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The Arabidopsis *ARGOS-LIKE* gene regulates cell expansion during organ growth

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Summary

Cell expansion, and its coordination with cell division, plays a critical role in the growth and development of plant organs. However, the genes controlling cell expansion during organogenesis are largely unknown. Here, we demonstrate that a novel Arabidopsis gene, *ARGOS-LIKE* (*ARL*), which has some sequence homology to the *ARGOS* gene, is involved in this process. Reduced expression or overexpression of *ARL* in Arabidopsis results in smaller or larger cotyledons and leaves as well as other lateral organs, respectively. Anatomical examination of cotyledons and leaves in *ARL* transgenic plants demonstrates that the alteration in size can be attributed to changes in cell size rather than cell number, indicating that *ARL* plays a role in cell expansion-dependent organ growth. *ARL* is upregulated by brassinosteroid (BR) and this induction is impaired in the BR-insensitive mutant *bri1*, but not in the BR-deficient mutant *det2*. Ectopic expression of *ARL* in *bri1–119* partially restores cell growth in cotyledons and leaves. Our results suggest that ARL acts downstream of BRI1 and partially mediates BR-related cell expansion signals during organ growth.

Keywords: *Arabidopsis thaliana*, *ARL*, cell expansion, organ growth, brassinosteroid.

Introduction

During post-embryonic development, plant organ growth depends on cell proliferation, expansion and differentiation, and the final size of an organ is determined by the resulting cell number and cell size (Mizukami, 2001). Early studies suggested that cell division plays a fundamental role in organ growth (Vernoux *et al.*, 2000). However, experimental manipulations of most cell cycle regulators, such as CDKA and CycD3, do not always alter organ size because of compensatory changes in cell differentiation and expansion (Dewitte *et al.*, 2003; Hemerly *et al.*, 1995; Riou-Khamlichi *et al.*, 1999). Therefore, there must be an intrinsic mechanism for coordinating cell division and expansion during the growth of an organ. Some recent experiments have begun to uncover regulators that control organ growth by just altering cell proliferation. For instance, the Arabidopsis *AINTEGUMENTA* (*ANT*) gene, which encodes an AP2-domain protein, prolongs the duration of cell division in a coordinated way by sustaining *CycD3* expression, suggesting that ANT may act as a coordinator between cell proliferation and organ growth (Mizukami and Fischer, 2000). Our previous observation that *ARGOS* is induced by auxin and functions upstream of *ANT* uncovers the role of this plant hormone in the control of organ growth (Hu *et al.*, 2003). The identification of ERECTA-family receptor-like kinases in Arabidopsis defines another family of genes that regulates organ growth by affecting cell proliferation through a pathway different from that of ANT and ARGOS (Shpak *et al.*, 2004).

In contrast to cell division, cell expansion seems to affect organ growth in more complicated ways. First, although the molecular basis is still elusive, the final size of a plant cell seems to be correlated with its ploidy, and endoreduplication, which has recently been found to exist widely in plant cells, appears to be an important factor for determining cell size in particular organs (Kondorosi *et al.*, 2000; Sugimoto-Shirasu and Roberts, 2003). Second, the size and shape of some aerial organs, especially leaves, are determined by polar cell expansion. For example, the

Arabidopsis *angustifolia* (*an*) mutant has narrower and thicker leaves resulting from a specific defect in cell expansion in the direction of the leaf width (Kim *et al.*, 2002). Conversely, *rotundifolia 3* (*rot3*) displays broader leaves and flower organs, owing to a defect in cell expansion in the direction of the leaf length (Kim *et al.*, 1998). Finally, ectopic expression of the Arabidopsis *auxin-binding protein 1* gene (*ABP1*), which mediates the auxin signal in cell expansion, does not affect the overall organ size because of a compensatory decrease in cell number (Jones *et al.*, 1998). Nevertheless, the altered expression of the *AtGRF* gene family, which encodes a family of putative transcription factors, indeed changes cell size in cotyledons and leaves, and thereby the final size of these organs (Kim *et al.*, 2003), suggesting that cell expansion alone also contributes to the overall size of the organ.

Cell elongation and expansion in plants are modulated by plant hormones, such as auxin, giberellic acid (GA), ethylene and brassinosteroid (BR) (Davies, 1995). These signals may regulate cell expansion and/or differentiation through their specific or cross-talk pathways, thus influencing organ growth and/or size (Mizukami, 2001). For instance, ABP1 is essential for auxin-regulated cell expansion (Chen *et al.*, 2001; Jones *et al.*, 1998). Brassinosteroid is an important plant hormone required for normal cell expansion (Altmann, 1998; Clouse and Sasse, 1998), and all mutants related to BR biosynthesis and signaling develop smaller leaves and other aerial organs, which are mainly due to a defect in cell expansion (Altmann, 1999; Azpiroz *et al.*, 1998; Fujioka *et al.*, 1997; Kauschmann *et al.*, 1996; Szekeres *et al.*, 1996). The finding that *ROT3* encodes a cytochrome P450 and is involved in BR biosynthesis implies that BR plays a role in controlling polar cell expansion during organ growth (Bancos *et al.*, 2002; Kim *et al.*, 1999). Moreover, the expressions of some genes involved in cell wall modification in expanding cells, such as *TCH4* and *KOR*, have been found to be upregulated by BR and altered in some BR-related mutants (Nicol *et al.*, 1998; Xu *et al.*, 1995). In addition, BR is also essential for elongation of the hypocotyl during light-dependent development (Kang *et al.*, 2001; Li *et al.*, 1996). Therefore, it is believed that BR is required for developmental processes related to cell expansion and elongation.

We previously reported that the Arabidopsis *ARGOS* gene transduces the auxin signal to affect cell proliferation of organs, thus controlling organ size (Hu *et al.*, 2003). Here we show that a gene with sequence homology to *ARGOS*, called *ARGOS-LIKE* (*ARL*), is involved in the regulation of cell expansion-dependent organ growth. Altered expression of *ARL* in Arabidopsis causes changes in organ size, which results from alterations in cell size. Our results also suggest that ARL functions downstream of BRI1 and partially mediates BR-related cell expansion signals in organ growth.

Results

ARL and its expression

Our previous studies demonstrated that Arabidopsis *ARGOS*, a novel gene that is induced by auxin, controls lateral organ size by affecting cell proliferation in aerial organs (Hu *et al.*, 2003). To extend our understanding of the molecular basis underlying organ growth, we performed a Blast search in the Arabidopsis genome with the *ARGOS* cDNA sequence. We found that another Arabidopsis gene, *At2g44080*, has sequence homology to the *ARGOS* gene in its coding region. The predicted ARL protein contains 135 amino acids, and has a leucine-rich motif near the C-terminal, which is highly conserved between ARL and ARGOS and predicted to be a transmembrane domain (Figure 1a). This related gene was cloned with reverse-transcriptional PCR and thus named *ARGOS-LIKE* (or *ARL*).

To investigate whether *ARL* is redundant to *ARGOS* or functions differently from the latter in plant growth and development, we first examined *ARL* expression with transgenic plants expressing an *ARL* promoter – glucuronidase (GUS) fusion gene. We found that the organ and tissue expression patterns of *ARL* were apparently different from those of *ARGOS*. Unlike the lower expression level of *ARGOS* observed only in juvenile leaves, vascular tissues of cotyledons and roots (Hu *et al.*, 2003), strong *ARL* expression was detected throughout the cotyledons as well as in the mature regions of roots in young seedlings (Figure 1b), and obvious GUS staining was also observed in expanding leaves, but not in leaf primordia and juvenile leaves (Figure 1b). In flowers, *ARL* was expressed in sepals and stamen filaments, as well as at the apices and bases of siliques (Figure 1b). We also investigated the intracellular localization of ARL by transient expression of a *35S-ARL–GFP* or a *35S-GFP–ARL* fusion gene in onion epidermal cells using green fluorescent protein (GFP) alone as a control. The intracellular distribution of both fusion proteins was similar to that of GFP alone (Figure 1c), indicating no specific cellular localization of ARL to any cellular compartment.

Reduced expression or overexpression of ARL affects organ growth

To explore the function of *ARL* during plant growth and development, we generated transgenic plants expressing an *ARL*-specific RNA interference (ARLi) sequence fragment or overexpressing *ARL* cDNA (ARL-OE). Among 15 lines examined for each transgenic construct, at least six ARLi lines and nine ARL-OE lines displayed similarly smaller or larger cotyledons and leaves, respectively, among all transformants (Figure 3a,b; see below for details), compared with control plants (CK) which contained

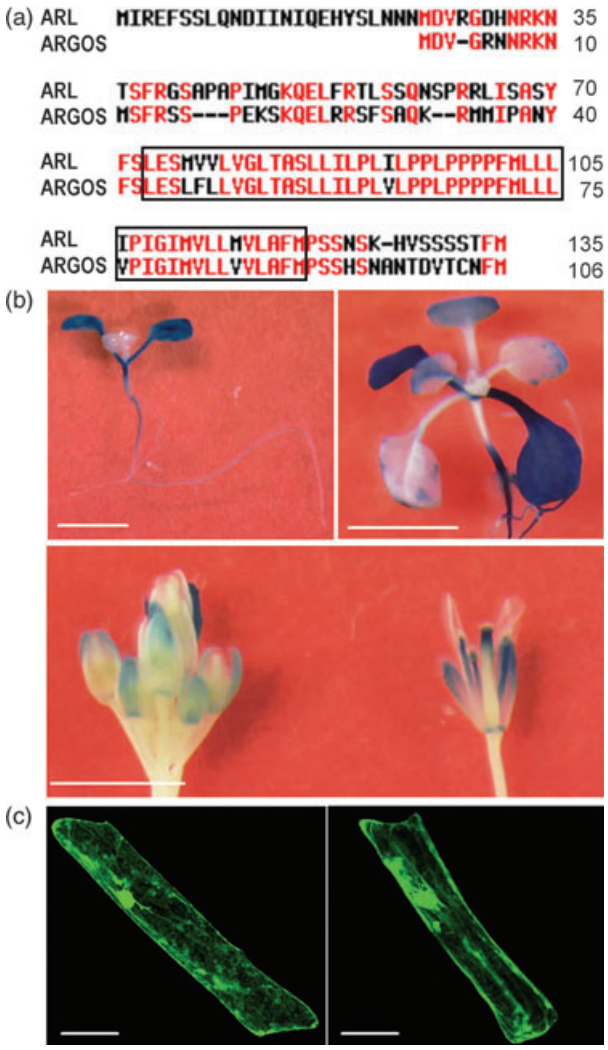

Figure 1. Alignment of derived amino acids of ARL with ARGOS, expression of the *ARL* gene, and the intracellular localization of ARL.
(a) The alignment of ARL encoded by *ARL* (*At2g44080*) with ARGOS. Identical amino acids are indicated with red letters and the putative transmembrane domain is boxed.
(b) GUS expression patterns in 10-day-old seedlings (top left), 15-day-old seedlings (top right) and floral organs of *ARL* promoter–GUS transgenic plants. Scale bars = 5 mm.
(c) Intracellular localization of GFP (control, left) and ARL–GFP fusion protein (right). Onion epidermal cells expressing either a *35S-GFP* (pGFP-2) or a *35S-ARL–GFP* fusion gene were analyzed by confocal microscopy. Scale bar = 100 μm.

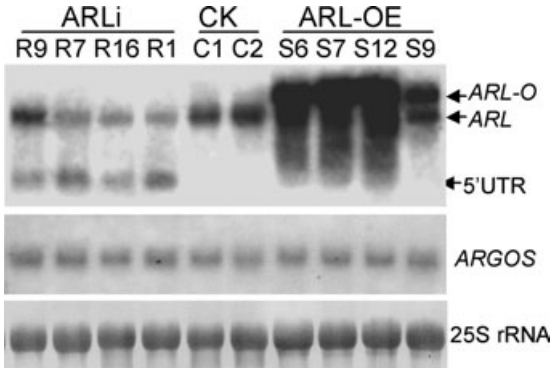

Figure 2. RNA gel blot analyses of *ARL* transgenic plants.
Twenty-day-old plants from four independent *35S-ARL–RNAi* (ARLi) and *35S-ARL* (ARL-OE) lines as well as two vector control (CK) lines were subjected to total RNA isolation and Northern blot analysis. Lines R9 and S9 were chosen as representatives of ARLi and ARL-OE lines with a less visible phenotype, respectively. The blot was probed with full-length *ARL* cDNA for the expression of the endogenous (*ARL*), the transgenic *ARL* (*ARL-O*) genes and of the RNA-interference fragment [5′-untranslated region (UTR)]. An *ARGOS* specific probe was used to detect *ARGOS* expression.

an empty vector. To verify that the morphological changes in these transgenic plants are due to the reduced or overexpressed *ARL*, RNA gel blot analyses were performed with two independent control lines and four independent lines of both ARLi and ARL-OE, including one line (R9 or S9) with less visible phenotypes (Figure 2). In comparison to those in control lines (CK), the obviously decreased *ARL* mRNA and highly expressed *ARL* transgene were detected in ARLi and ARL-OE plants, respectively. Consistently, the two lines (R9 and S9) with the less visible phenotype had comparatively higher *ARL* or lower transgene transcript (Figure 2). In addition, the transcripts of the fragment for RNAi were also detected in ARLi lines. Nevertheless, *ARGOS* expression remained unchanged among these transgenic lines (Figure 2). These results demonstrated that the phenotypes in *ARL* transgenic plants are due to the specific reduced or overexpressed *ARL*.

The most noticeable difference amongst *ARL* transgenic seedlings was the size of cotyledons and leaves (Figure 3a,b). Compared with that in control seedlings, the area of a fully expanded cotyledon in ARLi plants decreased by about 25% but increased by about 30% in ARL-OE plants (Figure 3a). Detailed examinations of fully expanded fifth rosette leaves showed that the width and length of the leaf blade and the length of the petiole were apparently reduced in ARLi plants but increased in ARL-OE plants (Figure 3b). Meanwhile, the size of flower organs, such as sepals, petals and siliques and the length of roots and hypocotyls of etiolated seedlings were also slightly decreased or increased correspondingly (data not shown). These results indicate that changes in *ARL* transcript levels influence organ size.

Next, we focused on further analysis of leaves and cotyledons because they are easier to characterize than other aerial organs and their differences are more distinguishable among *ARL* transgenic plants. A comparison of the growth kinetics of fifth rosette leaves revealed that size differences among *ARL* transgenic leaves resulted mainly from an altered growth rate rather than an alteration in the duration of growth (Figure 3c). This is in contrast to that previously observed in *ARGOS* transgenic plants (Hu *et al.*, 2003).

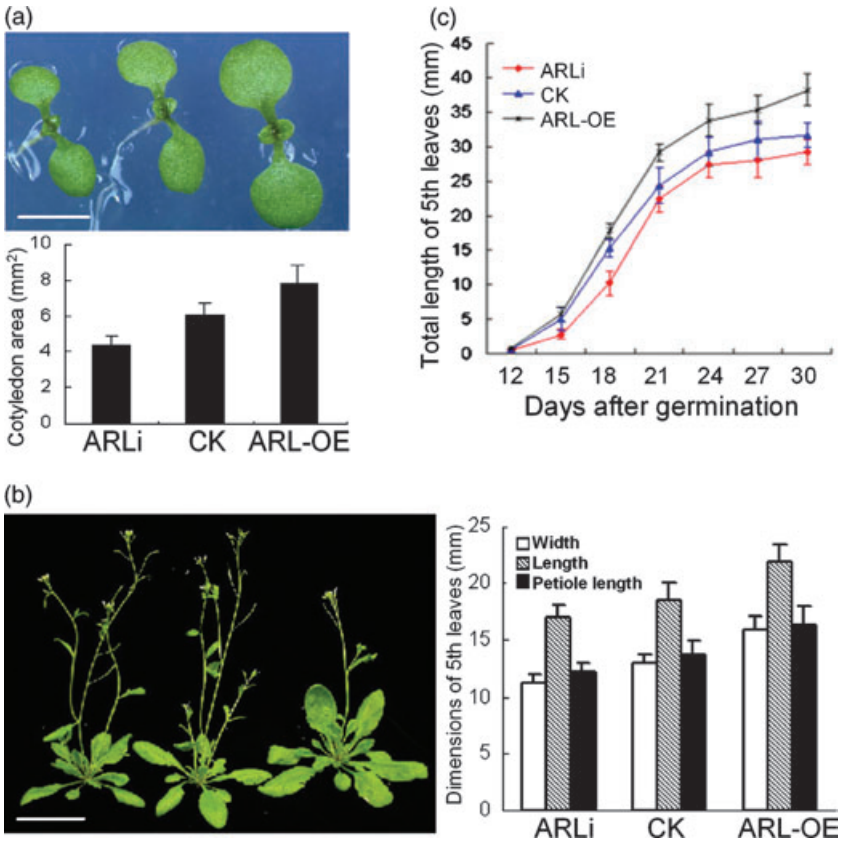

Figure 3. Phenotypes of *ARL* transgenic plants.
(a) Eight-day-old seedlings of ARLi, control (CK) and ARL-OE (top panel, from left to right) lines and cotyledon areas of 15-day-old seedlings (bottom panel, $n = 20$) of the same lines. Scale bar = 5 mm.
(b) Thirty-day-old plants of ARLi, CK and ARL-OE (left panel, from left to right) lines and the dimensions of their fifth rosette leaves (right panel, $n = 15$). Scale bar = 50 mm.
(c) Growth kinetics of fifth rosette leaves of *ARL* transgenic plants. Total leaf lengths (including blade and petiole) from plants of the three genotypes were measured starting from 12 days after germination in a 3-day interval ($n = 15$).

Cell size accounts for the change in size of organs in ARL transgenic plants

In Arabidopsis and other higher plants, cotyledons develop during embryogenesis, and cotyledon growth after seed germination depends mostly on cell expansion (Tsukaya, 2002). Thus, cotyledons were proposed to be a good model for studying the role of cell expansion during morphogenesis. The strong expression of *ARL* in cotyledons and the apparent size change in cotyledons of *ARL* transgenic seedlings led us to reason that *ARL* may function differently from *ARGOS*, which controls later organ growth by affecting the duration of cell proliferation but does not affect cotyledon size when it is over- or under-expressed in plants (Hu *et al.*, 2003). It is likely that the alteration in size of organs in *ARL* transgenic plants is not due to changes in cell number but to changes in cell size. To test this possibility, we first analyzed epidermal cells of cotyledons in *ARL* transgenic plants using scanning electron microscopy (SEM). Although epidermal pavement cells are not well shaped, the comparison of cotyledon cells among the three types of transgenic plants indicated that epidermal cells appeared smaller in seedlings of ARLi lines but larger in seedlings of ARL-OE lines compared with those in control (CK) seedlings (Figure 4a). Cell numbers counted within a fixed area of cotyledons increased about 25% in ARLi lines but decreased about 23% in ARL-OE lines (Figure 4b). Similar differences were also observed in epidermal pavement cells of fully expanded 5th rosette leaves of these transgenic lines (Figure 4c). To further analyze leaf mesophyll cells, we made thin sections in the directions of both leaf width and leaf length of fully expanded fifth rosette leaves. Sizes of palisade cells, which are the main contributors to leaf size, were variable among the three types of transgenic plants. Palisade cells of ARLi leaves were apparently smaller, whereas cells of ARL-OE leaves were bigger than those of control (Figure 4d). However, the numbers of palisade and total mesophyll cell aligning in directions of both the leaf width and leaf length remained almost same among the three types of transgenic leaves (Figure 4d). These findings demonstrate that cell size is mainly responsible for the changes in size in organs of *ARL* transgenic plants.

We further investigated the expression of *TCH4* in *ARL* transgenic seedlings. The *TCH4* gene is expressed in expanding cells and presumably plays a role in the cell expansion process, and is responsive to some plant hormones and stimuli, including auxin, BR and touch (Iliev *et al.*, 2002; Xu *et al.*, 1995). Figure 5 shows that transcript levels of *TCH4* were reduced in ARLi lines but elevated in ARL-OE lines, which is consistent with the alteration of cell size observed above.

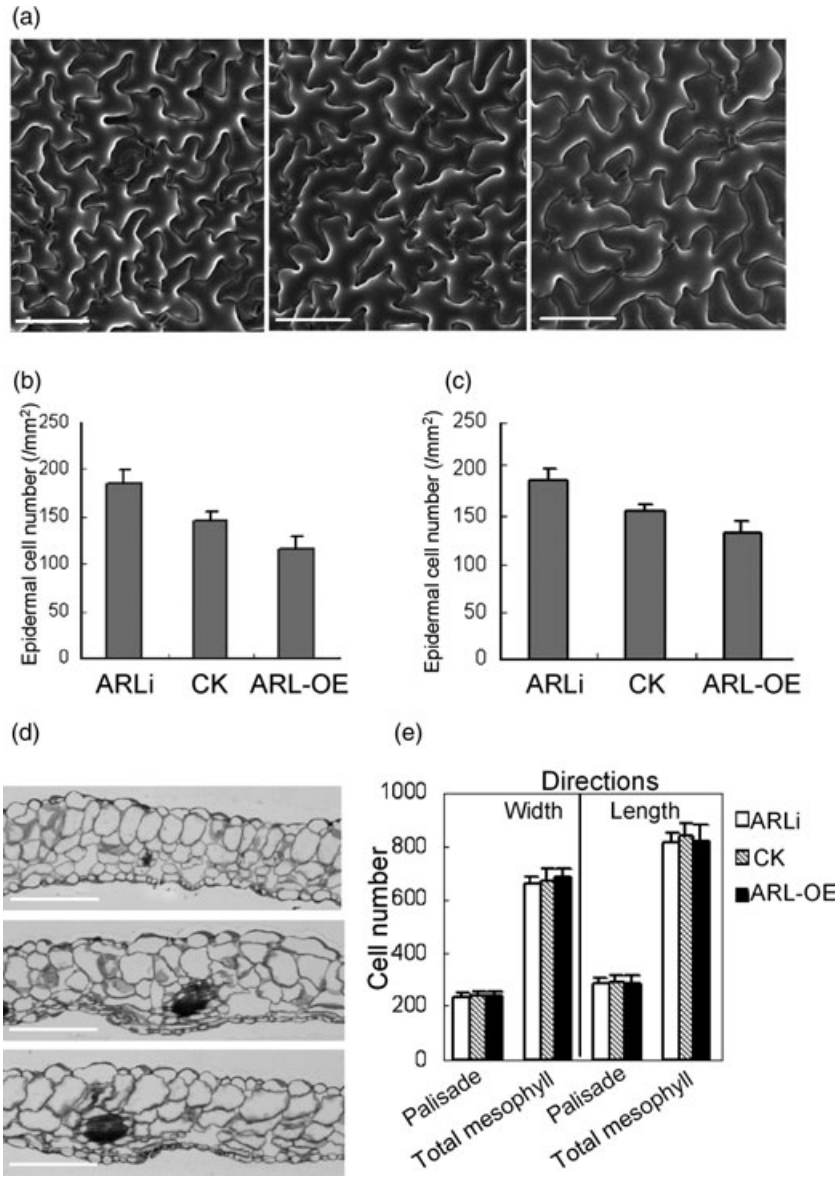

Figure 4. Anatomical analysis of cotyledons and fifth leaves of *ARL* transgenic plants.
(a) Scanning electron micrograph of adaxial epidermal cells of fully expanded cotyledons from ARLi, CK and ARL-OE seedlings (from left to right). Scale bar = 100 μm.
(b) Number of epidermal cells per area in the adaxial central region of fully expanded cotyledons of plants from the three genotypes ($n = 5$).
(c) Number of epidermal cells per area in the adaxial surface of fully expanded fifth rosette leaves of plants from the three genotypes. The positions examined were the middle area of a half leaf beside the midvein ($n = 5$).
(d) Transverse sections of fully expanded fifth rosette leaves of ARLi, control (CK) and ARL-OE plants (from top to bottom). Scale bar = 100 μm.
(e) Palisade and total mesophyll cell numbers in transverse sections across the middle region (width direction) or in longitudinal sections 1–2 mm from the midvein (length direction) ($n = 5$).

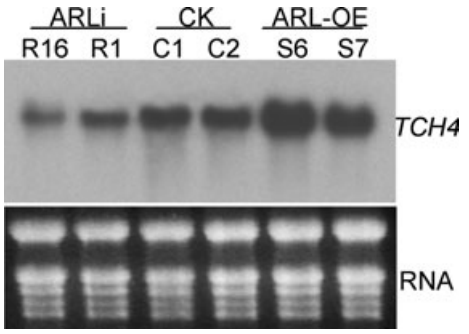

Figure 5. *TCH4* expression in *ARL* transgenic plants.
RNA isolation and Northern blot analysis was carried out with 10-day-old seedlings from two independent lines of each type of transgenic plant. Each lane contained 10 μg RNA. Stained bands of RNAs were used as loading controls.

ARL is upregulated by epi-brassinolide

Some plant hormones, such as auxin, BR, GA and ethylene, play an important role in cell expansion or elongation during plant growth and development (Davies, 1995). To test whether plant hormones are involved in *ARL*-related cell expansion, we examined *ARL* expression in seedlings treated with various hormones. Figure 6 shows that levels of *ARL* transcript were just slightly elevated by auxin and cytokinin but somewhat repressed by ABA and GA treatments. On the other hand, an apparent induction of *ARL* (2.2-fold) was found when seedlings were treated with epi-brassinolide (epi-BL) (Figure 6), indicating that *ARL* is BR-inducible. Controlled experiments showed that *ARGOS* transcripts were highly induced by auxin and cytokinin, but

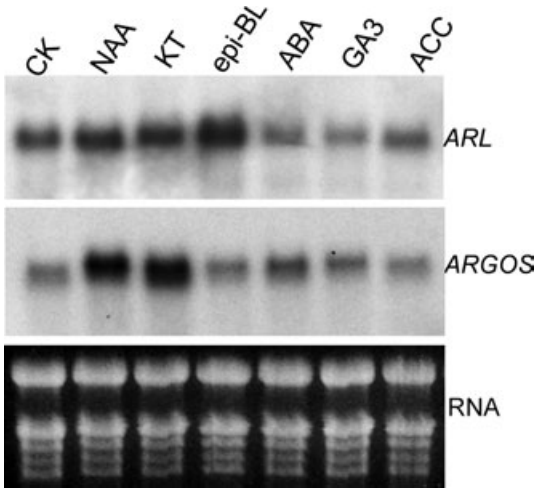

Figure 6. Transcriptional regulation of *ARL* by plant hormones.
Ten-day-old wildtype seedlings were sprayed with 2 μm NAA, 5 μm kinetin (KT), 1 μm 24-epi-brassinolide (epi-BL), 50 μm ABA, 100 μm GA3, 10 μm ACC or water [with the same concentration of DMSO (control, CK)], respectively, and harvested after 3 h for RNA isolation. Specific *ARL* and *ARGOS* probes were prepared from sequences in their 5′-untranslated regions. Similar results were obtained with samples from other independent biological experiments.

remained almost unchanged under epi-BL treatment (Figure 6). This result suggests that unlike *ARGOS*, which may mediate auxin and cytokinin signals, *ARL* is probably involved in BR-related signals.

Induction of ARL by epi-brassinolide is impaired in bri1–119 but not in det2-1

To investigate the involvement of *ARL* in BR-regulated cell expansion, we analyzed *ARL* expression in the BR-insensitive mutant *bri1* and the BR-deficient mutant *det2*. *BRI1* encodes a BR membrane-located receptor, which is essential for most BR-regulated processes, including cell expansion and elongation (Bishop and Koncz, 2002; Li and Chory, 1997; Wang *et al.*, 2001). If *ARL* mediates BR-related cell expansion signals, it is reasonable that ARL functions downstream of BRI1 and the induction of *ARL* by epi-BL should be attenuated or disrupted in *bri1* but not in BR-biosynthesis mutants. As expected, the induction of *ARL* by epi-BL was disrupted in *bri1–119*, but still detectable in the BR-deficient mutant *det2-1* (Figure 7a). This observation, along with observations that *TCH4* is a BR-inducible gene and its expression is altered in *ARL* transgenic seedlings, suggests that ARL probably regulates BR-related cell expansion downstream of BRI1.

Ectopic expression of ARL partially restores cotyledon and leaf growth of bri1–119

Next, we introduced *35S-ARL* into *bri1–119* to examine the impact of overexpressed *ARL* on cotyledon and leaf growth. Compared with control *bri1–119* seedlings, which contained an empty vector and displayed the mutant

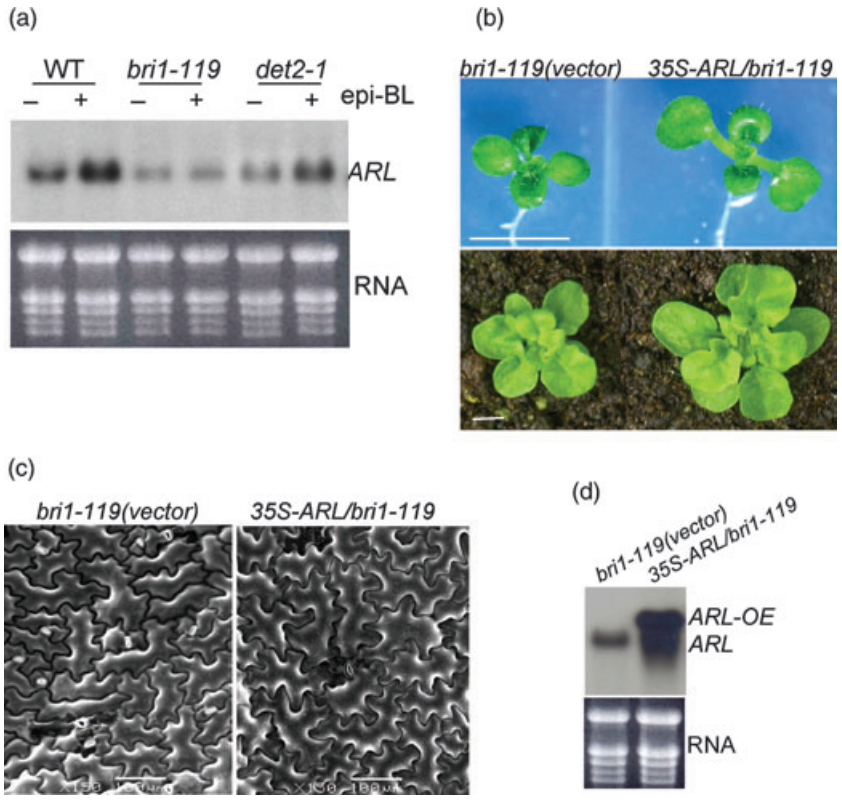

Figure 7. ARL operates downstream of BRI1 in BR signaling.
(a) The *ARL* induction by epi-BL was disrupted in BR-insensitive mutant *bri1–119* but not in BR-deficient mutant *det2-1*.
(b) Morphology of cotyledons (top, 12 days after germination) and leaves (bottom, 4 weeks after germination) of *bri1–119* containing an empty vector (*bri1–119/vector*) or *35S-ARL* (*35S-ARL/bri1–119*). Scale bars = 5 mm.
(c) SEM diagram of adaxial epidermal cells of cotyledons in two *bri1–119* transgenic plant lines. Note that the defects in epidermal cell expansion in *bri-119* cotyledons were almost restored in *35S-ARL/bri1–119*. Scale bars = 100 μm.
(d) Expression of endogenous and transgenic *ARL* in transgenic *bri1–119* plants.
Stained RNA bands were used as loading controls. Each lane contained 10 μg RNA.

phenotype with smaller cotyledons and compact leaves, overexpression of *ARL* apparently rescued cotyledon growth, and in part also restored the *bri1–119* leaf growth (Figure 7b). Scanning electron micrographs and RNA blot analysis verified that the defect in epidermal pavement cells of cotyledons in *bri1–119* was almost restored (Figure 7c) by overexpression of *ARL* (Figure 7d). However, transgenic *bri1–119* plants overexpressing *ARL* still exhibited relatively compact leaves, especially in the direction of leaf length (Figure 7b). This is because leaf development is influenced by many factors, and the *ARL*-mediated cell expansion may be just a part of this process (see below).

Discussion

ARL plays a role in cell expansion-dependent organ growth

In plants, organ growth is dependent on durable cell division and expansion. Our previous work identified Arabidopsis ARGOS as a regulator for controlling aerial organ growth (Hu *et al.*, 2003). Here, we show that ARL, which shares some sequence homology to ARGOS, is also involved in the control of organ growth. Although overexpression of *ARL* and RNAi-mediated reduced expression of *ARL* in Arabidopsis result in phenotypes similar to those of sense and antisense *ARGOS* transgenic plants, our further analyses demonstrated that these two genes actually regulate different processes. *ARGOS* controls the development of organs by affecting the duration of organ cell proliferation (Hu *et al.*, 2003) whereas *ARL* influences the growth of organs through the regulation of cell expansion.

ARL is expressed mainly in cotyledons and expanding leaves as well as elongating and mature regions in roots. In contrast to the related gene, *ARGOS*, which is highly induced by auxin and cytokinin (Figure 6), the two hormones essential for cell division *in vitro* (Krikorian, 1995), *ARL* is upregulated by BR, a plant hormone that plays a main role in cell elongation and/or expansion (Altmann, 1998; Clouse and Sasse, 1998). All these observations are consistent with their specific roles in regulating cell expansion or division during organ growth. Recent studies on some genes, such *AN*, *ROT3*, *SCD1* and *AtGRF*, have revealed that both polar cell elongation and general cell expansion are involved in the control of some organ development (Falbel *et al.*, 2003; Kim *et al.*, 1998, 2002, 2003). Our current characterization of *ARL* defines another gene that regulates organ growth by affecting cell expansion in a general manner.

ARL may mediate some BR-related cell expansion signals

Brassinosteroids are steroid hormones that are distributed widely in the plant kingdom and are essential for plant growth and development. Brassinosteroid deficient and insensitive mutants exhibit dramatic phenotypes, including dwarfism, epinastic round leaves, delayed flowering and de-etiolation in the dark (Altmann, 1999; Clouse and Sasse, 1998). Extensive studies on plant responsiveness to BR and BR-related mutants demonstrate that BR plays a critical role in polar cell expansion/elongation, which may be responsible for hypocotyl elongation and some organ growth (Clouse and Sasse, 1998; Kang *et al.*, 2001; Li *et al.*, 1996; Pérez-Pérez *et al.*, 2002). Our observation that *ARL*, which is induced by epi-BL, regulates the expansion of organ cells in a general manner suggests that this gene may be involved in BR-related control of cell expansion. Further analyses with the BR-insensitive mutant *bri1–119* and the BR-deficient mutant *det2-1* reinforced this notion. Therefore, our data show that, apart from its role in polar cell expansion, BR also functions in general cell expansion during organ growth, and *ARL* may mediate BR-dependent general cell expansion signaling. During Arabidopsis development, leaf growth in the length direction involves BR-regulated polar cell expansion and cell proliferation in lamina (Nakaya *et al.*, 2002; Pérez-Pérez *et al.*, 2002). This may explain why *ARL* overexpression simply enlarges the leaves of *bri-119* in general but does not totally restore leaf growth in the direction of the leaf length.

Regulatory roles of ARGOS and ARL in organ growth

During the growth of plant organs, cell division and cell expansion are key processes that are strictly coordinated and greatly influenced by plant hormones, such as auxin, cytokinin and BR (Mizukami, 2001). However, in general cell division and expansion are separately controlled during developmental process (Neufeld *et al.*, 1998). Obviously, plant hormone signals must execute their regulatory roles through specific or shared signaling components to influence either cell division or cell expansion or both processes, thereby affecting developmental progression. Considering their expression and functions, ARGOS and ARL may mediate such signals. ARGOS and ARL are small proteins, with just 106 and 135 amino acids, respectively, and both contain a putative transmembrane domain. The expressions of *ARGOS* and *ARGOSL* are induced by different plant hormones, indicating that they mediate different hormone signals. In addition, a loss-of-function mutant *argos* has recently been identified, and this mutant exhibits smaller organs with normal morphology (Hu and Chua, unpublished data), suggesting that ARGOS and ARL may play regulatory roles in lateral organ development.

Along with a previous report (Hu *et al.*, 2003) our data demonstrate that ARL and ARGOS act as new mediators of plant hormone signals to regulate organ growth. Further studies are needed to investigate how these two homologous genes are involved in two specific processes in organ growth and what other components are needed for these processes. In this regard, identification of molecules that

may interact with ARGOS and ARL will further our understanding of the molecular mechanisms that govern organ development.

Experimental procedures

Plant materials

Arabidopsis thaliana ecotype Columbia was used in all experiments. Seeds were surface sterilized and plated on Murashige and Skoog (MS) medium. Plates were kept at 4°C in darkness for 2–4 days and then moved to a culture room at 23°C under a photoperiod of 16 h light and 8 h darkness. For morphological and histological analyses, seedlings were transferred to soil in a growth chamber with a temperature and photoperiod the same as the culture room.

Generation of transgenic plants

To generate *ARL* promoter – GUS fusion construct, a 1.7 kb DNA fragment of the promoter region including the first intron of the *ARL* gene (*At2g44080*) was amplified by PCR from genomic DNA, and ligated into pBI101.1. To create transgenic plants constitutively under- or overexpressing the *ARL* gene, a 200 bp fragment from the 5′-untranslated region of the *ARL* cDNA was amplified and cloned into pSK-int in forward and reverse orientations at the 5′ and 3′ of an actin intron sequence (Guo *et al.*, 2003), to form an intermediate construct of pSK-ARLi. A 450 bp full-length *ARL* cDNA and an *Xho*I/*Xba*I fragment from pSK-ARLi were cloned to pVIP96 (Leu *et al.*, 1995) to generate *35S-ARL* and *35S-ARLi* constructs, respectively. The resulting plasmids were introduced into Arabidopsis by *Agrobacterium*-mediated transformation (Bechtold *et al.*, 1993). About 15 independent lines for each construct were selected on MS agar plates containing 50 mg l^{-1} kanamycin, and homozygous T_3 generations of transgenic plants were subjected to GUS staining or phenotypic and RNA blot analyses.

Gene expression analysis

To monitor the expression of *ARL*, seedlings and young organs of transgenic plants carrying *ARL* promoter – GUS fusion gene were incubated for a few hours at 37°C in the staining buffer described previously (Hu *et al.*, 2003). Total RNA isolation and RNA gel blot were performed as described (Hu *et al.*, 2000). The full-length cDNA of *ARL* was used as a probe to monitor expression of the endogenous and overexpressed *ARL* and the *ARL* RNAi fragments. An *ARGOS* non-coding fragment was used to analyze *ARGOS* expression. To examine the intracellular localization of ARL, both *35S-ARL–GFP* and *35S-GFP–ARL* fusion genes were generated with pGFP-2 and transiently expressed in onion epidermal cells by bombardment. The GFP florescence was visualized under confocal microscopy as described previously (Xie *et al.*, 2000).

Measurement of dimensional parameters of leaves and cotyledons

Fully expanded cotyledon and fifth rosette leaves were chosen as representatives for dimensional and histological analyses (Tsuge *et al.*, 1996). The initial images of 20 fully expanded cotyledons from each type of transgenic seedling were taken and cotyledon areas were determined on images. Leaf blade width, length and petiole length were measured on 30-day-old plants, and the total leaf length (including leaf blade and petiole length) was used to investigate the kinetics of leaf growth.

Scanning electron microscopy and histological analysis

To examine epidermal cells, the detached fully expanded cotyledons and fifth leaves were cryofixed in liquid nitrogen and scanned using scanning electron microscopy (SEM). Epidermal cell size and number on the adaxial side were determined at the position of a central region of a cotyledon or in a middle region of a half leaf by the midvein. At least five cotyledons and leaves from each type of transgenic plant were selected for counting epidermal cell number in a fixed area on the SEM images. Thin sections of fifth rosette leaves were prepared and stained according to the method described previously (Hu *et al.*, 2003). The palisade and total mesophyll cell numbers were determined in transverse sections across the middle region or longitudinal sections at 1–2 mm from the midvein of the blades.

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