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SEMAPHORE1 functions during the regulation of ancestrally duplicated *knox* genes and polar auxin transport in maize

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SUMMARY

The expression of class 1 *knotted1*-like homeobox (*knox*) genes affects numerous plant developmental processes, including cell-fate acquisition, lateral organ initiation, and maintenance of shoot apical meristems. The SEMAPHORE1 gene product is required for the negative regulation of a subset of maize *knox* genes, the duplicated loci *rough sheath 1* and *gnarley1* (*knox4*). Recessive mutations in *semaphore1* result in the ectopic expression of *knox* genes in leaf and endosperm tissue. Genetic analyses suggest that SEMAPHORE1 may regulate *knox* gene expression in a different developmental pathway than ROUGH SHEATH2, the first-identified regulator of *knox* gene expression in maize. Mutations at *semaphore1* are pleiotropic, disrupting specific domains of the shoot. However, unlike previously described mutations that cause ectopic *knox* gene expression, *semaphore1* mutations affect development of the embryo, endosperm, lateral roots, and pollen. Moreover, polar transport of the phytohormone auxin is significantly reduced in semaphore1 mutant shoots. The data suggest that many of the pleiotropic semaphore1 phenotypes result from defective polar auxin transport (PAT) in sem1 mutant shoots, and support models correlating down-regulated *knox* gene expression and PAT in maize shoots.

Key words: KNOX, *semaphore1*, Leaf development, Auxin transport

INTRODUCTION

The precisely regulated expression of *homeobox* (*hox*) genes and gene complexes is a fundamental feature of animal development (reviewed by McGinnis and Krumlauf, 1992). Hox proteins are transcription factors containing a homeodomain, an evolutionarily conserved motif comprising 60 amino acids that functions to control pattern formation and cell specification during development (Laughon and Scott, 1984; McGinnis et al., 1984). First discovered by molecular genetic analyses of homeotic (segment identity) mutants in *Drosophila melanogaster*, evolutionarily-conserved relatives of the homeobox have since been identified in widespread phylogenetic groups including vertebrates, invertebrates, yeasts and plants (Murtha et al., 1991; Patel et al., 1991; Vollbrecht et al., 1991; Ruberti et al., 1991). Moreover, the residual homology of the metazoan homeodomain to transcriptional regulatory proteins from bacteria and phage lambda suggests that the role of *hox* genes during development has evolved subsequent to a primordial role in DNA recognition and binding (Laughon and Scott, 1984; Pabo and Sauer, 1984). In lieu of the extended evolutionary history of *hox*-like genes, the pathways regulating homeobox gene expression may be extremely variable across disparate taxonomic groups.

The maize gene *knotted1* is the first homoebox gene identified in plants (Vollbrecht et al., 1991). Dominant, over-expressing alleles of *Knotted1* (Smith et al., 1992) and related class 1 *KNotted1*-like homeobOX (*Knox*) (Kerstetter et al., 1994) genes in maize (Freeling, 1992; Schneeberger et al., 1995; Muehlbauer et al., 1999; Foster et al., 1999), barley (Müller et al., 1995), rice (Matsuoka et al., 1993), *Arabidopsis* (Lincoln et al., 1994; Chuck et al., 1996), tobacco (Sinha et al., 1993; Sakamoto et al., 2001) and tomato (Haraven et al., 1996; Chen et al., 1997) condition seemingly disparate, species-specific phenotypes including ectopic meristem formation, delayed cell-fate acquisition, indeterminate growth patterns, increased leaf lobing and super-compound leaf morphology. Null mutations of *knox* genes may abort the development and/or maintenance of shoot apical meristems (SAMs) (Long et al., 1996; Kerstetter et al., 1997; Vollbrecht et al., 2000), organogenic centers that initiate lateral organs in plant shoots.

The structure of plant meristems is correlated with their function. The central zone (CZ) of the SAM is occupied by large, slowly dividing stem cells, which are surrounded by a peripheral zone (PZ) of smaller, mitotically active cells. Plant lateral organs are formed from founder cells recruited from the peripheral zone (PZ) of the SAM. Anlagen lost from the PZ during organogenesis are replenished from stem cells supplied by the CZ. Thus, the SAM maintains an equilibrium during the production of each plant segment, balancing the production of stem cells in the CZ with the loss of founder cells from the PZ (reviewed by Fletcher and Meyerowitz, 2000). In plants with simple (undissected) leaves, KNOX proteins accumulate in the CZ, but are excluded from lateral organ primordia and also from the organogenic PZ. Patterns of *knox* gene expression in

the SAM support a model whereby KNOX proteins function to promote developmental indeterminacy in plant cells. Consequently, the precise, epigenetic regulation of *knox* gene expression affects cell-fate acquisition and lateral organ formation during plant development.

The MYB-domain protein ROUGH SHEATH2 (RS2) is the first regulator of *knox* gene expression found in plants (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999b). Recessive mutations in the maize *rs2* gene result in the ectopic expression of at least three *knox* genes (*liguless3*, *knotted1* and *rough sheath1*) in shoot lateral organs. Correlated with the mis-regulation of these *knox* genes is an array of mutant shoot phenotypes; many of which mimic those conferred by dominant, neomorphic mutations in *knox* genes such as *rough sheath1* and *knotted1* (reviewed by Freeling, 1992). Orthologous mutations are described in *myb* genes from *Arabidopsis* (*asymmetric leaves1, as1*) (Ori et al., 2000; Byrne et al., 2000) and *Antirrhinum* (*phantastica1, phan1*) (Waites and Hudson, 1995; Waites et al., 1998). The expression patterns of the *rs2/phan/as1* gene family are complimentary to those of *knox* genes, consistent with their role during negative regulation of *knox* gene expression. Furthermore, *rs2* mutants are defective in polar auxin transport (PAT) (Tsiantis et al., 1999b), which may account for many morphological abnormalities of *rs2* mutant shoots.

Another intriguing finding is that although the *rs2/as1/phan1* mutations all cause ectopic *knox* expression, their mutant phenotypes are not fully analogous (Schneeberger et al., 1998; Ori et al., 2000; Waites and Hudson, 1995). Differential defects in proximal-distal (enhanced in *rs2*), dorsal-ventral (enhanced in *phan1*), and mediolateral patterning (enhanced in *as1*) are noted in the three mutants. The variable phenotypes of *knox* regulatory mutants observed in maize, *Antirrhinum* and *Arabidopsis* may be attributed to differences in the specific *knox* gene targets, differential expression patterns of *knox* and downstream genes, and/or divergent strategies employed during development of eudicot and monocot leaf morphology (Timmermans et al., 1999; Tsiantis et al., 1999b; Ori et al., 2000).

The accumulated data suggest a model whereby the RS2/PHAN1/AS1 family regulates *knox* gene expression via the epigenetic control of transcriptional memory in differentiating cells (Ori et al., 2000) (reviewed by Byrne et al., 2001). In support of this model, mutations such as *pickel1* and *serrate1*, both of which affect genes predicted to encode chromatin remodeling factors in *Arabidopsis*, enhance the phenotypes of *knox* regulatory mutants but do not themselves condition *knox* gene mis-expression (Ori et al., 2000). A similar scenario is reported for the *handlebars1* mutant (Waites and Hudson, 2001), which greatly enhances the phan1 mutant phenotype. Ultimately, these data suggest that the epigenetic control of *knox* gene expression during plant development is a complex interaction of multiple genes and gene networks, many of which exhibit phase-specific, organ specific, as well as organ-domain specific effects.

Here we report the function of SEMAPHORE1 (SEM1), the second regulator of *knox* gene expression identified in maize. The unique and pleiotropic *semaphore1* mutant phenotypes include abnormalities in the development of the embryo and endosperm, roots, stem, leaves, vasculature and flowers. These mutant phenotypes are correlated with the mis-expression of the ancestrally duplicated *knox* genes (Kerstetter et al., 1994; Foster et al., 1999) *rough sheath1* and *gnarley1*, and also with impaired polar auxin transport in *semaphore1* mutant shoots. The phenotypic, molecular and genetic data suggest that RS2 and SEM1 function in separate pathways to control *knox* gene expression in maize shoots. Furthermore, these data support a model in which suppression of *knox* gene expression promotes basipetal transport of auxin in maize leaves.

MATERIALS AND METHODS

Maize stocks

The *sem1-R* mutant (previously *dek*Mu1364*) was first identified as a recessive, defective kernel mutant phenotype obtained from Robertson's *Mutator* stocks (Scanlon et al., 1994). The mutation is introgressed 6 times into the inbred B73, and 5 times into the Q66 inbred line. The genetically unlinked *rs2-R* mutation (isolated by M. I. Hajidov in 1937) was donated by P. Becraft (Iowa State University).

The *sem1*Mu12* allele was obtained via transposon-tagging with Robertson's *Mutator* (Robertson, 1978). Homozygous *sem1-R* plants are male sterile; therefore *sem1-R* heterozygous males were crossed to plants containing *Mutator* transposon activity. Among approximately 25,000 F_1 kernels, 18 were selected on the basis of their small endosperm/small embryo phenotype, planted, and grown to maturity. A single plant among the 18, designated as *sem1*Mu12*, exhibited a small stature and displaced ligule phenotype, and was propagated by out-crossing to inbred W23. The *sem1*Mu9* allele was obtained by directed transposon-tagging utilizing plants homozygous for the *sem1-R* mutation as female. Mutant *sem1-R* plants were de-tasseled to eliminate self-contamination, and crossed to pollen from *Mu*-active plants. Among approximately 2,000 F_1 progeny kernels, two were selected on the basis of the small endosperm/small embryo phenotype. The selected seed were sown, and a single plant, designated *sem1*Mu9*, which displayed the small stature/displaced ligule phenotype was out-crossed to inbred W23.

Genetic analyses and double mutant construction

In order to locate the *sem1-R* mutation to a particular chromosome arm, a series of plants heterozygous for B/A translocations (Beckett, 1994) (for details, see Scanlon et al., 2000) were crossed as male to plants heterozygous for *sem1-R*. B/A translocations are useful for genetic mapping (reviewed by Beckett, 1994), and in analyses of gene dosage (reviewed by Birchler, 1994). B/A translocations are reciprocal exchanges between a normal (A) maize chromosome and a supernumerary (B) chromosome. The centromere of the supernumerary B chromosome inherently undergoes non-disjunction at high frequency during the second mitotic division of microsporogenesis. Therefore, plants harboring a B/A translocation typically produce discordant pollen grains, in which the two sperm nuclei are unbalanced with respect to the dosage of genes linked to the B centromere. Specifically, one sperm nucleus contains two doses of genes linked to the B centromere (hyperploid) whereas the other sperm nucleus is deficient (hypoploid). Following double fertilization by a male plant carrying a B/A translocation, discordant aneuploid kernels are generated in which the embryo may be hypoploid and the endosperm hyperploid, and vice versa. In this way, recessive mutations located distal to the breakpoint in the A chromosome will be "uncovered" in hypoploid F_1 progeny.

F_1 progeny of crosses between *sem1-R/B73* heterozygotes and male plants hyperploid for TB-9Sd (which carried the linked anthocyanin marker *C1*) segregated kernels that were discordant for sem1 mutant phenotypes (Fig. 1C). Two discordant kernel phenotypes were noted: (1) kernels with hypoploid, colorless sem1 mutant embryos (genotype *sem1-R*/–) and purple, non-mutant hyperploid endosperm (genotype *sem1-R*/*sem1-R*/*Sem1*/*Sem1*); and (2) kernels with purple, non-mutant

hyperploid embryos (genotype *sem1-R*/*Sem1*/*Sem1*) and colorless, hypoploid mutant endosperm (genotype *sem1-R*/*sem1-R*/–).

Allele tests of *sem1-R* and both mutant isolates, *sem1*Mu12* and *sem1*Mu9*, were performed by reciprocal crosses between heterozygous plants as described previously (Scanlon et al., 1994). Newly identified *sem1* mutants are each expected to be heterozygous for both the *sem1-R* allele and the newly tagged *sem1*Mu* allele. Following out-crossing to non-mutant standard lines, the progeny of the newly tagged mutant plants are predicted to segregate 1:1 for heterozygosity for either *sem1-R* or the newly tagged *sem1*Mu* allele. RFLP analyses using a genetic marker tightly linked to *sem1-R* (HN900, no recombinants have been detected among over 250 progeny, unpublished data) were used to distinguish plants heterozygous for the newly tagged mutant *sem1* alleles from those harboring the reference allele *sem1-R*. Positive allelism tests were indicated by the segregation of approximately one in four mutant kernels on ears test-crossed to *sem1-R/B73* heterozygous plants.

For the construction of semaphore1-R/rough sheath2-R double mutants, plants heterozygous for the *sem1-R* mutation were crossed to plants heterozygous for the unlinked *rs2-R* mutation, and the F_1 plants were self-pollinated. The resulting F_2 progeny were scored for the presence of one in four *sem1* mutant kernels. F_2 *sem1* mutant kernels obtained from self-pollinated ears that also segregated approximately one in four *rs2* mutant seedlings were screened for segregation of putative *sem/rs2* double mutants. The genotypes of *rs2/sem1* double mutants identified in the progeny were verified by DNA gel-blot analyses using a 0.5 kb fragment of the *rs2* gene (gift from M. Timmermans), and the *sem1-R* linked RFLP marker HN900 as probes.

Immunohistology and light microscopy

Immunohistolocalization of KNOX proteins in 14-day post-germination maize seedlings and in maize seeds collected 12 days after pollination were performed as described previously (Scanlon et al., 1996) using the KNOX polyclonal antibody (gift from R. Schneeberger). Hypocotyl samples were dissected from *sem1-R* mutant and non-mutant seedlings (5 samples each), fixed in FAA, paraffin-embedded, sectioned and stained in Safranin-Fast Green as described (Sylvester and Ruzin, 1994).

Clearing of maize tissues

Leaves were cleared of cytoplasmic components as described by Sylvester and Ruzin (Sylvester and Ruzin, 1994). Harvested leaves were treated in 5% NaOH for 24 hours. Following 2 rinses in distilled H_2O, leaves were incubated in chloral hydrate (250 g/100 ml) overnight. Cleared leaves were stained in 1% saffranin, destained in xylene, mounted in Permount™ (Fisher Scientific) and photographed.

Polar auxin transport (PAT) assays

Analyses of polar auxin transport were performed on hypocotyls harvested from dark grown, 5 day-old non-mutant and *semaphore1* mutant seedlings as described previously (Tsiantis et al., 1999), with the following modifications. Assays were performed by incubating 2.5 cm segments of hypocotyls oriented base down (acropetal transport) or base up (basipetal transport) in 60 μl of nutrient solution (Gil et al., 2001) containing 12.5 μCi of tritiated indol-acetic acid [^{3}H]IAA for 16 hours. Measurements were performed on 2 mm tip segments taken from 5 hypocotyl segments for each treatment, using a LKB Wallac 1218 Rackbeta scintillation counter.

Scanning electron microscopy (SEM) analyses

Scanning electron microscopy (SEM) was performed on epidermal replicas of mature maize leaves and developing leaf primordia as described previously (Sylvester et al., 1990).

Analyses of *knox* gene transcripts

Semi-quantitative RT-PCR analyses were performed on cDNA prepared from non-mutant, *sem1-R* mutant and *rs2-R* mutant seedlings leaves as described by Bauer et al. (Bauer et al., 1994). Gene-specific primers for *rough sheath1*, *knotted1*, *liguless3* and *ubiquitin* were the same as those employed by Schneeberger et al. (Schneeberger et al., 1998). Gene-specific primer sequences for *gnarley1* (*knox4*) provided by J. Yamaguchi and S. Hake (USDA, Albany, CA) were as follows:

knox4-A 5′-tatcgcctcggttcatga-3′
knox4-B 5′-ccgcaccccgccttggtt-3′

First-strand cDNA was prepared from RNA extracted from seedling leaves (dissected away from internode and shoot meristems tissue) or the ligule/auricle region of unexpanded, adult leaves. RT-PCR was performed using gene-specific primers for 25, 30 and 40 cycles (cycle=94°C for 30 seconds; 60°C for 30 seconds; 72°C for 60 seconds) and the products were analyzed by gel-electrophoresis in 1.5% agarose. In order to control for discrepancies in gel mobility or ethidium bromide staining, all PCR products shown in Fig. 4C were loaded onto the same agarose gel. The specificity of the *knox* gene primers was verified by sequencing cloned PCR products.

RESULTS

Recessive *semaphore1* mutations cause defective kernel phenotypes

The *sem1-R* mutation was identified in a screen for defective kernel phenotypes derived from maize *Mutator*-transposon backgrounds (Scanlon et al., 1994). Plants heterozygous for *sem1* mutations segregate approximately one to four mutant kernel phenotypes in self-pollinated ears (Fig. 1A). Homozygous *sem1-R* mutant kernels have both a smaller endosperm and embryo than non-mutant sibling seed (Fig. 1B), and kernel viability is dependent upon genetic background. Following six generations of introgression into the maize inbred B73 approximately 70% of *sem1-R* mutant kernels are viable. However, five introgressions into the inbred Q66 result in greater than 90% mortality of *sem1-R* homozygous seed. Included among the lethal sem1-R mutant phenotypes are kernels that fail to germinate, as well as seedling-lethals that develop an abnormal fused coleoptile (described below) but no leaves.

The maize B/A translocations (described in Materials and Methods) (reviewed by Birchler, 1994; Beckett, 1994) were used to locate the *sem1-R* mutation to the short arm of chromosome 9 (Fig. 1C). Complementation analyses with previously described seed mutations in this map location detected no allelism. Thus, *semaphore1* denotes a novel, defective-kernel maize mutation located on the short arm of chromosome 9.

semaphore1 mutant seedlings ectopically express two maize *knox* genes

Immunohistochemical analyses were performed on *semaphore* mutant seedlings using a polyclonal antibody that recognizes class 1 KNotted1-like homeobOX (KNOX) proteins (Schneeberger et al., 1998). KNOX proteins accumulate in the shoot apical meristem (SAM) and stems of non-mutant maize seedlings, but are not detected in leaf primordia or founder cells of the incipient leaf (Fig. 2A,D). In contrast, viable *sem1* mutant seedlings exhibit ectopic accumulation of KNOX protein(s) in leaf primordia, predominately in the epidermis (Fig. 2B). Interestingly, down-regulation of KNOX

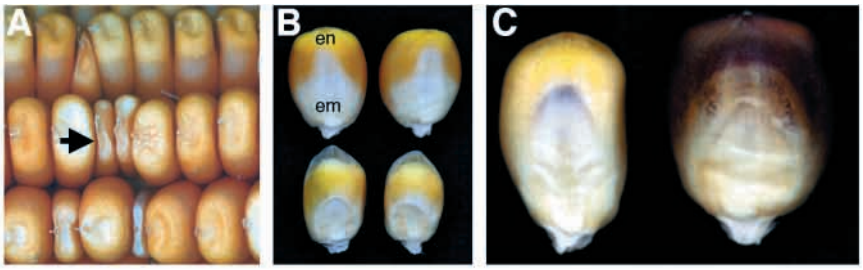

Fig. 1. Kernel phenotypes of *semaphore1* mutants. (A) Portion of a self-pollinated ear from a *sem1-R/Sem1* heterozygous plant, segregating for approximately one in four mutant kernels (arrow). (B) Mutant kernels (lower tier) contain both a smaller embryo (em) and endosperm (en) than non-mutant sibling kernels (upper tier). (C) Non-discordant kernels are generated following pollination of *sem1-R/Sem1* heterozygous plants by plants harboring the B/A translocation TB-9Sd. The kernel on the left contains a hypoploid, mutant endosperm (genotype *sem1-R/sem1-R/–)* and a purple, hyperploid non-mutant embryo (genotype *sem1-R/Sem1/Sem1*). The sibling kernel on the right contains a hyperploid, purple non-mutant endosperm (genotype *sem1-R/sem1-R/Sem1/Sem1*) and a hypoploid, mutant embryo (genotype *sem1-R–)*.

accumulation appears normal in the incipient mutant leaf (Fig. 2E), implying that leaf founder-cell recruitment is not disrupted in viable *sem1-R* mutants. Furthermore, the aberrant accumulation of KNOX protein(s) is absent from younger mutant primordia (P1-P3), but increases in later stages of leaf development (P4-P5 and older). These data indicate that SEMAPHORE is not essential for repression of KNOX protein accumulation during early stages of leaf development.

Seedling-lethal *sem1-R* mutants display a novel phenotype (Fig. 2C,F). The first leaf-like lateral organ of non-mutant maize shoots is the coleoptile, a hollow tube that encloses and protects the young leaf primordia during germination. In severe *sem1-R* mutants, however, the coleoptile develops as a fused shaft that accumulates high levels of KNOX protein throughout. Moreover, seedling-lethal *sem1-R* mutants produce no lateral organs subsequent to this mutant coleoptile, and no organized SAM is present in the mutant apex (Fig. 2F). This extreme phenotype is more prevalent in the Q66 genetic background, although seedling-lethal phenotypes are seen in <10% of *sem1-R* mutant seed introgressed into inbred B73. In summary, immunohistolocalization data indicate that SEMAPHORE1 is required for negative regulation of KNOX accumulation in maize seedlings.

The phenotypic variation seen in *sem1-R* homozygous seedlings is reflected in *sem1-R* mutant embryos harvested at 12 days after pollination (DAP). Among 10 individual non-mutant sibling embryos examined at 12 DAP all had progressed to stage 2 (Abbe and Stein, 1954), during which two leaf primordia are enclosed within the coleoptile (Fig. 3A,B). In contrast, two distinct classes of embryo phenotype were observed in 12 DAP *sem1-R* mutant kernels. Moreover, all *sem1-R* mutant embryos were developmentally retarded. Specifically, seven out of 12 mutant embryos had reached stage 1, during which the coleoptile surrounds a single leaf primordium (Fig. 3C,D). Intriguingly, five of 12 mutant embryos had progressed only to the coleoptilar stage (Fig. 3E-H). Moreover two of these severe mutants displayed aberrant embryo morphology, including an enlarged coleoptile and a broad apex that does not exhibit normal tunica-corpus morphology (Fig. 3G,H). These results suggest that developmentally aberrant embryos such as those shown in Fig. 3G,H may develop into lethal seedlings with abnormal coleoptile and SAM morphology, as shown in Fig. 2C,F.

Immunolocalization studies of maize kernels reveal that KNOX proteins accumulate in sem1 mutant endosperm at 12 DAP (Fig. 3C,E,G), but not in non-mutant endosperm (Fig. 3A) (Smith et al., 1995). Also, the degree of KNOX accumulation in the endosperm is correlated with the severity of the mutant embryo phenotype; extremely retarded mutant embryos (Fig. 3E,G) contain more immunopositive endosperm nuclei than mutant siblings with milder embryo phenotypes (Fig. 3C). However, no abnormal KNOX accumulation is detected in lateral organs of 12 DAP *sem1* mutant embryos (Fig. 3). As in non-mutant siblings, *sem1* mutants accumulate high levels of KNOX in the shoot apex and embryonic stem, but do not accumulate KNOX proteins in the mutant scutellum, coleoptile or developing leaf primordium. These data are

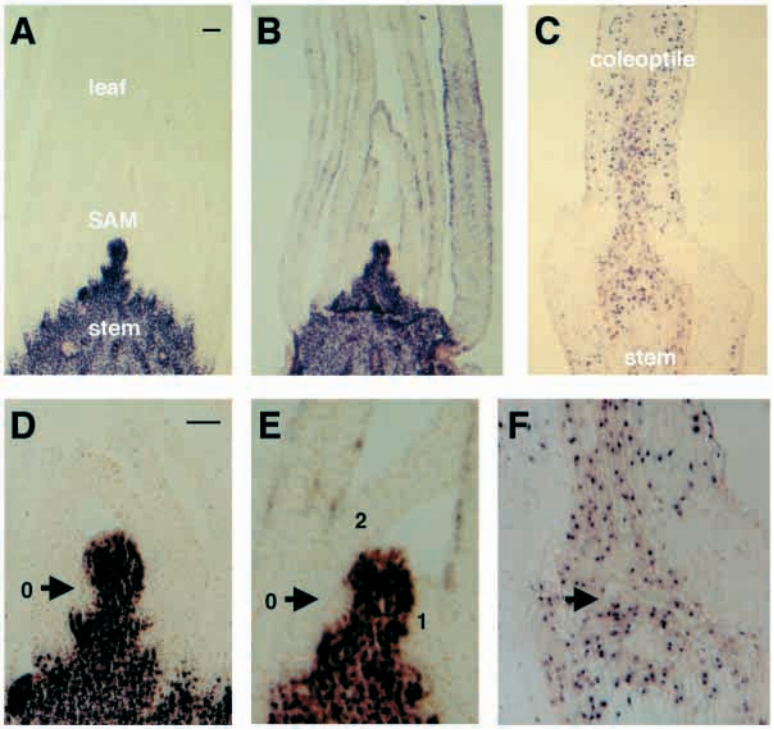

Fig. 2. Ectopic KNOX accumulation in semaphore1 mutant seedlings. Immunohistolocalization of vegetative shoots using a polyclonal KNOX antibody. (A,D) non-mutant siblings, (B,E) moderate *sem1* mutant seedling, (C,F) severe mutant seedling. The arrows in D and E indicate the position of the incipient leaf primordium (labeled 0); the two youngest leaf primordia in E are numbered. The arrow in F indicates the expected position of the shoot apex. Scale bars: 200 μm, in A for A-C and in D for D-F.

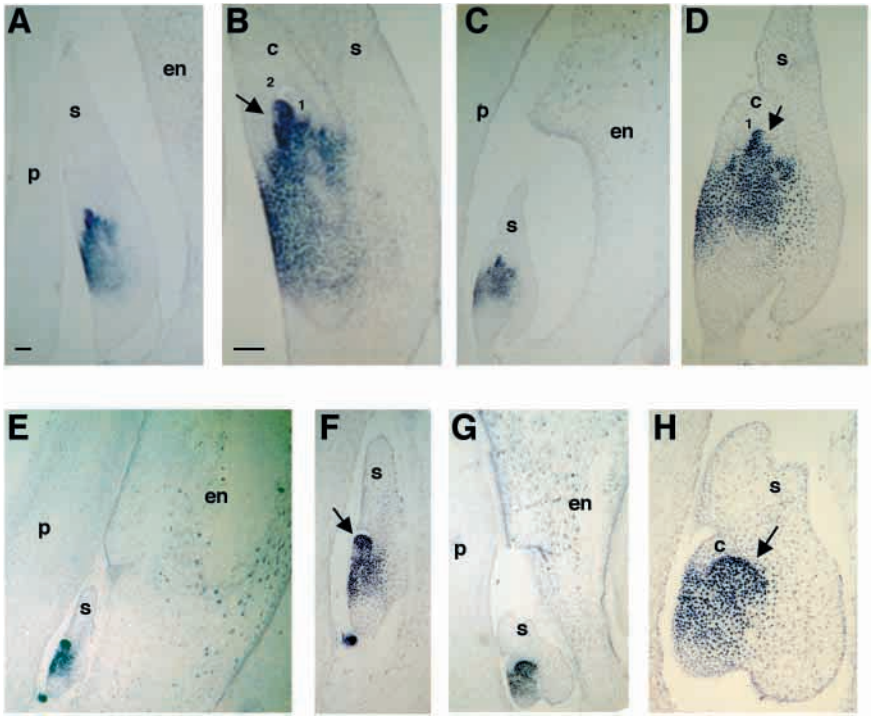

Fig. 3. Embryo development is abnormal in *semaphore* mutants. KNOX immunohistolocalizations of 12 DAP embryos. Non-mutant embryos (A,B) are larger than sem1 mutant siblings (C-H) and show no KNOX accumulation in the incipient leaf (arrow in B and D), leaf primordia (numbered), pericarp (p), scutellum (s), coleoptile (c) or endosperm (en). Moderately retarded *sem1* mutants (C-D) and more severely retarded mutants (E-H) exhibit ectopic KNOX accumulation in the endosperm and fewer (if any) leaf primordia than non-mutant siblings. The arrows in F and H indicate the shoot meristem. Scale bars: 200 μm, in A for A,C,E,G and in B for B,D,F,H.

consistent with KNOX accumulation patterns observed in semaphore mutant seedlings; no ectopic KNOX proteins are observed in young, mutant leaf primordia.

The KNOX antibody is not gene specific (Scanlon et al., 1996). Therefore, RT-PCR was used to determine the relative abundance of specific, *knox* gene transcripts in *sem1-R* mutant, *rs2-R* mutant and non-mutant leaves (Fig. 4). Primers specific for the maize *ubiquitin* gene were used to control for approximately equal concentrations of cDNA in the RT-PCR reactions. RT-PCR reveals that ectopic transcripts of *gnarley1* (*gn1*), and to a lesser degree *rough sheath1* (*rs1*) accumulate in unexpanded leaves extracted from the whorl of young adult mutant plants (Fig. 4A,B). No *knotted1* (*kn1*) RT-PCR products are detected in adult *sem* mutant leaves (data not shown), whereas no *rs1*-, *gn1*-, or *kn1*-specific RT-PCR products are amplified in non-mutant adult leaves (Fig. 4A,B). Inconsistent results are obtained in our RT-PCR analyses using *lg3* primers (Fig. 4B), such that weakly amplified transcripts are occasionally detected in both non-mutant and *sem1-R* mutant leaves. These observations are in agreement with previous RT-PCR analyses of non-mutant leaves utilizing *lg3* primers (G. Muehlbauer, personal communication). Time-course RT-PCR (Bauer et al., 1994) was used to compare *knox* gene transcript accumulation in cDNA prepared from *sem1-R*, *rs2-R* and non-mutant B73 seedling leaves (Fig. 4C). Transcripts from both *gn1* and *rs1* are amplified from *sem1-R* seedling leaves following 40 and 30 PCR cycles respectively (Fig. 4C top). Therefore, whereas ectopic *gn1* transcripts predominate over *rs1* transcripts in *sem1-R* adult leaves, *rs1* is more abundant in *sem1-R* seedlings. In comparison, abundant transcripts of *rs1* are amplified from *rs2-R* leaves

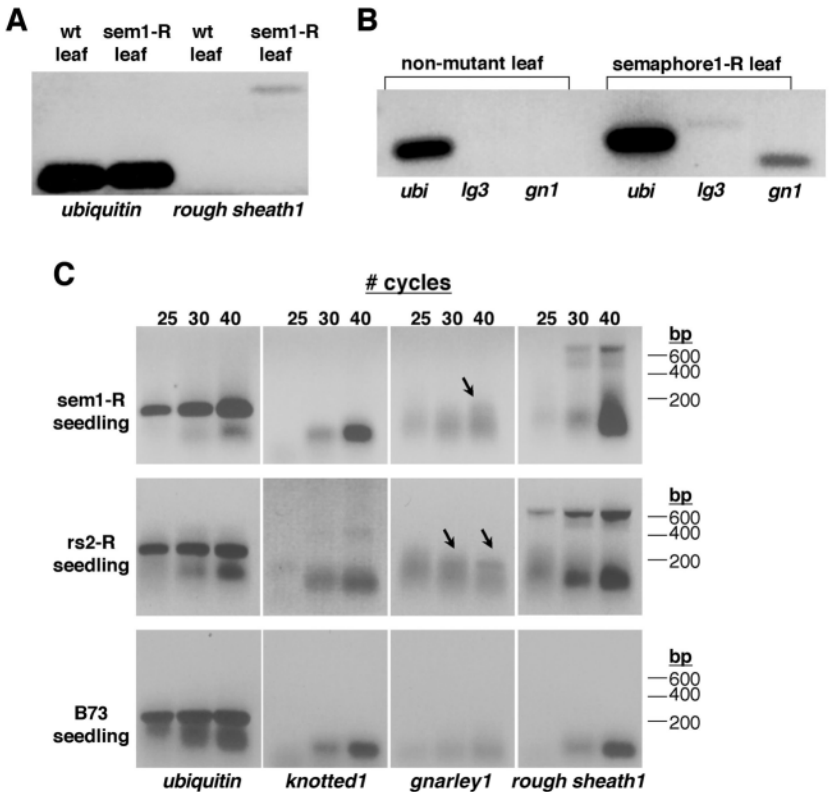

Fig. 4. Mutant leaves show ectopic expression of *rough sheath1* and *gnarley1* transcripts. Following 40 cycles of RT-PCR, *rs1* (A) and *gn1* (B) transcripts are detected in *sem* mutant adult leaves. Inconsistent results are obtained using *lg3*-specific primers (B). (C) Semi-quantitative RT-PCR of *kn1*, *gn1* and *kn1* transcripts in *sem1-R* mutant (top), rs2-R mutant (center) and non-mutant B73 seedling leaves (bottom). No *knox* transcripts are detected in non-mutant leaves (Fig. 4A, B, C bottom), even after 50 RT-PCR cycles (data not shown). Sizes of predicted RT-PCR products are: ubiquitin, 208 bp; rough sheath1, 625 bp; knotted 1, 342 bp; liguless3, 248 bp; gnarley, 190 bp, indicated by arrows to distinguish from primer-dimers.

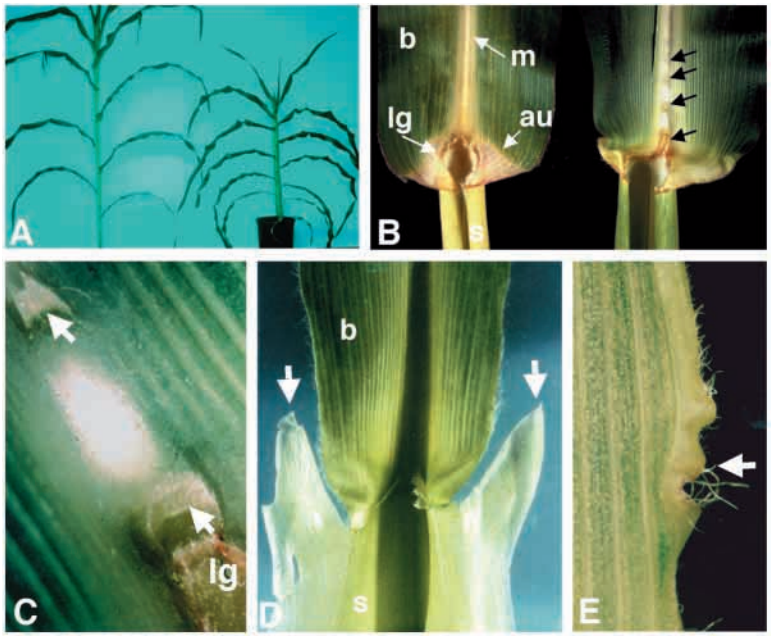

Fig. 5. Pleiotropic shoot phenotypes of sem1 mutants. (A) Adult sem1 mutant plants (right) have much shorter stems than non-mutant siblings (left). (B) The leaf blade (b), midrib (m), ligule (lg), auricle (au) and sheath of the non-mutant sibling leaf (left) are indicated. The black arrows in the mutant leaf (right) indicate the displaced and ectopic ligules. (C) Close up of the displaced ligule (lg) and two ectopic ligules (arrows) of a *sem1* mutant leaf. (D) Ectopic sheath extensions (arrows) at the margins of the blade-sheath boundary in a *sem1* mutant leaf. (E) Ectopic auricle formation (arrow) at the margin of a *sem1* mutant leaf blade.

after just 25 cycles, whereas *gn1* amplicons are first detected after 30 cycles (Fig. 4C middle). In addition, *kn1* transcripts are weakly amplified following 30-40 cycles of RT-PCR in *rs2* seedlings, whereas no *kn1* is detected in *sem1-R* seedlings (Fig. 4C). Thus, *sem1-R* mutant seedlings exhibit a slightly different profile of ectopic *knox* expression than *rs2-R*, the first-identified *knox*-regulatory mutant of maize (Schneeberger et al., 1998). Both mutants mis-regulate *rs1* and *gn1*, whereas ectopic expression of *kn1* is detected in *rs2-R* seedlings only. Moreover, the *rs2-R* shoot phenotypes are more extreme than in viable *sem1-R* seedlings, and are correlated with higher levels of ectopic *knox* expression (Fig. 4C).

semaphore1 mutations have pleiotropic effects on maize shoot development

After introgression into the inbred B73 genetic background, the *sem1-R* mutation conditions specific abnormalities in shoot development. Homozygous *sem1-R* mutant plants have short internodes (brachytic), although they develop the same number of leaves as non-mutant sibling plants (Fig. 5A). Non-mutant maize leaves comprise several domain-specific structures, which display distinct epidermal cell morphologies (Sylvester et al., 1990). The proximal sheath surrounds the stem and is separated from the distal blade by the wedge-shaped auricle and the ligule, an epidermally derived fringe of tissue (Fig. 5B, left). The *sem1-R* mutant ligule is displaced distally, but only along the midrib (Fig. 5B, right). Multiple ectopic ligules may form distal to the primary ligule, forming a stack of ligules centered over the midrib (Fig. 4B,C). Scanning electron microscopy (Fig. 6) reveals that in contrast to the narrow fringe of ligule that forms in non-mutant leaves, the *sem1-R* mutant ligule is a broad patch of tissue (Fig. 6E). Furthermore, cell-fate acquisition is altered in-between the stacks of ectopic ligules (Fig. 6F-H), whereas normal midrib cell types are found distal to these disturbed regions (Fig. 6I). This polarized pattern of mutant cell-fate acquisition, in which proximal tissues (i.e. ligule and sheath) are displaced into distal domains (i.e. blade), is a common phenotype in maize mutants that ectopically express *knox* genes (reviewed by Freeling, 1992). Moreover, the *sem1-R* ligule phenotype is detectable at very early stages (Fig. 6A,B) [normal ligule development is described by Walsh et al. (Walsh et al., 1998)], suggesting a defect in ligule initiation rather than later-staged, distorted growth along the blade/sheath boundary.

Abnormal tissue proliferation also occurs at the margins of

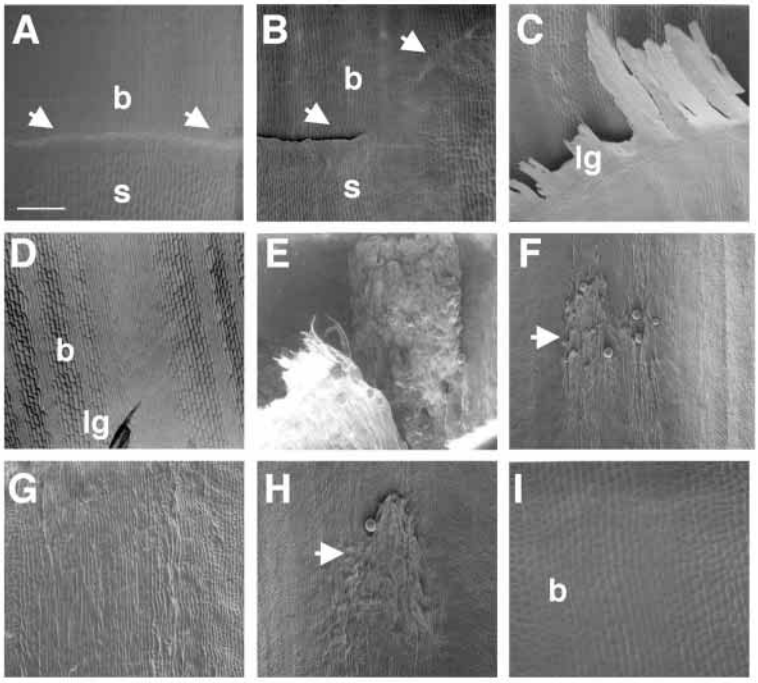

Fig. 6. Disruptions of the blade-sheath boundary in *sem1* mutant leaves. Scanning electron micrograph images of the epidermal surfaces of non-mutant (A), and mutant (B) leaf primordia soon after initiation of the ligule (arrows), separating the blade (b) from the sheath (s). The mutant ligule (B) is displaced distally in the midrib region. (C) Non-mutant young adult leaf at maturity. lg, ligule. (D) The non-mutant midrib and blade (b) distal to the ligule depicted in C. (E-I) Images of a *sem1* mutant, young adult leaf showing ligule displacement over the midrib (E), an ectopic second ligule (F), the region distal to the ectopic second ligule (G), an ectopic third ligule (H), and (I) normal cell types located distal to the disturbed region. Scale bar: 300 μm.

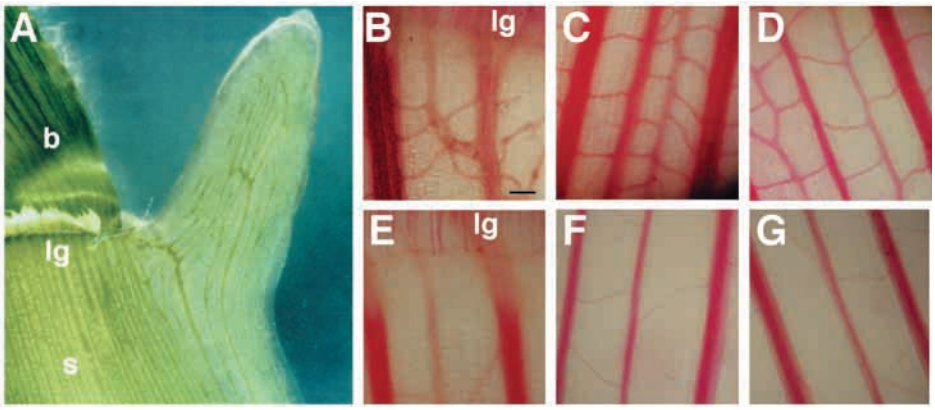

Fig. 7. Abnormal vasculature in *sem1* mutant leaves. (A) Ectopic proliferation of margin tissue at the blade-sheath boundary of a mutant leaf shows extensive transverse vasculature elements. b, blade; s, sheath; l, ligule. (B-G) Cleared and stained sheath tissue of (B-D) non-mutant and (E-G) *sem1* mutant leaves. (B,E) Just proximal to the ligule and midway between the midrib and margin. (C,F) As in B and E, but 4 cm proximal to the ligule. (D,G) Near the margin, 4 cm proximal to the ligule. The *sem1* mutant sheath shows poor development of transverse veins. Scale bar in B: 50 μm for B-G.

semaphore mutant leaves, near the blade-sheath boundary (Fig. 5D). Ectopic outgrowths of sheath tissue form highly vascularized, symmetrical pairs at the leaf edge. Sheath outgrowths are never observed at only a single leaf margin. Close examination of the sheath outgrowths reveals atypical vascularization patterns. In contrast to the parallel arrangement of major vessels present in non-mutant maize (Sharman, 1942) (and described below), the *sem1-R* sheath outgrowths are highly reticulated and form large, transverse branch vessels (Fig. 7A). Although the *sem1-R* sheath outgrowths are hyper-vascularized, overall transverse vein development in the mutant sheath is markedly deficient. In contrast to the thick transverse vessels found in sem1 mutant sheath outgrowths, transverse veins in non-mutant sheaths are thinner than the parallel, lateral veins (Fig. 7B-D). Non-mutant maize leaves form narrow, yet proliferative transverse connections between the larger lateral vessels in the sheath (Fig. 7B-D). Normally, the elaboration of minor, transverse vessels begins almost immediately proximal to the ligule (Fig. 7B); maize leaf blades develop far fewer transverse connections (Sharman, 1942) (and data not shown). However, transverse vessels in the *sem1-R* mutant leaf sheath are smaller and less abundant than in non-mutant leaves, especially near the sheath margins (Fig. 7E-G). No gross abnormalities are observed in the leaf-blade vasculature of *semaphore1* mutants (data not shown).

In addition, viable *sem1-R* mutant plants are male-sterile. Fertile ears do develop however, such that homozygous mutant plants may be propagated as female. More variable mutant phenotypes are seen in non-introgressed genetic stocks. Additional *sem1-R* mutant phenotypes may include the formation of ectopic auricle or ligule tissue on leaf margins (Fig. 5E), and extremely narrow leaves (data not shown). Genetic background strongly influences the *sem1-R* mutant phenotype, which suggests that many unidentified genetic factors contribute to SEMAPHORE1 function, KNOX accumulation, and *knox* gene regulation.

semaphore1 mutations disturb lateral root development

A prominent phenotype of *sem1-R* mutant plants is the formation of yellow stripes on vegetative leaves (Fig. 8A). This pattern of leaf-striping is a diagnostic feature of plants deficient in the uptake of soil magnesium (Olsen and Sander, 1988). However, the leaf-striping phenotype of sem mutant leaves is only partially alleviated by magnesium supplementation. Therefore, we suspected that *sem1-R* mutant plants might have root abnormalities that result in magnesium-deficient plants. When compared to non-mutant siblings at an equivalent stage of shoot development (determined by the total number of leaves produced), *sem1-R* mutant seedlings had fewer and severely stunted primary, seminal, and adventitious roots (Fig. 8B,C). Furthermore, there is a striking reduction in the development of lateral roots. As such, *sem1* represents the first maize *knox*-expression mutant shown to disrupt both shoot and root development. Transverse sections of the embryonic stem (Fig. 8D-E) of *sem1-R* and non-mutant seedlings reveal no gross abnormalities in vascular development of the mutant hypocotyl. However, the hypocotyls and vessel elements in the smaller, *sem1-R* mutant seedlings are thinner in diameter than those of larger, non-mutant siblings.

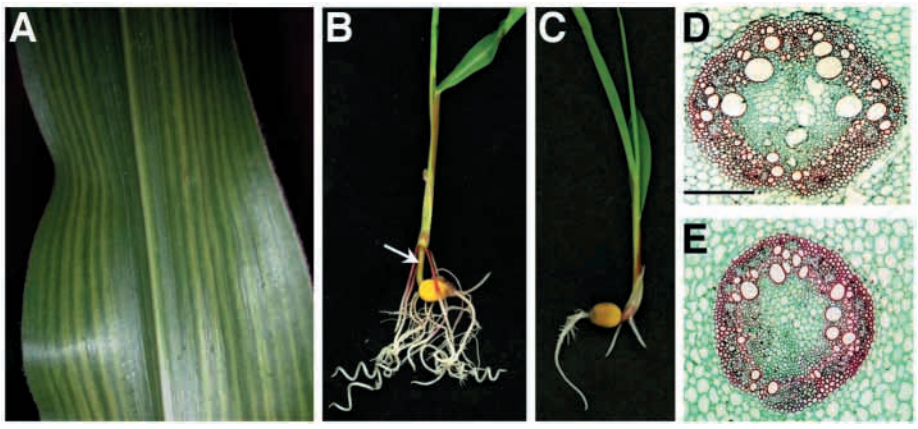

Fig. 8. (A) Adult *sem1* mutant leaf showing yellow, zebra striping. (B,C) Impaired root development in *sem1* mutants. (B) Root development in a non-mutant seedling. Arrow, hypocotyl (seedling stem). (C) Root development in a *sem1* mutant seedling. (D,E) Transverse sections of the hypocotyl in non-mutant siblings (D) and in smaller mutant plants (E) reveal no gross abnormalities in vascular development in *sem1-R* mutant seedlings. Scale bar for D and E, 50 μm.

semaphore1 mutants show a marked reduction in polar auxin transport

The phytohormone auxin undergoes basipetal transport in plant shoots, from the tip to the base of the plant (reviewed by Berleth and Sachs, 2001). The major bio-active auxin in plants is indole-acetic acid (IAA). IAA is known to affect an array of plant developmental processes, including embryonic pattern formation, lateral organ initiation, suppression of lateral branching, vascular development and lateral root development. Regulation of IAA function in plant shoots is proposed to depend upon an equilibrium maintained between auxin biosynthesis and polar auxin transport (PAT). Measures of PAT in *sem1* and non-mutant shoots reveal a significant reduction in basipetal transport of IAA in mutant hypocotyls (Fig. 9). The ratio of basipetal:acropetal transport of ^{3}H-labeled IAA in non-mutant sibling shoots is approximately 9.7:1, whereas this ratio is reduced to 2.7:1 in *sem1* mutant shoots (Fig. 9). Moreover, the vascular development in the *sem1-R* hypocotyl is not overtly different than non-mutant siblings (Fig. 8D,E). Therefore, the reduction in PAT in sem1-R mutant shoots cannot be attributed solely to aberrant vasculature in *sem1-R* hypocotyls.

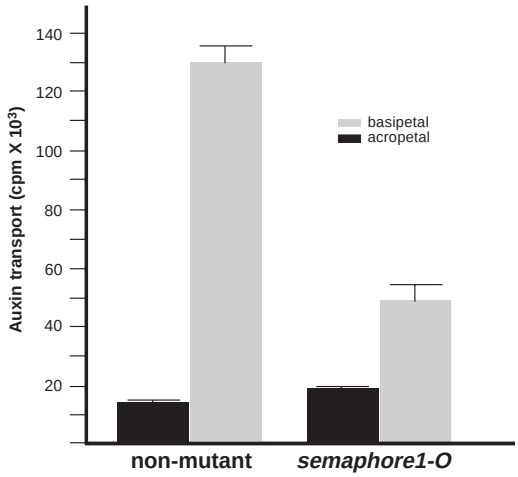

Fig. 9. Basipetal auxin transport is impaired in hypocotyls of *sem1* mutant shoots. Each value represents the mean of 5 individual hypocotyl segments, as described in Materials and Methods.

Genetic analyses: SEMAPHORE1 is a novel gene function that regulates *knox* gene expression at a different level than ROUGH SHEATH2

Analyses of genetically discordant kernels generated by the B/A translocation TB-9Sd (described in Materials and Methods) reveal that the *sem1* locus maps to the short arm of chromosome 9, distal to the breakpoint of TB-9Sd (Fig. 1C). No class 1 *knox* genes of maize have been mapped to this genetic location (Kerstetter et al., 1994). Furthermore, homozygous (*sem1-R/sem1-R*) mutant plants have comparable phenotypes to plants derived from all 23 *semaphore1*-hypoploid embryos (genotype *sem1-R/–*) tested in this study. Therefore, plants harboring a single dose (and no non-mutant copies) of the *semaphore1-R* mutation are phenotypically indistinguishable from plants harboring two doses of *sem1-R*. These dosage analyses strongly suggest that the *sem1-R* allele is a null, recessive mutation.

A directed *Mutator* transposon-tagging strategy was employed (described in Materials and Methods) in order to generate two independently isolated mutants that exhibit sem1-like phenotypes. Subsequent testcrosses verified that two new *sem1*-allelic mutations, designated *sem1-Mu9* and *sem1-Mu12*, have been identified. Following a single generation of out-crossing, the genetically heterogeneous *sem1-Mu9* mutants are predominantly lethal kernel phenotypes; introgression into B73 and Q66 is in progress. At an equally early stage of introgression, viable *sem1-Mu12* mutant plants are obtained in low frequency. The mutant phenotype of viable *sem1-Mu12* and *sem1-Mu9* plants resembles that of the reference mutant, *sem1-R*.

SEMAPHORE1 represents only the second *knox* gene regulatory function described in maize. Accordingly, *sem1-rs2* double mutant plants were constructed (described in Materials and Methods) in order to test whether SEMAPHORE1 and ROUGH SHEATH2 function in a shared developmental pathway(s). RFLP analyses confirmed the double-mutation genotypes. The leaf phenotypes of *rs2* mutants are more extreme than viable *sem1-R* mutants (this work) (Schneeberger et al., 1998). Moreover, disruptions of the blade/sheath boundary conditioned by the *rs2-R* mutation are so severe as to mask the displaced ligule phenotype of *sem1-R* mutant plants. Therefore, analyses of epistasis are difficult in *sem1-rs2* double mutants. However, the appearance of numerous sem1-specific phenotypes (embryo and endosperm defects, extreme brachytic stature, ectopic sheath extensions, zebra-striped leaves and male sterility) in combination with rs2-specific leaf phenotypes indicate that the double mutant phenotype is additive. Furthermore, sem1 and rs2 mutants ectopically express slightly different profiles of *knox* genes. These results suggest that SEMAPHORE1 and ROUGH SHEATH2 may regulate maize *knox* gene expression in separable developmental genetic pathways.

DISCUSSION

SEMAPHORE1 functions in the epigenetic regulation of a subclass of Class 1 *knox* genes

semaphore1 mutants ectopically express at least two class 1 *knox* genes, the homologues *rough sheath1* and *gnarley1* (*knox4*) (Kertstetter et al., 1994; Schneeberger et al., 1995; Foster et al., 1999). The coding regions of RS1 and GN1 share greater than 90% similarity, and these loci map to duplicated regions of the maize genome. Therefore, SEMAPHORE1 may function to down-regulate the expression of a specific, subset of *knox* gene homologues. Correspondingly, many of the shoot phenotypes observed in *semaphore1* mutant plants are similar to those previously described for dominant mutations in maize *knox* genes (reviewed by Freeling et al., 1992), as well as the recessive, *knox*-regulatory mutant *rough sheath2* (Schneeberger et al., 1998). In addition, although the leaf phenotypes of *sem1* mutants are restricted to specific domains of the midrib and margins (Fig. 5), RT-PCR assays and KNOX immunohistolocalization assays reveal that ectopic *knox* gene expression transcends these domains and includes leaf regions that exhibit no mutant phenotype (Fig. 2 and data not shown). These results concur with those reported for dominant *knox*

mutations such as *Rs1* and *Lg3*, and further suggest that the phenotypic consequences of *knox* gene expression in maize leaf cells may be influenced by their competency to respond to ectopic signals (Schneeberger et al., 1995; Muehlbauer et al., 1997).

Moreover, KNOX down-regulation is normal in the founder cells of *sem1* mutant leaves; ectopic KNOX accumulation is not observed until later primordial stages of leaf development (Fig. 2B,E). These data imply that the initial down-regulation of KNOX accumulation in maize founder cells and young leaf primordia is controlled by separate gene function(s), whereas SEMAPHORE1 is required to maintain *knox* transcriptional repression during later stages of lateral organ development. An intriguing aspect of the *semaphore1* mutation is the phenotypic variation of semaphore homozygotes. Even in introgressed genetic backgrounds, homozygous *sem1* mutant phenotypes range from seedling/embryo-lethals to viable, more-moderately affected plants. In less-introgressed genetic backgrounds, phenotypic variability is summarily increased (Fig. 5E). Furthermore, dosage analyses reveal that *sem1-R* is a null allele (Fig. 1C), indicating that this phenotypic variation is not attributable to a 'leaky' gene product. Phenotypic variability is also noted in *knox*-regulatory null mutants of the *phan1*/*as1*/*rs2* family (Waites and Hudson, 1995; Ori et al., 2000; Schneeberger et al., 1998), as well as the maize dominant, neomorphs *Kn1, Rs1, Lg3* and *Gn1* (Freeling, 1992). These data indicate that complex epigenetic pathways interact during the regulation of – and responses to – ectopic *knox* gene expression in plant shoots (reviewed by Byrne et al., 2001).

Unlike previously described *knox* mutants in maize, the sem1 phenotype is not restricted to shoot lateral organs. Thus, sem1 presents the first known correlation between ectopic *knox* gene expression and abnormal endosperm, embryo and root development. Furthermore, the ectopic accumulation of KNOX proteins in sem1 mutant endosperm represents the first observation of ectopic *knox* expression in this tissue (Fig. 3C,E,G), and further suggests that SEMAPHORE1 function is required to maintain *knox* transcriptional repression. Interestingly, sem1 mutant embryo development is disrupted prior to the abnormal accumulation of KNOX proteins in shoot lateral organs. These results suggest at least two possible causes of defective patterning in sem1 embryos. Perhaps embryo development in *sem1* mutants is hampered by interaction with an ectopic-*knox*-expressing endosperm? Alternatively, SEMAPHORE1 may have an additional function(s), which is required for embryonic pattern formation.

Pleiotropic semaphore mutant phenotypes may be attributed to defects in auxin transport

In addition to the classical 'knox' mutant shoot phenotypes described above, *sem* mutants also exhibit distinct phenotypes that have not been described previously for maize *knox* expression mutants. These include defects in endosperm development, embryonic patterning, lateral root initiation, vascular development and pollen viability. Mutations at *semaphore1* also disrupt PAT (Fig. 9), as do maize *rough sheath2* mutations (Tsiantis et al., 1999b). Consequently, it is possible that defective PAT may be responsible for many of the developmental defects conditioned by *sem1* mutations. For example, defective basipetal (tip to base) transport of auxin is known to affect internode elongation, lateral root initiation, vascular development and embryonic patterning (reviewed by Berleth and Sachs, 2001). In addition, lethal *sem1* seedlings form a fused coleoptile and no SAM (Fig. 2C,F). Likewise, aberrant PAT is associated with abnormal meristem and coleoptile development in wheat embryos (Fischer and Neuhaus, 1996), as well as organ fusion in the cotyledons of *Arabidopsis* and *Brassica* (Okada et al., 1991; Bennett et al., 1995; Liu et al., 1993; Hadfi et al., 1998).

Furthermore, vascularization of sem1 mutant leaves is markedly deficient in sheath tissue. In contrast no variations are noted in the mutant leaf blade, whereas hypervascularization is observed in sheath extensions located at the marginal, blade-sheath boundary (Fig. 7). Taken together, these data suggest a model in which defective PAT in the sheath may result in localized pooling of auxin concentrations at the blade-sheath boundary. Moreover, local maxima of auxin concentrations may accumulate along the vasculature of the midrib and margin domains, putative routes of auxin transport in shoots (Berleth and Sachs, 2000). This model may explain the generation of an auxin deficit in the basal regions of the phytomer, perhaps resulting in hypovascularization of the sheath, short stems and reduced lateral root formation. Likewise, pooling of auxin over the midrib and margins of the blade/sheath boundary may cause proliferative growth over the midrib and hypervascularized extensions at the margins, all of which are nominal phenotypes of *sem1* mutants (Fig. 5).

Is *knox* gene expression related to defective auxin transport?

Numerous reports suggest a link between ectopic *knox* gene expression and defective regulation of hormone concentrations in plant shoots. In particular, *knox* over-expression results in elevated levels of cytokinin (Frugis et al., 1999; Ori et al., 1999), whereas the KNOX protein NTH15 has been shown to directly suppress the accumulation of gibberellin in the tobacco SAM (Sakamoto et al., 2001). Moreover, recessive mutations in the *knox* regulator *rs2* are correlated with defective auxin transport in maize shoots (Tsiantis et al., 1999a). Tsiantis et al. also showed that chemically induced disruptions of PAT induce rs2-like 'knox' phenotypes in maize seedlings. Intriguingly, no ectopic *knox* gene expression is detected in maize seedlings treated with PAT inhibitors (Tsiantis et al., 1999a); these data suggest that defective PAT is a downstream effect of ectopic *knox* expression, rather than vice-versa. The sem1 mutant phenotypes provide further evidence that the regulation of *knox* gene expression impacts plant hormone levels.

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