth kinetics of fifth rosette leaves. Under our growth condition, fifth rosette leaves emerged at ∼11 days after germination. The total leaf length of vector control and *ARGOS* transgenic plants increased similarly in the subsequent 12 days. Leaves of antisense plants ceased to elongate at ∼24 days after germination, followed by control leaves at 27 days. However, *35S-ARGOS*

Table 1. Morphology of *ARGOS* Transgenic Plants

Variable	*35S-anti-ARGOS*	Vector Control	*35S-ARGOS*
Cotyledon width (mm)	3.50 ± 0.32 (*n* = 6)	3.42 ± 0.37 (*n* = 6)	3.56 ± 0.38 (*n* = 12)
Cotyledon length (mm)	6.50 ± 0.55	7.33 ± 0.61	5.58 ± 0.38
Hypocotyl length[a] (mm)	12.6 ± 2.2 (*n* = 22)	14.6 ± 1.7 (*n* = 29)	17.4 ± 3.1 (*n* = 33)
Flowering time (days)	23.1 ± 1.8 (*n* = 15)	26.6 ± 2.3 (*n* = 15)	33.8 ± 1.7 (*n* = 15)
Silique length (mm)	7.46 ± 0.75 (*n* = 20)	13.0 ± 1.04 (*n* = 20)	16.92 ± 1.07 (*n* = 20)
Seeds/silique	31.5 ± 6.7	55.6 ± 2.54	67.4 ± 5.1
Plant height (cm)	33.7 ± 3.31 (*n* = 5)	44.3 ± 1.94 (*n* = 5)	57.3 ± 1.25 (*n* = 5)

[a] Seedlings grown in darkness for 5 days.

leaves continued to grow even after 33 days (Figure 4B). These data demonstrate that variations in the growth duration, rather than the growth rate, contribute to the differences in organ size in *ARGOS* transgenic plants.

To determine the role of cell division in the altered duration of leaf development, we introduced a *CycB1-GUS* transgene into vector control and *35S-ARGOS* transgenic plants. *CycB1* is expressed at the G2/M-phase of the cell cycle, and CycB1-GUS has been used as a marker for studies of cell proliferation in lateral organ development (Ferreira et al., 1994; Donnelly et al., 1999; Casimiro et al., 2001). At 16 days after germination, GUS staining in transgenic vector control seedlings was observed in leaf primordia and marginal meristems of young leaves, but less marginal and diffused submarginal cell division occurred in expanding leaves (Figure 4C). By contrast, *35S-ARGOS* seedlings displayed prolonged cell division in marginal meristems as well as in leaf blades, giving more visible marginal meristems and more diffused cell division in expanding leaves (Figure 4C). Besides cell expansion, the lateral growth of the leaf was thought to depend on marginal and submarginal cell proliferation within the leaf blade (Donnelly et al., 1999). Thus, our observation suggests that the ectopic expression of *ARGOS* extends the period of cell proliferation in organs.

ARGOS Influences *ANT* Expression and Cell Competence

Because the phenotypes of *ARGOS* transgenic plants are similar to those obtained from the loss and gain of function of *ANT* (Krizek, 1999; Mizukami and Fischer, 2000), we surmised that, like ANT, ARGOS may affect organ cell competence, thus influencing the duration of cell proliferation. To test this hypothesis, we analyzed the expression of *ANT* and *CycD3;1* in fully expanded and young rosette leaves of vector control and *ARGOS* transgenic plants. Compared with the undetectable expression in vector control and *ARGOS* antisense leaves, *ANT* expression persisted in rosette leaves of 40-day-old *35S-ARGOS* plants (Figure 4D). Meanwhile, similar to that in *35S-ANT* plants (Mizukami and Fischer, 2000), prolonged expression of *CycD3;1* also was detected in *35S-ARGOS* leaves (Figure 4D). However, in 12-day-old rosette leaves, *ANT* and *CycD3;1* transcript levels were comparable in the three genotypes (Figure 4D). These results suggest that during development, ARGOS may prolong the expression duration of *ANT* and *CycD3;1*.

Similar to the situation in *35S-ANT* plants, neoplasia was observed in *35S-ARGOS* leaf cells. When cultured on hormone-free

B5 medium, *35S-ARGOS* leaf explants but not explants from vector control or antisense plants generated small calli after 1 week (Figure 4E). Moreover, striking differences were seen when the leaf explants were cultured on a callus induction medium for a longer time period without being transferred to fresh medium. All explants produced calli in 1 week, but *ARGOS* antisense calli ceased to grow at ~20 days, as did the control calli at ~30 days. By contrast, calli of the *35S-ARGOS* sense line continued to grow even after 40 days (Figure 4F). These results show that, indeed, overexpression and underexpression of *ARGOS* affect cell competence.

ARGOS Mediates Organ Growth Upstream of *ANT*

The prolonged expression of *ANT* and the altered cell competence in *35S-ARGOS* transgenic plants imply that ARGOS may function through the regulation of *ANT* expression. To address this possibility, we crossed homozygous *35S-ARGOS* transgenic plants with heterozygous *ANT/ant-1* and analyzed the F2 progeny. Among 121 *35S-ARGOS* plants from two independently segregating lines, 86 displayed enlarged leaves and floral organs of *35S-ARGOS* plants; however, 35 exhibited the *ant-1* phenotype, with small leaves and floral organs (Figure 5A). These results approximate the expected genetic segregation ratio of 3:1. RNA gel blot analyses showed that these two types of plants expressed comparably high levels of *ARGOS-OE* (Figure 5B). Thus, our findings indicate that the loss of function of ANT can block ARGOS-mediated organ growth and hence ARGOS functions upstream of ANT.

Induction of *ARGOS* by Auxin Is Attenuated or Abolished in *axr1* Mutants

Because *ARGOS* is highly induced by auxin and involved in cell division–related organ development, it is possible that it, at least in part, mediates the auxin signal during organogenesis. A number of mutants involved in auxin transport and signaling have been identified, but only the *axr1* mutant was reported to have smaller leaves and shorter inflorescence stems resulting from decreased cell numbers (Lincoln et al., 1990). To determine whether ARGOS is involved in the AXR1-related control of organ development, we examined *ARGOS* expression in seedlings treated with or without NAA in *axr1-3* and *axr1-12* as well as some auxin-related mutants. Compared with the wild type, apparently decreased levels of *ARGOS* expression were observed in *axr1-3* and *axr1-12*, and the induction of *ARGOS* by NAA was attenu-
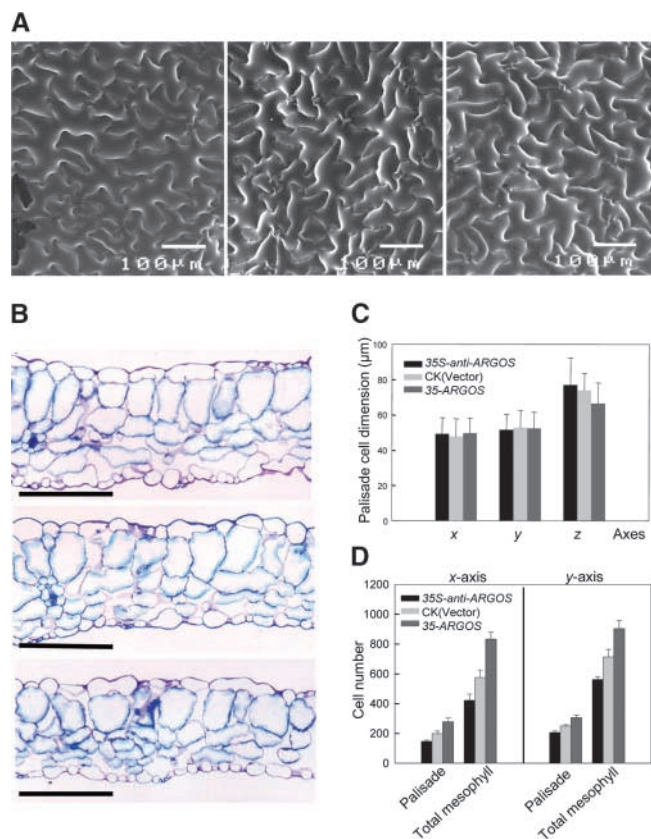
Figure 3. Anatomical Analysis of Fifth Leaves in Transgenic *ARGOS* Plants.

(A) Adaxial epidermal pavement cells of fully expanded fifth leaves of *35S-anti-ARGOS* (left), vector control (middle), and *35S-ARGOS* (right) plants. Bars = 100 μm.

(B) Transverse sections of leaf blades of *35S-anti-ARGOS* (top), vector control (middle), and *35S-ARGOS* (bottom) plants. Bars = 100 μm.

(C) Dimensions of palisade cells. X, Y, and Z axes correspond to the directions of leaf width, length, and thickness, respectively. At least 40 cells of each line were measured with a microscope.

(D) Number of palisade cells and total number of mesophyll cells in X and Y axes of leaves. Four leaves of each line were sectioned, and the cells were counted in the middle of each leaf in the X axis and ∼1 mm from the midvein in the Y axis.

ated in the weak allele *axr1-3* and abolished completely in *axr1-12* (Figure 6A). These results are consistent with the observation that the defects in organ development are more severe in *axr1-12* than in *axr1-3* (Lincoln et al., 1990). On the other hand, *ARGOS* induction by NAA still was observed in *axr2*, *tir1-3*, *aux1-7*, and *iaa28* (data not shown). Our results suggest that the auxin induction of *ARGOS* requires *AXR1*.

Overexpression of *ARGOS* Partially Restores Organ Development in *axr1*

Because it is difficult to obtain transgenic seeds from the strong allele *axr1-12*, we introduced *35S-ARGOS* into the weak allele *axr1-3* to further investigate the role of ARGOS in auxin signaling.

Although the morphology of leaves in transgenic *35S-ARGOS/axr1-3* plants was similar to that in *axr1-3*, overexpression of *ARGOS* apparently restored a part of the *axr1-3* leaf development, producing similarly sized or even larger leaves compared with wild-type leaves (Figures 6B and 6C). As a negative control, transgenic *axr1-3* plants carrying an empty vector still displayed the mutant phenotype of small leaves (Figure 6B). RNA gel blot analysis verified the correlation between the leaf phenotype and *ARGOS* expression levels in these plants (Figure 6D). This finding, together with the observation that *ARGOS* induction by NAA was reduced or blocked in *axr1* mutants, suggests that ARGOS functions downstream of AXR1 to mediate auxin-related cell proliferation and organ growth. Interestingly, the retarded lateral root development in *axr1-3* seedlings was not rescued by overexpressing *ARGOS* (data not shown).

DISCUSSION

ARGOS Defines a Novel Gene Involved in Plant Development

Using a DNA microarray approach, we identified the *ARGOS* gene whose expression is highly induced by auxin. Further characterization of sense and antisense transgenic plants showed that ARGOS is involved in the regulation of cell proliferation during organ development, thereby affecting plant organ size. *ARGOS* encodes a small protein with no recognizable functional domain. Only one putative homolog is present in the recently disclosed rice genome, suggesting that *ARGOS* might be plant specific. The Arabidopsis genome contains another putative gene sharing some sequence identity with *ARGOS*, but its expression pattern is different from that of *ARGOS* (our unpublished data) and its function remains unknown. Therefore, our studies of *ARGOS* have revealed a novel gene that plays an important role in plant growth and development.

ARGOS Regulates ANT-Mediated Organ Size Control

The control of organ size is a fundamental aspect of growth and development, and the mechanism of this process remains largely unknown in animals and plants (Mizukami, 2001; Potter and Xu, 2001). In plants, recent studies of *ANT* have revealed a gene that defines organ size through regulation of the duration of cell proliferation. Unlike overexpression of some cell cycle genes, which increases the total cell number in organs but inhibits differentiation, overexpression of *ANT* prolongs cell proliferation and enlarges an organ in a coordinate manner, suggesting that ANT may act at a checkpoint in organ size control (Mizukami and Fischer, 2000). Our data suggest that *ARGOS* is a novel gene involved in the control of this process. First, reduced expression or overexpression of *ARGOS* and the loss or gain of function of *ANT* lead to similar phenotypic changes in aerial parts, which are caused by alteration in the duration of cell proliferation. Interestingly, although *ARGOS* and *ANT* are expressed in roots, the root phenotype was not observed in early developmental stages of *ARGOS* transgenic plants or in the loss or gain of function of *ANT* plants. Second, prolonged expression of *CycD3;1* and neoplasia were observed in leaf explants overexpressing either
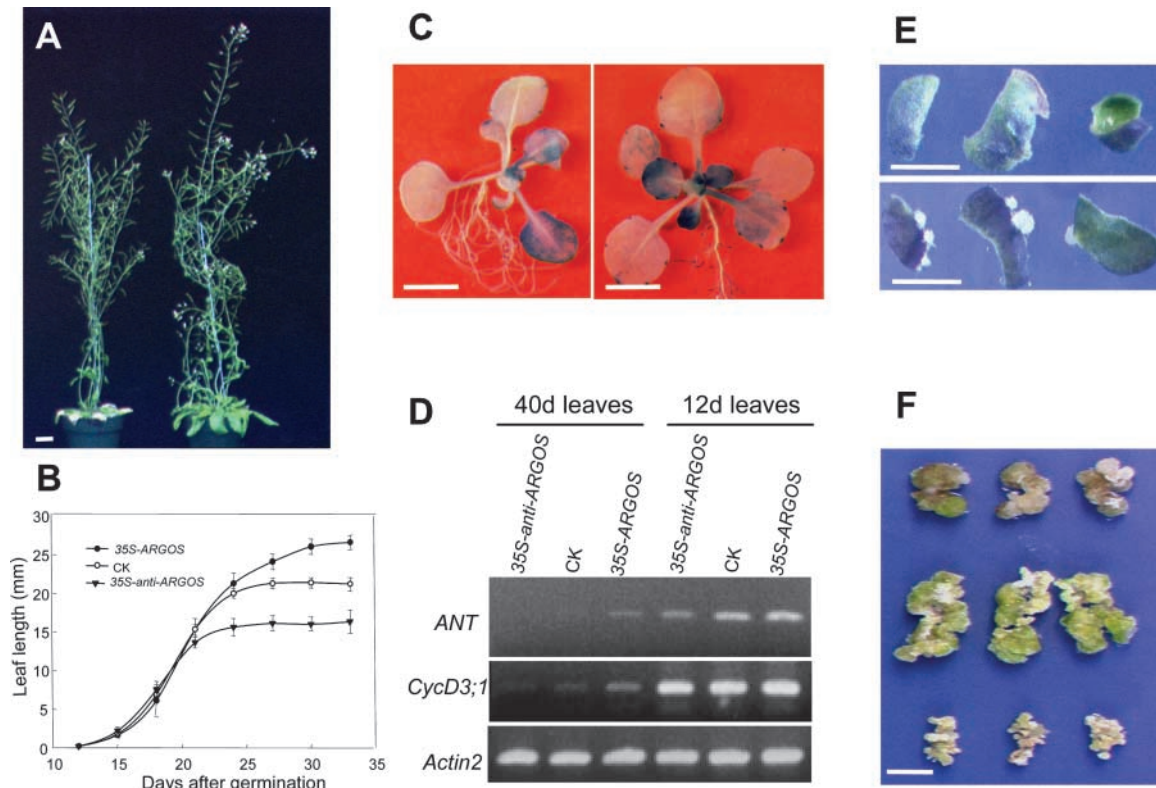
Figure 4. Effect of ARGOS on Growth and Cell Meristematic Competence.

(A) Eight-week-old vector control (left) and *35S-ARGOS* (right) plants grown in a growth chamber. Bar = 1 cm.

(B) Growth kinetics of the fifth leaves in *35S-anti-ARGOS*, vector control (CK), and *35S-ARGOS* plants. After emergence, leaf length was measured every 3 days (*n* = 10).

(C) *CycB1-GUS* activity in 16-day-old seedlings of CK (left) and *35S-ARGOS* (right) plants. Bars = 5 mm.

(D) *ANT* and *CycD3;1* transcript levels in juvenile and fully expanded rosette leaves of vector control (CK) and *ARGOS* transgenic plants.

(E) and **(F)** Neoplasia in leaf explants of *ARGOS* transgenic plants. Bars = 5 mm.

(E) Leaf explants from 4-week-old transgenic plants were cultured on hormone-free B5 medium, and photographs were taken at 10 days after excision. Note the callus formation in *35S-ARGOS* (bottom) but not in the vector control (top).

(F) Callus growth in leaf explants of vector control (top), *35S-ARGOS* (middle), and *35S-anti-ARGOS* (bottom) plants. The explants were cultured on B5 medium containing 4.5 μM 2,4-D and 0.5 μM kinetin and photographed at 40 days without changing the medium.

ARGOS or *ANT*. A recent study of transgenic plants with ectopic *CycD3;1* expression suggested that *CycD3;1* acts downstream of *ANT* to determine leaf cell number (Dewitte et al., 2003). Most importantly, the prolonged expression of *ANT* is observed in fully expanded *35S-ARGOS* leaves, and the loss of function of ANT blocked organ enlargement in *35S-ARGOS* plants. All of these results are consistent with the notion that ARGOS acts upstream of ANT to regulate organ size. Although the molecular mechanism remains unclear, the function of *ARGOS* likely is executed through the regulation or maintenance of *ANT* expression.

ARGOS Mediates the Auxin Signal to Affect Lateral Organ Development

Little is known about how organ development is controlled by plant growth signals. There is strong evidence that plant hormones, such as auxin, play a very important role in this process. Auxin is essential for cell division and expansion in vitro and has been used widely in cell culture and plant regeneration in concert with cytokinin. The expression of mitotic genes, including *CycD3;1* and *CDKA;1*, has been found to be regulated by auxin (Hemerly et al., 1993; Doerner et al., 1996; Oakenfull et al., 2002). At the whole-plant level, some auxin mutants display a dramatic alteration in aerial organ development. Unlike *axr2*, a mutation in an auxin/indoleacetic acid gene that causes a dramatic decrease in cell length in stems (Timpte et al., 1992), *axr1* significantly reduces organ size by decreasing cell number (Lincoln et al., 1990). This finding suggests that AXR1 might be involved in auxin signaling that mediates cell proliferation in development.

Our observations that the overexpression and reduced expression of *ARGOS* altered cell proliferation and thus organ size indicate that ARGOS is involved in the control of cell division–related organ development. The high induction of *ARGOS* by auxin suggests that ARGOS could mediate auxin signaling. These results provide molecular evidence that auxin influences
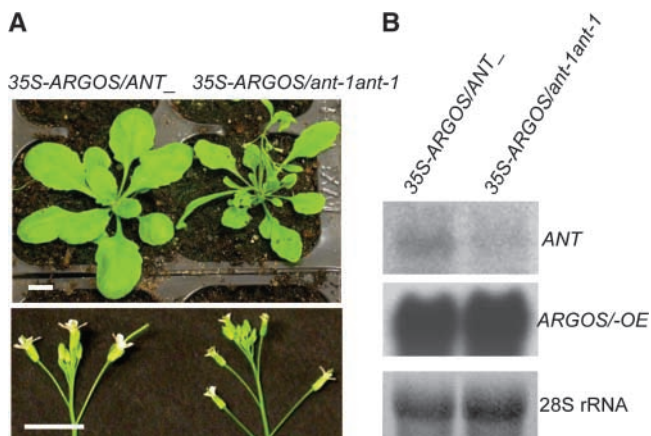
A   **B**

35S-ARGOS/ANT_   35S-ARGOS/ant-1ant-1

Figure 5. Loss of Function of ANT Blocks Organ Enlargement in *35S-ARGOS* Transgenic Plants.

(A) Morphology of 4-week-old plants (top) and inflorescences (bottom) of *35S-ARGOS/ANTANT* or *35S-ARGOS/ANTant-1* (*35S-ARGOS/ANT_*) and *35S-ARGOS/ant-1ant-1* plants in line L2-4. Bars = 10 mm.
(B) Endogenous *ANT* mRNA and transgenic *ARGOS* mRNA levels in the two types of plants shown in **(A)**.

cell proliferation during organ development, although this process is difficult to mimic by the application of exogenous auxin. The involvement of ARGOS in auxin signaling is strengthened further by the finding that *ARGOS* induction by auxin is attenuated in the weak allele *axr1-3* and abolished in the strong allele *axr1-12*; moreover, overexpression of *ARGOS* partially restores organ growth in *axr1-3* (Figure 6). AXR1 is a component of the ubiquitin-activating enzyme E1 (Leyser et al., 1993), which functions in an early step of auxin signaling (Leyser, 2002) and also may mediate other plant signals, such as jasmonate and light (Schwechheimer et al., 2002; Xu et al., 2002). With respect to *ARGOS* expression and function, we suspect the existence of as yet unidentified signaling components between AXR1 and ARGOS. Identification and characterization of these mediators will further our understanding of auxin signaling in lateral organ development.

Putative Roles of ARGOS in Plant Organogenesis

During organ development, it is possible that there is a developmental switch to regulate the extent of cell competence, thus determining the duration of cell proliferation and organ growth. *ANT* is a plant-specific gene, and studies of the loss and gain of function of ANT suggest that it might be such a factor (Mizukami and Fischer, 2000). On the other hand, how growth signals, such as auxin, affect organ cell proliferation during organogenesis remains unclear. If ANT functions as a checkpoint, there should exist some mediators that transduce auxin-triggered developmental signals to this checkpoint. Our observations strongly suggest that ARGOS might be such a signaling component. It is likely that the endogenous auxin signal might first regulate *ARGOS* expression, thereby regulating and/or maintaining *ANT* expression to affect the duration of organ cell proliferation, thus determining the final organ size (Figure 7) (Mizukami and Fischer, 2000).

Our studies reveal ARGOS to be a novel regulator in auxin signaling and organ size control and to link auxin signaling with aerial organ development. Further studies are needed to identify other components of this signaling network to understand how ARGOS functions and whether it mediates other signals as well. Finally, the increased lateral organ mass, as well as the increased seed number in each silique in *35S-ARGOS* plants, could lead to biotechnological applications to improve the yield of agronomic crops.
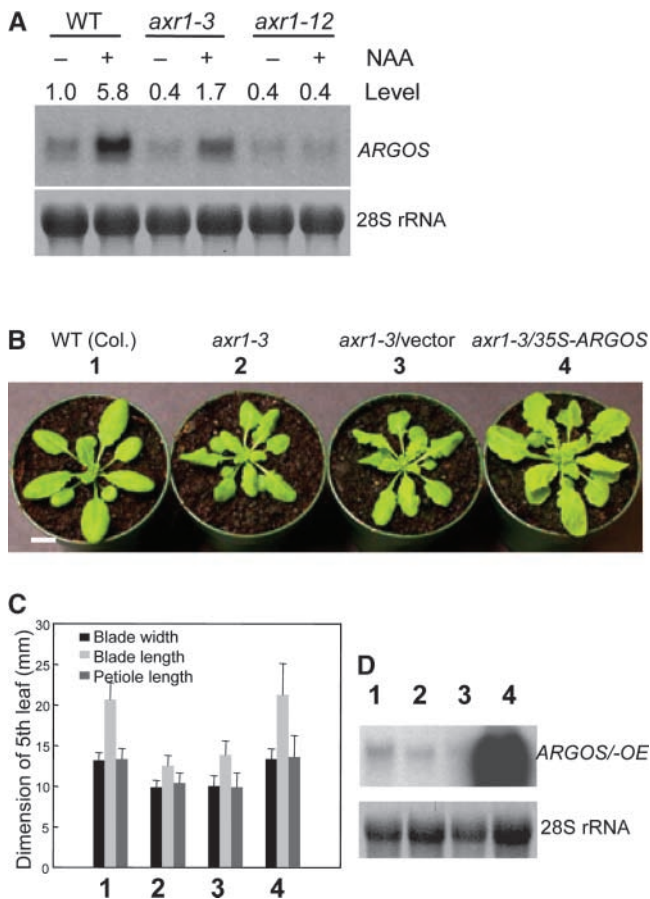

Figure 6. ARGOS Acts Downstream of AXR1.

(A) *ARGOS* expression in wild-type (WT), *axr1-3*, and *axr1-12* plants with and without auxin treatment. RNA was extracted from 12-day-old seedlings treated with 5 μM NAA (+) or water (−) for 3 h. Numbers above the lanes refer to *ARGOS* expression levels relative to 28S rRNA levels.
(B) Three-week-old plants of wild-type Columbia [WT (Col.)], *axr1-3*, transgenic *axr1-3* carrying an empty vector, and transgenic *axr1-3* carrying a *35S-ARGOS* transgene. Bar = 1 cm.
(C) Dimensions of fifth leaves of 5-week-old wild-type (1), *axr1-3* (2), *axr1-3*/vector (3), and *axr1-3/35S-ARGOS* (4) plants The corresponding fresh weights (mg) are as follows: (1) 29.8 ± 4.1; (2) 15.5 ± 5.2; (3) 16.1 ± 3.8 ; and (4) 32.8 ± 5.2. At least 12 leaves from each genotype were measured.
(D) Endogenous *ARGOS* mRNA and transgenic *ARGOS* (*ARGOS-OE*) mRNA levels in the wild type (lane 1), *axr1-3* (lane 2), *axr1-3*/vector (lane 3), and *axr1-3/35S-ARGOS* (lane 4).
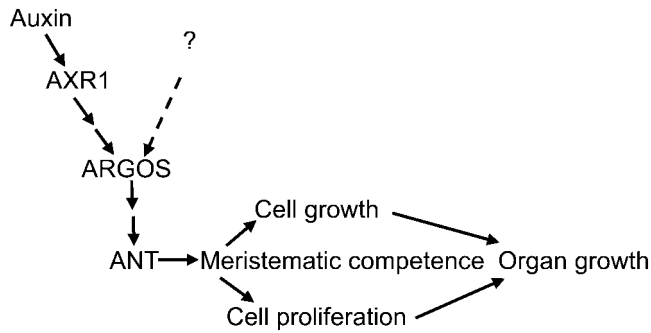
Figure 7. A Proposed Model for ARGOS Function in Plant Organ Size Control.

The model was adapted from Mizukami and Fischer (2000).

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used in this study. Seeds were sterilized in 50% bleach with 0.01% Triton X-100 for 10 min and washed three times with sterilized water. Sterilized seeds were plated on Murashige and Skoog (1962) (MS) medium and vernalized in darkness at 4°C for 2 days before the plate was transferred to a culture room at 23°C under a 16-h-light/8-h-dark photoperiod. For naphthylacetic acid treatment or root observation, seeds were germinated and seedlings were grown vertically for 7 to 14 days. For morphological examination of aerial parts, seedlings were transferred to soil 7 to 10 days after germination and placed in a growth chamber at 23°C under a 16-h-light/8-h-dark photoperiod.

Plant Transformation

A cDNA fragment containing the *ARGOS* open reading frame was cloned into pVIP96 (Leu et al., 1995) in both sense and antisense orientations. Transgenic plants were generated by *Agrobacterium tumefaciens*–mediated transformation via vacuum infiltration (Bechtold et al., 1993). T1 seeds and progeny were germinated on MS medium containing 50 mg/L kanamycin for the selection of transformants. At least two independent T3 homozygous lines with a single T-DNA insertion were used for detailed analysis.

Gene Expression Analysis

Total RNA was isolated using the guanidine thiocyanate extraction buffer as described (Hu et al., 2000). Each gel lane contained ~10 μg of total RNA. RNA gel blots were probed with an *ARGOS* cDNA fragment labeled with α-^{32}P-dCTP using the Ready Primer Labeling Kit (Amersham). To identify the expression of the *ARGOS* sense transgene, an *ARGOS* RNA complementary probe was synthesized using T3 RNA polymerase (Promega) and labeled with α-^{32}P-UTP. Endogenous *ARGOS* expression in transgenic plants was detected using as a probe a 250-bp fragment from the *ARGOS* 5′ nontranslated region. Hybridizations were performed as described previously (Church and Gilbert, 1984). Reverse transcriptase–mediated PCR was performed with the QIAGENE One-Step RT-PCR Kit (Valencia, CA).

A 2.1-kb DNA fragment containing the 5′ upstream region of *ARGOS* was cloned into pBI101 for construction of the *ARGOS-GUS* fusion gene. The SalI-BamHI fragment containing *CycB1;1-GUS* (Ferreira et al.,

1994) was cloned into pCAMBIA1300 and transformed into plants carrying either a *35S-ARGOS* transgene or an empty vector for the examination of cell proliferation in leaf development. To monitor GUS expression, seedlings or young organs of transgenic plants were incubated in a 50 mM Na-phosphate, pH 7.0, solution containing 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid at 37°C for several hours.

Histological Analysis

Fully expended fifth leaves and flower organs were cryofixed in liquid nitrogen and scanned with a scanning electron microscope for the examination of epidermal cells. To determine cell size and number in thin section, specimens of fully expanded fifth leaves (35 days after germination) and the second internodes of inflorescence stems were fixed in 100 mM K-phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde. After dehydration with an ethanol series, samples were infiltrated and embedded with a Leica Historesin Embedding Kit (Wetzlar, Germany) according to the manufacturer's instructions. The thin sections were stained with 0.05% toluidine blue O before microscopic observation. Cell size was measured and cell number was determined in the middle region of the fifth leaf blade in a transverse section or ~1 mm from the midvein in longitudinal sections.

Leaf Explant Culture

To test leaf cell competence, rosette leaves of 4-week-old control and *ARGOS* transgenic plants were sterilized and excised. The explants were cultured on hormone-free B5 medium and callus induction medium under a 16-h-light/8-h-dark photoperiod at 23°C. The callus induction medium used was B5 medium containing 4.5 μM 2,4-D and 0.5 μM kinetin (Hu et al., 2000).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact N.-H. Chua, chua@mail.rockefeller.edu.

Accession Number

The GenBank accession number for *ARGOS* is AY305869.

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The Arabidopsis Auxin-Inducible Gene *ARGOS* Controls Lateral Organ Size
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