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BOLITA, an Arabidopsis AP2/ERF-like transcription factor that affects cell expansion and proliferation/differentiation pathways

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Abstract The *BOLITA (BOL)* gene, an *AP2/ERF* transcription factor, was characterized with the help of an activation tag mutant and overexpression lines in Arabidopsis and tobacco. The leaf size of plants overexpressing *BOL* was smaller than wild type plants due to a reduction in both cell size and cell number. Moreover, severe overexpressors showed ectopic callus formation in roots. Accordingly, global gene expression analysis using the overexpression mutant reflected the alterations in cell proliferation, differentiation and growth through expression changes in *RBR, CYCD*, and *TCP* genes, as well as genes involved in cell expansion (i.e. expansins and the actin remodeling factor *ADF5*). Furthermore, the expression of hormone signaling (i.e. auxin and cytokinin), biosynthesis (i.e. ethylene and jasmonic acid) and regulatory genes was found to be perturbed in *bol-D* mutant leaves.

Keywords AP2/ERF transcription factor · Organ size · Cell growth · Cell proliferation and differentiation · Cell cycle · CyclinD/retinoblastoma pathway

Abbreviations

BL22-23	Brassinolide22-23
EBR	24-Epibrassinolide
BAP	Benzyl-amino-purine
NAA	Naphthalenacetic acid
IAA	Indole-3-acetic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
AVG	Aminoethoxyvinylglycine
STS	Silver thiosulphate
SAUR	Small auxin up-regulated RNAs

Introduction

The AP2/ERF transcription factor family is one of the largest in Arabidopsis, comprising of almost 150 genes that are differentially expressed (database of Arabidopsis transcription factors: http://datf.cbi.pku.edu.cn; (Riechmann et al. 2000; Sakuma 2002; Alonso et al. 2003; Kim et al. 2006; Nakano et al. 2006). They have been primarily studied as transcriptional regulators in plants, although proteins that contain the AP2 domain are also coded in the genomes of viruses, cyanobacteria and a ciliate, where they are thought to function as endonucleases (Magnani et al. 2004). The AP2/ERF family members are classified in groups based on the number of AP2/ERF domains and the presence of other domains. AP2 members have two, while ERF members have only one AP2 domain (Riechmann and Meyerowitz 1998). The consensus sequence of the AP2

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and the ERF domains are also slightly different, and they have been suggested to belong to distinct families (Okamuro et al. 1997; Riechmann and Meyerowitz 1998; Fujimoto et al. 2000).

While genes belonging to the AP2 family have been shown to play a developmental role, most of the ERF proteins have been studied in relation to biotic and abiotic stress (Riechmann and Meyerowitz 1998). ERFs (Ethylene response factors, also known as EREBP—ERE binding proteins) were first isolated as proteins that could bind to the Ethylene responsive element (ERE) sequence, present in promoters of a number of ethylene-responsive pathogenesis-related (PR) genes (Riechmann and Meyerowitz 1998). The EREBP/ERF domain has been shown to bind the GCC box in promoters of tobacco genes and to regulate genes containing the GCC box in Arabidopsis (Allen et al. 1998; Fujimoto et al. 2000). The study of five Arabidopsis *ERF* genes by Fujimoto and colleagues showed that they could act either as transcriptional activators or repressors (Fujimoto et al. 2000).

Interestingly, some members of the ERF subfamily in Arabidopsis, i.e. *TINY* and *DORNROSCHEN/ENHANCER OF SHOOT REGENERATION1* (*DRN/ESR1*), have not been described in context to stress, but have been rather related to developmental roles. In the case of *TINY*, its overexpression leads to plants that have organs of reduced size, due to a reduction in cell elongation (Wilson et al. 1996). *DRN/ESR1* overexpression enhances shoot regeneration from roots and leads to shoot apical meristem consumption (Banno et al. 2001; Kirch et al. 2003).

Leaf development requires the co-ordinated activity of genes that determine dorsoventrality of the primordia, switch from indeterminate to determinate growth, and regulate cell cycling and cell elongation (reviewed in Tsukaya 2005). Organ size is finally determined by cell size in combination with cell number (Mizukami 2001). Cell size increases through cell expansion, and is affected by alterations in cell wall biosynthesis enzymes and remodeling proteins like expansins (reviewed in Fleming 2006), cytoskeleton (Smith 2003; Wasteneys and Fujita 2006), and nuclear DNA content, which can be increased by endoreduplication (Sugimoto-Shirasu and Roberts 2003). Other factors, like sterols and hormones also affect cell growth (Timpte et al. 1992; Kieber et al. 1993; Klahre et al. 1998; Schrick et al. 2004). Cell proliferation, closely linked to the cell cycle, is controlled by different genes (e.g. *AINTEGUMENTA*, an AP2 gene, *ARGOS*, an auxin regulated gene, and *TCP* genes among others (Mizukami and Fischer 2000; Mizukami 2001; Hu et al. 2003; Nath et al. 2003). Like for cell expansion, changes in hormonal pathways also affect cell proliferation, leading to altered cell numbers (reviewed in Dewitte and Murray 2003). Auxin in particular has broad effects in plants and is also important in leaf development, since its accumulation leads to leaf formation in the apical meristem (Kuhlemeier and Reinhardt 2001). Transcription factors play an important role in hormone signal transduction, and they interconnect different hormone pathways (Vogler and Kuhlemeier 2003). Key effects of hormones in development have been found to be mediated by transcription factors. The *PLETHORA* genes mediate root stem cell specification in response to auxin (Aida et al. 2004), and *WUSCHEL* controls shoot meristem function by direct regulation of the cytokinin-inducible response regulators *ARR5*, *ARR6*, *ARR7* and *ARR15* (Leibfried et al. 2005).

The processes of cell proliferation and differentiation are balanced by cell cycle regulators together with other genes (reviewed in Ramirez-Parra et al. 2005). For example, the cell cycle component RBR1 (Retinoblastoma-related protein) has been shown to control nuclear proliferation in the female gametophyte and to regulate stem cell fate in the root (Ebel et al. 2004; Wildwater et al. 2005).

Here, we describe the characterization of *BOLITA (BOL)*, an Arabidopsis AP2/ERF like gene that affects cell proliferation and size, which when overexpressed in Arabidopsis leads to reduced organ size and affects cell differentiation, inducing the formation of ectopic green callus in roots. Some of its effects might be due to both perturbations of cell cycle regulators like *RBR1*, *CyclinD* and *TCP* (named after *teosinte branched 1*, *cycloidea* and *pcf1* and *2*) genes and hormone signaling alterations.

Materials and methods

Mutant identification

The original *bolita (bol-D)* mutant was first identified as a leaf mutant in a collection of plants with stable activation tag transposon insertions in ecotype Wassiliewskija (Ws) (Marsch-Martinez et al. 2002). A single plant with the *bolita* phenotype was observed among the progeny of the original parental line. Seed obtained from self-fertilized plants were sown in soil in the greenhouse and the number of plants showing the *bolita* or wild type phenotype scored.

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Plant growth

Arabidopsis seeds received a cold treatment (4°C for at least 3 nights) in a wet filter paper in petri dishes before being sown in soil. Plants were grown in the greenhouse at 22°C, mostly during long day conditions. Tobacco (*Nicotiana tabacum* cv SR1) plants were transferred from medium to soil and grown in a temperature-controlled greenhouse.

For transformant selection, Arabidopsis seeds were surface sterilized with bleach, and sown in medium containing ½ MS, 50 mg/l kanamycin, 1% sucrose, 0.8% purified agar. For phenotypic analysis of seedlings, medium lacking kanamycin, with 1% agarose was used. The plates used for these analyses were placed almost vertically in the growth chamber. Plates were kept at 4°C for at least 3 nights before transferring to the growth chamber. Tobacco seeds were sown in MS medium containing 3% sucrose and 1% agarose. Both plants were grown in a growth chamber at 22–23°C, with 16 h of light per day.

DNA analysis and plant transformation

The isolation of the sequence flanking the transposon was done by TAIL-PCR (Liu and Whittier 1995; Tsugeki et al. 1996) as described in (Marsch-Martinez et al. 2002). The *BOLITA* coding sequence (*At1g24590)* was amplified from Ws genomic DNA by PCR using the following primers: EREBP-Xba: 5′-TAT ATC TAG AAG GTC AAC CAT GGA AGA AGC-3′ and EREBP-Sst: 5′-TAT AGA GCT CTT GTC TTC ATC CAG CAC CTC-3′. The PCR was performed using Pfu polymerase (PfuUltra, Stratagene) with the following conditions: 94°3′, (94°1′, 60°1′, 72°2′30″) 35 cycles, 72°10′. The 1.2-kb product was cloned first into the pGEM-T easy (Promega) and then directionally behind the CaMV*35S* promoter in a modified pBI121 binary vector (Clontech). For the *BOL* promoter—*GUS* fusion, a 1550 bp DNA sequence upstream of the predicted translation start was also amplified by PCR from genomic Ws DNA. The following primers were used: AP2-p-Xba F: 5′-TAA TCT AGA GCT CAC GAC TTC TCT TCC TTC-3′ and AP2-p-Nco R: 5′-ATT GCT TCT TCC ATG GTT GAC CT-3′. The fragment was subsequently cloned into pGEM-T easy and then in front of the *GUS* gene in the pBINplus vector (Engelen et al. 1995). Both constructs were transformed in *A. tumefaciens* C58 for Arabidopsis and tobacco transformation. The constructs were introduced into Arabidopsis, ecotype Ws using the floral dip method with some modifications (Clough and Bent 1998). Tobacco (*Nicotiana tabacum*) transformations were done as described (Horsch et al. 1985; Mlynarova et al. 1994).

RNA isolation and gene expression analysis

RNA was isolated using either LiCl (Verwoerd et al. 1989), Trizol reagent, following the protocol supplied by the provider (Life Technologies) or with the QIAGEN RNeasy plant mini kit. Around 1 μg RNA was treated with DNAse I (Invitrogen), and 1/10 of the treated RNA was used for cDNA synthesis with M-MLV Reverse Transcriptase or Superscript II Rnase H-Reverse Transcriptase (both from Invitrogen), following the supplier's instructions.

The cDNA obtained was used for gene expression analysis. PCR were performed using cDNA from wild type and mutant tissues (wild type roots, rosette leaves, cauline leaves, stem, flower buds, flowers; mutant roots, rosette leaves, cauline leaves and flowers; and leaves from *BOL* overexpression lines -A, -B, and -C). The reactions were performed in the following conditions: 94°C 3′, (94°C 30″, 60°C 1′, 72°C 2′) 35 or 40 cycles, 72°C 10′. The following primers for the *BOL* gene were used: EREBP-Xba: 5′-TAT ATC TAG AAG GTC AAC CAT GGA AGA AGC-3′; and BL-AP2-R2: 5′-CAA TAC TGA TAA AAC ATT CCA CCAT-3′. A PCR using *ACTIN* primers for all the samples was used as a control. The reaction was performed as follows: 94°C 3′, (94°C 30″, 55°C 1′, 72°C 2′) 35 cycles, 72°C 10′. The primers were: Actin-forward: 5′-GTGTTGGACTC-TGGAGATGGTGTG -3′; and Actin-reverse 5′-GCCAAAGCAGTGATCTCTTTGCTC-3′.

Analysis of an insertion line containing an insertion in the *BOL* gene

A L*er* line containing multiple *I* element insertions was used to study the effects of gene disruption. The line was identified as containing the *Inhibitor* Tagged Site 75 (Speulman et al. 1999), indicating an insertion in the *At1g24590* exon. The position of the insertion is near nucleotide 775 in the only exon of the gene. The plants were assayed with primers itir3 (5′-CTTACCTTTTTTCTTGTAGTG-3′) and EREBP-Xba for the presence of the insertion, and with primers EREBP-Xba and EREBP-Sst to assess for plant homo or heterozygosis.

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Histological analysis and GUS staining

Impressions of leaf epidermis were done either using foam dissolved in xylene or domestic nail polish (HEMA, The Netherlands) for Arabidopsis leaves. The liquid solution or polish was applied to the adaxial surface of tobacco and Arabidopsis leaves. The dry layer was removed after 3–15 min and observed under a light microscope. Arabidopsis rosette leaves from 5 weeks old *bol-D* and wild type plants were used. The adaxial epidermis of the middle region of the leaves was analyzed at 40× magnification. GUS staining of all lines was done overnight at 37°C in a standard X-gluc solution (Gallaher 1992).

Hormone and etiolation experiments

Seed were treated at 4°C for 3 nights and the seedlings grown in a 22°C growth chamber. The ''basic'' medium used was ½ MS, 1% sucrose, 1% agarose. Two sets of experiments were done. In the first, seed were directly germinated in medium supplemented with hormones (EBR—Epibrasinolide, 5 nM; BL22-23—Brassinolide, 5 nM; BAP—Benzyl amino purine, 0.5 uM; Kinetin, 0.5 uM; GA_3—Gibberellin, 0.5 uM; and IAA—Indole-3-acetic acid, 0.5 uM, and no hormones). These seedlings were observed after 7, 11, and 33 days. In the second set, seed were first germinated in medium without hormones, and then transferred after 5 days to medium supplemented with hormones (NAA-1—Naphthaleneacetic acid, 100 nM; Kinetin, 5 uM; IAA, 5 uM; and no hormones). The seedlings were observed just before transfer, 6 days and 25 days after transfer.

The etiolation experiments were done by placing the stratified plates for 3 days in dark conditions in half-strength MS, 0.8% or 0.7% agar medium supplemented with STS 0.1 mM, AVG 5 uM, and ACC 5 uM or not supplemented. For the spraying experiments in the greenhouse, GA_3 was dissolved in 1 mM KOH, and diluted further with water, a 100 mM solution containing triton was used. The plants were sprayed just before flowering (before 4 weeks after sowing) and twice a week onwards.

Flow cytometry

Pieces of the internal area (closest to the middle vein) or to the edge of wild type and *35S-BOL* tobacco leaves were chopped in 1 ml PBS buffer (pH 6.8). The suspension was passed through a 50 μm mesh and 20 μl propidium iodine/ml was added. After 10 min the DNA content per nucleus was measured using a Beckman Coulter Epics XL-MCL flow cytometer. Different experiments were performed using independent samples, and to each sample isolated nuclei of tomato seeds or sunflower embryos were added as internal markers for DNA content.

RNA isolation, target synthesis and hybridization to Affymetrix GeneChips

Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany). The plants were grown under normal greenhouse conditions (23–25°C, 14 h light). The youngest leaves larger than 2 mm emerging from the rosette of 4 weeks old plants were used. For the biological replicates, 3–4 mutant or two wild type leaves from different plants were pooled for one sample, and the same amount from different plants for the second sample.

Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Each GeneChip experiment was performed with biological duplicates. The hybridizations were performed at the Affymetrix Core Facility in the Instituto Gulbenkian de Ciência (Oeiras, Portugal). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) Arabidopsis ATH1 Genome Arrays, according to the manufacturer's One-Cycle Target Labeling Assay. Briefly, 2.5 μg of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (One-Cycle DNA synthesis kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription (IVT) reaction to generate biotinylated cRNA (GeneChip Expression 3′-Amplification Reagents for IVT-Labeling; Affymetrix). Size distribution of the cRNA and fragmented cRNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. Ten micrograms of fragmented cRNA was used in a 200-μl hybridization containing added hybridization controls for 16 h at 45°C. Standard post-hybridization wash and double-stain protocols (EukGE-WS2v4) were used on an Affymetrix GeneChip Fluidics Station 400. Arrays were scanned on an Affymetrix GeneChip scanner 3000.

GeneChip data analysis

Scanned arrays were analyzed first with Affymetrix MAS 5.0 software to obtain Absent/Present calls and

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subsequently with DNA-Chip Analyzer (dChip) Version 1.3 (http://www.dchip.org, Wong Lab, Harvard). The arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li and Wong 2001b). Normalized CEL intensities were used to obtain model-based gene expression indices based on a PM (Perfect Match)-only model (Li and Wong 2001a). Replicate data for the same sample type were weighted gene-wise by using inverse squared standard error as weights. Only genes called Present in at least one of the four arrays and within replicate arrays called Present within a variation of $0 <$ Median (Standard Deviation/Mean) < 0.5 were kept for downstream analysis (14.474 genes). Thus, genes called Absent in all arrays and genes with highly inconsistent expression levels within replicate arrays were excluded. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was above 1.3 (Median false discovery rate of 0%). The lower confidence bound criterion means that we can be 90% confident that the fold change is a value between the lower confidence bound and a variable upper confidence bound. Li and Wong (2001a, b) have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression (Li and Wong 2001a).

Annotations for the ~22.750 genes represented on the Arabidopsis ATH1 Genome Array were obtained from the NetAffx database (www.affymetrix.com) as of April 2005 and imported into dChip using ChipInfo software (Zhong et al. 2003). All GeneChip datasets are available in a MIAME-compliant format through ArrayExpress (Accession No. XXX).

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Results

Mutant identification and description

An Arabidopsis mutant with a rosette of reduced size and extremely short stem (Fig. 1A, B, E and F) was identified from an *En-I* transposon activation tagging population (Marsch-Martinez et al. 2002). The mutant was named "*bolita*" *(bol-D)*, which means "small ball" in Spanish. Segregation analysis of *bol-D* selfings or crosses to wild type revealed wild type and mutant plants of varied severity and suggested that the mutation was semi-dominant. Among the selfed progeny, mutant plants of different sizes were observed to segregate, ranging from medium-sized plants (with a rosette diameter larger than 1 cm) to some extremely small plants (less than 0.5 cm in diameter), most probably homozygotes (Supplementary Fig. 1G). The original mutant plant and selfed progeny had small epinastic (curved downwards) rosette and cauline leaves without petioles (Fig. 1A, B and F). The leaves could not be flattened without folding or cutting the lamina, as occurs with surfaces having positive Gaussian curvature (Nath et al. 2003).

Moreover, stem elongation was severely affected in the *bol* mutant, resulting in a mature plant height of about 3 cm (Fig. 1B), representing more than 10-fold reduction compared to a 6 weeks old wild type plant (Fig. 1B and Supplementary Fig. 1J).

Bol-D flower buds were therefore compacted in a short axis (Fig. 1B and Supplementary Fig. 1C, D, I and J). They were rounder and smaller than wild type buds and they opened later, though the flowering time was not affected. Mature flowers of young plants had shorter, sometimes greenish petals and shorter anthers with no visible pollen (Fig. 1C and Supplementary Fig. 1H). In older plants, the flowers recovered the wild type petal and anther phenotype, but they had reduced male fertility. Therefore, whenever necessary, crosses were done using *bol-D* as the female parent. In spite of the reduced fertility, the medium-sized mutant progeny plants produced some selfed seed when allowed to grow for longer times than wild type plants. The siliques of the mutant remained shorter and broader than wild type, some being club-shaped (Supplementary Fig. 1F). Most of them were partially empty and contained less than half the normal amount of seeds, both in the case of crosses or selfings. In extreme cases, only one or two seeds were present. Moreover, *bol-D* seeds were larger than wild type seeds (Supplementary Fig. 1K). Finally, though roots were not strongly affected 1 week after germination, after 15 days they showed a decrease in the number of lateral roots when compared to wild type plants (Fig. 1D).

Reduction in cell size and number in the *bol-D* leaves

Leaf size depends both on cell size and cell number. Therefore, both parameters were analyzed in the small sized *bol-D* leaves. First, to assess whether cell size was affected, the epidermis was imprinted and observed

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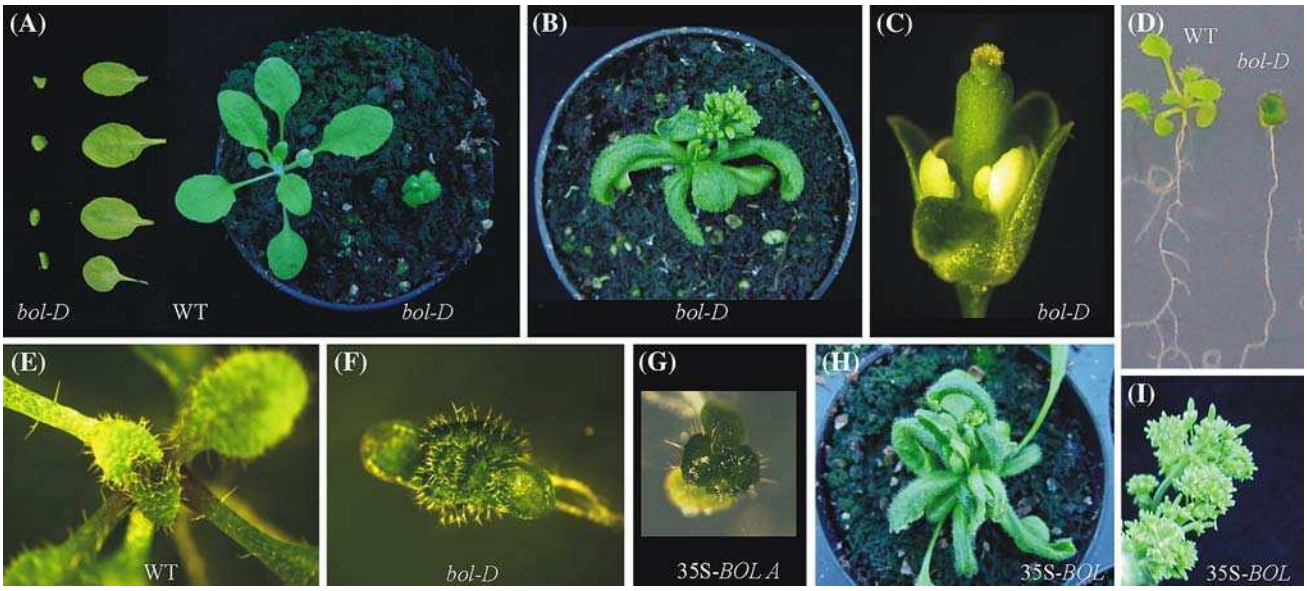

Fig. 1 Phenotypes of the original gain of function *bol-D* mutant and 35S-*BOL* lines. (**A**) Comparison of *bol-D* and wild type rosette leaves and soil grown wild type Ws and *bol-D* plants, just flowering. (**B**) Mature *bol-D* plant (older than 4 weeks) showing extremely reduced elongation of the main stem, while a wild type plant had a height of at least 30 cm (not shown). Sometimes, the first leaves elongated spirally. In general, leaves senesced slowly and the oldest leaves were thick, with severe curling that caused breaks in the leaf lamina (**C**), *bol-D* flower. (**D**) In vitro grown wild type and *bol-D* plants. *bol-D* roots have less lateral roots than wild type. (**E–G**) In vitro grown wild type (**E**), *bol-D* (**F**), and 35S-*BOL*-A (**G**) young plants. (**H**) Mature 35S-*BOL* plant.

under a light microscope. Interestingly, imprints from Arabidopsis *bol-D* leaves revealed cells of reduced size in comparison to wild type leaf cells (Table 1; Fig. 2A and B). The reduction in leaf size observed among segregating *bol-D* progeny correlated with the reduction of cell size, as leaves of smaller *bol-D* plants had smaller cells than leaves of medium-sized *bol-D* plants.

Next, the number of cells per leaf was determined (Table 1) in wild type Ws and *bol-D* plants. In the examined leaves, wild type leaf area was about 5.7 times larger than *bol-D* leaf area. The density of *bol-D* cells was almost three times the density of wild type cells, and remarkably, the total number of cells per leaf was only the half. Therefore, both cell size and cell number reduction led to the smaller leaf size in *bol-D* mutants.

Since some mutants affected in hormone pathways resemble the *bol-D* phenotype (i.e. dwarfism caused by brassinosteroid or gibberellin deficiencies (Helliwell et al. 1998; Choe et al. 2000), we tested whether hormone application would restore its leaf phenotype. None of the hormone treatments given in the conditions tested restored the leaf phenotype (See Supplementary text). However, while gibberellin sprayed to greenhouse grown plants at flowering time did not restore leaf expansion or stem elongation, it resulted in the earlier elongation of petals and anthers of *bol-D* flowers (Supplementary Fig. 1I and J).

Molecular analysis and gene isolation

Southern blot analysis of the *bol-D* activation tag mutant showed a single transposon insertion present in the genome. Isolation, sequencing of the flanking DNA, and comparison to the Arabidopsis genome sequence using BLAST (Altschul et al. 1997), revealed

Table 1 Cell density, size and number in mature leaves

Plant line (Arabidopsis)	Pavement cell density (cells/mm^2 ± SD)	Average cell size (μm^2 ± SD)	Cell number per leaf (±SD)
Wild type	140.625 ± 18.60	7214 ± 896	43031 ± 5691
bol-D	403.12 ± 33.90	2496 ± 210	21688 ± 1824
Ratio *bol-D*:Ws	2.87	0.35	0.5

The data represents eight measurements from the middle region of mature rosette leaves (adaxial epidermis) of WT and *bol-D* soil grown plants

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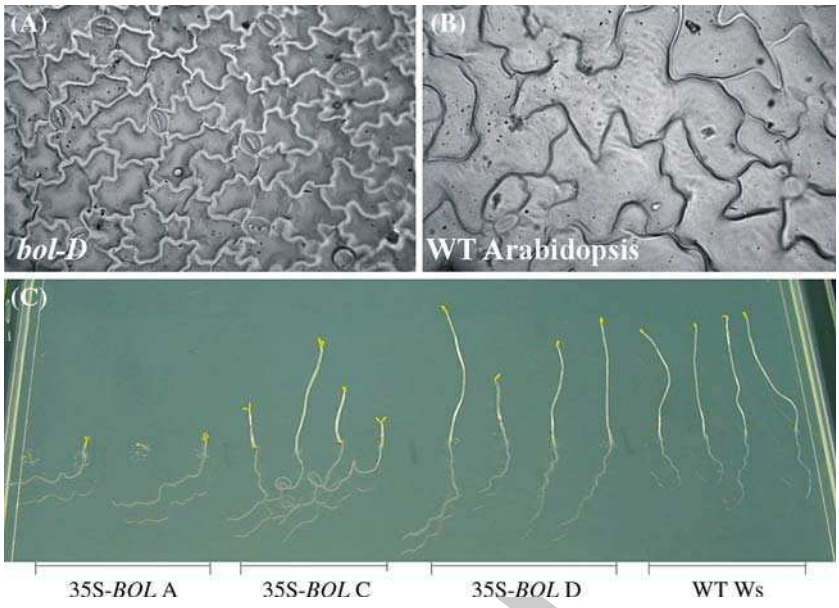

Fig. 2 Comparison of wild type Arabidopsis leaf epidermal cells to *bol-D* leaf cells. (**A** and **B**) Epidermal cells of *bol-D* (**A**) and wild type (**B**) Arabidopsis leaves (both mature rosette leaves). (**C**) Dark germinated seedlings of different 35S-*BOL* lines compared to wild type seedlings showing altered etiolation response at different degrees

that the insert was present on chromosome I, between two predicted genes transcribing outwards with respect to the insertion (Fig. 3A). The translation start of the nearer gene (*At1g24590*), encoding a putative AP2/ERF transcription factor, was situated 600 bp away from the right border of the transposon insert. The more distant gene (*At1g24600*), annotated as an expressed protein, was situated 4.5 kb from the left border, adjacent to the transposon end bearing the 35S enhancer tetramer.

RT-PCR experiments were then performed to assess expression of AP2/ERF gene *At1g24590*, representing the best candidate based on the position of the insert in the activation tag mutant and the nature of the gene itself. While in wild type plants the presence of its transcript was detected only in flower buds, in the *bol-D* mutant hyper-accumulation of this transcript occurred in roots, rosette and cauline leaves, flowers buds and open flowers (Fig. 3B). This intronless gene, henceforth named *BOLITA* (*BOL*), was predicted to encode a 306 aa protein that belongs to the ERF family, as it contains a single AP2/ERF domain. The closest homolog of BOL in the Arabidopsis genome is DRN/ESR1, which led to it being referred to as DRN-like (Kirch et al. 2003). To test whether the change in expression of this gene was causing the observed *bol-D* phenotype, an overexpression construct with the *BOL* coding sequence driven by the 35S promoter (*35S-BOL*) was introduced into wild type Arabidopsis and tobacco plants. The plants containing the overexpression construct showed leaves with the *bol-D* leaf phenotype (Figs. 1E–I and 4B and F) suggesting that *BOL* overexpression was indeed causing it.

Gene expression analysis in Arabidopsis

The RT-PCR experiment previously described showed that *BOL* transcript accumulation occurred mainly in flower buds in wild type plants, and was not detected in other tissues in the conditions tested (Fig. 3B). In addition, a BOLpromoter-*GUS* construct was used to study further the temporal and spatial pattern of expression. In plants containing the construct, GUS staining was detected at different stages of development (Fig. 5A–D and Supplementary Fig. 2A and B). In the first 2 days after germination, staining occurred at the shoot apical meristem (SAM, Fig. 5A and B) in 5 out of 6 independent transfomants, and at the distal regions of the cotyledons and the inner cell layers of the root meristematic zone (Fig. 5B and Supplementary Fig. 2A) in 3 and 2 lines, respectively. The root expression pattern, both in primary and secondary roots, was also observed in older plants. Five days after germination, seedlings showed mild staining at the SAM and intense staining at leaf primordia (Fig. 5C). Emerging leaves from older seedlings stained first at the tip and later at separated spots at the leaf periphery (hydatodes). Mature plants also showed staining at young axillary buds (Fig. 5D) and the internal organs of young flower buds, confirming the RT-PCR results and in accordance with in situ hybridization data reported by Kirch and colleagues for *DRN*-like, showing expression in young petals and stamens (Kirch et al. 2003). In mature flowers, half of the *BOL-GUS* lines showed stained anthers. Moreover, *BOL* appeared to be expressed in the embryo and seed according to a study analyzing gene expression during fruit develop-

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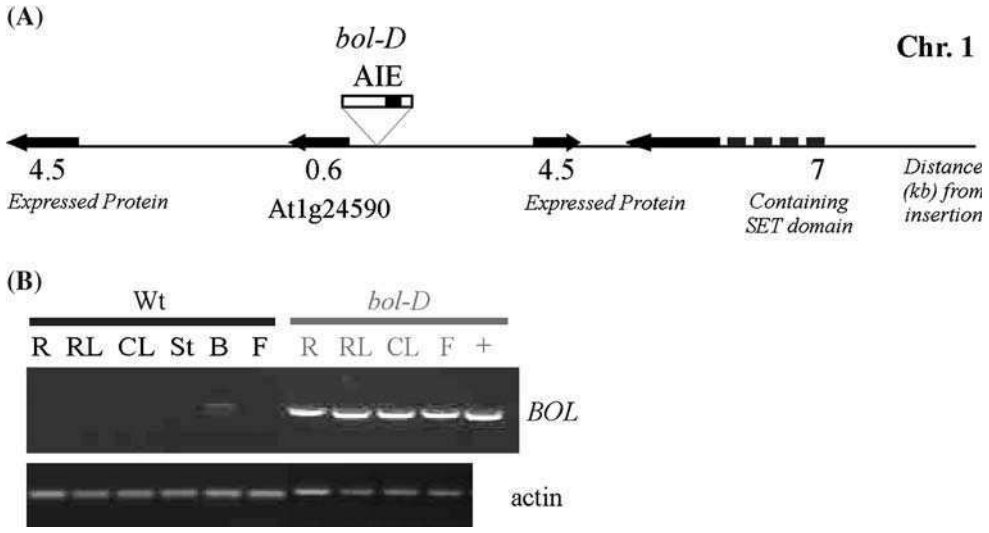

Fig. 3 Position of the Activating I Element (AIE) in *bol-D*, and expression analysis of adjacent gene. (**A**) AIE insertion in the *bolita* mutant, the dark box inside the ''insertion'' represents the 35S enhancer tetramer. (**B**) Semi-quantitative RT-PCR analysis of the AP2-ERF-like gene next to the AIE insertion. The RT-PCR shows higher accumulation of the *BOL* transcript in different tissues of the activation mutant than in wild type tissues. R, roots; RL, rosette leaves; CL, cauline leaves; St, stem; B, flower buds; F, flowers

ment (de Folter et al. 2004). Unfortunately, neither *BOL* nor *DRN*, its closest homolog, are represented in Affymetrix chips, and therefore the accumulating expression data in public databases could not be used.

Analysis of an insertion mutant in the *BOL* gene

In order to assess the effect of the *BOL* loss of function, a plant containing a transposon insertion in the gene was studied. T-DNA insertions within the *BOL* coding region were not available. The transposon insertion was identified with the adjacent sequence ITS75 in a multiple *I-dSpm* insertion population in the ecotype L*er* (Speulman et al. 1999), and was positioned at approximately 775 nucleotides after the translational start of the gene (921 nucleotides long), corresponding to the C-terminal region beyond the AP2 domain in the protein. Progenies from this line were genotyped by PCR to identify homozygous and heterozygous plants. When compared to wild type plants, the homo- and heterozygote progeny lines did not reveal fully penetrant major alterations in the general aerial architecture in mature stages or in early root development (first 3 weeks) that could be associated with the presence of the insert.

Overexpression of *BOL* in Arabidopsis induces formation of ectopic calli in vitro

Since the insertion mutant allele studied did not provide further information about the gene function, the Arabidopsis and tobacco overexpression lines were analyzed in more detail. Additional phenotypes were observed when the *35S-BOL* Arabidopsis lines were grown in vitro. Three lines out of four showed callus formation when grown on medium containing kanamycin. Ten days after germination, different tissues from the affected seedlings (i.e. cotyledons, new leaves, hypocotyl) were vitrified (Fig. 5F). Four weeks after germination, the organization of their aerial tissues was lost (Fig. 5G) and root regions, particularly above the tip, had formed callus. Some seedlings were totally converted into green callus (Fig. 5H) by this time. One of the callus forming lines, the *35S-BOL*-A line representing the most severe phenotype (with multiple loci), showed callus formation also on media lacking kanamycin. Approximately one-quarter of the *35S-BOL*-A seeds produced stunted seedlings that were yellowish/white, and did not form true leaves or a root (Supplementary Fig. 2J). The remaining seedlings developed green cotyledons and started to form true leaves, but 2 weeks after germination their aerial organs were vitrified (Fig. 5J and Supplementary Fig. 2M). After 3–4 weeks, some seedlings were almost completely converted into callus and could not survive when transferred to soil. The phenotype of callus formation was also observed in roots, which were very reduced in length and had very few lateral roots in comparison to wild type plants (Supplementary Fig. 2N and Q), an enhanced phenotype of the original *bol-D* roots. Green sectors started to form near 35S-*BOL*-A root tips (shown in Fig. 5I). These sectors were first visible as a few green

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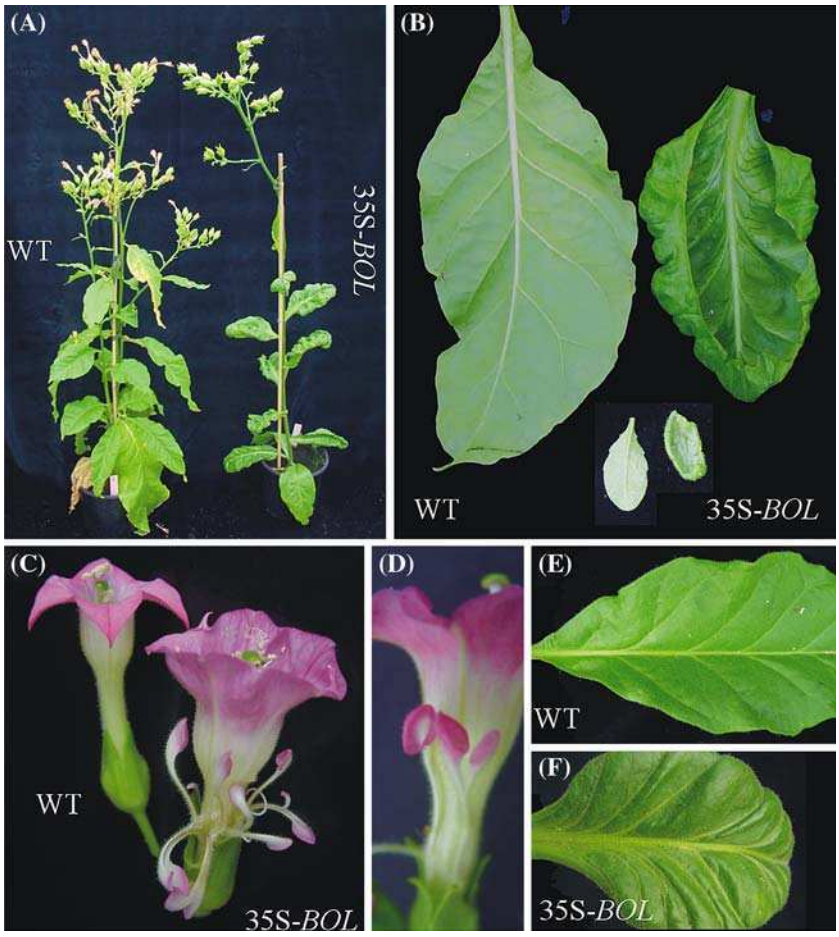

Fig. 4 Phenotype of 35S-*BOL* tobacco plants. (**A**) 35S-*BOL* compared to Wild type plant. The curved leaf phenotype present in the original *bol-D* mutant is also present in the tobacco transformants. (**B**, **E** and **F**) Wild type and 35S-*BOL* tobacco leaves. The positive curvature of the 35S-*BOL* leaf is shown in **B**. Below, a comparison of the original *bol-D* (left) and wild type (right) Arabidopsis leaves. The venation pattern of 35S-*BOL* leaves is disorganized in comparison of wild type leaves (**E** and **F**). (**C** and **D**) Comparison of 35S-*BOL* and wild type flowers. Most 35S-*BOL* flowers had increased size and some had extra petals developing between the normal petals and sepals. The small ectopic petals were closed or half closed as a tube (**D**), reminiscent of the wild type corolla

cells contrasting with the colorless root, and started to proliferate above the root surface after several days (Supplementary Fig. 2H, I and K). Noteworthy, when the green callus was detached from the root and placed again in medium devoid of hormones, it proliferated and gave rise (at least in one-fourth of the cases) to leaves and later stems and flowers, though roots were rarely observed in these conditions (Fig. 4K and Supplementary Fig. 2L).

This observation of callus formation with shoot identity close to the root tip of *35S-BOL*-A seedlings led us to study the effect of different hormones on the frequency and time of on the process. This was analyzed by seed germination directly, or seedling transfer after 5 days, to medium supplemented with different hormones. As shown in Table 2, calli initiation was observed after 7–9 days when the seedlings were grown in medium supplemented with brassinosteroids (BL22-23 and EBR) and cytokinins, whereas it required at least 14 days to observe them in untreated seedlings, a 25–50% reduction in time. More lateral roots developed in auxin treated seedlings, which resulted in a total higher number of calli per seedling. When seedlings were transferred to medium supplemented with hormones after germination, at 11 days after transfer, kinetin treated seedlings had a very defined callus at the root tip (single root) whereas NAA and IAA treated seedlings had many secondary greenish roots that were beginning to fuse with each other (data not shown). Milder *35S-BOL*-B and -C lines produced shoot tissues in the region between the hypocotyl and the root when transferred to medium supplemented with kinetin after being germinated in the presence of IAA (data

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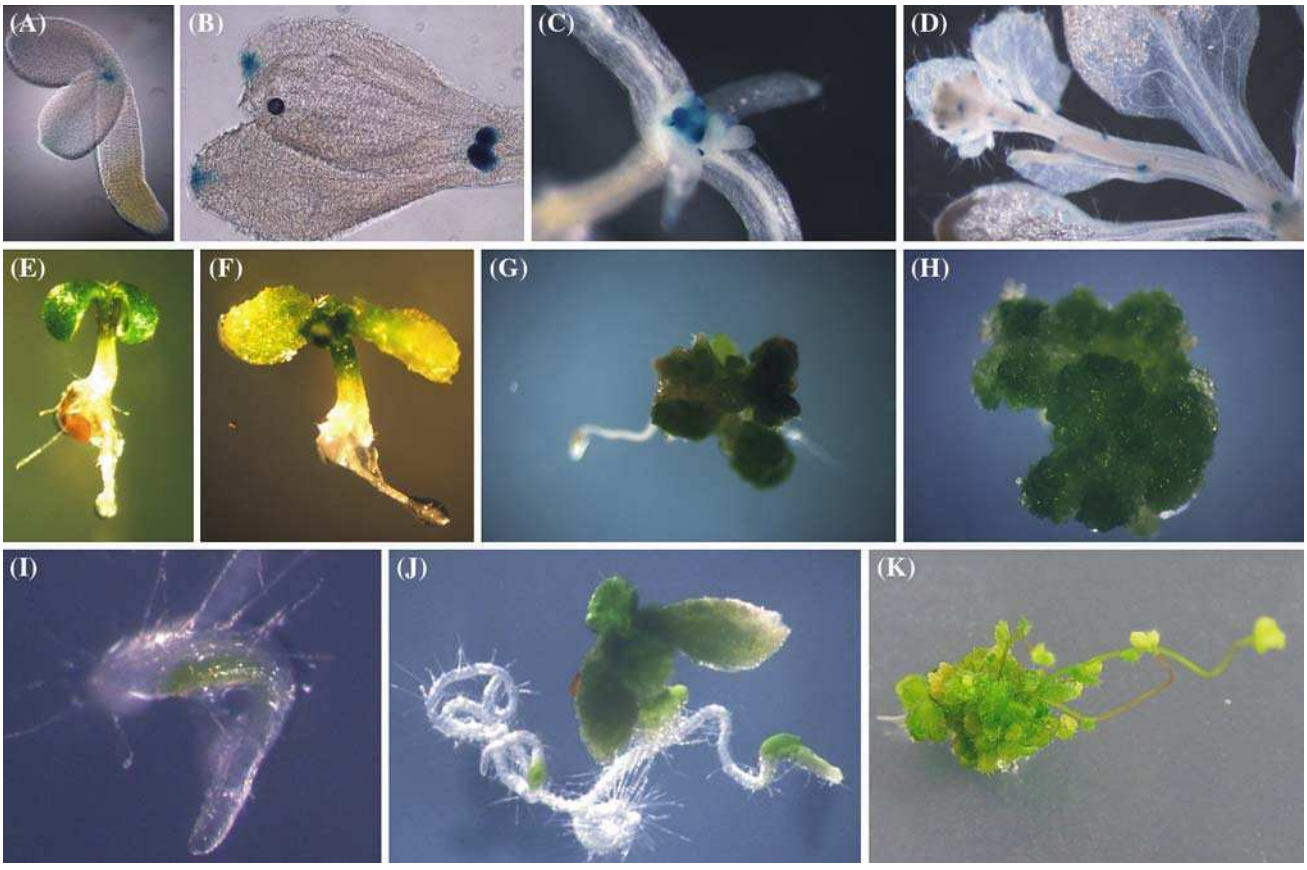

Fig. 5 Callus formation in 35S-*BOL* without the addition of hormones to the medium and X-gluc staining of *BOL* promoter-*GUS* plants. (**A**–**D**) GUS staining of *BOL* promoter-*GUS*. (**A**) One day after germination, seedlings showed staining between the cotyledons. (**B**) Two- to three-day-old seedlings showed staining of leaf primordia and the tip of the cotyledons. (**C**) Around 13 days after germination, seedlings showed staining at the tip of the young new leaves, intense staining at leaf primordia and milder staining at the meristem. (**D**) Flowering plant showing staining at the axillary meristems, young leaf stipules and flower bud internal organs. (**E**–**H**) 35S-*BOL* seedlings grown on medium containing kanamycin. (**E** and **F**) Ten days old 35S-*BOL*-B seedlings: (**E**) showing alterations in the shape of the cotyledons and (**F**) showing vitrification in the aerial tissues and thickening of the root tip. (**G** and **H**) Four weeks old plants, grown for 17 days in kanamycin and transferred afterwards to medium lacking the antibiotic, showing conversion into callus. (**G**) separate aerial organs and (**H**) conversion of the whole plant. (**I**–**K**) 35S-*BOL*-A seedlings grown on medium without kanamycin or hormones. (**I**) Initiation of green sector near the root tip. (**J**) Conversion of 35S-*BOL*-A aerial organs and root regions into callus-like tissue. (**K**) A callus detached from the root started developing shoots, leaves and flowers without the addition of hormones

not shown). This was reminiscent of the features observed in the severe *35S-BOL*-A and was also observed in some *bol-D* plants directly grown in BAP (Supplementary Fig. 2G). Noteworthy, while callus formation appeared to be enhanced in medium supplemented with 5 uM kinetin, we observed that leaves were insensitive to the treatment, since serrations at the leaf edge shown by wild type plants 4 weeks after germination were not observed in the *35S-BOL* or *bol-D* plants (Supplementary Fig. 2F).

Overexpression phenotype of *BOL* in tobacco

Tobacco plants overexpressing At*BOL* were generated and their leaves showed a cupped phenotype (positive curvature, Fig. 4B) and had smaller cells than wild type leaves (data not shown), as occurred in the original *bol-D* Arabidopsis mutant. Since nuclear DNA content, which can be increased through endoreduplication cycles, is commonly related to cell size (Kondorosi et al. 2000; Sugimoto-Shirasu and Roberts 2003), the DNA content per nucleus was measured in wild type and *35S-BOL* tobacco leaves with a flow cytometer. Characteristically, these measurements revealed a relatively higher proportion of 4C cells in 35S-*BOL* than wild type mature tobacco leaves (Supplementary Fig. 3).

Moreover, the flowers of the tobacco 35S-*BOL* lines revealed interesting morphological changes. They had broader petals than wild type flowers, with

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Table 2 Effect of hormone treatment in the frequency of callus formation at the root tip of 35S-*BOL*-A seedlings[a]

Hormone	Number of seedlings with callus at Day 7	Number of seedlings with callus at Day 9	Total germinated seedlings
EBR 5 nM	5 (71%)	7 (100%)	7
BL22-23 5 nM	3 (42%)	7 (100%)	7
BAP 0.5 uM	3 (25%)	10 (83%)	12
Kin 0.5 uM	0 (0%)	4 (33%)	12
Gib 0.5 uM	0 (0%)	1 (9%)	11
IAA 0.5 uM	0 (0%)	0 (0%)	11
Control (no hormone)	0 (0%)	1 (9%)	11

[a]The seedlings were germinated directly on medium supplemented with hormones and observed at 7 days and 9 days after germination. The number of seedlings showing visible green sectors (later calli) in the main root is indicated, and in parenthesis the percentage that it represents from the total seedlings assayed (given in the last column)

edges curving towards the inner part of the flower rather than the outside in two out of three transformants (-a and -c, Fig. 4C). Furthermore, these transformants showed an extra whorl of petals, present between the petals and the sepals in most flowers of transformant -a, and some of transformant -c (Fig. 4C). These ectopic petals were smaller than the wild type petals, longitudinally curved and sometimes forming a closed circle, as the normal tobacco fused corolla (Fig. 4D).

Effects of *BOL* activation on the expression of other genes

In order to investigate whether *BOL* overexpression resulted in changes in the expression of other genes that could explain the leaf phenotype, gene expression in *bol-D* and wild type Arabidopsis leaves was studied. Leaves were chosen for these experiments because they showed a clear, consistent phenotype that was reproduced by overexpression of the gene in tobacco, suggesting that there could be comparable effects in both plants. RNA from the youngest leaves from 4 weeks old plants was hybridized to Affymetrix Arabidopsis ATH1 Genome Arrays. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change—further referred as ''fold'' for simplicity—between experiment and baseline was above 1.3 (Median false discovery rate of 0%).

The genes differentially changed above a threshold of 2 were first analyzed. Genes involved in particular processes were overrepresented either in the up or downregulated groups. Many upregulated genes were related to lipid metabolism and transport, and histone genes were exclusively present among the upregulated genes. On the other hand, genes involved (or putatively involved) in signaling (e.g. calcium-dependent signaling), transcriptional regulation and hormone biosynthesis/signaling were prominently repressed (Table 3). Genes related to stress, transport and metabolism were present to an equal extent in both groups. The highest upregulated genes included lipid related genes, while cell wall remodeling genes were among the genes showing the highest downregulation, changing from present calls in the wild type to absent calls in the mutant. Remarkably, nine auxin responsive genes belonging to the Aux/IAA (three genes: *IAA7/AXR2*, *IAA17/AXR3*, and *IAA3/SHY2*) and SAUR (Small Auxin Up-regulated RNAs, six genes) families were changed above 2-fold. Interestingly, four of the six changed SAUR genes (*At1g29440*, *At1g29450*, *At1g29460*, *At1g29500*) belong to a cluster of eight SAURs in chromosome 1 (Scherer 2002) http://kty12.sci.hokudai.ac.jp/plant_physiol/SAUR.htm), while SHY2 and AXR3 are also located next to each other in the genome (*At1g04240* and *At1g04250*). SAUR proteins are suggested to have a role in auxin signaling involving calcium and calmodulin (Hagen and Guilfoyle 2002). In congruence, many calcium or calmodulin binding genes, including the calcium dependent protein kinase *CPK32*, were also downregulated (Cheng et al. 2002). The auxin induced genes *TCH3* and *PBP1* which contain calcium binding motives and interact in a calcium dependent manner with the PINOID kinase, a key component in auxin signaling, were also repressed (Benjamins et al. 2003).

In a deeper survey of the differentially regulated genes due to *BOL* overexpression, we lowered the threshold to 1.3-fold in order to look for genes with modest changes, which could be still informative about the role of *BOL* (Supplementary Table I). We were particularly interested in regulatory genes that might have a role in determining cell size, division, hormonal regulation and that could explain the leaf curvature. A selection of relevant genes annotated as cyclins, *RBR1*, *TCP*, and histones are shown in Table 3.

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Table 3 Selected genes with transcript level fold changes more/less than 1.3

Locus	Annotation	Probe set	WT	call WT	*bol-D*	call *bol-D*	FC
Auxin related							
At1g75580	Auxin-responsive protein	257460_at	126	A	630	P	3.55
At1g29450	Auxin-responsive protein	259784_at	427	P	142	A	–2.19
At3g03840	Auxin-responsive protein	259331_at	217	P	73	A	–2.31
At1g04250	Auxin-responsive protein/IAA induced protein 17 (IAA17/AXR3)	263664_at	614	P	191	P	–2.48
At5g18060	Auxin-responsive protein	250012_x_at	2965	P	871	P	–2.63
At1g04240	Indoleacetic acid-induced protein 3 (IAA3/SHY2)	263656_at	632	P	183	P	–2.83
At3g23050	Indoleacetic acid-induced protein 7 (IAA7/AXR2)	257769_at	2465	P	580	P	–3.64
At1g29440	Auxin-responsive family	257506_at	604	P	113	P	–3.95
At1g29500	Auxin-responsive protein	259773_at	857	P	115	P	–5.34
At1g29460	Auxin-responsive protein	259787_at	556	P	62	A	–5.82
Plus 16 more							
Ethylene related							
At4g37580	Hookless1 (HLS1)	253054_at	97	A	492	P	4.03
At4g37770	ACC synthase	253066_at	414	P	1124	P	2
At1g05010	ACC oxidase (ACO) (EAT1)	265194_at	6433	P	2089	P	–2.46
At5g47220	Ethylene-responsive element-binding factor 2 (ERF2)	248794_at	1331	P	332	P	–2.5
At1g62380	ACC oxidase	260637_at	6525	P	2024	P	–2.56
Plus 7 more							
Jasmonate related							
At1g32640	bHLH protein (RAP-1) ATMYC2, JAI1, JIN1, RD22BP1	261713_at	1021	P	429	P	–2.02
At1g17990	12-oxophytodienoate reductase	255895_at	1854	P	792	P	–2.08
At5g42650	Allene oxide synthase (AOS)	249208_at	2466	P	715	P	–2.36
At2g06050	12-oxophytodienoate reductase (OPR3) (DDE1)	265530_at	994	P	356	P	–2.57
At1g76690	12-oxophytodienoate reductase (OPR2)	259875_s_at	2931	P	772	P	–3.04
Gibberellin regulation							
At1g66350	Gibberellin regulatory protein (RGL1)	260141_at	445	P	143	P	–2.49
Cytokinin signaling							
At1g19050	Two-component responsive regulator 7 (ARR7)	259466_at	570	P	1649	P	1.9
At1g74890	Two-component responsive regulator 15 (ARR15)	262212_at	159	P	316	P	1.45
At1g10470	Two-component responsive regulator 4 (ARR4)	263236_at	688	P	1413	P	1.39
TCP, cyclin and RB							
At5g60970	TCP family transcription factor 5	247605_at	140	P	239	P	1.41
At1g69690	TCP family transcription factor 15	260371_at	320	P	486	P	1.33
At5g08330	TCP family transcription factor 21	246011_at	2312	P	976	P	–1.6
At3g50070	CYCD3.1-like	252189_at	353	A	747	P	1.77
At5g67260	CYCD3.1-like	247034_at	871	P	1586	P	1.48
At5g65420	CYCD4-1	247190_at	74	P	130	P	1.42
At3g12280	Retinoblastoma-related protein	256268_at	465	P	828	P	1.33
Nucleosome assembly							
At5g65360	Histone H3	247192_at	1013	P	2751	P	2.21
At5g10390	Histone H3	250434_at	345	P	938	P	2.08
At1g09200	Histone H3	264262_at	488	P	1071	P	1.93
At1g14900	High-mobility-group protein/HMG-I/Y protein	262840_at	176	P	427	P	1.82
At3g45930	Histone H4 /// histone H4	252562_s_at	110	P	265	P	1.81
At5g59870	Histone H2A, putative	247651_at	994	P	2198	P	1.79
At3g27360	Histone H3	257714_at	86	P	190	P	1.62
At1g74560	Nucleosome assembly protein (NAP) family protein	260235_at	396	P	747	P	1.53
At5g59690	Histone H4	247692_s_at	1538	P	2815	P	1.51
At1g07790	Histone H2B, putative	261411_at	491	P	847	P	1.43
At2g19480	Nucleosome assembly protein (NAP), putative	265940_at	1865	P	2779	P	1.42
At1g51060	Histone H2A, putative	245750_at	1259	P	1937	P	1.38
At2g38810	Histone H2A, putative	263264_at	125	A	197	P	1.37
At2g37470	Histone H2B, putative	265960_at	418	P	660	P	1.36
At4g26110	Nucleosome assembly protein (NAP), putative	253996_at	361	P	629	P	1.33

Genes with 90% lower confidence bound of fold change (FC) more than 1.3. The first three columns describe the TAIR locus (AGI ID), the gene annotation and the Affymetrix probe set. The following columns give the expression value of the gene for the wild type (WT) and *bol-D* mutant followed by the detection call (present/absent as P/A) and the FC

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In the group of genes changing from 1.3- to 2-fold, many other transcription factors (including MYB, AP2, NAM and WRKY families, and the abaxial cell fate regulator *YABBY3* (Siegfried et al. 1999), auxin and ethylene-related genes, and expansin genes were altered significantly. The auxin-related genes with altered expression summed up to a total of 25 (including those above 2-fold). Twelve ethylene-related, seven expansins and three genes involved in cytokinin signaling (two-component responsive regulators) (Hwang et al. 2002), were also altered.

The Supplementary Table II shows a gene ontology classification of all genes showing fold changes above 1.3. A significant enrichment of genes involved in the ribosome, nucleosome, cell wall catabolism, and phosphorylation was observed, as shown in Table 4. All the histone and ribosomal genes altered in expression were upregulated.

Discussion

BOL affects cell growth, cell number and differentiation

Cell proliferation and differentiation are developmentally regulated in leaves (Donnelly et al. 1999; Desvoyes et al. 2006) that reveal an organized pattern of development from the axillary meristem (Meijer and Murray 2001). To identify genes involved in this process, an activation tagging approach (Marsch-Martinez et al. 2002) was used to identify mutants with altered cell size or number, revealed as changes in leaf morphology. A small sized mutant plant isolated in this screen, named *bolita*, had petiole-less, small epinastic leaves, and a major reduction in stem elongation. In leaves, both cell expansion and cell proliferation were affected: A reduction of about three-times cell size and twice cell number accounted for the almost six times total area reduction in *bol-D* leaves when compared to wild type.

The *BOLITA* gene belongs to the *ERF* gene subfamily of transcriptional regulators and contains a single AP2 domain. Independent lines containing a *35S-BOL* construct reproduced the *bol-D* phenotype with different degrees of severity in wild type Arabidopsis, which could be due to differences in expression of *BOL* in the activation tag mutant and in the *35S* driven overexpressors. The overexpression approach allowed the phenotypic comparison to close homologs that had been studied previously in the same way, like *LEAFY PETIOLE* (*LEP*) (van der Graaff et al. 2000) and *DRN/ESR1* (Banno et al. 2001; Kirch et al. 2003). The closest homolog of the *BOL* gene in the Arabidopsis genome is *DRN/ESR1*, and had therefore been identified as *DRN-like*. *DRN/ESR1* is involved in meristem and lateral organ development. Kirch et al. (2003) reported that plants containing an insertion in the *DRN/ESR* coding sequences did not show any phenotypic alterations, possibly due to redundancy with *BOL* (*DRN-like*). However, they also indicate that since *DRN-like* (*BOL*) is not expressed in the same as *DRN/ESR1* (stem cell domain of meristems) their functions might be only partially overlapping. The overexpression phenotypes of *BOL* and *DRN/ESR1* confirm this suggestion. They share similarities that include plant dwarfism, siliques of altered shape and reduced size and formation of green calli in roots, also enhanced by cytokinin application (Banno et al. 2001; Kirch et al. 2003). However, *drn-D*, also an activation tagging mutant, prematurely arrests organ formation at the shoot meristem: It begins to form radialized lateral organs after producing 4 or 5 leaves (Kirch et al. 2003). In *bol-D* mutants such radialized organs were not ob-

Table 4 Gene ontologies enriched in the group of genes with altered expression in *bol-D*

Gene ontology	Genes found in 1144 annotated genes	Total in 17457	*P* value
Nucleosome	13	74	0.000950
Chromosome organization & biogenesis	13	72	0.000727
Nucleosome assembly	15	61	0.000007
Nucleolus	4	6	0.000247
Structural constituent of ribosome	134	578	0.000000
Ribosome	135	576	0.000000
Protein biosynthesis	137	780	0.000000
Translational elongation	11	45	0.000117
Large ribosomal unit	19	54	0.000000
Intracellular	149	873	0.000000
Protein amino acid phosphorylation	91	922	0.000052
Protein kinase activity	62	616	0.000488
Chitinase activity	6	14	0.000149
Cell Wall catabolism	7	24	0.000656

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served and the meristem did not seem to be affected as in *drn-D*, since flower buds were observed at a similar time and position as in wild type plants.

BOL transcripts were found in young stamen and petals, embryo and seed (Kirch et al. 2003; de Folter et al. 2004), with expression generally observed in meristematic regions and intensely in organ primordia. Cells in these tissues are small in comparison to cells in mature tissues. Accordingly, *BOL* confers reduced cell size in mature leaves of both Arabidopsis and tobacco overexpressors, implying a conserved function of *BOL* in cell growth regulation during development. Moreover, the cell size reduction phenotype was reflected by changes in expression of cell wall remodeling genes and the actin depolymerizing factor *ADF5*. Cell wall remodeling genes were among the most repressed genes, and the highest downregulated gene was an expansin. Expansins are key regulators of cell wall extension during growth (Li et al. 2003), and *ADF* family members are considered to be key regulators of cell and organ expansion in Arabidopsis (Dong et al. 2001; Smith 2003).

Differentiation programs were also clearly affected as revealed by changes in organ identity. The most conspicuous changes were the development of callus with shoot identity at the root tip, and vitrification of aerial organs in the most severe *BOL* overexpressor, without the addition of hormones. Milder lines showed also callus formation on aerial parts when grown on medium containing kanamycin, suggesting that the antibiotic triggered the process (probably by reducing chlorophyll and inducing redifferentiation). Remarkably, the abaxial cell fate regulator *YABBY3* was downregulated almost 2-fold in *bol-D*. This polarity gene, required for proper leaf outgrowth, also prevents cells at the leaf margins—the last to differentiate—from reverting to stem cells (Siegfried et al. 1999; Kumaran et al. 2002).

BOL causes changes in the expression of cell cycle regulators

A relationship with the cell cycle was suggested by the *BOL* pattern of expression, together with the reduced cell numbers and the leaf curvature phenotype (reproduced in a heterologous species) upon *BOL* overexpression. Support of this relationship comes from 15 nucleosome components that were upregulated in *bol-D* leaves. These included histone *H4* genes, which are also altered in the *Antirrhinum majus cin* mutant (Nath et al. 2003) and in *CYCD3;1* overexpressors (Riou-Khamlichi et al. 1999). The expression of a large number of ribosomal components was also changed. Both the changes in expression of nucleosome and ribosomal components could be related to the higher proportion of 4C cells observed in 35S-*BOL* tobacco leaves. Remarkably, this increase in the 4C cells in 35S-*BOL* tobacco leaves had also been observed in tobacco leaves overexpressing both E2Fa and DPa (Kosugi and Ohashi 2003), involved in cell cycle regulation. Additionally, other key features of their phenotypes were markedly similar in both plants: the morphology of their organs, and the small sized cells in leaves.

E2F genes are the final component of the E2F/cyclin D/retinoblastoma pathway of cell proliferation and differentiation control, where CYCD proteins inhibit RBR1 through phosphorylation, derepressing E2F regulated genes and promoting S-Phase entrance (reviewed in Dewitte and Murray 2003). Remarkably, three cyclin D (*CYCD*) genes and the single Arabidopsis *RBR1* gene were upregulated in *bol-D*, supporting a role for *BOL* in cell proliferation. A key step in the cell cycle is the G1–S transition, and it is dominantly driven by the CYCD3;1 D type cyclin (Menges et al. 2006). *CYCD* genes are expressed in different tissues and cell suspension lines (Menges et al. 2005). *CYCD3* genes are activated by cytokinins (Riou-Khamlichi et al. 1999; Gaudin et al. 2000), and are associated to proliferating, undifferentiated cells (reviewed in Dewitte and Murray 2003). During leaf development, *CYCD* transcripts are found at the proliferation stage (Beemster et al. 2005), and *CYCD3;1* is expressed at the periphery of the shoot meristem and young organ primordia (Dewitte et al. 2003), similarly to *BOL*. Moreover, some phenotypic features of plants overexpressing *CYCD3;1* (Dewitte et al. 2003) were observed in *BOL* overexpressors. For example, *CYCD3;1* overexpression can bypass the hormone requirement for the growth of Arabidopsis calli (Riou-Khamlichi et al. 1999). Moreover, the leaves of plants overexpressing *CYCD3;1* are small, curled, have asymmetries in their venation pattern and their cells have a reduced size (Dewitte et al. 2003). However, while leaves overexpressing *CYCD3;1* showed an increase in cell number, *bol-D* leaves had less cells. However, in *BOL* not only *CYCD3s* but also *RBR1* are upregulated. Dewitte and colleagues showed that *RBR1* mRNA levels were also upregulated in plants overexpressing *CYCD3;1* suggesting a possible feedback mechanism (Dewitte et al. 2003). *RBR1*, in contrast to cycD genes, is associated with the promotion of cell differentiation (Huntley et al. 1998; Wildwater et al. 2005). Moreover, it restricts

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cell division in the early stages of leaf development, but this effect largely depends on the developmental stage, the tissue and cell type, due to their distinct proliferative potential (Desvoyes et al. 2006). In this context, the reduced cell number observed in *bol-D* leaves more closely reflects the *RBR1* overexpression phenotype.

On the other hand, the ''less-cells'' phenotype could also be related to alterations in the expression of *TCP*s. Characterized members of this family of DNA-binding proteins are organ growth modifiers that function in processes related to cell proliferation, either influencing it positively (Type I) or negatively (Type II TCP genes) (Cubas et al. 1999). For example, the Antirrhinum *cyc* and *cin* mutants, defective in Type II TCP gene, show ectopic cycD3 expression (Gaudin et al. 2000; Nath et al. 2003). Type I and II TCP genes bind to different motifs in promoters (Cubas et al. 1999; Kosugi and Ohashi 2002; Li et al. 2005). Using the Pattern Match tool from the TAIR database (www.arabidopsis.org), among the *CYCD* genes, a TCP I binding site was found 1000 upstream of the *CYCD4;1* gene (GGCCCAC), and most interestingly, a TCP II binding site upstream of the *RBR1* gene (GTGGGCCC), both upregulated in *bol-D*.

In the Arabidopsis *jaw* and the Antirrhinum *cin* mutants, the absence of type II TCP gene function causes unrestrained cell division at the edges of leaves (Nath et al. 2003; Palatnik et al. 2003). The result is faster growth at the edge than inside the leaf that leads to a negative curvature phenotype. In *bol-D* leaves, three TCP genes are affected, and one of the upregulated genes belongs to class II. Accordingly, the phenotype showed by *bol-D* is exactly opposite to *jaw* and *cin* mutants: The edge seems to grow slower than the inner lamina. The altered *TCP* genes are different from those affected by the *jaw* miRNA, so their regulation might be different.

Furthermore, post-transcriptional modifications, e.g. protein degradation and phosphorylation among others, are pivotal cell cycle regulatory mechanisms. In fact, a significant enrichment of genes involved in phosphorylation was observed, though it was not further investigated. Therefore, it cannot be ruled out from the present results that these or other cell cycle components are also post-transcriptionally modified.

The perturbations in the normal cell proliferation and differentiation programs observed in different tissues of *BOL* overexpressors together with the misregulation of the *RBR1*, *CYC-D* and *TCP* genes in Arabidopsis, suggests that even if the changes just reflect a secondary or compensatory response, *BOL* is clearly capable of affecting proliferation processes.

Interaction of hormonal pathways and *BOL* expression

Some *bol-D* phenotypic features, e.g. less lateral roots in *bol-D* and photomorphogenesis in 35S-*BOL*-A hypocotyls suggested alterations in hormonal pathways (Bhalerao et al. 2002; Alabadi et al. 2004). However, hormonal treatments at concentrations that induced a response in wild type plants and partial responses on the *BOL* overexpressors, did not restore the mutant phenotype to wild type. For auxin in particular, the staining of the *DR5-GUS* reporter was not diminished in the mutant leaves, suggesting at least that the phenotype was not caused by a reduction in auxin content or in auxin transport. The microarray experiment revealed changes in many genes involved in auxin signaling rather than biosynthesis, which could explain why hormone application did not restore the mutant phenotype. Most down-regulated early auxin responsive genes from the *SAUR* and *Aux/IAA* gene families corresponded to clusters in the genome, and this co-regulation was also observed for the interacting protein pairs TCH3-PBP1 and AXR3-SHY2 (Ouellet et al. 2001; Benjamins et al. 2003), suggesting that auxin signaling was altered. The finding of particular subsets of genes from each auxin responsive family could indicate a role in the mediation of specific responses. Accordingly with perturbations in auxin signaling, the expression of a number of auxin-influenced genes was also affected in *bol-D* (e.g. cell wall, and ethylene and jasmonate related genes, among others).

Dark-grown 35S-BOL seedlings had short hypocotyls (Fig. 2C) and they showed reduced ACC sensitivity (impaired formation of an exaggerated hook, a feature of the triple response, see supplementary text) (Guzman and Ecker 1990). These features indicated possible alterations in the ethylene pathway. Accordingly, there were changes in the expression of the ethylene related genes *ERF2* (ethylene response factor 2), *HLS1* (Lehman et al. 1996), and three ACC synthases involved in ethylene biosynthesis. Both brassinosteroid and cytokinin treatments shortened the time at which green calli appeared at the *35S-BOL*-A root tip (Table 2). Cytokinin treatments had been reported to enhance shoot formation in roots of *DRN/ESR1* overexpressors (Banno et al. 2001), but the effect of brassinosteroids was not reported. Since brassinosteroids have been suggested to alter the ratio

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of cytokinin:auxin, this could explain the effect of brassinosteroids enhancing callus formation. However, a lack of response to cytokinins in leaves was observed in all overexpressors, which could suggest that the cytokinin signaling in this tissue was impaired. Three *ARR* genes (two-component responsive regulator genes involved in cytokinin signaling) were affected in *bol-D* leaves (Kiba et al. 2003b; To et al. 2004). Remarkably, two of them, *ARR4* and *ARR15*, have been characterized and have opposite effects, promoting or reducing sensitivity to exogenously applied cytokinin, respectively (Osakabe 2002; Kiba et al. 2003a).

Gibberellins specifically enhanced *bol-D* petal and anther elongation, a feature similar to gibberellin deficient mutant plants. However, young leaves showed the downregulation of the *RGL1* gene (a negative modulator of gibberellin response) (Wen and Chang 2002), reflecting a more general imbalance in the gibberellin pathway. Interestingly, *LEP*, a closely related gene to *BOL*, is a positive regulator of GA-induced germination (Ward et al. 2006).

Finally, imbalances in the jasmonic acid pathway in *bol-D* leaves were also revealed by the microarray analysis. The jasmonic acid response mediator *MYC2/JAI1* (Berger et al. 1996), and four jasmonic acid biosynthetic enzymes (Table 3) were downregulated, including the auxin induced oxide synthase (*AOS*) gene that is a major control point in octadecanoid signaling (Laudert and Weiler 1998; Tiryaki and Staswick 2002).

Notably, regulatory genes involved in the different hormonal pathways were affected. Therefore, *BOL* might connect these diverse pathways, though the gene itself did not seem to be directly regulated by short hormonal treatments in young seedlings (data not shown). On the other hand, given the complex interplay between plant hormones, this could be an indirect effect from alterations in a single hormonal pathway (e.g. auxin or cytokinin signaling) (Van Zhong and Burns 2003; De Paepe et al. 2004). The expression changes displayed could also reflect a secondary alteration caused by *BOL* misexpression, but they are indications of *BOL* influence on them.

BOL influences flower organ development

Both the expression pattern and the typical flower phenotypes observed in Arabidopsis and tobacco overexpressors suggested that *BOL* is also involved in floral organ development. Even though young petals and stamens are part of the usual expression pattern of the gene, *BOL* overexpression has an effect on them, which could be caused by ectopic expression. *35S-BOL* floral organs are altered in shape and size in both plants, with greenish petals in Arabidopsis that indicate alteration in organ identity. Moreover, the role of *BOL* in floral organ development is strongly supported by the appearance of a new petal whorl in the *35S-BOL* tobacco flowers. In this way, the comparison of the overall 35S-BOL tobacco and Arabidopsis phenotypes suggest both the presence of conserved *BOL* interactions that lead to similar phenotypes, and provides indications of new interactions in flower development.

Integrated view of the role of *BOL* in organ development

The *BOL* overexpression phenotype and the global expression data together suggest that *BOL* modulates cell growth and affects proliferation/differentiation processes. BOL overexpression also had effect in the expression of genes involved in auxin and cytokinin signaling and other hormonal pathways revealing the possibility that the effects of BOL are related to one or more hormonal signaling cascades. This is not unlikely, since there are many interconnections between different hormones themselves and with the cell cycle (Vogler and Kuhlemeier 2003; Ramirez-Parra et al. 2005). In this regard, the altered expression of three cytokinin signaling regulators (*ARR*s) and *TCP*s could be correlated with the three *CYCD* and the *RBR1* genes as a consequence of *BOL* overexpression. Noteworthy, the contrasting phenotype of arrested growth in certain tissues and excess proliferation on others seen in *BOL* overexpressors had been also observed in plants overexpressing its close homolog *DRN/ESRI*. In these plants the formation of lateral organs is arrested in the SAM, but the shoot apex has extra layers of cells, which have lost their stem cell identity (Kirch et al. 2003), while callus with shoot identity proliferates in 35S-*ESR1* roots (Banno et al. 2001). Moreover, the shoot regeneration experiments reported by Banno and colleagues suggested that *DRN/ESR1* acts synergistically with cytokinins (Banno et al. 2001). Therefore, the proposed role of *BOL* in proliferation/differentiation pathways possibly linked with hormones could be a basic function shared by *DRN/ESR1* and *BOL*. Variations, e.g. in the expression pattern and/or certain gene interactions, could account for the differences in their individual roles during development.

Organ development proceeds through different stages that involve the concerted operation of prolif-

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eration, expansion and differentiation processes (Beemster et al. 2005). Each process is temporally and spatially controlled, and the action of components like the CYCDs, RBR1 and TCPs are required for their correct succession to give rise to the final shape and size of an organ. BOL, most likely together with other genes, including some hormone signaling regulators (i.e. ARRs), is involved in the initiation of the proliferation–differentiation process from meristematic zones like the organ primordia that develops into lateral organs such as the leaf.

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