Development 133, 3955-3961 (2006) doi:10.1242/dev.02545

ASYMMETRIC LEAVES1 and auxin activities converge to repress *BREVIPEDICELLUS* expression and promote leaf development in *Arabidopsis*

Angela Hay*, Michalis Barkoulas* and Miltos Tsiantis†

Leaf development in higher plants requires the specification of leaf initials at the flanks of a pluripotent structure termed the shoot apical meristem. In *Arabidopsis*, this process is facilitated by negative interactions between class I KNOTTED1-like homeobox (KNOX) and ASYMMETRIC LEAVES1 (AS1) transcription factors, such that KNOX proteins are confined to the meristem and AS1 to leaf initials. Sites of leaf inception are also defined by local accumulation of the hormone auxin; however, it is unknown how auxin and AS1 activities are integrated to control leaf development. Here, we show that auxin and AS1 pathways converge to repress expression of the KNOX gene *BREVIPEDICELLUS* (*BP*) and thus promote leaf fate. We also demonstrate that regulated auxin gradients control leaf shape in a KNOX-independent fashion and that inappropriate KNOX activity in leaves perturbs these gradients, hence altering leaf shape. We propose that regulatory interactions between auxin, AS1 and KNOX activities may both direct leaf initiation and sculpt leaf form.

KEY WORDS: KNOX, AS1, Auxin, AXR1, Leaf development

INTRODUCTION

Correct cell fate allocation in the *Arabidopsis* shoot depends upon mutual repression between the AS1 Myb protein, which promotes leaf fate, and class I KNOX transcription factors, which promote meristem activity (Long et al., 1996; Ori et al., 2000; Byrne et al., 2000; Byrne et al., 2002). These interactions result in the delimitation of AS1-expressing leaf founder cells in the meristem. Leaf initials are also defined by local auxin maxima generated by activity of the PINFORMED1 (PIN1) auxin efflux facilitator protein (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005). However, it is not known how the promotion of organ growth by auxin is integrated with the cell fate allocation pathway defined by AS1/KNOX proteins.

The disruption of mechanisms repressing *KNOX* expression in leaves is associated with perturbations in leaf development and disruption in auxin homeostasis (Tsiantis et al., 1999; Scanlon et al., 2002; Zgurski et al., 2005), indicating that *KNOX* misexpression may perturb an auxin-directed mechanism controlling leaf morphogenesis. However, whether regulated auxin gradients sculpt leaf shape and how KNOX activity in leaves may disrupt such auxin gradients is unclear.

Here, we investigate these questions by examining genetic interactions between components of the AS1/KNOX and auxin regulatory pathways in *Arabidopsis*. We show that auxin activity acts together with AS1 to repress expression of the *KNOX* gene *BP* and hence promote leaf development. We also provide evidence that local auxin maxima are required to initiate marginal serrations in the wild-type *Arabidopsis* leaf, indicating that auxin acts not only to define leaf inception at the meristem but also later in development to control leaf shape. We also show that ectopic *BP* expression in *Arabidopsis* leaves alters leaf shape, at least in part by perturbing these local auxin gradients that shape the leaf margin. Thus, the combined action of AS1 and auxin to repress *BP* expression in leaves plays a key role in safeguarding leaf fate and controlling leaf shape.

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK.

*These authors contributed equally to this work
†Author for correspondence (e-mail: miltos.tsiantis@plants.ox.ac.uk)

Accepted 21 July 2006

MATERIALS AND METHODS

Plant material and genetics

All mutant alleles and transgenic lines are listed in Table 1. *as1;axr1*: *axr1* plants from the F2 generation of a cross between *axr1-12* and *as1-1* homozygotes were self-pollinated to generate F3 families that segregated *as1;axr1* double mutants. *bp;axr1: bp* plants from the F2 generation of a cross between *bp-9* and *axr1-12* homozygotes were self-pollinated to generate F3 families that segregated *bp;axr1* double mutants. No obvious difference in leaf phenotype was observed between *axr1-12* and *bp-9;axr1-12* leaves by visual inspection of 50 single and double mutants. *bp;as1;axr1: as1;axr1* plants from the F2 generation of a cross between *as1-1;axr1-12* and *bp-9;axr1-12* plants were self-pollinated to generate F3 families that segregated *bp;as1;axr1* triple mutants. *axr1;BP::GUS*: GUS-positive *axr1* plants from the F2 generation of a cross between *axr1-3* and *BP::GUS* homozygotes were self-pollinated to generate *axr1;BP::GUS* F3 families. *as1;axr1;BP::GUS*: *axr1* plants from the F2 generation of a cross between *axr1-3;BP::GUS* and *as1-1;BP::GUS* (Ori et al., 2000) homozygotes were self-pollinated to generate F3 families that segregated *as1;axr1;BP::GUS* double mutants. *as1;axr1;BP::GUS* double mutants were also constructed with the *axr1-12* allele and showed an identical pattern of *BP::GUS* expression to double mutants constructed with the *axr1-3* allele. *pin1-6* double mutant combinations were generated by crossing heterozygous plants with *as1-1*, *bp-9* or *blr-126* mutants. *as1*, *bp* or *blr* plants from the F2 generation were self-pollinated to generate respective F3 families that segregated *as1;pin1-6*, *bp;pin1-6* or *blr;pin1-6* double mutants. We excluded any effects of mixed backgrounds on the phenotypes by obtaining similar results using *pin1-En134*, which was generated in the Col ecotype, in crosses with *as1-1* and *bp-9*. *pid;bp: bp* plants from the F2 generation of a cross between a heterozygous *pid-3* and homozygous *bp-9* plant were self-pollinated to generate F3 families that segregated *bp;pid* double mutants. All reporter lines were crossed into respective mutant or transgenic lines and expression analysis was performed in segregating F3 families.

Plant growth conditions

Plants on soil were grown in a greenhouse with supplemental lighting (days: 18 hours, 20°C; nights: 6 hours, 16°C) or in growth cabinets under the same conditions.

DEVELOPMENT

Table 1. Plant materials

Allele	Background	Reference
as1-1	Col	CS3374, ABRC
axr1-3	Intermediate allele, Col	CS3075, ABRC
axr1-12	Strong allele, Col	Lincoln et al., 1990
PIN1:GFP	Col	Benkova et al., 2003
DR5rev::GFP	Col	Benkova et al., 2003
PIN1::GUS	Col	CS9374, ABRC
DR5::GUS	Col	Ulmasov et al., 1997
pin1-6	Strong allele, Ws	Vernoux et al., 2000
pin1En134	Null allele, Col	Galweiler et al., 1998
bp-9	Col	Smith and Hake, 2003
blr-126	SALK_040126, Col	Smith and Hake, 2003
BP::GUS	Col	Ori et al., 2000
STM::GUS	Col	Kirch et al., 2003
35S::BP	No-0	Chuck et al., 1996
ANT::GFP	Col	Grandjean et al., 2004
FIL>>BP	*Ler*	Hay and Tsiantis, 2006
IAA2::GUS	Col	Luschnig et al., 1998

Chemical treatments
1-N-naphthylphthalamic acid (NPA; Duchefa) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 500 mM and added to Murishuge Skoog (MS) medium to a final concentration of 5 or 10 μM. 2,3,5-Triiodobenzoic acid (TIBA; Sigma) was also dissolved in DMSO to a stock concentration of 500 mM and added to MS medium to a final concentration of 20 μM. In all experiments, MS plates with the same concentration of DMSO were used as controls.

Microscopy
SEM and confocal microscopy were carried out as previously described (Bowman et al., 1991; Running and Meyerowitz, 1995). Seedlings for confocal microscopy were mounted and observed in water without fixation using the 458 nm argon laser of a Zeiss LSM510 microscope.

Quantitative RT-PCR analysis
Total RNA (1 μg) extracted from mature leaf tissue was DNaseI treated and used for cDNA synthesis with an oligo(dT) primer and Superscript reverse transcriptase (Invitrogen). cDNA was amplified on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Amplification reactions were prepared with the SYBR-Green PCR Master Kit (Applied Biosystems), according to manufacturer's specifications, with 0.4 μM of primers and 10 μl of cDNA per reaction. Each reaction was made in triplicate, and each experiment was repeated three times. The efficiency of each set of primers and calculation of the level of induction was determined according to Pfaffl (Pfaffl, 2001). Error bars represent the standard error calculated on experiment repetitions. Expression levels were normalized with values obtained for the *ORNITHINE TRANSCARBAMILASE* (*OTC*) gene, which was used as an internal reference gene as described by Cnops et al. (Cnops et al., 2004). Primers are listed in Table 2.

GUS analysis
GUS activity was detected as described (Scarpella et al., 2004). Tissue was fixed in 90% acetone at –20°C for one hour, washed briefly with 100 mM phosphate buffer, and stained overnight in freshly prepared 100 mM sodium phosphate buffer with 10 mM sodium EDTA, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Molecular Probes) and ferrocyanide and ferricyanide salts (1 mM PIN1::GUS, 2 mM other GUS lines). Reactions were terminated with 95% ethanol, and leaves were dissected, mounted in 50% glycerol and viewed with dark-field microscopy.

Table 2. Quantitative RT-PCR primers

Primer name	Primer sequence
OTC-F	5′-TGAAGGGACAAAGGTTGTGTATGTT-3′
OTC-R	5′-CGCAGACAAAGTGGAATGGA-3′
STM-F	5′-TGGTGCTCCAACCTTCTGACA-3′
STM-R	5′-GTCAAGGCCAAGATCATGGCT-3′
BP-F	5′-CCATTCAGGAAGCAATGGAGTT-3′
BP-R	5′-ACTCTTCCCATCAGGATTGTTGA-3′
AS1-F	5′-CGGTCTAACGTTGTCCCTGC-3′
AS1-R	5′-AGCCATCACAACCGTTGCA-3′
PIN1-F	5′-TGCAGGTCTAGGCATGGCTA-3′
PIN1-R	5′-TTTAACGCCATGAACAACCCA-3′

Leaf silhouettes
Leaves were flattened onto clear adhesive, adhered to white paper and digitally scanned.

RESULTS AND DISCUSSION
AXR1 acts alongside AS1 to exclude *BP* expression from leaves
To investigate whether AS1 and auxin act in the same or distinct pathways to control leaf development, we examined genetic interactions between *as1* and *axr1*, which confers a primary defect in auxin signalling (Lincoln et al., 1990). *AXR1* encodes a subunit of the RUB1 activating enzyme that regulates the protein degradation activity of Skp1-Cullin-F box complexes, primarily, but not exclusively, affecting auxin responses (Leyser et al., 1993; Pozo et al., 1998; Gray et al., 2001; Schwechheimer et al., 2002; Xu et al., 2002). Leaf phenotypes of the *as1;axr1* double mutant were enhanced with respect to either single mutant, with deeply lobed margins and ectopic stipules present in the sinus of each lobe (arrowheads, Fig. 1A-L). These novel phenotypes observed in *as1;axr1* double mutants, but not in either single mutant, suggest that AS1 and auxin may act in overlapping pathways to direct leaf development.

To investigate the basis of this genetic interaction, we examined whether auxin signalling is required to repress *KNOX* expression in a manner similar to AS1. We observed inappropriate expression of the *KNOX* gene *BP* but not *SHOOTMERISTEMLESS* (*STM*) in *axr1* mutant leaves, although the level of *BP* expression was substantially higher in *as1* than in *axr1* leaves (Fig. 1M). This ectopic *BP* expression in *axr1* leaves was not accompanied by a reduction in *AS1* transcripts (Fig. 1M), indicating that AXR1 is unlikely to repress *BP* by promoting *AS1* transcription. We observed a similar expression profile of ectopic *BP* but not *STM* in leaves of a dominant *Aux/IAA17* mutant, *axr3-1* (data not shown), suggesting that regulation of *BP* by AXR1 probably reflects SCF activity related to auxin, rather than other signalling pathways. Notably, ectopic expression of *BP* in the *axr1* leaf is not responsible for the mild leaf phenotypes that distinguish *axr1* from wild type (Lincoln et al., 1990), as *bp;axr1* double mutant leaves appeared identical to *axr1* single mutants (Fig. 1C,J,N,P).

To determine whether the convergence of AS1 and AXR1 activities on *BP* regulation might account for the novel phenotypes observed in *as1;axr1* double mutants, we examined the pattern of *BP* expression in *axr1*, *as1* and *as1;axr1* leaves. Although *BP::GUS* was absent from wild-type leaves (Fig. 1R), expression was observed in the serration tips of *axr1* leaves (Fig. 1S), and in the petiole, midvein and serration tips of *as1* leaves (Fig. 1T). However, the pattern of *BP::GUS* expression in *as1;axr1* double mutant leaves was different than that of either single mutant, being sharply localised to margin cells in the sinus of every lobe from an early stage in leaf development (arrows, Fig. 1U,V), correlating with the ectopic initiation of stipules. These results indicate that both AS1 and auxin signalling are required to exclude *BP* expression from leaves, and that, in their absence, *BP* is misexpressed at the leaf margin. To test whether this novel pattern of *BP* expression observed in *as1;axr1* leaves is responsible for the ectopic initiation of stipules, we analysed *as1;axr1;bp* triple mutants. Ectopic stipules observed in cauline leaves of *as1;axr1* double mutants, but not in *as1* or *axr1*

DEVELOPMENT

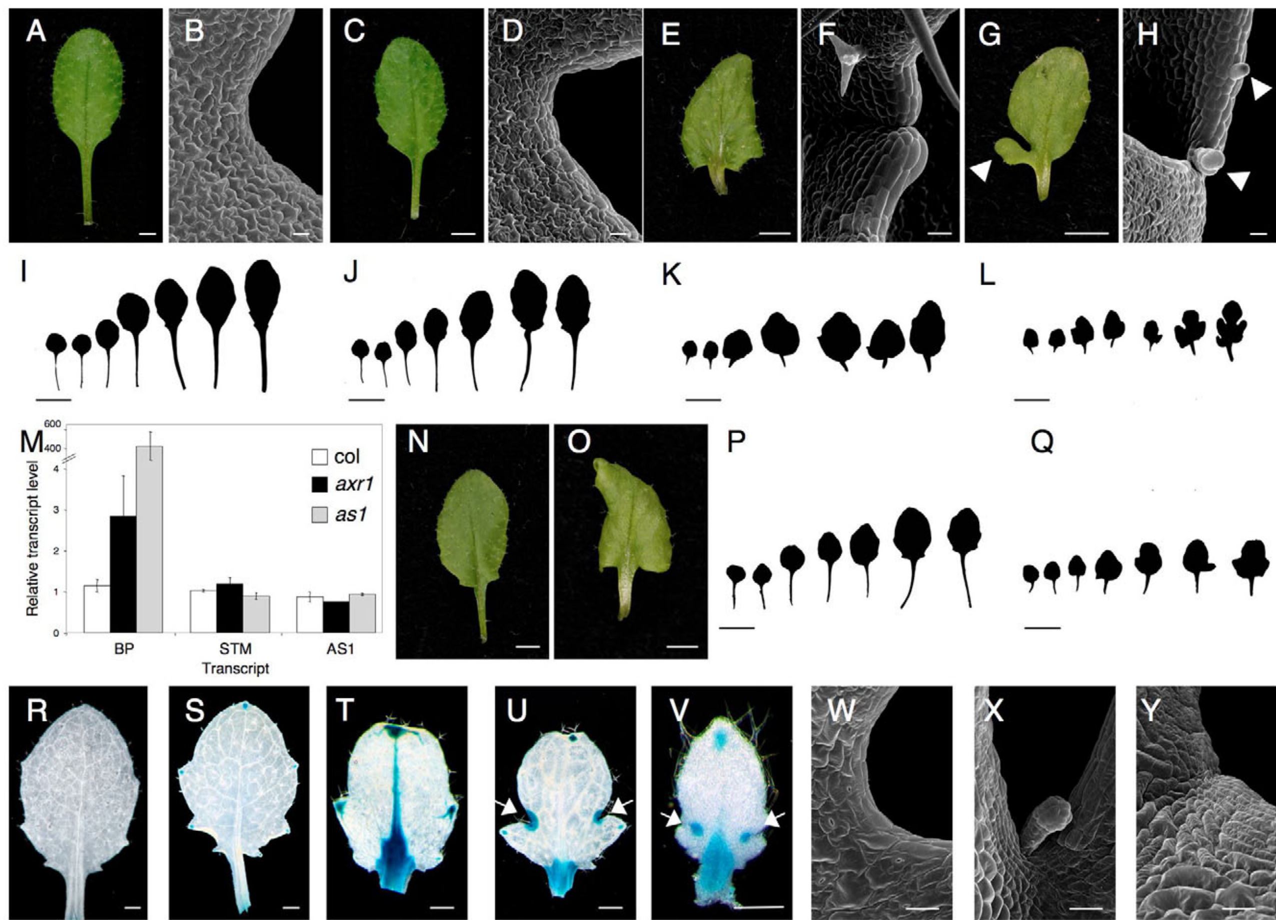

Fig. 1. AXR1 acts redundantly with AS1 to exclude *BP* expression from leaves. (**A-H**) Fourth rosette leaf (A,C,E,G) and scanning electron micrographs of the sinus region (B,D,F,H) of (A,B) Col, (C,D) *axr1-12*, (E,F) *as1-1* and (G,H) *as1-1;axr1-12* plants; arrowheads indicate lobe (G) and ectopic stipules (H). (**I-L**) Rosette leaves 1-7 of (I) Col, (J) *axr1-12*, (K) *as1-1* and (L) *as1-1;axr1-12.* (**M**) Quantitative RT-PCR analysis of *BP*, *STM* and *AS1* expression in mature leaves of Col (white bars), *axr1-3* (black bars) and *as1-1* (grey bars). Error bars indicate s.e.m. (**N**,**O**) Fourth rosette leaf of (N) *axr1-12;bp-9* double and (O) *as1-1;axr1-12;bp-9* triple mutants. (**P**,**Q**) Leaves 1-7 of (P) *axr1-12;bp-9* and (Q) *as1-1;axr1-12;bp-9*. (**R-V**) Leaves stained for *BP::GUS* expression in (R) Col, (S) *axr1-3*, (T) *as1-1* and (U,V) *axr1-3;as1-1*; arrows indicate staining in sinus regions. (R-U) Fourth leaves and (V) eighth leaf dissected from 14-day old plants. (**W-Y**) Scanning electron micrographs of the sinus region of (W) *as1-1*, (X) *as1-1;axr1-12* and (Y) *as1-1;axr1-12;bp-9* cauline leaves. Scale bars: 0.5 cm in A,C,E,G,N,O; 25 μm in B,D,F,H,W-Y; 1 cm in I-L,P,Q; 200 μm in R-V.

single mutants, were not found in *as1;axr1;bp* triple mutants (Fig. 1W-Y, *n*=20 triple mutant leaves), indicating that their initiation is likely to depend on BP activity.

It is of note that *as1* mutants do not initiate ectopic stipules, which are a hallmark of *BP* overexpression in *Arabidopsis* (Chuck et al., 1996), nor does loss of BP function suppress the *as1* mutant phenotype (Byrne et al., 2002). By contrast, in *as1;axr1* double mutants, ectopic stipules initiate at the sites of ectopic *BP* expression, and loss of BP function suppresses this phenotype. These observations suggest that, in *as1* mutant leaves, the inability of ectopic *BP* expression to elicit ectopic stipule initiation reflects the additional repression of *BP* by AXR1. As previously suggested, the ectopic initiation of stipules may reflect the formation of ectopic meristem-leaf boundaries within the leaf, because normal stipule formation occurs at the boundary between initiating leaves and the shoot apical meristem (SAM) (Ori et al., 2000). Notably, *as1;axr1;bp* triple mutant leaves do not revert to wild type, but rather to an *as1*-like morphology (Fig. 1O,Q), possibly because of the effects of additional *KNOX* genes misexpressed in *as1* or because AS1 also regulates KNOX-independent pathways to promote leaf development.

PIN1 acts with AS1 to repress *BP* and promote lateral organ development

To test whether polar transport of auxin at the shoot apex acts in concert with AS1 to promote leaf development, we analysed *pin1;as1* double mutants. The auxin efflux facilitator PIN1 transports auxin in the epidermis towards leaf initial cells that then act as auxin sinks, and this local accumulation of auxin triggers organ initiation (Benkova et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005). Strong *pin1* mutants with impaired auxin transport, therefore, initiate a reduced number of leaves and no flowers (Okada et al., 1991). However, *pin1;as1* double mutants initiate significantly fewer leaves than do *pin1* single mutants (Fig. 2A-E, Student's *t*-test, $P=2.4\times10^{-3}$), demonstrating that AS1 and PIN1 function redundantly to promote leaf development.

To test whether PIN1 promotes lateral organ development by regulating *BP*, we analysed *pin1;bp* double mutants, predicting that those aspects of the *pin1* phenotype that are BP dependent will be suppressed. We observed that the failure of *pin1* mutants to initiate both leaves and flowers is partially rescued in these double mutants (Fig. 2F-H,J,K, Student's *t*-test, *P*=0.025; see also Fig. S1 in the supplementary material, Student's *t*-test, *P*=0.0025), indicating that

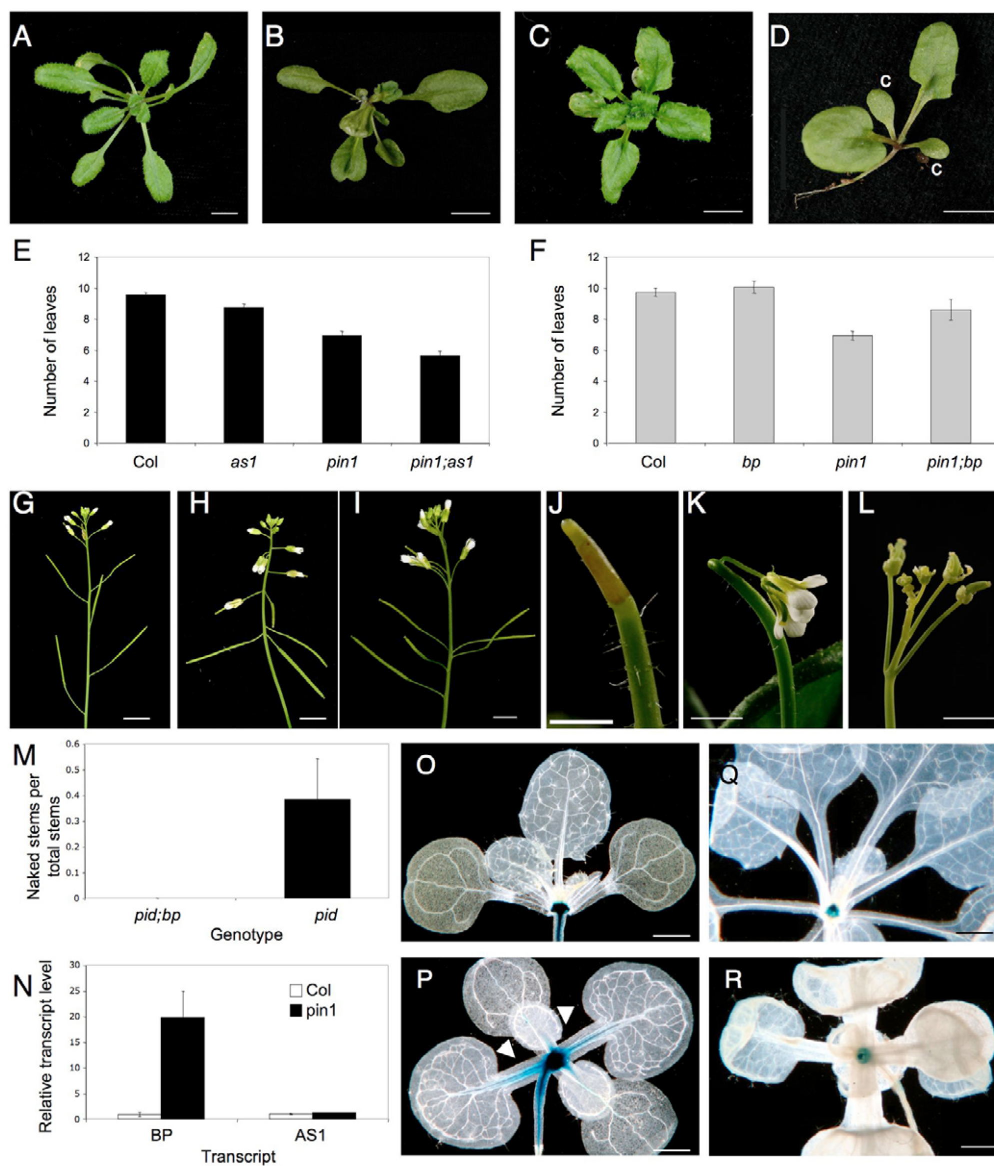

Fig. 2. PIN1 acts redundantly with AS1 to exclude *BP* expression from leaves and promote lateral organ initiation. (**A-D**) Rosettes just before bolting of (A) Col, (B) *pin1-6*, (C) *as1-1* and (D) *pin1-6;as1-1*. c, cotyledon. (**E**,**F**) The number of rosette leaves in (E) Col (*n*=68), *as1-1* (*n*=56), *pin1-En134* (*n*=30) and *pin1-En134;as1-1* (*n*=19; note, *pin1;as1* double mutants were never observed to flower earlier than *pin1* or *as1* single mutants), and (F) Col (*n*=68), *bp-9* (*n*=17), *pin1-En134* (*n*=30) and *pin1-En134;bp-9* (*n*=5) plants. (**G-L**) Inflorescences of (G) Col, (H) *bp-9*, (I) *blr-126*, (J) *pin1-6*, (K) *pin1-6;bp-9* and (L) *pin1-6;blr-126*. (**M**) The number of naked branches lacking flowers as a fraction of the total number of branches counted for *pid* (*n*=28) and *bp;pid* (*n*=29) mutants. (**N**) Quantitative RT-PCR analysis of *BP* and *AS1* expression in mature leaves of Col (white bars) and *pin1-En134* (black bars) plants. Error bars indicate s.e.m. (**O-R**) GUS-stained seedlings of *BP::GUS* grown on (O) MS media and (P) MS media supplemented with 20 μM TIBA (arrowheads indicate ectopic expression of *BP::GUS* in leaves), and *STM::GUS* grown on (Q) MS medium and (R) MS medium supplemented with 20 μM TIBA. Scale bars: 1 cm in A-D,G-L; 200 μm in O-R.

PIN1 activity in lateral organ formation involves the repression of BP activity. BP can act as a dimer with a related homeobox protein, BELLRINGER (BLR)/PENNYWISE (Byrne et al., 2003; Smith and Hake, 2003), and the flower initiation defects of *pin1* were also suppressed in *blr;pin1* double mutants (Fig. 2G,I,J,L), indicating that PIN1-mediated auxin action to promote lateral organ initiation is antagonised by both BLR and BP activities. Antagonistic actions of BP towards auxin-mediated organogenesis were also observed in double mutants between *bp* and *pinoid* (*pid*) (Bennett et al., 1995; Benjamins et al., 2001), in which organ initiation defects of *pid* mutants were partially suppressed (Fig. 2M, Student's *t*-test, P=2.1442$\times 10^{-6}$).

To test whether regulated auxin transport is required to exclude *BP* expression from leaves, we analysed *BP* expression in *pin1* mutants and plants where auxin transport was perturbed by treatment with auxin transport inhibitors. We observed misexpression of *BP* transcripts in *pin1* leaves, relative to wild type, whereas *AS1* transcript levels were unaltered (Fig. 2N), which indicates that PIN1 activity is required to repress *BP* expression, but not via promoting *AS1* transcription. Similarly, we observed ectopic *BP::GUS* expression in the leaves of plants treated with the auxin transport inhibitors 1-N-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) (Fig. 2O,P and data not shown), whereas *STM::GUS* expression was unaltered by these treatments (Fig. 2Q,R and data not shown). This suggests that the correct regulation of auxin transport is required to repress *BP* expression independently of other meristem-expressed genes.

PIN1 regulates leaf margin development

Our results suggest that auxin and AS1 activities promote leaf fate, in part by excluding meristem-expressed *BP* transcripts from leaves. Previous work has shown that aberrant leaf development resulting from inappropriate *KNOX* expression is associated with reduced polar auxin transport and altered auxin distribution (Tsiantis et al., 1999; Scanlon et al., 2002; Zgurski et al., 2005). Thus, regulated auxin transport may be an important determinant of leaf shape. To investigate whether PIN1-directed auxin flux controls leaf shape, we compared the margin configuration of *pin1* and wild-type leaves. Whereas wild-type (Col ecotype) rosette leaves have a serrated margin (Fig. 3A), *pin1-En134* mutants (Col ecotype) have a smooth margin (Fig. 3B), indicating that PIN1 activity promotes the development of leaf marginal serrations, hence determining leaf

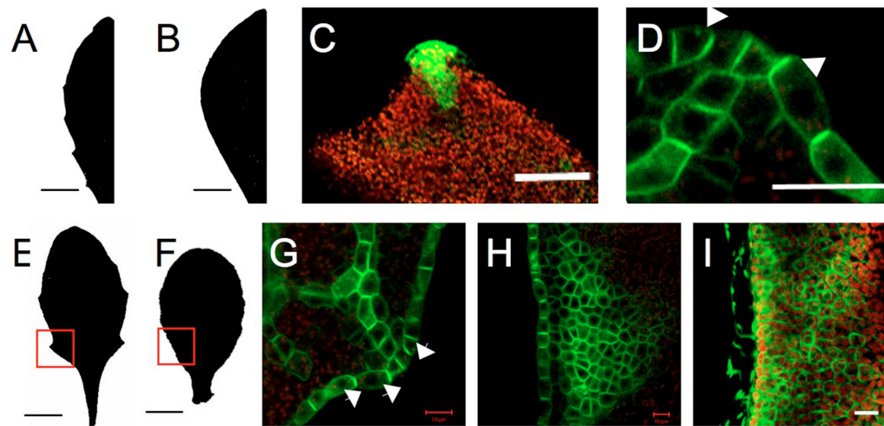

Fig. 3. PIN1 regulates leaf margin development. (**A**,**B**) Half-leaf silhouettes of the seventh rosette leaf of (A) wild type and (B) *pin1-En134*. (**C**,**D**) Confocal micrographs of GFP expression (green) in wild-type developing leaf margins. (C) *DR5rev::GFP* expression maximum at the tip of a serration, and (D) PIN1:GFP localisation indicates the direction of auxin flux (arrowheads) towards the serration tip. (**E**,**F**) Whole-leaf silhouettes of the seventh rosette leaf of wild-type (Col) plants grown on (E) MS medium and (F) MS medium supplemented with 5 μM NPA. (**G-I**) Confocal micrographs of GFP expression (green) in developing leaf margins indicated by boxes in E,F. (G) PIN1:GFP localisation indicates the direction of auxin flux (arrows) towards the serration tip in plants gown on MS (box in E), whereas, in the margin of NPA-treated plants (box in F), PIN1:GFP localisation is non-polar (H) and *DR5rev::GFP* expression is diffuse (I). Red indicates chlorophyll autofluorescence. Scale bars: 0.5 cm in A,B,E,F; 20 μm in C,D; 10 μm in G-I.

shape. This effect of loss of *PIN1* function on the leaf margin is independent of the ectopic *BP* expression observed in *pin1* leaves, as the margins of *bp;pin* double mutant leaves are indistinguishable from those of *pin1* single mutants (data not shown).

To investigate whether serrations are elaborated from the leaf margin through the generation of PIN1-directed auxin maxima, similar to those driving organogenesis at the tips of emerging organs (Benkova et al., 2003), we assayed *DR5rev::GFP* and PIN1:GFP expression in wild-type leaf margins. *DR5rev::GFP* was localised in the tips of initiating serrations (Fig. 3C), and polar expression of PIN1:GFP in the epidermis indicated that these auxin maxima may be generated by PIN1-directed auxin efflux (arrowheads Fig. 3D). This observation suggests that aspects of the mechanism whereby PIN1-dependent auxin activity gradients trigger leaf initiation at the SAM may be recapitulated within the *Arabidopsis* leaf to determine the shape of the leaf margin. These local auxin maxima correlated with epidermal convergence points of PIN1 polarity in the leaf margin have recently been shown to control vein positioning (Scarpella et al., 2006), suggesting that a common auxin-mediated mechanism may underlie patterning of leaf venation and elaboration of leaf shape.

To further test whether local auxin activity gradients active in the developing leaf margin are required to initiate serrations, we perturbed these gradients by growing wild-type plants (Col ecotype) on NPA. Compared with the serrated leaf margin of plants grown on MS medium (Fig. 3E), a smoother leaf margin developed when these plants were grown on MS medium supplemented with NPA (Fig. 3F). Local auxin activity gradients and polar localisation of PIN1:GFP in margin cells of plants grown on MS medium (arrowheads, Fig. 3G) were abolished in NPA-grown plants and *pin1* mutants (Fig. 3H,I, see also Fig. S2 in the supplementary material), demonstrating that these local gradients of auxin activity, generated by PIN1 polarity, are required for the development of a serrated wild-type leaf margin.

To test whether auxin activity in the developing leaf margin responds to ectopic *KNOX* expression, we examined *PIN1::GUS* and *DR5::GUS* expression in wild-type and *35S::BP* leaves. In comparison with wild-type leaves (Fig. 4A,C), *PIN1::GUS* and *DR5::GUS* expression was repressed in the distal lamina and concentrated in developing lobes of *35S::BP* leaves (Fig. 4B,D). Therefore, KNOX exclusion from leaves is required to establish the wild-type pattern of auxin activity gradients and *PIN1* expression in leaves. To further examine whether PIN1 localisation in the leaf margin is altered in response to ectopic KNOX activity, we assayed PIN1:GFP expression during the development of wild-type and dissected leaves that result from ectopically expressing *BP* under the control of the *FILAMENTOUS FLOWER* (*FIL*) promoter (Hay and Tsiantis, 2006). PIN1:GFP expression maxima were observed at sites of developing serrations along the wild-type leaf margin (arrow Fig. 4E), and were shifted basipetally as new serrations were initiated (arrow Fig. 4F). PIN1:GFP expression in initiating leaflets of *FIL>>BP* leaves was indistinguishable from that of wild type; however, expression persisted as leaflets developed in *FIL>>BP* leaves (arrows Fig. 4G), suggesting that KNOX activity in the leaf prevents the normal basipetal displacement of PIN1:GFP expression maxima, correlating with prolonged localised growth and leaflet formation. BP-induced alterations in PIN1:GFP expression were mirrored by similar alterations in expression of the *AINTEGUMENTA* (*ANT*) gene (arrows, Fig. 4H-J), which promotes growth in *Arabidopsis* lateral organs (Krizek, 1999; Mizukami and Fischer, 2000; Grandjean et al., 2004). These observations suggest that auxin-mediated reorganisation of growth at the leaf margins underpins leaflet formation in *FIL>>BP* leaves.

To test whether perturbation of such auxin activity gradients contributes to KNOX-dependent alterations in leaf shape, we analysed leaflet formation in *FIL>>BP* plants grown on MS medium (Fig. 4K) or MS medium supplemented with NPA. Strikingly, we observed that NPA treatment completely blocked leaflet initiation (Fig. 4L; a similar suppression of lobe initiation by NPA treatment was observed in *35S::BP* plants, data not shown), and prevented the generation of PIN1-directed auxin maxima in the leaf margin (Fig. 4M and data not shown). The smooth margin formed in both wild-type and *FIL>>BP* plants as a result of NPA treatment indicates that local auxin maxima generated by polar auxin transport may stimulate the localised growth required for

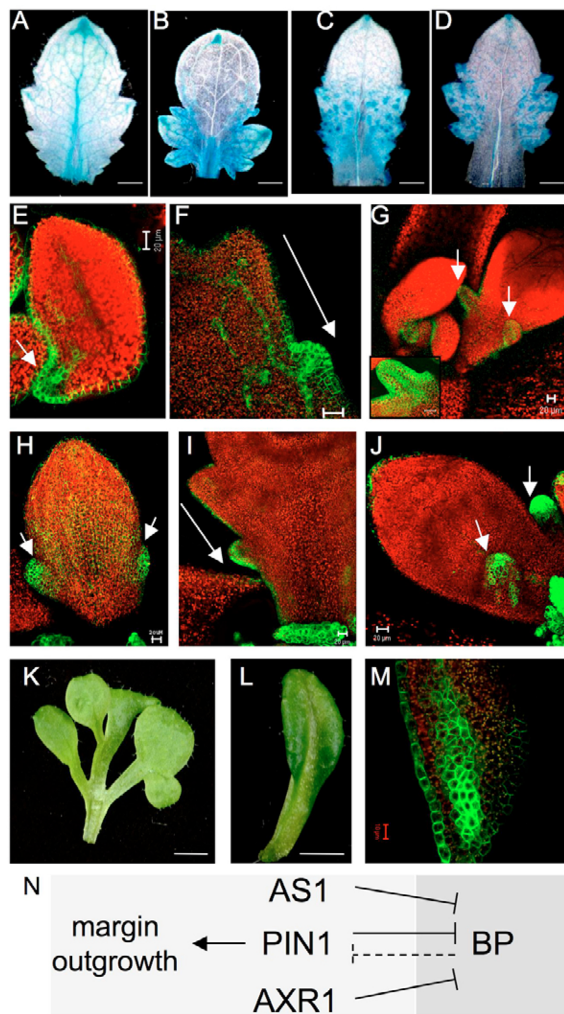

Fig. 4. Prolonged expression of PIN1 at the margin accompanies leaflet initiation in *FIL>>BP* leaves. (**A-D**) Young rosette leaves stained for GUS expression of (A,B) *PIN1::GUS* in wild type (A) and *35S::BP* (B), and (C,D) *DR5::GUS* in wild type (C) and *35S::BP* (D). (**E-J**) Confocal micrographs of GFP expression (green) in developing leaf margins, 7 (E,H), 10 (F,I) and 12 (G,J) days after germination. (E-G) PIN1:GFP in (E) wild-type (arrow indicates GFP expression in initiating serration), (F) wild-type (arrow indicates a basal shift in GFP expression as a second serration initiates), and (G) *FIL>>BP* (arrows indicate prolonged GFP expression in developing lobes; inset shows a magnification of one of these lobes). (H-J) *ANT::GFP* is expressed in a similar manner in (H) wild type (arrows indicate GFP expression in initiating serrations), (I) wild-type (arrow indicates a basal shift in GFP expression as a second serration initiates), and (J) *FIL>>BP* (arrows indicate prolonged GFP expression in developing lobes). (**K**,**L**) Rosette leaves of *FIL>>BP* plants grown on (K) MS medium and (L) MS medium supplemented with 10 μM NPA. (**M**) Confocal micrograph of PIN1:GFP expression in a developing leaf margin of *FIL>>BP* plants grown on MS medium supplemented with 5 μM NPA. (**N**) Proposed role of auxin in regulating BP activity and leaf shape. *AS1* acts in overlapping pathways with *PIN1* and *AXR1* to repress *BP* expression in *Arabidopsis* leaves (solid barred lines), thus contributing to definition of the leaf-meristem boundary and control of leaf development. *PIN1* is also required to elaborate margin outgrowths (arrow) in wild-type leaves and in leaves in which *BP* is ectopically expressed. Such BP-mediated changes in leaf shape may involve restriction of the *PIN1* expression domain by BP (dotted barred line). Arrows and barred lines denote genetic and not physical interactions. Red indicates chlorophyll autofluorescence. Scale bars: 200 μm in A-D; 20 μm in E-J; 0.5 cm in K,L; 10 μm in M.

development of both a wild-type serrated margin, when *BP* is absent from the leaf, and a dissected leaf margin, when *BP* is ectopically expressed in the leaf. Thus, although auxin activity gradients acting in the leaf margin to control leaf shape are sensitive to ectopic BP activity, their effects on leaf shape are mediated by factors that remain unknown.

By contrast, BP antagonises PIN1 activity in leaf initiation, and the repression of *BP* expression in leaves requires both PIN1 and AXR1 activities (Fig. 4N), suggesting that auxin-dependent repression of *BP* may be a component of leaf initiation processes. Alternatively, the suppression of *pin1* defects observed in *bp;pin1* double mutants may reflect post-transcriptional or post-translational regulation of *BP*, or the repression of BP-dependent processes by auxin. High-resolution studies of *BP* expression in the apices of wild type and *pin1* mutants (e.g. Heisler et al., 2005), and the characterization of downstream targets of BP, will help to distinguish between these two possibilities that are not mutually exclusive. The partial recovery of organ initiation defects of *pin1* and *pid* mutants in *bp;pin1* and *bp;pid* double mutants is reminiscent of the suppression of cotyledon boundary formation and the growth defects of *pin1;pid* double mutants in *stm;pin;pid* triple mutants (Furutani et al., 2004). These genetic interactions may indicate that the antagonism between KNOX and auxin activities operates in multiple contexts throughout *Arabidopsis* development to promote organ initiation and the associated elaboration of organ boundaries. Taken together, these observations highlight the modular nature of auxin action, and emphasize the significance of identifying factors that contextualise auxin action in distinct tissues at different developmental stages and possibly at various auxin concentrations (Ljung et al., 2001; Xu et al., 2006). For example, the auxin response factors (ARF) ETTIN/ARF3 and ARF4 act with KANADI proteins to facilitate axial patterning of lateral organs (Pekker et al., 2005); therefore, it will be interesting to determine whether the same or different transcriptional components mediate the repression of *BP* by auxin.

Conclusions

Our results establish two novel points about developmental patterning in plants. First, we show that auxin activity, directed by PIN1-dependent fluxes, is required together with AS1 to repress *BP* expression and promote leaf development. Secondly, we show that PIN1 activity is required later in leaf development to control leaf shape by regulating the initiation of marginal serrations (Fig. 4N). Ectopic *KNOX* expression in leaves perturbs these PIN1-dependent local gradients of auxin activity, resulting in lobe or leaflet outgrowth. Both KNOX activity in leaves and auxin signalling are involved in the development of dissected leaf forms in nature (Bharathan et al., 2002; Wang et al., 2005; Hay and Tsiantis, 2006); therefore, it is possible that the differential regulation of auxin activity gradients by KNOX proteins mediates natural variation in leaf form.

We thank O. Leyser, A. Hudson, Y. Eshed and S. Hake for critical reading of the manuscript. We thank J. Friml for PIN1:GFP and *DR5rev::GFP* seeds, O. Leyser for *axr1-12* seeds, Y. Mizukami for *ANT::GFP* seeds, B. Scheres and I. Blilou for *pin1En134* seeds, J. Traas for *pin1-6* seeds, W. Werr for *STM::GUS* seeds, S. Hake and N. Ori for *bp-9* and *35S::BP* seeds, J. Craft for *FIL>>BP* lines, and Y. Eshed and J. Bowman for *FIL::LhG4* seeds. We also thank J. Baker for photography, I. Moore for assistance with confocal microscopy and the *Arabidopsis* Biological Resource Center for seeds. M.T. receives support from the BBSRC and the Gatsby Charitable foundation. A.H. is the recipient of a University of Oxford Glasstone Research Fellowship and a Balliol College Junior Research Fellowship. M.B. is the recipient of a Bodossakis Foundation Award. This work was also funded by an EU MECHPLANT project.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/20/3955/DC1

References

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001). The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. *Development* **128**, 4057-4067.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591-602.

Bennett, S. R. M., Alvarez, J., Bossinger, G. and Smyth, D. R. (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. *Plant J.* **8**, 505-520.

Bharathan, G., Goliber, T. E., Moore, C., Kessler, S., Pham, T. and Sinha, N. R. (2002). Homologies in leaf form inferred from KNOXI gene expression during development. *Science* **296**, 1858-1860.

Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1991). Genetic interactions among floral homeotic genes of Arabidopsis. *Development* **112**, 1-20.

Byrne, M. E., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A. and Martienssen, R. A. (2000). Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. *Nature* **408**, 967-971.

Byrne, M. E., Simorowski, J. and Martienssen, R. A. (2002). ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. *Development* **129**, 1957-1965.

Byrne, M. E., Groover, A. T., Fontana, J. R. and Martienssen, R. A. (2003). Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. *Development* **130**, 3941-3950.

Chuck, G., Lincoln, C. and Hake, S. (1996). KNAT1 induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. *Plant Cell* **8**, 1277-1289.

Cnops, G., Jover-Gil, S., Peters, J. L., Neyt, P., De Block, S., Robles, P., Ponce, M. R., Gerats, T., Micol, J. L. and Van Lijsebettens, M. (2004). The rotunda2 mutants identify a role for the LEUNIG gene in vegetative leaf morphogenesis. *J. Exp. Bot.* **55**, 1529-1539.

Furutani, M., Vernoux, T., Traas, J., Kato, T., Tasaka, M. and Aida, M. (2004). PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. *Development* **131**, 5021-5030.

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226-2230.

Grandjean, O., Vernoux, T., Laufs, P., Belcram, K., Mizukami, Y. and Traas, J. (2004). In vivo analysis of cell division, cell growth, and differentiation at the shoot apical meristem in Arabidopsis. *Plant Cell* **16**, 74-87.

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271-276.

Hay, A. and Tsiantis, M. (2006). The genetic basis for differences in leaf form between Arabidopsis thaliana and its wild relative Cardamine hirsuta. *Nat. Genet.* **38**, 942-947.

Heisler, M. G., Ohno, C., Das, P., Sieber, P., Reddy, G. V., Long, J. A. and Meyerowitz, E. M. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. *Curr. Biol.* **15**, 1899-1911.

Kirch, T., Simon, R., Grunewald, M. and Werr, W. (2003). The DORNROSCHEN/ENHANCER OF SHOOT REGENERATION1 gene of Arabidopsis acts in the control of meristem ccll fate and lateral organ development. *Plant Cell* **15**, 694-705.

Krizek, B. A. (1999). Ectopic expression of AINTEGUMENTA in Arabidopsis plants results in increased growth of floral organs. *Dev. Genet.* **25**, 224-236.

Leyser, H. M., Lincoln, C. A., Timpte, C., Lammer, D., Turner, J. and Estelle, M. (1993). Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* **364**, 161-164.

Lincoln, C., Britton, J. H. and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. *Plant Cell* **2**, 1071-1080.

Ljung, K., Bhalerao, R. P. and Sandberg, G. (2001). Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. *Plant J.* **28**, 465-474.

Long, J. A., Moan, E. I., Medford, J. I. and Barton, M. K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the SHOOTMERISTEMLESS gene of Arabidopsis. *Nature* **379**, 66-69.

Luschnig, C., Gaxiola, R. A., Grisafi, P. and Fink, G. R. (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. *Genes Dev.* **12**, 2175-2187.

Mizukami, Y. and Fischer, R. L. (2000). Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proc. Natl. Acad. Sci. USA* **97**, 942-947.

Okada, K., Ueda, J., Komaki, M. K., Bell, C. J. and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. *Plant Cell* **3**, 677-684.

Ori, N., Eshed, Y., Chuck, G., Bowman, J. L. and Hake, S. (2000). Mechanisms that control knox gene expression in the Arabidopsis shoot. *Development* **127**, 5523-5532.

Pekker, I., Alvarez, J. P. and Eshed, Y. (2005). Auxin response factors mediate Arabidopsis organ asymmetry via modulation of KANADI activity. *Plant Cell* **17**, 2899-2910.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Pozo, J. C., Timpte, C., Tan, S., Callis, J. and Estelle, M. (1998). The ubiquitin-related protein RUB1 and auxin response in Arabidopsis. *Science* **280**, 1760-1763.

Reinhardt, D., Pesce, E., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J. and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. *Nature* **426**, 255-260.

Running, M. P. and Meyerowitz, E. M. (1995). Using confocal microscopy in the study of plant structure and development. *Aliso* **14**, 263-270.

Scanlon, M. J., Henderson, D. C. and Bernstein, B. (2002). SEMAPHORE1 functions during the regulation of ancestrally duplicated knox genes and polar auxin transport in maize. *Development* **129**, 2663-2673.

Scarpella, E., Francis, P. and Berleth, T. (2004). Stage-specific markers define early steps of procambium development in Arabidopsis leaves and correlate termination of vein formation with mesophyll differentiation. *Development* **131**, 3445-3455.

Scarpella, E., Marcos, D., Friml, J. and Berleth, T. (2006). Control of leaf vascular patterning by polar auxin transport. *Genes Dev.* **20**, 1015-1027.

Schwechheimer, C., Serino, G. and Deng, X. W. (2002). Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell* **14**, 2553-2563.

Smith, H. M. and Hake, S. (2003). The interaction of two homeobox genes, BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the Arabidopsis inflorescence. *Plant Cell* **15**, 1717-1727.

Tsiantis, M., Brown, M. I., Skibinski, G. and Langdale, J. A. (1999). Disruption of auxin transport is associated with aberrant leaf development in maize. *Plant Physiol.* **121**, 1163-1168.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**, 1963-1971.

Vernoux, T., Kronenberger, J., Grandjean, O., Laufs, P. and Traas, J. (2000). PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development* **127**, 5157-5165.

Wang, H., Jones, B., Li, Z., Frasse, P., Delalande, C., Regad, F., Chaabouni, S., Latche, A., Pech, J. C. and Bouzayen, M. (2005). The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *Plant Cell* **17**, 2676-2692.

Xu, J., Hofhuis, H., Heidstra, R., Sauer, M., Friml, J. and Scheres, B. (2006). A molecular framework for plant regeneration. *Science* **311**, 385-388.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W. L., Ma, H., Peng, W., Huang, D. and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell* **14**, 1919-1935.

Zgurski, J. M., Sharma, R., Bolokoski, D. A. and Schultz, E. A. (2005). Asymmetric auxin response precedes asymmetric growth and differentiation of asymmetric leaf1 and asymmetric leaf2 Arabidopsis leaves. *Plant Cell* **17**, 77-91.