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 homeobox 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; Muchhal, 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...
Orthologous mutations are described in
conferred by dominant, neomorphic mutations in
with the mis-regulation of these
knox
and
such as
rough sheath1
mutant phenotypes are correlated with the mis-expression of
endosperm, roots, stem, leaves, vasculature and flowers. These
include abnormalities in the development of the embryo and
The unique and pleiotropic
second regulator of
gene expression identified in maize.

Differential defects in proximal-distal (enhanced in
rs2
expression,
and downstream genes, and/or
diversity of the
gene expression found in plants
Arabidopsis
Antirrhinum
phantastica1, phan1
knox
differences in the specific
expression patterns of
and 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
F1
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
F1
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*Mu8,
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 (hyperdiploid) whereas the other sperm nucleus is deficient (hypodiploid). Following double fertilization by a male plant carrying a B/A translocation, discordant aneuploid kernels are generated in which the embryo may be hypodiploid and the endosperm hyperdiploid, and vice versa. In this way, recessive mutations located distal to the breakpoint in the A chromosome will be “uncovered” in hypodiploid
F1
progeny.

F1
progeny of crosses between
sem1-R/B73
heterozygotes and male plants heterozygous for
TB-95d
(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 hypodiploid, colorless
sem1
mutant embryos (genotype
sem1-R/–
) and purple, non-mutant hyperdiploid endosperm (genotype
sem1-R/sem1-R/sem1*sem1)
; and (2) kernels with purple, non-mutant

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 (liguleless3, 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
conferring by dominant, neomorphic mutations in
knox
genes such as
rough sheath1
and
knot1
(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/phan1
family are complimentary to those of
knox
genes, consistent with their role during negative regulation of
knox
gene expression. Furthermore, the
rs2
mutants are defective in polar auxin transport (PAT) (Tsiantis et al., 1999b), which may account for many of their phenotypes 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
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
handlebar1
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
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 F1 plants were self-pollinated. The resulting F2 progeny were scored for hyperploid embryos (genotype heterozygous 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.

Immunohistology and light microscopy

Immunohistochemical localization 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 linked RFLP marker HN900 as probes.

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 H2O, 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 [3H]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, liguleless3 and ubiquitin were the same as those employed by Schneeberger et al. (Schneeberger et al., 1998). Gene-specific primer sequences for

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 semaphore1 mutant seedlings using a polyclonal antibody that recognizes class 1 KNotted1-like homeoBQX (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
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. In contrast, two distinct classes of embryo phenotype were observed in 12 DAP sem1-R mutant embryos. 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
SEMAPHORE1 regulates knox gene expression

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, 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.

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 gnrarley1 (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.
after just 25 cycles, whereas *gnl* amplicons are first detected after 30 cycles (Fig. 4C middle). In addition, *knl* transcripts are weakly amplified following 30-40 cycles of RT-PCR in *rs2* seedlings, whereas no *knl* 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 *gnl*, 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

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**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.

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**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.
Semaphore1 regulates knox gene expression

Semaphore1 mutations disturb lateral root development

A prominent phenotype of semaphore1-R mutant plants is the formation of yellow stripes on vegetative leaves (Fig. 8A). This pattern of leaf-stripping is a diagnostic feature of plants deficient in the uptake of soil magnesium (Olsen and Sander, 1988). Therefore, we suspected that semaphore1-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), semaphore1-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, semaphore1 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 semaphore1-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, semaphore1-R mutant seedlings are thinner in diameter than those of larger, non-mutant siblings.

Fig. 7. Abnormal vasculature in semaphore1-R 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) semaphore1-R 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 semaphore1-R mutant sheath shows poor development of transverse veins. Scale bar in B: 50 μm for B-G.

Semaphore1 mutations disturb lateral root development

A prominent phenotype of semaphore1-R mutant plants is the formation of yellow stripes on vegetative leaves (Fig. 8A). This pattern of leaf-stripping is a diagnostic feature of plants deficient in the uptake of soil magnesium (Olsen and Sander, 1988). Therefore, we suspected that semaphore1-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), semaphore1-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, semaphore1 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 semaphore1-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, semaphore1-R mutant seedlings are thinner in diameter than those of larger, non-mutant siblings.

Fig. 8. (A) Adult semaphore1-R mutant leaf showing yellow, zebra striping. (B,C) Impaired root development in semaphore1-R mutants. (B) Root development in a non-mutant seedling. Arrow, hypocotyl (seedling stem). (C) Root development in a semaphore1-R 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 semaphore1-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:acropletal transport of 3H-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.

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 outcrossing, 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) (Kerstetter et al., 1994; Schneeberger et al., 1995; Foster et al., 1999). The coding regions of RSI 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 k 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 moderately affected plants. In less-introgressed genetic backgrounds, phenotypic variability is also noted in semaphore1/semaphore1 vari-ability is also noted in 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.

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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