### 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 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
other *knox* genes might replace *STM* in vegetative but not in floral meristems, accounting for the phenotype of *asl* *stm-1* plants (Byrne et al., 2000).

The mutant asymmetric leaves2 (*as2*) has a leaf phenotype comparable to *asl*, 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 *ASI*. We used second-site suppressor mutagenesis to identify meristem factors that replace *STM* in *asl* *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 *ASI*. Gene trap and enhancer trap lines were used to show that *KNAT2* and the novel gene *LATERAL ORGAN BOUNDARIES* (LOB) are also regulated by *ASI* but do not contribute significantly to the *asl* 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 *asl-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. The *stm-11* allele was used to show that *KNAT2* is the absence of *STM*. Mutant alleles of *asl* and *as2* interact similarly with *stm*, but do not contribute significantly to the *asl* 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 *F2* populations a novel phenotype segregated at a low frequency. *F3* plants from individuals of the genotype *as1asl* *stm-1*/*+* were 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 methanesulfonate (EMS) mutagenesis was carried out by treatment of seed from *as1asl* *stm-1*/*+* plants with 0.5% EMS for 8 hours. Approximately 80 *F2* seeds from each of 1200 fertile individuals, of the genotype *as1asl* *stm-1*/*+* or *as1asl*+/+, were screened on soil for a shoot meristemless phenotype.

**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 *asl* 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 *asl* (Ori et al., 2000; Semiarti et al., 2001).
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 hypcotyl, 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).

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.

**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.

**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)
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.

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**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*. 
that like some homeotic conversions (Fig. 2F). This result demonstrates AS2 interaction indicates that is negatively regulated by as2 (Semiarti et al., 2001) this epistatic in stm-2 (Fig. 2D) double mutants. As with as1 stm-1 mutant is sterile. Progeny from 1200 F1 individuals, two-thirds were mutagenized with EMS, since the double homozygous phenotype. Seed from plants of the genotype stm-1 and pedicels and downward-hanging flowers resembling the double mutants as expected, as well as plants with reduced vegetative shoots of and an independently derived triple mutants between as1 stm-1 bp/bp stm-1 mutants with nucleotide disruptions in KNAT1 (Fig. 3). In one line carried bp mutants with nucleotide disruptions in 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 homoyzogotes, 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 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).

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.

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 F1 individuals, two-thirds of which were heterozygous for stm-1, were screened for a abortive shoot (Fig. 4B,C). Flowers of which were heterozygous for STM. Upon flowering EMS202 also segregated as1 stm-1 double mutants as expected, as well as plants with reduced vegetative shoots of and downward-hanging flowers resembling 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.
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-1 double mutants were indistinguishable from examined the interaction between STM single and double mutants of origin proposed based on molecular characterization of homozygous for is enhanced in plants that are also stm-2 is epistatic to stm demonstrating that bp stm-11. 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 florescence 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...
and is not required for these phenotypes. Nonetheless, in both as1 mutants, KNAT2 was misexpressed in as1 double mutants (data not shown). However, the transgene::GUS transgene somewhat into the base of the leaves (Fig. 7E,F). These results indicate that misexpression of KNAT2 in as1 mutants is consistent with a genetic hierarchy whereby STI 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 STI.

**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 STI 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 STI (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, ASI 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 STI, 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 sepalas 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 (Rennardiz 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 subependimal cells of the stem and pedicel (Lincoln et al., 1994; Long et al., 1996). The stem and pedicel are affected in hp and as1 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 API, 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).

We thank Dan Riggs and Kathy Barton for providing alleles of hp and stm, respectively, and also Dan Riggs and Raju Datla for sharing unpublished results. Thanks to Marja Timmermans and Erik Vollbrecht for helpful discussion and critical reading of the manuscript. We also thank Tim Mulligan for plant care, and Manisha Lotlikar and Anupa Mandava for lab assistance. This work was supported by grants from National Science Foundation, Department of Energy and United States Department of Agriculture.

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