Development 127, 5523-5532 (2000)
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DEV0336

Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot

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Accepted 2 October; published on WWW 14 November 2000

SUMMARY

***Knotted1*-like homeobox (*knox*) genes are expressed in specific patterns within shoot meristems and play an important role in meristem maintenance. Misexpression of the *knox* genes, *KNAT1* or *KNAT2*, in *Arabidopsis* produces a variety of phenotypes, including lobed leaves and ectopic stipules and meristems in the sinus, the region between lobes. We sought to determine the mechanisms that control *knox* gene expression in the shoot by examining recessive mutants that share phenotypic characteristics with *35S::KNAT1* plants. Double mutants of *serrate* (*se*) with either *asymmetric1* (*as1*) or *asymmetric2* (*as2*) showed lobed leaves, ectopic stipules in the sinuses and defects in the timely elongation of sepals, petals and stamens, similar to *35S::KNAT1* plants. Ectopic stipules and in rare cases, ectopic meristems, were detected in the sinuses on plants that were mutant for *pickle* and either *as1* or *as2*. *KNAT1* and *KNAT2* were misexpressed in the leaves and flowers of single *as1* and *as2* mutants and in the sinuses of leaves of the different double mutants, but not in *se* or *pickle* single mutants. These results suggest that AS1 and AS2 promote leaf differentiation through repression of *knox* expression in leaves, and that SE and PKL globally restrict the competence to respond to genes that promote morphogenesis.**

Key words: *asymmetric1*, *asymmetric2*, *knox* repressors, *pickle*, Meristem, Leaf development, Leaf shape

INTRODUCTION

Most of the plant body is formed postembryonically by apical meristems. The shoot apical meristem (SAM) produces leaves from its flanks, and at the same time maintains a pool of undifferentiated cells. Based on histological and molecular studies (Foster, 1938; Ledin, 1954; Kaplan and Cooke, 1997; Lenhard and Laux, 1999) the SAM is thought to contain two major functional domains, the central zone and the peripheral zone. The central zone consists of slowly dividing, less cytoplasmic cells, while the peripheral zone surrounds the central zone, and consists of more densely cytoplasmic cells. Leaves are initiated within the peripheral zone in a regular pattern and within defined time intervals. The time interval between the initiation of two successive leaves is referred to as the plastochron index and is useful for following the progression of leaf development. A Plastochron 1 (P_1) leaf is visible as a bump on the flank of the meristem. A P_2 leaf is the next oldest leaf, whereas a P_0 leaf is not yet visible but its position within the meristem can be predicted on the basis of the regular pattern of leaf initiation.

A number of genes are expressed in the SAM and are required for meristem function (Lenhard and Laux, 1999). *knotted1*-like homeobox (*knox*) genes are expressed in shoot meristems (Jackson et al., 1994; Long et al., 1996; Nishimura et al., 1999; Sentoku et al., 1999). In many species, such as maize and *Arabidopsis*, *knox* expression disappears in the P_0 region of the meristem, prior to any visible protrusion of leaf primordia, and remains absent as leaves develop. Three different *knox* genes have been described in *Arabidopsis* that are expressed in the SAM but not in leaves, *KNAT1*, *KNAT2* and *SHOOTMERISTEMLESS* (*STM*) (Lincoln et al., 1994; Long et al., 1996). *STM* is expressed in the central and peripheral zones whereas *KNAT1* is expressed in the peripheral zone. *STM*, as well as the maize *knox* gene *knotted1*, have been shown by mutational analysis to be essential for meristem maintenance. *stm* mutants produce normal cotyledons, which are the first leaves of the embryo, but fail to produce any other organs from the SAM (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996). The phenotype of maize *knotted1* mutants is similar but depends on the genetic background (Vollbrecht et al., 2000).

Misexpression of *knox* genes in leaves causes a variety of phenotypes, depending on the species (for review, see Reiser et al., 2000). In maize plants carrying dominant *knox* mutations, leaf tissues are delayed in differentiation and acquire altered cell fates (Freeling, 1992). Similar leaf phenotypes have been seen in rice leaves as a result of misexpression of *knox* genes driven by the 35S promoter (Matsuoka et al., 1993). More dramatic phenotypes, in which

ectopic shoots form from the adaxial surface of leaves, have been documented using the actin promoter in rice or the 35S promoter in tobacco (Sinha et al., 1993; Sentoku et al., 2000). Tomato leaves are normally dissected and shown to express *knox* genes. The level of dissection increases a hundred fold when plants express *knotted1* or a tomato *knox* gene from the 35S promoter (Hareven et al., 1996; Chen et al., 1997). In *Arabidopsis*, the mildly serrated, spoon-shaped leaf develops deep lobes when misexpressing *knotted1* or *KNAT1*. The lobes form at the position of the serrations and appear to result from a failure to expand in the sinus, the region between the lobes. Stipules, which are normally found at the base of leaves, are found in the sinus of *35S:KNAT1* plants, and express stipule-specific markers (data not shown). Under specific environmental conditions, ectopic meristems can also be found in this region (Lincoln et al., 1994; Chuck et al., 1996). Plants misexpressing *KNAT2* show a range of phenotypes from mildly lobed to dissected leaves (Jeff Long, G. C. and S. H., unpublished data). *35S:STM* transgenic plants were reported to be severely stunted with a disorganized shoot, which produces leaf like bulges that do not develop into mature leaves (Williams, 1998).

In order to find genes that negatively regulate *knox* genes in *Arabidopsis*, we searched for recessive mutations that share phenotypic aspects with transgenic plants that misexpress *knox* genes. We reasoned that the normal function of such genes would be to restrict expression of *knox* genes to the meristem. Similar rationales have led to the successful isolation of genes that repress *knox* expression in maize (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). We show that *asymmetric1* (*as1*), or *asymmetric2* (*as2*) mutant plants ectopically express *KNAT1* and *KNAT2* in leaves and flowers, and that, depending on additional genetic components, the leaf shape can be dramatically altered, and ectopic growth of stipules and meristems can be detected on leaves.

MATERIALS AND METHODS

Plant material

The *as1-1*, *as2-2* and *se* mutants (Redei, 1965) were obtained from the *Arabidopsis* Biological Resource Center. Backgrounds and accession numbers are as follows: *as1-1*, accession number CS3774, background Col-1; *as2-2*, accession number CS 3118, background An; *se*, accession number CS3257, background Col-1. *as2-14* is a new allele, generated by T-DNA mutagenesis in the Ler background. *pkl-15* was previously described as *gym-5* and is in the Ler background (Eshed et al., 1999). The KNAT2*::GUS* transgenic plants (Dockx et al., 1995; Laufs et al., 1998) are in the C24 background and were kindly provided by Jan Traas (INRA, Versailles, France). *KNAT1::GUS* transgenic plants are in the Col background. Plants were grown in a greenhouse under long days or in a growth chamber under short days, where indicated. Long days were 16 hours light and 8 hours dark, short days were 16 hours dark and 8 hours light. Day temperature was 20°C and night temperature was 18°C in both short and long days.

Construction of double mutants

as1-1, *as2-2*, *as2-14*, *se* and *pkl-15* mutants were crossed to each other and to *KNAT1::GUS* and *KNAT2::GUS* plants. F_2 populations of 180 individuals were examined to identify double mutants. Potential double mutants of *as1-1* × se, *as2-2* × se, *as1-1* × *pkl-15* and *as2-14* × *pkl-15* were found at the ratio of 1:16 or less, and were back-crossed to both single mutants to verify their genetic identity. *as1-1*, *as2-2* and *se* single mutants were found in the F_2 populations at the expected ratio of 1:4, and no enhancement of their phenotypes due to background effects was observed in the *as2-2* × *se* F_2 population. No obvious double mutant was detected in the F_2 from the cross of *as1-1* × *as2-2*. In order to determine if the double mutant was masked by epistasis, F_3 seeds were collected from single F_2 plants with either *as1-1* or *as2-2* phenotypes. F_3 progeny of *as1-1* plants occasionally segregated the *as2-2* phenotype in a 1:3 ratio, but F_3 progeny of *as2-2* never revealed the *as1-1* phenotype. This result suggested that the double mutant was identical to *as2-2*. We crossed potential double mutants to both *as1-1* and *as2-2* and found that some of the *as2-2* mutants were also homozygous for *as1-1*, whereas none of the *as1-1* mutants were homozygous for *as2-2*.

SEM analysis and in situ hybridization

SEM analysis was carried out according to the method of Siegfried et al. (1999). Plants for in situ hybridization were grown in short day conditions. In situ hybridization was performed as previously described (Jackson, 1991; Jackson et al., 1994). *KNAT1* and *STM* probes are as described by Chuck et al. (1996).

GUS staining

Plants were grown under long days conditions in the greenhouse. Plant tissue was vacuum infiltrated in a solution containing 25 mM phosphate buffer, pH 7, 0.25% Triton X-100, 1.25 mM potassium ferricyanide, 1.25 mM potassium ferrocyanide, 0.25 mM EDTA, 1 mg/ml 5 bromo-4 chloro 3 indolyl β-D-glucuronide (X-GlcU), and incubated overnight at 37°C. Tissue was then cleared for a few days in 95% ethanol, gradually brought to 50% ethanol and then to 50% glycerol. Tissue was photographed in 50% glycerol. The *KNAT1* promoter extends from −1 to −5000 base pairs, where 1 is the translation start site.

RESULTS

Recessive mutants that phenocopy the *35S::KNAT1* phenotype

In order to identify genes that negatively regulate *knox* expression, we investigated previously isolated mutants for phenotypes similar to plants expressing *KNAT1* from the 35S promoter. *35S::KNAT1* leaves are lobed and folded upwards with ectopic stipules and meristems in the sinus regions (Fig. 1; Lincoln et al., 1994; Chuck et al., 1996). The flowers show a delay in elongation and maturation of sepals, petals and stamens, which causes the flowers to open prematurely and results in reduced fertility. *35S::KNAT1* plants also exhibit a phyllotaxy defect on the inflorescence stem; flowers initiate in an irregular pattern in contrast to the spiral pattern of wild-type plants (data not shown).

No single mutant had been described with a deeply lobed leaf phenotype, but several mutants had some degree of lobing or serration in addition to one or two other aspects of the *35S::KNAT1* phenotype (Tsukaya and Uchimiya, 1997; Berna et al., 1999; Serrano-Cartagena et al., 1999). The *serrate* (*se*) mutant (Fig. 1B) showed strong serration of the leaves compared to wild-type (Fig. 1A). In addition, *se* mutants had phyllotaxy defects in the inflorescence, similar to those of *35S::KNAT1* (data not shown). The *asymmetric1* (*as1*) and *asymmetric2* (*as2*) mutants showed rumpled rosette leaves (Fig. 1C,E), reminiscent of tobacco leaves that overexpress *KNAT1* or its homolog from maize, *knotted1* (J. Long and G. C., unpublished observations; Sinha et al., 1993). In addition,

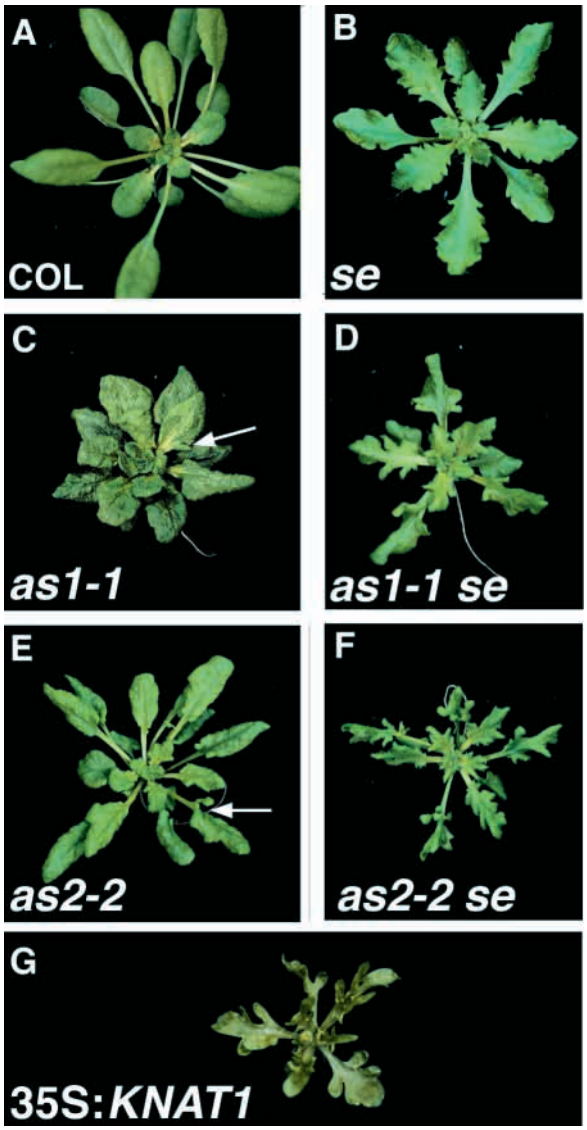

Fig. 1. *as1 se* and *as2 se* double mutants mimic aspects of the *35S::KNAT1* phenotype. Vegetative phenotypes of the wild-type Columbia (COL; A), and mutant plants (B-E) are shown. Plants were grown under short day conditions. Lobes are present at the base of the leaf in *as1* and *as2* leaves (C,E, arrow).

about half of the leaves of *as1* and *as2* had lobes close to the base of the leaf. The severity of the lobing phenotype increased gradually, such that the first formed leaves rarely had lobes but leaves that initiated later had one or more lobes. The lobing of cauline leaves on *as1* and *as2* plants was similar to that of *35S::KNAT1* plants. The phenotypes of *as1* and *as2* mutants were very similar, though distinguishable by longer petioles in *as2* mutants and a greater length/width ratio of *as2* mutant leaves (Fig. 1C,E).

We reasoned that the inability to find single mutants with a deeply lobed leaf phenotype could be due to multiple factors that acted to restrict homeobox gene expression and function. We therefore constructed double mutant combinations among *se*, *as1* and *as2*. As previously described (Serrano-Cartagena et al., 1999), the *as1 as2* double mutant phenotype was indistinguishable from the *as2* phenotype (Materials and Methods). Interestingly, double mutants between *se* and *as1* or *se* and *as2* showed dramatic lobing of leaves, similar to *35S::KNAT1* (Fig. 1D,F,G). To quantify the difference in lobing between the *as1* and *as2* single mutants and the double mutants with *se*, we counted the number of lobes on all the rosette leaves of 15 plants of each genotype. All leaves of the double mutants had more lobes than the single mutants, but the difference was greater for early initiating leaves (Table 1). The lobes in the double mutants appeared throughout the leaf and were not restricted to the basal part of the leaf, while in *as1* and *as2* the lobes only occurred at the base of the leaf (Fig. 1C,E). Thus, *se* quantitatively and qualitatively enhanced the lobing of *as1* and *as2* leaves.

SEM analysis of *as1 se* and *as2 se* double mutants

In order to further characterize the lobed leaf phenotypes we performed SEM analysis on leaves of the double and single mutants, and compared them to wild-type and *35S::KNAT1* plants (Fig. 2). We examined the phenotype of leaf margins, particularly in the sinuses (Fig. 2A), as these regions were known to contain ectopic stipules and meristems (Fig. 2B) (Chuck et al., 1996). Leaf margins of the Columbia ecotype (Fig. 2C) and the *se* mutant (Fig. 2D) appeared continuous, with two to three files of long straight cells running along the margins. More files of long straight cells could be seen along the margins of *as1* and *as2* single mutants, thus increasing the width of the margin. Most striking was the discontinuous nature of the margin in the sinus (Fig. 2A,E-H). A notch was visible in *as1* and *as2* leaves (arrows in Fig. 2E,G). Sinuses of leaves from *as1 se* and *as2 se* double mutants had an even deeper notch, and the long, straight cells that were normally confined to the margin increased in number and were found spreading onto both surfaces of the leaf. Cells closer to the notch were smaller than cells in the same file at a distance from the notch. One to three ectopic structures that resemble stipules formed in the sinus (Fig. 2F,H). Ectopic stipules were rarely detected in lobes of single *as1* or *as2* mutants. They occurred in approximately 20% of the few lobes that formed, compared to approximately 90% of all lobes on leaves of the double mutants. The proliferation of ectopic stipules was reminiscent of the *35S::KNAT1* margins (Fig. 2B). Other aspects of the *as1*

Table 1. *se* enhances lobing in *as1* and *as2* mutants

Genotype	No. of lobes on leaf1	leaf2	leaf3	leaf4	leaf5	leaf6	last leaf*	% with lobes
as1-1	0.0±0.0	0.0±0.0	0.1±0.1	0.7±0.2	1.7±0.3	1.9±0.4	2.7±0.3	52±3
as1-1 se	1.9±0.3	2.8±0.2	4.7±0.3	4.6±0.5	4.5±0.3	4.6±0.4	3.6±0.5	99±1
as2-2	0.0±0.0	0.0±0.0	0.5±0.2	1.1±0.2	1.1±0.3	1.5±0.2	1.9±0.3	60±4
as2-2 se	2.3±0.2	3.1±0.2	4.4±0.3	5.1±0.4	5.1±0.2	5.4±0.2	4.7±0.3	100±0

*The last leaf before bolting.

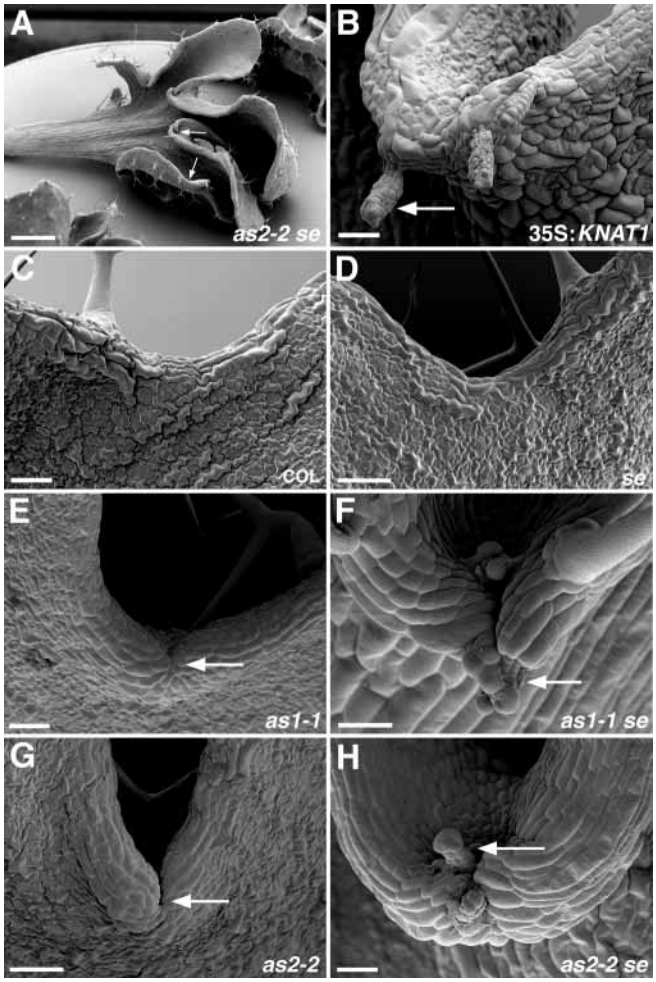

Fig. 2. Scanning electron micrographs of the sinus region. (A) An entire *as2 se* leaf, (B) sinus of a *35S::KNAT1* plant with three visible stipules, (C) margin of wild-type Columbia (COL) leaf, (D) margin of *se* leaf in the region of a serration, (E) sinus of *as1* leaf, (F) sinus of an *as1 se* leaf with stipules in the sinus, (G) sinus of an *as2* leaf, (H) sinus of an *as2 se* leaf with stipules in the sinus. Arrows indicate, sinuses (A), stipules (B,F,H), notch (E,G). Bars, 1 mm (A); 50 μm (B-H).

and *as2* leaf phenotype, including the notch and curling of the leaves downward (Fig. 1), differed from *35S::KNAT1*.

We also performed SEM analysis of mature flowers. The sepals and petals on *35S::KNAT1* plants were narrow, and either failed to elongate or were delayed in elongation, causing the flowers to open prematurely (Fig. 3E). Maturation and elongation of the stamens were delayed relative to the carpel, and consequently fertility was reduced. Flowers of *as1* and *as2* were similar in some aspects to those of *35S::KNAT1* plants; they opened prematurely owing to reduced elongation of the petals, sepals and stamens (Fig. 3C,D). Unlike *35S::KNAT1* or wild-type flowers (Fig. 3A), *as1* and *as2* flowers had much wider sepals. Occasionally their sepals were serrated, and the petals curled outward, opposite to the wild type. Flowers of *se* mutants appeared normal (Fig. 3B), but the stamens were less fertile because of reduced pollen shed. The phenotypes of *as1 se* and *as2 se* flowers were enhanced in comparison to *as1* and *as2* single mutants. The sepals, petals and stamens were shorter, the sepals were wider and more lobed, and the petals were often curled outward and occasionally serrated (Fig. 3F,G). The flowers opened prematurely and were less fertile.

Misexpression of *KNAT1* and *KNAT2* in *as1* and *as2* mutants

An appealing interpretation of the phenotypic similarities between *as1 se*, *as2 se* double mutants and *35S::KNAT1*, is that the normal function of *AS1*, *AS2* and *SE* is to restrict expression of *KNAT1* and/or other related homeobox genes. To test this hypothesis, we utilized transgenic plants that expressed the *iudA* gene reporter, which encodes β-glucuronidase (GUS; Jefferson et al., 1987) from the *KNAT1* or *KNAT2* promoter.

We used three different transgenic lines to examine expression of *KNAT1* in wild-type and mutant leaves. Similar to *KNAT1* expression detected by in situ hybridization (Lincoln et al., 1994), expression in the *KNAT1::GUS-1* line was detected in the hypocotyl, stem and shoot meristems but not in leaves (Fig. 4A). GUS expression in the *KNAT1::GUS-18* line was similar to the pattern seen in the *KNAT1::GUS-1* line except that weak GUS expression was also detected in some of the veins of cotyledons and occasional later leaves (Fig. 4G).

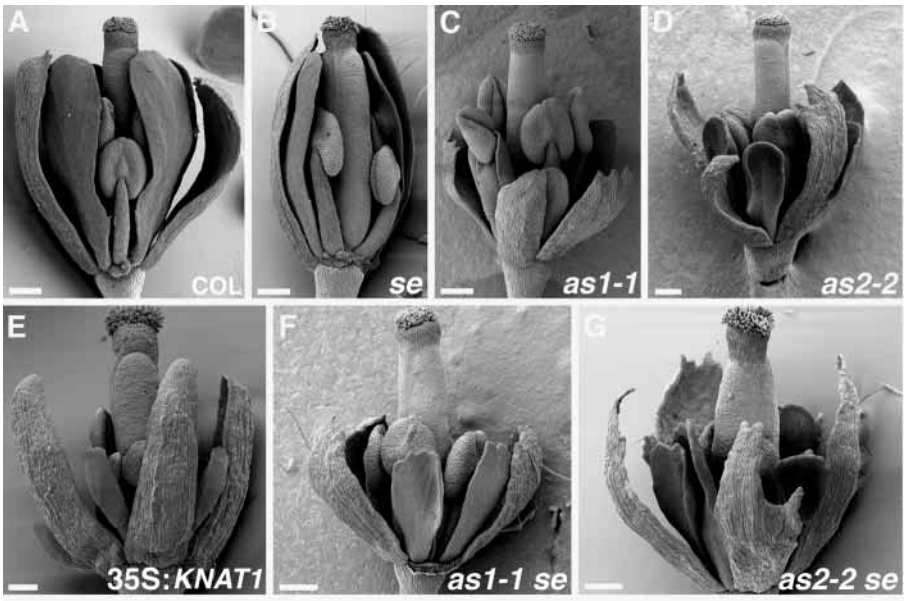

Fig. 3. Floral phenotypes of wild-type Columbia (COL; A) flower and the single and double mutants (B-G). The *se* flower (B) is similar to wild type. The *as1*, *as2* and *35S::KNAT* flowers (C-E) have shorter petals and stamens. The petals and sepals of *as1*, *as2* and their double mutants with *se* (F,G) are serrated and the petals fold outward. Bars, 200 μm.

The third line, *KNAT1::GUS-19*, was used to examine expression in *pickle* mutants (see later). *se* mutants appeared to express the *KNAT1::GUS-1* transgene normally (Fig. 4B). In *as2* mutants, *KNAT1::GUS-1* expression was detected in the petioles of leaves, in the main vein, sometimes in secondary veins, and occasionally in patches on the leaves (Fig. 4C). Leaf expression in *as2* mutants carrying the *KNAT1::GUS-18* line (Fig. 4I) was similar to the expression seen with *KNAT1::GUS-1*, but stronger, presumably because of the slightly enhanced expression seen in the *KNAT1::GUS-18* line. Due to linkage of *KNAT1::GUS-1* to *as1*, we could only use the *KNAT1::GUS-18* and *KNAT1::GUS-19* lines to examine expression in *as1*. Similar to the results found with *as2*, GUS expression was strong in the petioles and in the vasculature of *as1* leaves (Fig. 4H). Weak GUS expression was also seen in the sinus of the occasional lobes that formed on *as1* and *as2* leaves (Fig. 4E,K).

as1 se and *as2 se* double mutants also showed expression of *KNAT1::GUS* in leaves although the expression was not as intense as in the *as1* or *as2* single mutant (Fig. 4D,J). Expression in the sinuses, however, was enhanced in the double mutants (Fig. 4F,L). The ectopic growth of stipules in the double mutants was located in spots of strong GUS expression (not shown).

In wild-type plants carrying the *KNAT2::GUS* construct, staining was detected in meristems but not leaves (Fig. 4M). *KNAT2*::*GUS* expression appeared normal in *se* mutants (Fig. 4N). Leaves of *as1* and *as2* single mutants revealed GUS expression in the sinuses, and slightly in the basal portion of the leaf petioles (Fig. 4O,Q and inserts). *as1 se* and *as2 se* double mutants expressed *KNAT2::GUS* strongly in the sinuses of lobes (Fig. 4P,R and inserts).

We used in situ hybridization to determine if the pattern of *knox* expression was altered in the mutant meristems. Expression of *KNAT1* RNA in meristems of the single and double *as1*, *as2* and *se* mutants appeared similar to that of wild-type plants; expression was confined to the peripheral zone of the meristem and was not detected in the P_0 region nor in the youngest leaf primordia (Fig. 5A-G). These results suggested that *AS1* and *AS2* are not required for the down-regulation of *KNAT1* during leaf initiation. Sporadic expression of *KNAT1* was detected in the basal part of young leaves of *as1*, *as2*, *as1 se* and *as2 se* mutants (Fig. 5C-G), in agreement with the results obtained using the GUS transgenic lines. We concluded that *KNAT1* was negatively regulated in P_0 and P_1 leaf primordia of *as1* and *as2*, but not later in leaf development. The data are consistent with a role for AS1 and AS2 in keeping *KNAT1* off in leaves post-initiation.

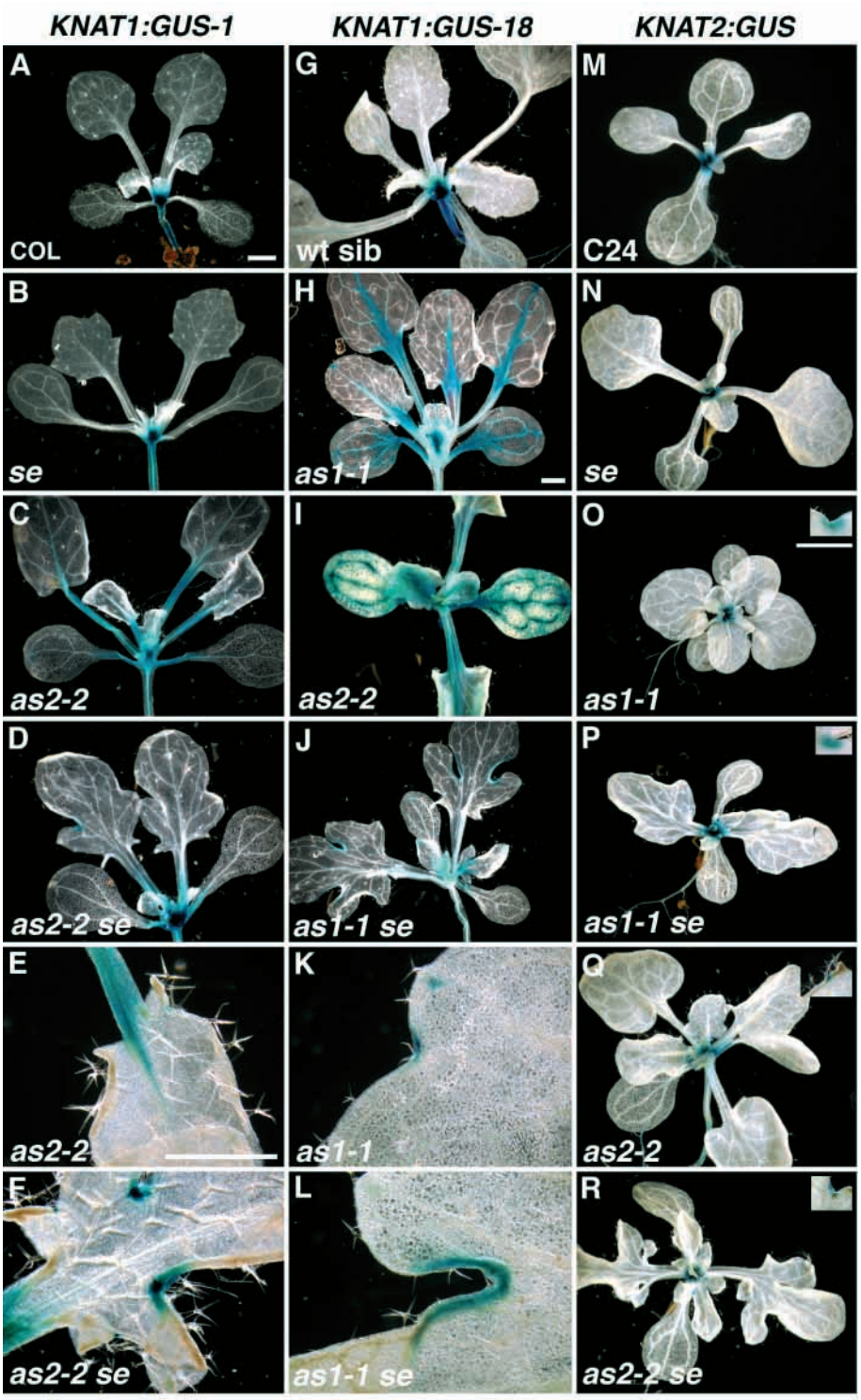

Fig. 4. Ectopic expression of *KNAT1::GUS* and *KNAT2::GUS* in *as1*, *as2* and *se* seedlings. Seedlings were grown in soil under long day conditions. The reporter line used is indicated at the top for each column. E,K,F,L and inserts in O-R show a higher magnification of a sinus. Bars, 1 mm (A-D,G,I,J,M-R); 1 mm (H); 1 mm (E,F,K,L); 1 mm (inserts in O-R).

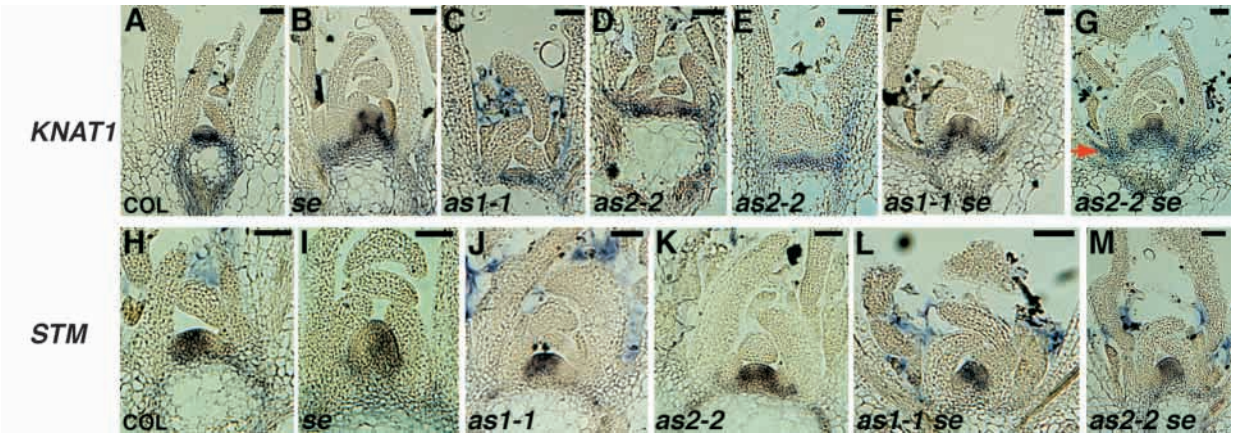

Fig. 5. *knox* expression is properly regulated in the mutant meristems. In situ hybridization of vegetative meristems, probed with *KNAT1* (A-G) or *STM* (H-M) probes. An example of ectopic expression is indicated with an arrow in G. Plants were grown in short days to assure that the meristems are vegetative. Bars, 50 μm.

Using in situ hybridization, we could not detect *SHOOTMERISTEMLESS* (*STM*) expression in leaves of *as1*, *as2* or *se* single or double mutants despite strong expression in the meristem (Fig. 5H-M). This result suggests that AS1 and AS2 do not negatively regulate *STM*; however, we cannot rule out the possibility that *STM* is expressed in occasional patches of older leaves, or is expressed below the level of detection.

We also noticed that the meristems of *se* mutants, whether as a single mutant or in combination with *as1* and *as2*, appeared taller than wild-type meristems in the Columbia ecotype. In *se* mutants, *KNAT1* expression appeared as two vertical stripes on the flanks of the meristem. Expression was not detected in the center of the meristem or in the P_0 cells (Fig. 5B). In the *se* mutant, the domain expressing *KNAT1* appeared taller because of the increased height of the meristem. Similarly, the group of meristem cells that do not express *STM*, marking the P_0 region, was taller (Fig. 5I).

After the transition to flowering, *KNAT1::GUS* was expressed in the stem and in the inflorescence meristem of wild-type plants. Expression in flowers was detected in the style, placenta, ovules and occasionally in the filaments of the stamens, but was absent from the sepals and petals and other parts of the carpels (Fig. 6A). *KNAT2::GUS* was expressed in wild-type flowers similar to *KNAT1::GUS* except that additional expression was detected in the replum (Fig. 6G). The expression of both constructs in *se* mutant flowers appeared similar to the wild-type pattern (Fig. 6B,H). In *as1* and *as2* flowers, as well as in the double mutants of *as1* and *as2* with *se*, *KNAT1::GUS* and *KNAT2::GUS* were expressed strongly throughout the sepals (Fig. 6C-F,I-L). Expression was not detected in the petals despite their altered morphology.

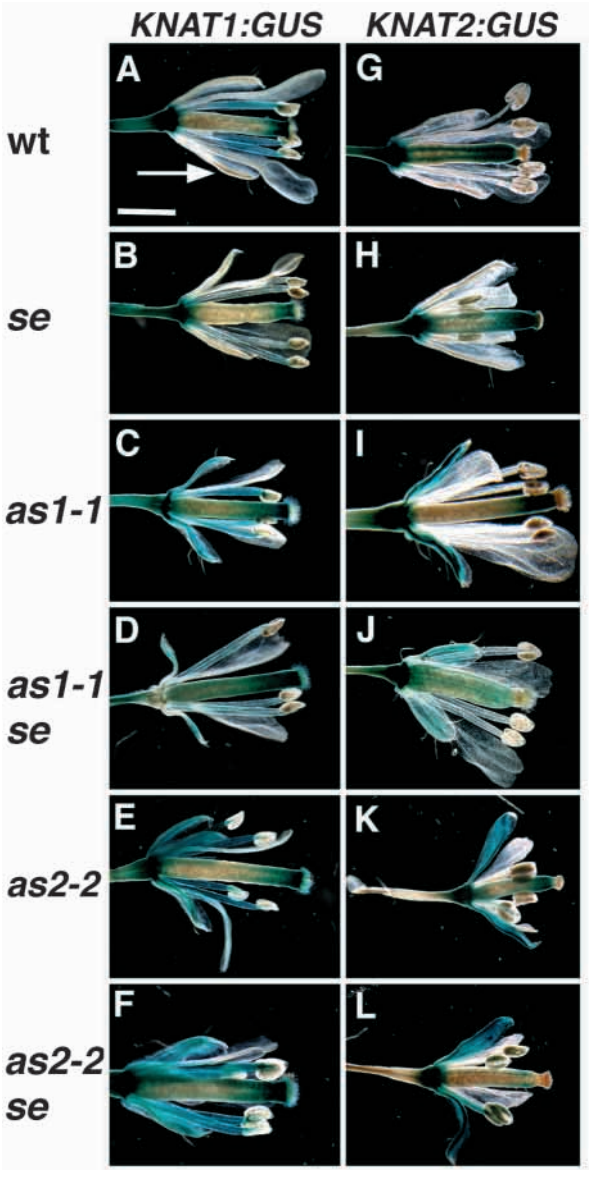

Fig. 6. Misexpression of *KNAT1::GUS* and *KNAT2::GUS* in flowers of *as* mutants. The wild type is Columbia for *KNAT1::GUS* and C24 for *KNAT2::GUS*. Bar, 1 mm. *KNAT1* and *KNAT2* are misexpressed in sepals of *as* mutants (C-F and I-L).

Interaction between *as1, as2* and the *pickle* mutants

PICKLE (PKL), also described as GYMNOS (GYM), is a

member of the CHD class of chromatin remodeling factors (Eshed et al., 1999; Ogas et al., 1999). It has been suggested that PKL plays a role in restricting expression of meristematic genes based on (1) the ectopic ovules that occur when plants are doubly mutant for *PKL* and *CRABSCLAW*, (2) the occasional ectopic embryos that can form on roots carrying mutant alleles (Ogas et al., 1997; Eshed et al., 1999), and (3) the sequence homology. To test this hypothesis, we generated double mutants between *as1-1* and *pkl-15* (previously referred to as *gym-5*), and *as2-14* and *pkl-15*. Shoots of single *pkl-15* mutants were mostly normal, except that they were slow growing and delayed in differentiation in most organs (Eshed et al., 1999). The leaf margins were not affected (Fig. 7A). Although the leaf shape of *as1-1 pkl-15* double mutants was similar to that of *as1-1* single mutants, close examination of the serrations and lobes of these leaves by SEM revealed ectopic growth of stipules in this region (Fig. 7B,C). Stipules were found on approximately 80% of the the sinuses, either singly or in clusters. On rare occasions, we detected ectopic meristems arising from the adaxial side of the leaf close to the sinus (Fig. 7C). Ectopic meristems were also occasionally detected on the adaxial surface of *as2-2 pkl-15* double mutants, near the sinus (Fig. 7D). These phenotypes are reminiscent of those found on *as se* double mutants and on *35S::KNAT1* plants. No synergistic interaction was found in the reproductive organs of the double mutants.

We also examined the expression of *KNAT1::GUS-19* in the *as1 pkl-15* double mutants. *KNAT1::GUS-19* was expressed in wild-type plants in a pattern similar to *KNAT1::GUS-18*; in the meristem and hypocotyl with weak expression in some veins (Fig. 7E). Similar to the findings for the *as1 se* double mutant, GUS expression in *as1-1 pkl-15* plants was concentrated in the sinus region and less intense throughout the leaf (Fig. 7H,I) in comparison to the strong expression in *as1* single mutants (Fig. 7G). *KNAT1::GUS-19* expression in *pkl-15* single mutants was indistinguishable from that of wild type (Fig. 7E,F). Nearly identical results were obtained with the *KNAT2::GUS* reporter in the *pkl-15 as1-1* mutant background (not shown).

DISCUSSION

Class I *knox* genes are expressed in shoot apical meristems and excluded from leaves in many species (Jackson et al., 1994; Lincoln et al., 1994; Long et al., 1996; Nishimura et al., 1999; Sentoku et al., 1999). When *knox* genes are misexpressed in leaves under control of constitutive promoters, leaf cell fates alter, including becoming meristematic, and the leaves are often misshapen (reviewed in Reiser et al., 2000). With the goal of dissecting the mechanisms that regulate the expression and activity of meristem-specific *knox* genes, we examined *Arabidopsis* recessive mutants that displayed phenotypes reminiscent of *knox* gene overexpression. *KNAT1* and *KNAT2* were misexpressed in leaves of *as1* and *as2* mutants, demonstrating that AS1 and AS2 function to restrict *knox* gene expression from leaves. *se* and *pkl* mutations enhanced the *as1* and *as2* mutant phenotypes but did not cause misexpression of *KNAT1* or *KNAT2* on their own. Expression of *KNAT1* and *KNAT2* was focused in the sinus region of the double mutants. We discuss the roles of these genes in *knox* regulation and leaf development.

The role of AS1 and AS2 in negative regulation of *KNOX* genes

We found that *KNAT1* and *KNAT2* were ectopically expressed in *as1* and *as2* mutants. *KNAT1* transcript accumulated in leaves of both mutants, but was down-regulated in the P_0 region as in normal meristems. This finding suggests that if AS1 and AS2 are involved in the initial down-regulation of *KNAT1* and *KNAT2* at the time of leaf initiation, they act redundantly with other factors at this stage. AS1 and AS2 are required, however, to keep *KNAT1* and *KNAT2* off later in leaf development. We have also noted downregulation of *knox* genes in the P_0 region of the meristem when ectopically expressed from constitutive promoters, suggesting that the down-regulation may function at the level of RNA turn-over (Chuck et al., 1996; Williams-Carrier et al., 1997).

The *as1 as2* double mutant appeared similar to *as2* single mutants (these data and Serrano-Cartagena et al., 1999), suggesting that *as2* is epistatic. All aspects of the *as2*

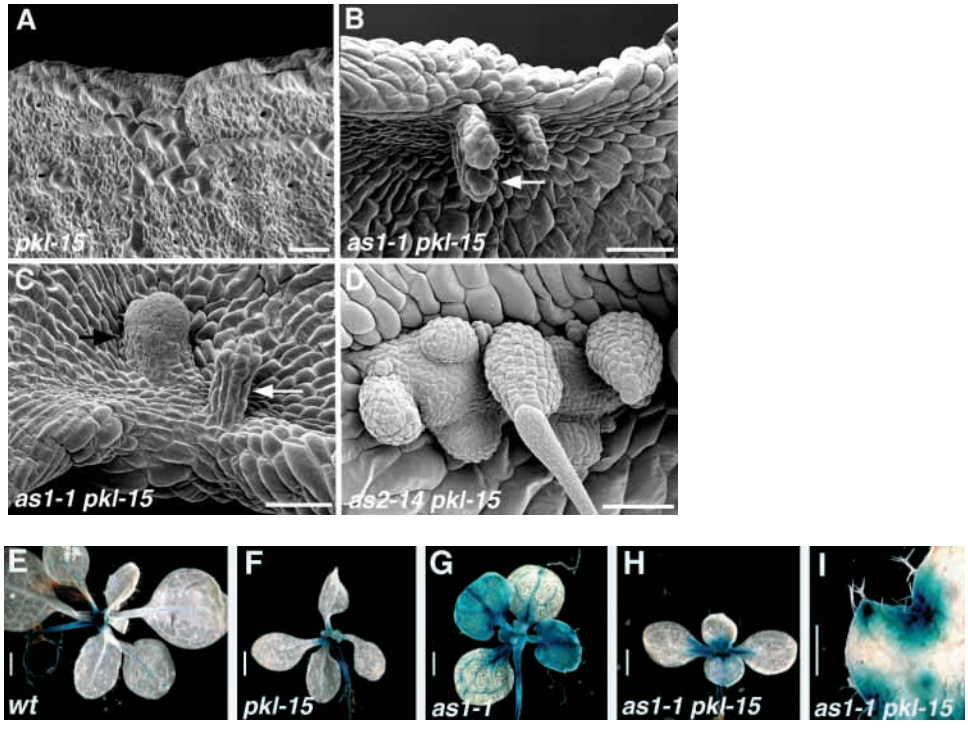

Fig. 7. Interactions of *pkl* with *as1* and *as2*. (A-D) Scanning electron micrograph images of the sinuses of *pkl* and *pkl as1*, *pkl as2* double mutants, showing the ectopic stipules (B,C, white arrows) and ectopic meristems (C, black arrows, and D). The meristem in D has initiated leaves. (E-I) GUS staining of wild type and mutant seedlings containing *KTP::GUS-19*. Bars, 50 μm (A-D); 1 mm (E-I).

phenotype were very similar to those of *as1*, including the interactions with *se* and *pkl*. In addition, ectopic expression of *KNAT1* and *KNAT2* was found in *as2* mutants, similar to the misexpression in *as1* mutants. The complete overlap of phenotypes and the epistasis of *as2* suggest that AS1 and AS2 act in a common pathway to regulate *knox* expression.

knox genes are also ectopically expressed in leaves of the maize mutant, *roughsheath2* (*rs2)* and the *Antirrhinum* mutant, *phantastica* (*phan*) (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). *rs2* and *phan* encode homologous myb-like transcription factors (Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). *rs2* mutants resemble dominant, gain-of-function mutations in *knox* genes (Freeling and Hake, 1985; Schneeberger et al., 1995; Muehlbauer et al., 1999; Foster et al., 1999). The maize mutants that misexpress *knox* genes are thought to be defective in the establishment of cell fates along a proximodistal axis (Freeling, 1992). *phan* mutant leaves are also uniquely characterized by alterations in their dorsoventral symmetry (Waites and Hudson, 1995; Waites et al., 1998). Normal leaves have an adaxial surface, which faces the meristem, and an abaxial surface, which faces away from the meristem (Sussex, 1955; Sessions and Yanofsky, 1999). *phan* mutant leaves have patches of abaxial cells on the adaxial surface and occasionally radial leaves, or portions of leaves. *as1* mutants also display some phenotypes that could be explained by defects in abaxial/adaxial polarity, such as curling of leaves downward and curling of petals outward. In addition, the ectopic stipules can be viewed as alterations of the proximal-distal leaf axis. Phenotypic differences between maize, *Arabidopsis* and *Antirrhinum* may result from differences in growth habit, temporal and spatial patterns of *knox* misexpression, and different downstream targets.

se and *pkl* enhance the phenotypes of *as1* and *as2* mutant

as1 and *as2* mutants lacked many of the phenotypes of *35S::KNAT1* plants despite missexpression of *KNAT1* and *KNAT2* in the leaves. This result suggests that AS1 and AS2 are only part of the regulation of *knox* expression and function. Strikingly, double mutants of *as1* or *as2* with the *se* mutant revealed phenotypes similar to those of *35S::KNAT1*. In both *35S::KNAT1* transgenic plants and *as1 se* and *as2 se* double mutants, lobes were found extensively on every leaf, and ectopic growth of stipules was detected in the sinuses. One possible explanation might have been that SE also negatively regulates *knox* genes and thus the absence of SE and either AS1 or AS2 leads to an increase in *knox* expression. However, we found no evidence for misexpression of *KNAT1* or *KNAT2* in *se* single mutants. In fact, expression of *KNAT1* and *KNAT2* in the *as se* double mutants was decreased in the petiole and veins while enhanced in the sinus relative to the *as1* or *as2* single mutants.

The *se* mutant is impaired in the regulation of phase length and phyllotaxy. Mutants make fewer leaves but flower at the same time as their normal siblings (Clarke et al., 1999). *se* may enhance the *as1* and *as2* phenotype by quickening the transition to adult leaves (Clarke et al., 1999) which are more likely to have deep lobes. However, even on adult leaves of *as1* or *as2* single mutants, the number of lobes is less than that of the double mutants and the presence of ectopic stipules is rare. A second possibility is that *SE* may negatively regulate genes that are downstream of *knox* genes. Such a rationale would explain why we do not see a change in *knox* expression in *se* mutants. These downstream genes may function in cytokinin metabolism (Ori et al., 1999), cell division (Meyerowitz, 1997), or cell fate acquisition (Muehlbauer et al., 1997). Leaves on both *se* mutants and *35S::KNAT1* plants also have an increase in number of veins which suggests that they may affect other pathways in common (Chuck et al., 1996; Clarke et al., 1999). A third explanation for the enhancement by *se*, is that *SE* may itself regulate meristem architecture or leaf initiation. We noted that *se* mutants have a taller meristem and previous workers noted a change in phyllotaxy (Clarke et al., 1999). One difficulty in assigning function to *SE* is that only a single allele has been described, despite several recent large-scale mutagenesis screens for leaf mutants (Berna et al., 1999). This finding suggests that the *se* null phenotype could be different from this allele. Additional alleles will help resolve these issues.

Ectopic growth in the sinuses was also detected in *as1 pkl* and *as2 pkl* double mutants, though the lobing pattern was similar to that of *as1* single mutants. Mostly stipules formed in the sinus but occasionally a meristem was detected in these mutants on the adaxial side of the leaves near the sinus. These regions had many small cells that did not expand. *35S::KNAT1* plants also consistently showed ectopic stipules and occasional meristems. The mixture of stipules and meristems suggests that the stipules are not just a marker for leaf bases, but possibly the signature of a passing meristem.

pkl alleles result in defects in maturation of various shoot and root tissues. *pkl* mutations enhance the *crabsclaw* mutant phenotype, leading to ectopic ovules on the outside of the carpel wall (Eshed et al., 1999). These mutants also acquire abnormal regeneration capacity in roots. When *pkl* roots are excised, they generate structures that resemble somatic embryos. The penetrance of this phenotype is very low but can be enhanced with gibberellic acid (GA) inhibitors (Ogas et al., 1997). Given that PKL may act through GA, it is interesting that *knox* overexpression in tobacco leads to a decrease in GA 20-oxidase and addition of GA suppresses *knox* overexpression phenotypes (Tanaka-Ueguchi et al., 1998). GA may therefore represent a point that is downstream of both PICKLE and AS1.

PICKLE encodes a chromatin remodeling factor of the CHD class (Eshed et al., 1999; Ogas et al., 1999). It is most closely related to the *Drosophila dMi2* gene, that is involved in the repression of *HOX* genes (Kehle et al., 1998). *SE* encodes a putative single 2Cys-2His zinc finger transcription factor, of a class known to regulate gene activity by modification of chromatin structure (M. Prigge and R. Wagner, personal communication). Thus, it is possible that both PKL and SE function to keep genes in an inactive state. The targets of PKL and SE are likely to be numerous. Their single and double mutant phenotypes suggest that at least some of these targets are genes that promote morphogenesis. A possible explanation for enhancement of the *as1* and *as2* phenotypes by *se* and *pkl* is that when *knox* genes are misexpressed in the leaf, SE and PKL keep a subset of *knox* targets inaccessible to activators, thus reducing the effect of *knox* misexpression. However, when *knox* genes are misexpressed and the chromatin is accessible due to the absence of SE, PKL, or other chromatin remodeling factors, KNOX proteins may be more able to activate their targets. This hypothesis would explain why *se* and *pkl* mutants

alone have a minimal phenotype and why *pkl* enhances other mutations such as *crabsclaw*, but does not explain the altered expression of *KNAT1:GUS* and *KNAT2:GUS* in the double mutants.

Ectopic boundaries

The expression of *KNAT1* in a ring within the peripheral zone of the meristem suggests that it may define a boundary, perhaps a border between undetermined cells and cells that are recruited to the initiating leaf. The expression patterns of other *knox* genes may define additional borders within the meristem (Jackson et al., 1994). Misregulation of *knox* genes could disrupt the establishment of boundaries or cause new boundaries to form, and accordingly affect pattern formation in the adaxial/abaxial and the proximal/distal dimensions. In both *35S::KNAT1* transgenic plants, which do not express *KNAT1* ubiquitously (Chuck et al., 1996), and *as se* double mutants, the sinuses of the lobes contain regions of small cells surrounded by elongated cells that have undergone more divisions. A similar, but temporal juxtaposition of cells exists during leaf initiation. Lobes may result from formation of an ectopic meristem boundary in the sinus region. Consistent with this idea is the expression of *KNAT1* and *KNAT2* in the sinus, as well as the presence of stipules, normally formed at leaf bases. This expression pattern transforms the leaf into distinct meristem-like and leaf-like zones. The boundaries formed by ectopic *knox* expression in the leaf could help explain the variable phenotypes that arise in *phan* and *rs2* mutants where *knox* expression was shown to be stochastic (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). We propose that the severity of the double mutants is not due to extra *knox* expression but the ectopic boundaries that are created.

We wish to thank Jan Doxck and Jan Traas for the *KNAT2::GUS* construct, Judy Yamaguchi for help in making the *KNAT1::GUS* construct, David Hantz and Catherine Choi for help in the greenhouse, and the Arabidopsis Stock Center for *as1 as2* and *se* seed stocks. We wish to thank Miltos Tsiantis, Mark Running and members of the Hake lab for thoughtful discussions. N.O. was funded by BARD and NIH. The work was supported by NSF grant MCB-9727611 to S. H. and 99 86054 to J. L. B.

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