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ASYMMETRIC LEAVES1 reveals *knox* gene redundancy in *Arabidopsis*

Mary E. Byrne, Joseph Simorowski and Robert A. Martienssen*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA
*Author for correspondence (e-mail: martiens@cshl.org)

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SUMMARY

The shoot apical meristem comprises undifferentiated stem cells and their derivatives, which include founder cells for lateral organs such as leaves. Meristem maintenance and lateral organ specification are regulated in part by negative interactions between the myb domain transcription factor *ASYMMETRIC LEAVES1*, which is expressed in lateral organ primordia, and homeobox transcription factors which are expressed in the shoot apical meristem (*knox* genes). The *knox* gene *SHOOT MERISTEMLESS* (*STM*) negatively regulates *ASYMMETRIC LEAVES1* (*AS1*) which, in turn, negatively regulates other *knox* genes including *KNAT1* and *KNAT2*, and positively regulates the novel gene *LATERAL ORGAN BOUNDARIES* (*LOB*). Genetic interactions with a second gene, *ASYMMETRIC LEAVES2* (*AS2*), indicate it acts at the same position in this hierarchy as *AS1*. We have used a second-site suppressor screen to isolate mutations in *KNAT1* and we show that *KNAT1* is partially redundant with *STM* in regulating stem cell function. Mutations in *KNAT2* show no such interaction. We discuss the regulation and evolution of redundancy among *knox* genes.

Key words: TALE class homeobox, shoot apical meristem, boundary, leaf shape, KNAT1, KNAT2

INTRODUCTION

The shoot apical meristem (SAM) of higher plants is divided histologically into a number of zones, which are also defined by gene expression patterns. The central zone contains undifferentiated, slowly dividing cells that give rise to daughter cells in the peripheral zone. Groups of cells in the peripheral zone (founder cells) are recruited into initiating lateral organs, and undergo rapid cell divisions, expansion and terminal differentiation. Meristem homeostasis is achieved by a balance between slow cell divisions in the central zone and displacement of cells into lateral organ primordia. Mutations leading to loss of meristem homeostasis have defined a number of genetic pathways for maintaining a balance between stem cells and their derivatives (reviewed by Bowman and Eshed, 2000; Clark, 2001).

One pathway involved in meristem initiation and maintenance involves a highly conserved class of homeodomain transcription factors encoded by *knox* genes. *knox* genes are defined by homology to the maize *knotted1* (*kn1*) gene and are separated into two classes based on sequence identity and conserved intron location (Bharathan et al., 1999; Kerstetter et al., 1994; Reiser et al., 2000). The *Arabidopsis* genome sequence has revealed 8 *knox* genes (The Arabidopsis Genome Initiative, 2000). Class I genes comprise *STM*, *KNAT1*, *KNAT2* and *KNAT6* (Lincoln et al., 1994; Long et al., 1996; Semiarti et al., 2001). Loss-of-function mutations in *STM* result in embryos that lack a SAM and so fail to develop any postembryonic vegetative tissue (Barton and Poethig, 1993; Clark et al., 1996; Long et al., 1996). *STM* is thus required to maintain proliferation of cells in the SAM and/or prevent their differentiation. Recessive mutations in the *kn1* gene of maize also condition defects in meristem maintenance (Kerstetter et al., 1997; Vollbrecht et al., 2000). Both *STM* and *kn1* are expressed throughout the SAM but are down-regulated in founder cells that are recruited to form lateral organs (Jackson et al., 1994; Long et al., 1996; Smith et al., 1992). Down regulation of *knox* genes in lateral organ primordia is a critical event in organ patterning as ectopic expression of *knox* genes disrupts normal leaf development (Byrne et al., 2001; Chuck et al., 1996; Reiser et al., 2000). In *Arabidopsis*, *KNAT1* and *KNAT2* are also expressed within the SAM and are down-regulated in lateral organ primordia, but so far there is no genetically defined role for these class I *knox* genes.

Mutations in *AS1* result in plants that have abnormal leaves, with marginal outgrowths or lobes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Tsukaya and Uchimiya, 1997). *AS1* is a myb domain transcription factor related to *ROUGH SHEATH2* (*RS2*) in maize and *PHANTASTICA* (*PHAN*) in *Antirrhinum* (Byrne et al., 2000). All three genes are expressed in lateral organ primordia and act as negative regulators of *knox* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Timmermans et al., 1999; Tsiantis et al., 1999; Waites et al., 1998). Unexpectedly, *as1* suppresses the *stm* mutant phenotype, so that double mutants have an *as1* vegetative shoot. Further, in *stm* mutant embryos, *AS1* expression spreads throughout the apical region. This genetic interaction indicates that *STM* prevents *AS1* expression in stem cells of the SAM and so maintains their undifferentiated state (Byrne et al., 2000). *STM* has additional roles in the inflorescence, since *as1 stm* mutants lack normal flowers. We previously proposed that other *knox* genes might replace *STM* in vegetative but not in floral meristems, accounting for the phenotype of *as1 stm-1* plants (Byrne et al., 2000).

The mutant *asymmetric leaves2* (*as2*) has a leaf phenotype comparable to *as1*, and *knox* genes are also mis-expressed (Ori et al., 2000; Semiarti et al., 2001). We show that *AS2* is also negatively regulated by *STM* and likely interacts with *AS1*. We used second-site suppressor mutagenesis to identify meristem factors that replace *STM* in *as1 stm* double mutants. In this screen we isolated mutations in the *KNAT1* gene, which corresponds to the classical locus *BREVIPEDICELLUS* (*BP*) (Douglas et al., 2002; Venglat et al., 2002). Thus *KNAT1* and *STM* are redundant in embryo and vegetative development in the absence of *AS1*. Gene trap and enhancer trap lines were used to show that *KNAT2* and the novel gene *LATERAL ORGAN BOUNDARIES* (*LOB*) are also regulated by *AS1* but do not contribute significantly to the *as1* phenotype. Interactions between leaves and meristems were first proposed to have a role in leaf patterning on the basis of surgical experiments (Sussex, 1954; Sussex, 1955). Our studies provide a molecular framework for some of these interactions.

MATERIALS AND METHODS

Plant material and growth conditions

Mutant alleles of *as1-1*, *as2-2*, *stm-1* and *stm-2* were obtained from the *Arabidopsis* Biological Resource Center (ABRC). *as2-2*, originally in the Er background, was backcrossed twice to Landsberg *erecta* prior to double mutant analysis. Kathy Barton kindly provided the *stm-11* allele. *bp-2* was kindly provided by Dan Riggs. Gene trap and enhancer trap lines were generated as previously described (Martienssen, 1998; Sundaresan et al., 1995). Plants were grown either on soil or on MS medium, supplemented with sucrose, with a minimum day length of 16 hours. Ethyl methanesulphonate (EMS) mutagenesis was carried out by treatment of seed from *as1*/*as1 stm-1*/+ plants with 0.5% EMS for 8 hours. Approximately 80 F_2 seeds from each of 1200 fertile individuals, of the genotype *as1*/*as1 stm-1*/+ or *as1*/*as1* +/+, were screened on soil for a shoot meristemless phenotype.

Plant genetics

To generate *as2 stm* double mutants homozygous *as2* plants were crossed to plants heterozygous for *stm*. *AS2* and *STM* are linked on chromosome 1 and in F_2 populations a novel phenotype segregated at a low frequency. F_3 plants from individuals of the genotype *as2 stm-1*/*as2* + segregated 1:3 for the double mutant phenotype [*as2* 159 (72.3%), *as2 stm-1* 61 (27.7%)]. To construct *as1 bp* and *as2 bp* double mutants, plants homozygous for *as1* or *as2* were crossed to plants homozygous for *bp*. Double *as1 bp* and *as2 bp* mutants segregated in the F_2 progeny in the expected 1:15 ratio. The number of plants in each phenotypic class segregating *as1* and *bp* were; wild type 182 (60.3%), *as1* 44 (14.5%), *bp* 58 (19.2%), *as1 bp* 18 (6.0%). The number of plants in each phenotypic class segregating *as2* and *bp* were; wild type 123 (57.2%), *as2* 40 (18.6%), *bp* 40 (18.6%), *as2 bp* 12 (5.6%). Double *stm-11 bp* and *stm-2 bp* mutants were generated by crossing plants homozygous for *bp* to plants heterozygous for *stm-11* or *stm-2*. Only *stm* and *bp* phenotypes segregated in the F_2 generation. F_3 seed from homozygous *bp* plants segregated 1:3 *stm* mutants. Segregation values for lines homozygous for *bp* and segregating *stm-11* were; *bp* 215 (72.6%), double *bp stm-11* 59 (27.4%). Segregation values for *bp* mutant lines segregating *stm-2* were; *bp* 229 (67.3%), double *bp stm-2* 75 (32.7%).

Triple *as1 stm-1 bp* mutants were generated by crossing plants homozygous for *as1* and heterozygous for *stm-1* to plants homozygous for *bp*. The F_3 generation from selfed *as1*/*as1 bp*/*bp stm-1*/+ individuals segregated 1 in 4 shoot meristemless individuals [*as1 bp* 113 (75.3%) and *as1 stm-1 bp* 37 (24.7%)]. Plants homozygous for the *Ds* insertion allele of *KNAT2* were crossed with homozygous mutants in the case of *as1*, *as2* and *bp*, and with heterozygous plants in the case of *stm-11* to generate double mutants. In F_3 lines homozygous for *as1*, *as2* or *bp* and segregating for *knat2* and in lines homozygous for the *knat2* allele and segregating for *stm*, no new phenotypes were observed. Triple *as1 stm-1 kt2* mutants were generated by crossing *as1*/*as1 stm-1*/+ plants with *kt2*/*kt2* plants. The F_3 progeny from selfed *as1*/*as1 stm-1*/+ *kt2*/*kt2* individuals segregated plants that were phenotypically *as1* and plants with an *as1 stm-1* phenotype in the ration 1:3.

Molecular biology

DNA extraction and manipulation were carried out using standard protocols (Sambrook et al., 1989). To sequence EMS-induced mutations, DNA from mutant plants was amplified with primer pairs encompassing the exon regions of *KNAT1*. PCR products were sequenced with internal primers, using dye terminator cycle sequencing (Applied Biosystems). For RT-PCR, total RNA was purified using Trizol reagent (GibcoBRL). Following treatment with DNase (Boehringer Mannheim) complementary DNA was synthesized using 100 Units of M-MuLV reverse transcriptase (New England Biolab) in 50 mM Tris-HCl (pH 8.3), 30 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, 1 mM each of dATP, dCTP, dGTP, dTTP, 1 μM oligo(dT), 50 Units RNasin and 0.1 μg BSA. RT-PCR reactions were performed with gene-specific primers. *KNAT2* primers (ACCACCGGAGACAATCAAAG and TCCGCTGCTATGTCATC-ATC) span the exon 3/exon 4 junction. PCR products were subject to Southern hybridization using gene-specific probes. ClustalW analysis of class I *knox* genes was performed using MacVector6.5.1 (Oxford Molecular Group).

Histology and microscopy

GUS staining was carried out as previously described (Gu et al., 1998) using a substrate solution containing 100 mM sodium phosphate pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D glucuronic acid (X-Gluc), 100 μg/ml chloramphenicol, 2 mM each of potassium ferricyanide and potassium ferrocyanide. Seedlings were mounted in 50% glycerol before viewing. Inflorescences from plants carrying a *DsG* element in *KNAT2* were first stained for GUS expression before fixing in FAA (50% ethanol, 5% glacial acetic acid, 3.7% formaldehyde), dehydrating through an ethanol series, embedding in paraffin and sectioning. Eight-day old seedlings were fixed in glutaraldehyde and dehydrated through an ethanol series prior to embedding in paraffin. 10 mm sections were cut and stained with Toluidine Blue. For scanning electron microscopy fresh material was mounted on silver tape (Electron Microscope Sciences) and viewed with an Hitachi S-3500N SEM using a beam voltage of 5 kV.

RESULTS

as1 and *as2* interact similarly with *stm*

Rosette leaves of wild-type plants are elongate, entire and spatulate in shape (Fig. 1A) whereas rosette leaves of *as1* are smaller and rounder (Fig. 1B) with the margins rolled downwards and lobed (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Lobing is variable and background dependent, but is most prominent in late rosette and cauline leaves. *as2* mutants have similar defects in leaf patterning (Fig. 1C), except rosette leaves and petioles are more elongate than *as1* (Ori et al., 2000; Semiarti et al., 2001).

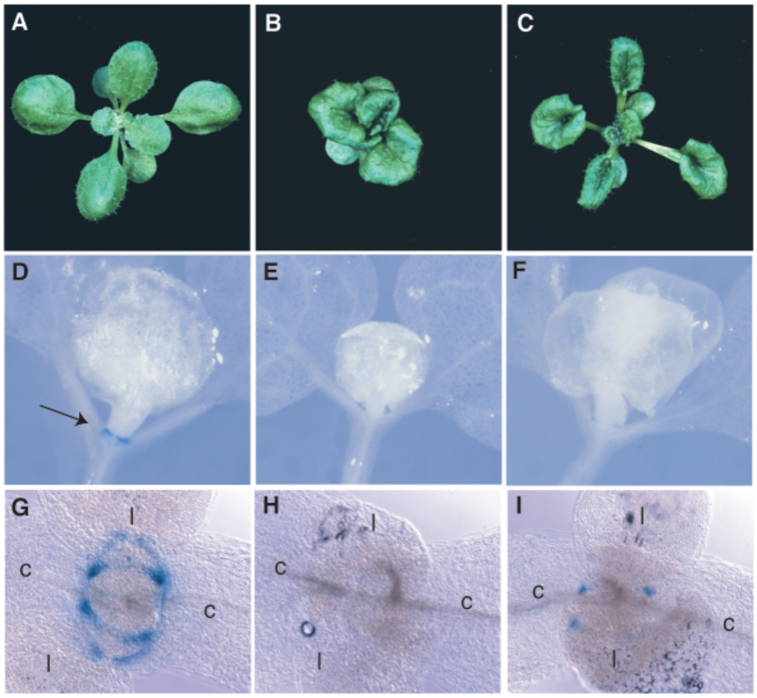

Fig. 1. Expression of *LATERAL ORGAN BOUNDARIES* (*LOB)* in *as1* and *as2*. (A-C) Vegetative shoot of wild-type (A), *as1* (B) and *as2* (C). Compared with wild-type rosette leaves, which are elongate and spatulate in shape, *as1* and *as2* rosette leaves are round, lobed and with margins rolled under. (D-F) Side view and (G-I) top view of *LOB* GUS enhancer trap expression in the shoot apex of 8-day old seedlings. In wild-type (D,G) expression is restricted to a band of cells at the boundary between developing organ primordia and the SAM (arrow). In *as1* seedlings (E,H) and *as2* seedlings (F,I) little or no *LOB* expression is detected in the SAM. c, cotyledon; l, young leaf.

AS1 is expressed in lateral organ primordia and negatively regulates *KNAT1* and *KNAT2*, which are mainly expressed in peripheral regions of the SAM. To identify additional targets in the shoot apex, *as1* plants were crossed with 10 gene trap and enhancer trap GUS reporter gene insertions (Springer et al., 1995; Sundaresan et al., 1995) that are expressed in this region (P. S. Springer, Q. Gu and R. A. M., unpublished). The only GUS reporter gene expression pattern that was altered in an *as1* background was ET22. ET22 disrupts the *LATERAL ORGAN BOUNDARIES* (*LOB*) gene, and is expressed in the shoot apex, the hypocotyl and the roots (Shuai et al., 2002). In the shoot apex, GUS localization is restricted to a band of cells at the boundary between developing organ primordia and the SAM (Fig. 1D). *LOB* expression in this region is found in vegetative, inflorescence and floral stages of growth and persists throughout development (Shuai et al., 2002). In *as1* mutants, expression of *LOB* in the vegetative shoot apex is absent in young seedlings (Fig. 1E) and reduced to two small patches at the outer margin of the leaf in older seedlings. *LOB* expression is also much reduced in the vegetative apex of *as2* (Fig. 1F), although weak GUS staining is observed at the boundary between organ primordia and the SAM in older seedlings. In contrast, *LOB* expression in the hypocotyl, the root and the inflorescence is unaltered in either mutant (data not shown). Thus, *as1* and *as2* affect *LOB* expression in the same manner, suggesting that both *AS1* and *AS2* positively regulate *LOB* within the shoot apex. Seedlings homozygous for the insertion allele of *LOB* have a wild-type phenotype (Shuai et al., 2002) and there is no effect on either *as1* or *as2*.

Given that *as1* and *as2* have similar phenotypes and are both required for normal expression of *knox* genes and *LOB*, we carried out double mutant analysis to determine if *as2* also interacts with *stm*. Embryos homozygous for strong *stm* alleles, including *stm-1* and *stm-11*, completely lack a SAM and develop cotyledons that are fused at their base (Barton and Poethig, 1993; Clark et al., 1996; Long and Barton, 1998).

Fig. 2. *as1* and *as2* suppress the *stm* mutant phenotype. Double mutants *as1 stm-1* (A) and *as2 stm-1* (B) have vegetative shoots and leaves similar to the single *as1* and *as2* mutants, respectively, but additional lateral shoots are formed in the place of flowers. Double mutants between the weak *stm-2* allele with *as1* (C) and *as2* (D) produce more flowers. Scanning electron micrographs of flowers from *as1 stm-2* (E) and *as2 stm-2* (F) reveal that terminal flowers are frequently fused along the pedicel. Floral organs, particularly reproductive organs, are reduced in number or absent. Scale bar, 2 mm.

Weak *stm* mutants, such as *stm-2*, also germinate with fused cotyledons, but subsequently form a SAM and initiate leaves (Clark et al., 1996; Endrizzi et al., 1996). In *as1 stm-1* double mutants vegetative shoots and leaves are indistinguishable from those of *as1* single mutants. In reproductive development *as1 stm-1* double mutants generate additional lateral shoots in the place of flowers. The phyllotaxy of lateral shoots in the inflorescence is also somewhat irregular compared with *as1* single mutants (Fig. 2A) (Byrne et al., 2000). Mutants homozygous for *as1* and the weaker *stm-2* allele are similar to *as1 stm-1* double mutants except that they form fewer lateral shoots and more flowers, most of which remain incomplete

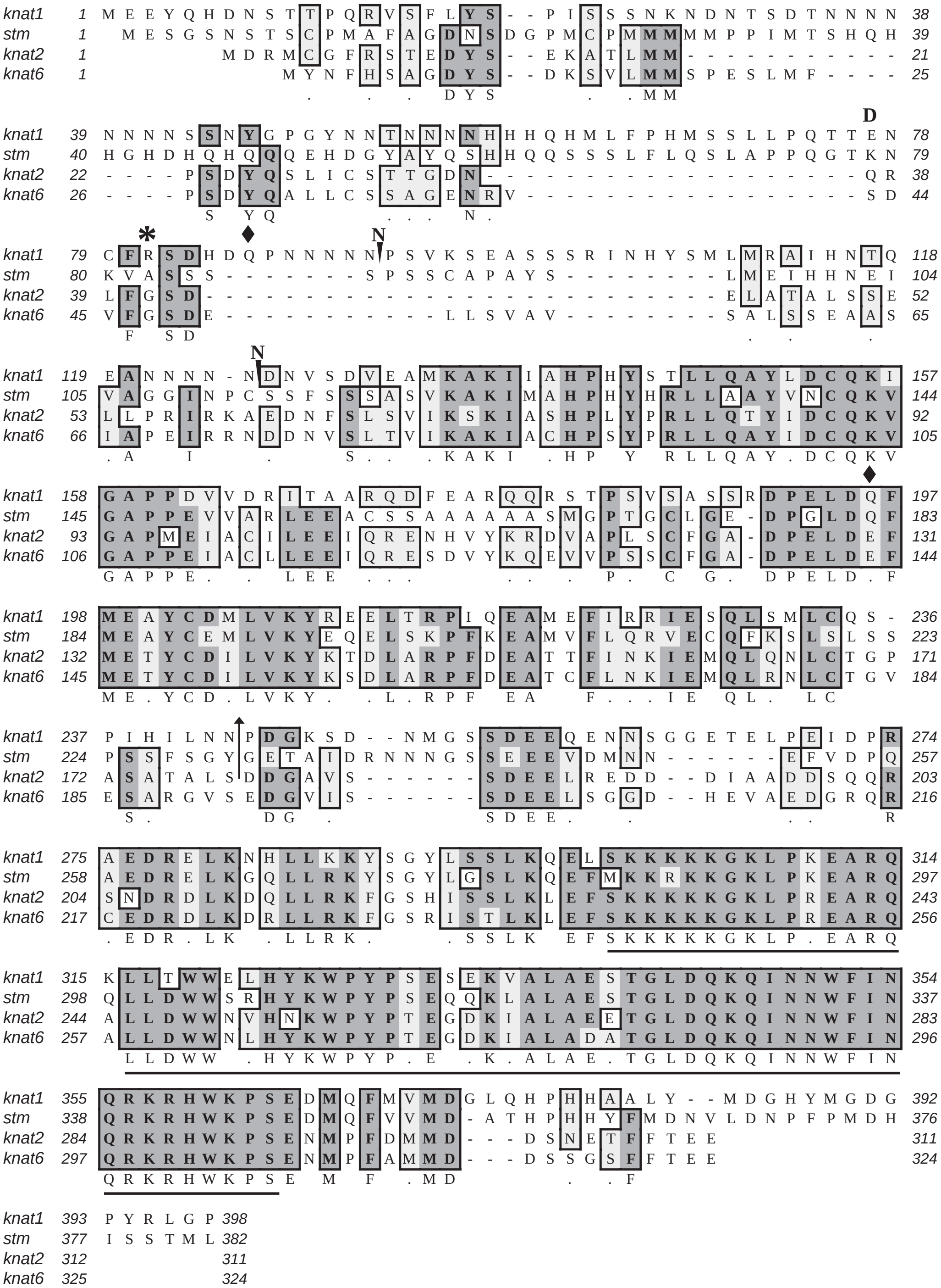

Fig. 3. Class I *knox* genes in *Arabidopsis*. Dark shading indicates identical amino acids, light shading indicates conserved amino acids. The consensus sequence is shown below the alignments. The homeodomain is underlined. Amino acid changes in new *bp* alleles are marked above the sequence. Diamonds indicate single base changes resulting in an amino acid change to a stop codon in *bp-6* and *bp-7*. In *bp-8*, D and * indicate single base changes leading to an amino acid substitution and creation of a stop codon, respectively. Two triplet nucleotide duplications result in amino acid insertions (N). An arrow marks the region where a *Ds* transposon disrupts *KNAT2*.

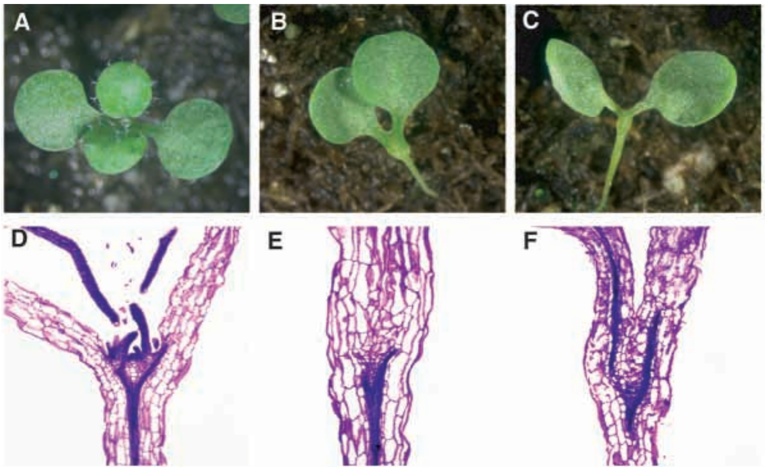

Fig. 4. *KNAT1* functions in SAM maintenance. (A-C) 8-day old whole seedlings of (A) wild type, with early vegetative leaves emerging, (B) the single *stm-1* mutant and (C) the triple *as1 stm-1 bp* mutant. Both mutants have two cotyledons fused at the base and lack a vegetative shoot. (D-F) Longitudinal sections. (D) Wild type, showing dense staining cells of the SAM and young leaf primordia at the base of the cotyledons. At the base of the fused cotyledons in *stm-1* (E) and *as1 stm-1 bp* (F) these cells are not found.

(Fig. 2C). Typically flowers have a normal number of sepals, a reduced number of petals and stamens and only occasionally form a central abnormal unfused carpel. Terminal flowers are often fusions of more than one flower (Fig. 2E).

Double mutants of *as2* with either strong or weak alleles of *stm* are comparable in phenotype to *as1 stm-1* and *as1 stm-2* mutants, respectively (Fig. 2B,D). Vegetative shoots of *as2 stm-1* and *as2 stm-2* are indistinguishable from *as2* alone. However, inflorescence development in double mutants is disrupted with only occasional and abnormal flowers produced in *as2 stm-1* double mutants (Fig. 2B) and more often in *as2 stm-2* (Fig. 2D) double mutants. As with *as1 stm*, the flowers in *as2 stm* double mutants lack reproductive organs and show some homeotic conversions (Fig. 2F). This result demonstrates that, like *as1*, *as2* suppresses *stm*. Because *STM* expression is unaffected in *as2* (Semiarti et al., 2001) this epistatic interaction indicates that *AS2* is negatively regulated by *STM*.

Screening for suppressors of *as1 stm-1*

One function of *STM* is to prevent *AS1* expression in stem cells of the SAM (Byrne et al., 2000). However, *STM* may have additional roles in meristem maintenance that are assumed by other factors redundant with *STM* that are only revealed in *as1 stm-1* double mutants. Likely candidates are the other class I *knox* genes expressed in the SAM, namely *KNAT1*, *KNAT2* and *KNAT6* (Lincoln et al., 1994; Long et al., 1996; Semiarti et al., 2001). In pairwise comparisons (Fig. 3) *STM* is most closely related to *KNAT1*, sharing 44% identity over all and 70% identity within the homeodomain. However, *KNAT2* is most closely related to *KNAT6* sharing overall 70% amino acid identity and 89% identity in the homeodomain.

In order to identify factors redundant with *STM* we carried out a screen for mutants that suppressed the *as1 stm-1* phenotype. Seed from plants of the genotype *as1/as1 stm-1*/+ were mutagenized with EMS, since the double homozygous mutant is sterile. Progeny from 1200 F_1 individuals, two-thirds of which were heterozygous for *stm-1*, were screened for a shoot meristemless phenotype. In one line, EMS202, approximately 1 in 16 seedlings lacked a shoot meristem and were indistinguishable from those carrying strong alleles of *stm*. Upon flowering EMS202 also segregated *as1 stm-1* double mutants as expected, as well as plants with reduced pedicels and downward-hanging flowers resembling the previously described mutant *brevipedicellus* (Koornneef et al., 1983). A likely candidate for mutation in EMS202 was *KNAT1* since *brevipedicellus* (*bp*) has recently been shown to coincide with the *KNAT1* locus (Douglas et al., 2002; Venglat et al., 2002). Sequence analysis revealed that EMS202 carries a single base change creating a stop codon in the first exon of *KNAT1* (Fig. 3). This allele is designated *bp-6*. Two additional lines carried *bp* mutants with nucleotide disruptions in *KNAT1* (Fig. 3). In one case (*bp-7*) a single nucleotide change creates a stop codon in the second exon. In the other (*bp-8*), multiple changes include two additional ACC repeats and two single nucleotide changes, which result in amino acid insertions, an amino acid substitution and a premature stop (Fig. 3).

To confirm that the shoot meristemless seedlings were derived from triple *as1 stm-1 bp* homozygotes, we constructed triple mutants between *as1 stm-1* and an independently derived deletion allele of *KNAT1* (Douglas et al., 2002). Progeny from *as1/as1 bp/bp stm-1*/+ mutants segregated 1 in 4 shoot meristemless plants, as expected. Like *stm-1*, these *as1 stm-1 bp* mutants have cotyledons fused at the base and no vegetative shoot (Fig. 4B,C), although rarely some leaves are formed. At 8 days after germination, the wild-type SAM is visible in sections as a dome of densely staining cells at the base of the

Fig. 5. *bp* enhances the weak allele *stm-2*. The weak *stm-2* mutant (A) produces a vegetative shoot with very few flowers. This phenotype is enhanced in the *bp stm-2* double mutant (B) where a much reduced vegetative shoot or only a few, abnormal, leaves are formed.

Fig. 6. *as1 bp* and *as2 bp* phenotypes are additive. (A-F) Whole plant phenotypes. Compared with wild-type (A), the single mutants *bp* (B), *as1* (C) and *as2* (E) are smaller in size. Double mutants *as1 bp* (D) and *as2 bp* (F) are smaller than any of the single mutants. Scale bar A-F, 4 cm. (G-R) Inflorescence and flower phenotypes. The short pedicels in *bp* (H,N) result in down-pointing flowers compared with wild type (G,M). In *as1* (I,O) reduced sepals and petals expose the inner reproductive organs in young flowers. *as1 bp* flowers (J,P) have both short pedicels and reduced sepals and petals. *as2* mutants have narrower sepals resulting in exposed inner floral organs in young flowers (K,Q). In the *as2 bp* double mutant (L,R) pedicels are short and sepals are reduced in size. Scale bar in M-R, 2 mm.

cotyledons (Fig. 4D). In contrast, sections through the apex of *as1 stm-1 bp* mutant seedlings have no SAM (Fig. 4F), and are comparable to *stm-1* single mutants (Fig. 4E). Thus *KNAT1* is required for SAM maintenance in the absence of *AS1* and *STM*.

Genetic interactions between *as1, as2* and *knox* genes

The genetic interaction between *STM*, *AS1* and *KNAT1* was originally proposed based on molecular characterization of single and double mutants of *as1* and *stm* (Byrne et al., 2000). To provide further support for this genetic pathway we also examined the interaction between *STM* and *KNAT1*. *bp stm-11* double mutants were indistinguishable from *stm* alone demonstrating that *stm* is epistatic to *bp*. On the other hand, the weak allele *stm-2* is enhanced in plants that are also homozygous for *bp*. Compared with *stm-2* mutants, *bp stm-2* double mutants have a much reduced vegetative shoot with many plants only giving rise to a few abnormal leaves and no flowers (Fig. 5). These interactions are consistent with *KNAT1* being downstream of *STM* and *AS1* (Byrne et al., 2000).

We also examined the genetic interaction of *bp* with *as1* and *as2* (Fig. 6). *as1*, *as2* and *bp* mutants are smaller than wild type and *bp* plants show a slight loss of apical dominance (Fig. 6A-C,E). There is no apparent affect of *bp* on leaf development. *as1 bp* and *as2 bp* double mutants are smaller than either single mutant alone (Fig. 6D,F). Leaves of *as1 bp* and *as2 bp* double mutants show no significant difference from that of *as1* and *as2* single mutants, respectively. In wild-type flowers, the sepals enclose the flower until just before anthesis (Fig. 6G,M). In contrast, *as1* sepals are reduced in size and do not enclose inner floral organs from an early stage of flower development, while petals do not elongate, and remain shorter than in wild type (Fig. 6I,O). The principal floral defect of *bp* is a reduction in the length of the pedicel (Fig. 6H,N). In *as1 bp* double mutants, floral organs are exposed from early in development, and both petals and pedicels are reduced in length (Fig. 6J,P). Sepals in *as2* are also reduced, such that developing floral organs are exposed (Fig. 6K,Q). *as2 bp* double mutants also show aspects of both single mutants (Fig. 6L,R). Thus both the *as1 bp* and *as2 bp* double mutant phenotypes are additive in all respects indicating that, although *KNAT1* is ectopically expressed in *as1* and *as2* mutants, it is not required for either phenotype.

Another *knox* gene, *KNAT2*, is misexpressed in *as1* mutants (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). A *Ds* transposon gene trap insertion allele of *KNAT2* (GT7953) was identified by systematic sequencing of gene and enhancer trap GUS reporter gene insertions (http://www.cshl.org/genetrap). The gene trap reporter is inserted in the third and largest intron of *KNAT2* in the sense orientation (Fig. 3) where it is expected to result in a GUS fusion protein (Springer et al., 1995). GUS activity is detected in the SAM region of embryos and vegetative plants (Fig. 7A,B). The expression pattern is broader in the reproductive shoot extending throughout the inflorescence and floral meristems. In flowers, GUS is initially detected in all organs, but is later confined to the carpels. In addition GUS is expressed in the inflorescence stem and in the pedicel of young flowers (Fig. 7C,D). This pattern closely parallels that reported for *KNAT2* mRNA by in situ hybridization and for plants carrying a *KNAT2* promoter-GUS transgene (Dockx et al., 1995; Pautot et al., 2001), except that gene trap expression in the inflorescence extends into the meristem. This slight difference may result from protein translocation, from disruption of a regulatory sequence, or from differences in the sensitivity of these experiments.

Full length *KNAT2* transcripts are undetectable in plants

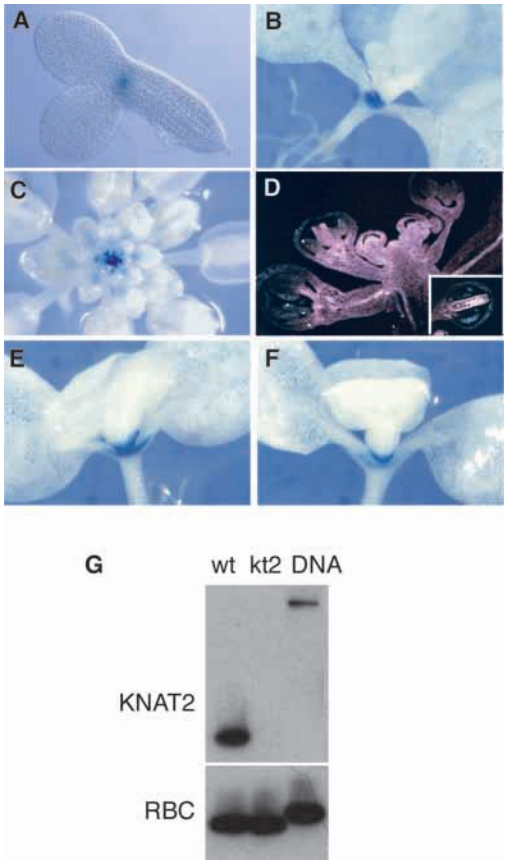

Fig. 7. *KNAT2* GUS reporter gene expression from the gene trap insertion GT7953. GUS reporter gene expression is observed in the embryonic (A) and seedling (B) SAM. In the inflorescence, GUS reporter gene expression is detected in the apex and young flowers but not in more mature flowers (C). Viewed under DIC microscopy (D), GUS activity (pink) is found in the inflorescence and floral meristems and in all organs of young flowers but in more mature flowers is confined to the carpels (inset). In *as1* (E) and *as2* (F), GUS reporter gene expression expands from the SAM into the base of the leaves. RT-PCR amplification using gene-specific primers and hybridization of products with gene-specific probes (G) show that *KNAT2* transcripts are detected in wild type but not in plants homozygous for the gene trap insertion (top panel). *RBC* transcripts were ampilfied as a control (bottom panel).

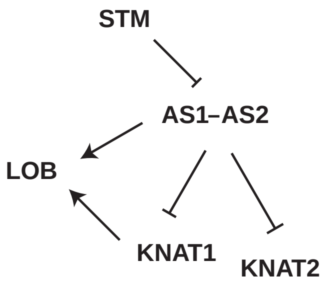

Fig. 8. A model of genetic interactions between stem cells and incipient leaf primordia in the SAM. *STM* represses *AS1* and *AS2* in stem cells and their immediate derivatives in the SAM. AS1 and AS2 together repress expression of *KNAT1* and *KNAT2* in organ primordia and may interact with each other. Expression of *KNAT1* and *KNAT2* is restricted to peripheral domains in the SAM. *LOB* is expressed in a region between the SAM and organ primordia and is activated by *AS1-AS2* and *KNAT1*.

homozygous for the *knat2* gene trap insertion allele (Fig. 7G) but this has no phenotypic effect, either alone or in combination with *as1* or *as2*. This indicates that misexpression of *KNAT2* is not required for these phenotypes. Nonetheless, in both *as1* and *as2*, the *KNAT2* gene trap reporter expression is expanded somewhat into the base of the leaves (Fig. 7E,F). These results are consistent with misexpression of a *KNAT2*::GUS transgene reported previously (Ori et al., 2000). However, the transgene was misexpressed in *as1* sepals, while gene trap reporter expression is unaltered (data not shown). The gene trap reporter is expressed normally in *bp knat2* double mutants which have a *bp* phenotype. However, *stm-11 knat2* double mutants have a *stm* phenotype and have no GUS expression (data not shown). The absence of *KNAT2* expression in *stm* mutants is consistent with a genetic hierarchy whereby *STM* negatively regulates *AS1*, which in turn negatively regulates *KNAT2*. In addition, the *KNAT2* gene trap is expressed in *as1 stm* double mutants (data not shown). However, the *knat2* gene trap insertion had no effect on *as1 stm* double mutants indicating that, unlike *KNAT1*, *KNAT2* could not functionally substitute for *STM*.

DISCUSSION

AS1 and *AS2* are in a common genetic hierarchy

as1 and *as2* have comparable defects in leaf development that are accompanied by misexpression of class I *knox* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). In addition, *AS1* and *AS2* positively regulate the gene *LOB*, which encodes a novel cysteine-rich protein and is a member of a large family of related genes (Shuai et al., 2002). *LOB* is normally expressed at the boundary between meristems and organ primordia but is absent in *as1* and greatly reduced in *as2*. *KNAT1* can also function as a positive regulator of *LOB* (P. S. Springer and R. A. M., unpublished) (Shuai et al., 2002), indicating that *KNAT1* and *AS1* are both required for *LOB* expression (Fig. 8). This could account for its localized expression at the boundary of *AS1* and *KNAT1* expression domains. Negative interactions between transcription factors in adjacent territories is a common mechanism for establishment of boundaries in animal systems and may be employed here (Byrne et al., 2001).

Both *AS1* and *AS2* interact with *STM* in a similar manner. Like *as1*, *as2* suppresses the *stm* mutant phenotype leading to vegetative and inflorescence development but little floral shoot development. This genetic interaction suggests that *AS1* and *AS2* function in a common genetic pathway, both being negatively regulated by *STM* (Fig. 8). Double mutants of either *as1* or *as2* with a weak allele of *stm* produce more flowers and correspondingly less lateral shoots than combinations with strong alleles, suggesting either direct or indirect dosage-dependent interactions. It has been previously reported that *as2* is epistatic to *as1* (Ori et al., 2000; Serrano-Cartagena et al., 1999). However, *AS1* transcripts can be detected at normal levels in *as2* mutants indicating *AS2* is not a negative regulator of *AS1* (data not shown). As both genes are regulated by *STM*, but neither regulates the other, a strong possibility is that AS1 and AS2 directly interact to repress *KNAT1*. The subtle difference in mutant phenotype might then be accounted for by additional non-overlapping functions.

Previously we have shown that the leaf phenotype in *as1 stm* double mutants is unaltered compared with *as1*, indicating that *STM* is not required for the *as1* phenotype (Byrne et al., 2000). Likewise, the *as2* leaf phenotype is unaltered in double mutants with *stm*. Surprisingly, mutations in *KNAT1* and *KNAT2* also have no effect on *as1* and *as2* phenotypes. One explanation is that misexpression of any one *knox* gene is sufficient for the phenotype, requiring a triple *knox* mutant to suppress *as1*. Alternatively, misexpression of other factors may contribute to *as1* and *as2*.

Redundancy of *knox* genes

The *Arabidopsis* genome sequence has revealed large-scale gene duplications that may reflect significant redundancy (The Arabidopsis Genome Initiative, 2000; Martienssen and Irish, 1999). For example, several closely related members of a large family of novel transcription factors, the *KANADI* genes, as well as members of the *YABBY* gene family play redundant roles in specification of organ polarity (Eshed et al., 2001; Siegfried et al., 1999). In flower development several groups of closely related MADS box transcription factor genes appear to be fully or partially redundant. Mutations in *SHATTERPROOF1* and *SHATTERPROOF2* have little phenotypic effect, but in combination they disrupt normal fruit development (Liljegren et al., 2000). Similarly, the three *SEPALLATA* genes have redundant roles, in that floral organs are replaced by sepals in the triple mutant but not in any other combination (Pelaz et al., 2000). A third group of closely related MADS box genes, *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*), have partially redundant functions in floral meristem identity (Ferrandiz et al., 2000; Gu et al., 1998; Mandel and Yanofsky, 1995).

In contrast to MADS box genes, Class I *knox* genes constitute a small family of only four genes in *Arabidopsis*. *KNAT2* and *KNAT6* share a high degree of amino acid sequence identity and, like *SHATTERPROOF* and *SEPALLATA*, they are located within segmental chromosomal duplications (The Arabidopsis Genome Initiative, 2000). Thus, redundancy probably accounts for the lack of phenotype we observed when a *Ds* transposon was inserted into *KNAT2*. *KNAT1* and *STM* are also closely related, but these genes are not part of a segmental duplication and were probably duplicated earlier than *KNAT2* and *KNAT6*. In the inflorescence, *STM* expression is found in all SAMs while *KNAT1* expression is restricted to subepidermal cells of the stem and pedicel (Lincoln et al., 1994; Long et al., 1996). The stem and pedicel are affected in *bp* mutants, consistent with this expression pattern (Douglas et al., 2002; Venglat et al., 2002). In the vegetative apex, both genes are down-regulated in leaf founder cells, but *KNAT1* expression is mainly in the peripheral zone while *STM* is expressed throughout the SAM (Lincoln et al., 1994; Long et al., 1996). Nonetheless, we have shown that *KNAT1* assumes a redundant role with *STM* in the vegetative SAM in the absence of *AS1*. The lack of flowers in *as1 stm* double mutants shows that *KNAT1* cannot substitute for *STM* in floral meristems, consistent with the lack of *KNAT1* expression in these cells. This situation resembles the partial redundancy and overlapping expression patterns exhibited by the MADS box genes *AP1*, *CAL* and *FUL*.

Evolutionary implications of *knox* gene duplications

Phylogenetic analysis of *knox* genes in plants suggests a monophyletic origin, but the ancestral gene expression pattern remains unresolved (Bharathan et al., 1999; Reiser et al., 2000). One possibility is that *STM* and *KNAT1* represent the ancient duplication of a gene involved in meristem maintenance that repressed *AS1*, a function that *KNAT1* has subsequently lost. Alternatively, *STM* has acquired a new function to negatively regulate *AS1*. We favor the former possibility since repression of *AS1* is critical to meristem maintenance. Following duplication, the differences between *STM* and *KNAT1* will have favored evolutionary stabilization of both genes (Cooke et al., 1997).

In general, screens for patterning mutants in the vegetative phase have typically recovered negative regulatory genes such as *AS1*, *CURLY LEAF*, *SERRATE* and *PICKLE* (Byrne et al., 2000; Goodrich et al., 1997; Ogas et al., 1999; Prigge and Wagner, 2001) rather than loss-of-function mutations in individual homeotic genes. One explanation is that genes controlling organogenesis in the vegetative apex have been duplicated over evolutionary time. If one copy of each of these duplicate pairs acquired additional functions in the flower, but still retained its vegetative role, then mutants in floral development would be readily obtained, but leaf mutants would be masked by redundancy (Martienssen and Dolan, 1998). Only genes that regulate this redundancy, such as *AS1*, could lose function with phenotypic effect. Of course, dominant and haplo-insufficient alleles of homeotic genes could still be recovered (McConnell et al., 2001).

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