The maize duplicate genes *narrow sheath1* and *narrow sheath2* encode a conserved homeobox gene function in a lateral domain of shoot apical meristems

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Summary

The narrow sheath (ns) phenotype of maize is a duplicate factor trait conferred by mutations at the unlinked loci *ns1* and *ns2*. Recessive mutations at each locus together confer the phenotypic deletion of a lateral compartment in maize leaves and leaf homologs. Previous analyses revealed that the mediolateral axis of maize leaves is comprised of at least two distinct compartments, and suggest a model whereby NS function is required to recruit leaf founder cells from a lateral compartment of maize meristems. Genomic clones of two maize homeodomain-encoding genes were isolated by homology to the *WUSCHEL*-related gene *PRESSED FLOWER* (*PRS*). *PRS* is required for lateral sepal development in *Arabidopsis*, although no leaf phenotype is reported. Co-segregation of the ns phenotype with multiple mutant alleles of two maize *PRS* homologs confirms their allelism to *ns1* and *ns2*. Analyses of NS protein accumulation verify that the *ns-R* mutations are null alleles. *ns* transcripts are detected in two lateral foci within maize meristems, and in the margins of lateral organ primordia. Whereas *ns1* and *ns2* transcripts accumulate to equivalent levels in shoot meristems of vegetative seedlings, *ns2* transcripts predominate in female inflorescences. Previously undiscovered phenotypes in the pressed flower mutant support a model whereby the morphology of eudicot leaves and monocot grass leaves has evolved via the differential elaboration of upper versus lower leaf zones. A model implicating an evolutionarily conserved NS/PRS function during recruitment of organ founder cells from a lateral domain of plant meristems is discussed.

Supplemental data available online

Key words: *narrow sheath*, *pressed flower*, Maize, Leaf development, SAM, Founder cells

Introduction

The formation of plant lateral organs is dependent upon shoot apical meristem (SAM) function. Founder cells of the incipient phytomer, which will eventually comprise the leaf, stem and lateral bud of an individual plant segment, are recruited from cells occupying the peripheral zone (PZ) of the SAM (reviewed by Fletcher and Meyerowitz, 2000). The mechanism of founder-cell recruitment is poorly understood. Fate-mapping analyses in maize have illustrated that founder-cell recruitment begins on one SAM flank, which will form the central domain of the leaf, and proceeds toward the opposite flank, from where both margins of the lower leaf arise (Poethig, 1984; Poethig and Szymkowiak, 1995). In plants with simple undissected leaves, founder-cell recruitment is correlated with the downregulated accumulation of KNOTTED1-like homeobox (KNOX) proteins in the PZ of the meristem (Jackson et al., 1994). The combined data demonstrate that the correlated processes of leaf founder-cell recruitment and KNOX downregulation are gradual; the central-midrib compartment of the leaf develops well before the margins of the same leaf. Recruitment of leaf founder cells from the SAM coincides with programs of development and differentiation along three axes, comprising the mediolateral, proximodistal, and dorsoventral axes of the leaf primordium. Although the molecular mechanisms governing formation of leaf axes remain unclear, genetic analyses of leaf developmental mutants have generated testable models for the generation of leaf pattern (reviewed by Byrne et al., 2001).

Mutations in the *narrow sheath* (*ns*) genes cause the deletion of a lateral domain in maize leaves that includes the margins of the lower leaf (Scanlon et al., 1996) (Fig. 1). The ns margin deletion phenotype does not extend into the upper portion of the leaf, and the leaf length is also unaffected (Fig. 1A-C). Both vegetative and floral phytomers are affected; whereas no ns mutant phenotype is observed in the embryonic coleoptile, shoot meristems or roots (Scanlon and Freeling, 1998). The ns phenotype is a duplicate factor trait controlled by recessive mutations at two unlinked loci, *ns1* and *ns2* (Scanlon et al., 2000). Heterozygous plants containing just a single, non-mutant copy of either *ns* gene display the non-mutant phenotype (Fig. 1G-H). KNOX immunolocalization studies and fate mapping of ns mutant meristems reveal that a meristematic founder-cell domain that normally contributes to the non-mutant leaf margins is not recruited in ns mutant leaves.

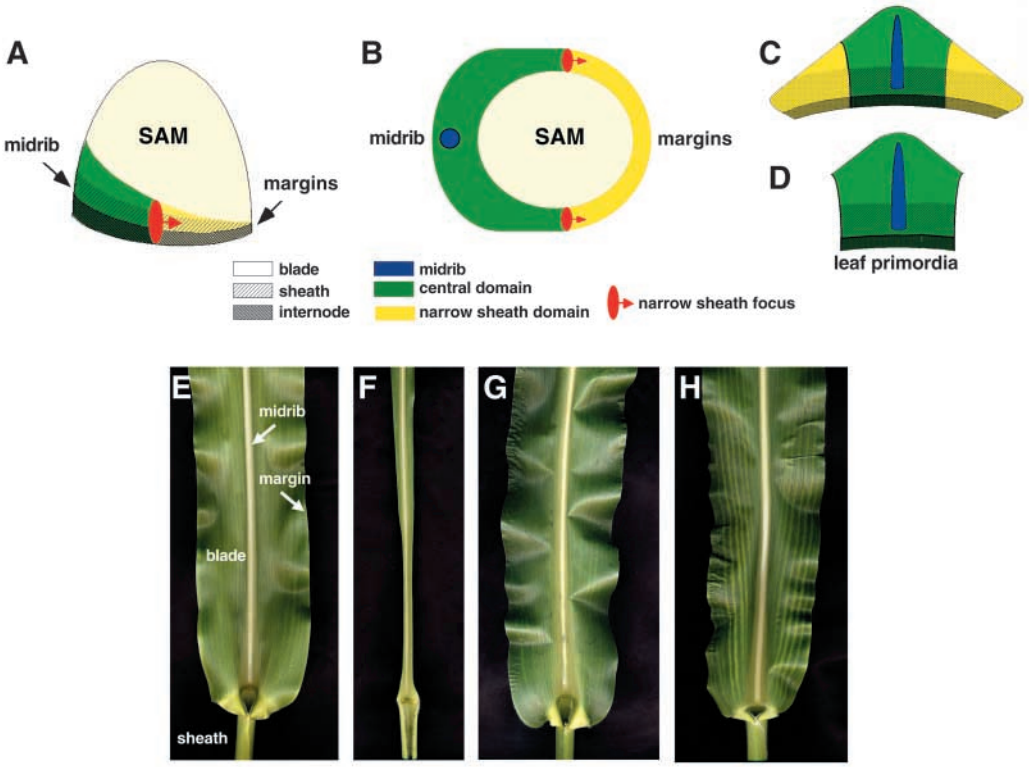

Fig. 1. The narrow sheath mutant phenotype is a deletion of a lateral compartment that includes the leaf margins. (A) Model depicting the recruitment of maize founder cells in two distinct compartments, corresponding to the central domain (green) and the *ns* lateral domain (yellow). (B) Cartoon of a transverse section through the maize founder cells. The model predicts that NS functions to recruit the lateral founder-cell domains from two distinct foci (red arrows), one corresponding to each side of the leaf. Cartoons model the ns mutant (D) and the non-mutant leaf primordium (C). Non-mutant leaves comprise at least two mediolateral compartments, the central domain (green) and the *ns* lateral domain (yellow). The ns mutant leaf exhibits a deletion of the lateral domain, which includes the margins of the leaf blade, the leaf sheath and internode. Note that the central compartment includes the midrib and the leaf tip, domains that are intact in ns mutant leaves. The ns mutant phenotype is a duplicate factor trait, dependent upon mutations in both *ns1* and *ns2*. (E) Mature maize leaves from plants homozygous for non-mutant alleles of *Ns1* and *Ns2*. (F) ns mutant leaves homozygous for mutations in both *ns1* and *ns2*. Non-mutant leaves from plants of the complimentary genotypes *Ns1*, *ns2* (G), and *ns1*, *Ns2* (H). SAM, shoot apical meristem.

Clonal mosaics demonstrate that NS1 recruits a lateral founder-cell compartment from two distinct foci (one focus for each leaf margin) in the maize shoot apex (Scanlon, 2000). NS1 function is not required for development of the central domain of maize leaves; likewise, loss of NS1 function during post-meristematic stages is non-phenotypic. These data suggest a model in which maize leaf initials are comprised of two distinct mediolateral compartments (Fig. 1A-D). The central compartment includes the midrib and distal leaf tip, and does not require NS function for its recruitment (green in Fig. 1). Thus, *ns* mutations do not affect leaf width or margin development in these upper leaf domains, which are derived from the central leaf compartment. By contrast, the *ns* lateral compartment includes all leaf domains extending from the central compartment to the leaf margin. Leaf domains contained within this lateral compartment include the margins of the lower portion of the leaf blade and the entire sheath (yellow in Fig.1).

We describe the cloning of the *ns* genes through homology to *PRESSED FLOWER* (*PRS*), a *WUSCHEL*-like homeobox gene that is required for development of lateral sepals in the *Arabidopsis* flower (Matsumoto and Okada, 2001). Sequence homology and mutational analyses suggest that the duplicated maize *ns* genes are the redundant, functional orthologs of *PRS*. Immunoblot analyses of maize proteins verify that the *ns-R* mutations are both null alleles. Furthermore, quantitative analyses of *ns* gene transcripts suggest that *ns1* and *ns2* are expressed redundantly in tissues enriched for shoot apical meristems, although differences in specific *ns* gene transcript abundance are detected in reproductive tissues. Moreover, previously undescribed phenotypes are discovered in the leaves and stamens of Prs^{-} mutant *Arabidopsis* plants. Together the ns and Prs^{-} mutant phenotypes support existing models for the evolution of angiosperm leaf morphology via the differential elaboration of distinct leaf zones, and suggest a model whereby orthologous NS/PRS proteins function to recruit organ founder cells in a lateral domain of shoot meristems.

Materials and methods

Maize and *Arabidopsis* genetic stocks

The maize *ns1-R* and *ns2-R* alleles were obtained from E. Elsing and M. Albertson, (Pioneer Hi-Bred, Johnston, IA, USA). *Arabidopsis* seeds segregating for the *prs-1* mutation in Landsberg *erecta* (*Ler*) ecotype were kindly supplied by K. Okada. The eight independent alleles of *ns1* and two alleles of *ns2* that were derived from *Mutator*

transposon stocks were generated using a directed transposon-tagging strategy, as described by Scanlon et al. (Scanlon et al., 2000) and outlined in the supplementary data (see Data S1 at http://dev.biologists.org/supplemental/).

Cloning of *narrow sheath* genomic DNA and cDNA

The homeodomain of the *ns1* gene (AC AJ536578) was isolated by PCR using degenerate primers designed from the conserved homeobox region of the *WUSCHEL* gene from *Arabidopsis*. The duplicated gene *ns2* (GenBank AC AJ472083) was cloned by homology to *ns1*, using primers designed from the conserved homeobox region of *ns1*. Detailed descriptions of the strategies, probes and primers employed to obtain full length genomic and cDNA clones of *ns1* and *ns2* alleles are provided (see Data S1 at http://dev.biologists.org/supplemental/).

Computational and database analysis

The multiple alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and BOXSHADE (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Phylogenetic trees were generated based on the Neighbor-Joining and Maximal Parsimony (MP) methods, using PAUP* Version 4.0b8 (SWOFFORD 1999) with default parameters. Sequences examined were as follows: AtPRS (*Arabidopsis thaliana*, BAB79446), AtWUS (*Arabidopsis thaliana*, CAA09986), AtHD (*Arabidopsis thaliana*, NP_188428), OsHD1 (*Oryza sativa*, CL042143.26.34) and OsHD2 (*Oryza sativa*, BAA90492).

DNA gel-blot analyses

Genomic DNA was isolated from maize seedlings and leaves, and analyzed by DNA gel blot hybridization analysis as described previously (James et al., 1995). Hybridization probes were radioactively labeled and column-purified as described (Fu et al., 2002). Specific probes used in these analyses were as follows. Probe 1 was a 479 base pair (bp) 5′ genome walker product (upstream of primer ZmPRSb, see Table S1 at http://dev.biologists.org/supplemental/) that hybridizes to both *ns* loci. Probe 2 was a 792 bp genomic PCR product (between primers ZmPRSc and ZmPRS2, see Table S1) that also includes the 5′ UTR of *ns1*.

Antibody production, protein extraction and immunoblot analyses

Affinity purified NS polyclonal antibodies were generated (Biosource) against a synthetic peptide comprising amino acid residues 235 to 251 of the predicted NS2 protein (N-LKTLDLFPTKSTGLKDE-C, see Fig. 2A), with a Cys residue added to the N terminus. This amino acid sequence is completely conserved in the predicted NS1 protein (amino acids 232 to 248). Thus, polyclonal antibodies raised against this peptide antigen are expected to identify epitopes in NS1 as well as NS2 proteins.

Proteins were extracted from immature (4-5 cm long) maize female inflorescences (ears) according to the procedures described in Fu et al. (Fu et al., 2002). Approximately 30 μg soluble protein was used in western blot analyses; protein gel electrophoresis, transfer, and Coomassie Brilliant Blue staining were performed as described (Sambrook et al., 1989). Immunoblot analyses were performed according to the manufacturer's protocol (ECL™, Amersham-Pharmacia) using a 1/5000 dilution of affinity-purified serum as the primary antibody.

In situ hybridization analyses

For non-radioactive in situ hybridization, samples were prepared following the protocol of Jackson (Jackson, 1991). For sections of maize embryos, kernels were trimmed on both sides of the embryo axis for better penetration of the formaldehyde fixative and the wax solution. Paraffin wax-embedded tissue was sectioned by the use of a rotary microtome and 7 μm sections were used for hybridization. The first 315 bp (upstream of primer ZmPRSb), including the whole 5′ UTR, of the *ns1* cDNA sequence, cloned either in the sense or antisense orientation to the T7 promotor, were used as a template for synthesis of digoxigenin-labelled RNA probes by T7 RNA polymerase as described (Bradley et al., 1993). This probe is predicted to hybridize to both *ns1* and *ns2* transcripts. The *PRS* probe corresponded to that used by Matsumoto and Okada (Matsumoto and Okada, 2001).

Quantification of *ns* transcript accumulation by real-time reverse transcriptase-mediated PCR

Total RNA was extracted from non-mutant B73 maize tissues with the TRIZOL™ Reagent (Invitrogen, Life Technology), according to the manufacturer's protocols. One μg of total RNA was treated with DNAseI (Promega) and was subsequently used to prepare first strand cDNA, as described by Bauer et al. (Bauer et al., 1994). The resultant cDNA was treated with RNaseH (GIBCO BRL) to remove residual mRNA and the concentration of all samples was adjusted to 50 ng/μl.

The cDNA was checked for residual genomic DNA contamination using the *ns2*-specific primer ns2F6, which is within the transcribed region of the gene, and a second primer, ns2R8, which is from the 3′ untranscribed region (see Table S1 at http://dev.biologists.org/supplemental/). Real-time RT-PCR amplification was performed in a volume of 25 μl using 100 ng cDNA template, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 250 nM of each primer and 1U of Platinum *Taq*™ DNA Polymerase (Invitrogen, Life technology), using a Cepheid Smart Cycler™. The cycling program was: 95°C for 2 minutes; and 45 cycles of 95°C for 10 seconds, 57°C for 10 seconds and 72°C for 15 seconds. The *LUX*™ primers were designed online (www.invitrogen.com/LUX) and are shown in Table S1.

The amplified fragments were analyzed by electrophoresis on 3% Agarose 1000™ (Invitrogen, Life Technology); a single band of the predicted size was obtained in all samples included in these assays. Each sample was assayed in triplicate, and analyses of relative *ns* gene expression data, normalized to control *ubiquitin* expression, was performed as described by Livak and Schmittgen (Livak and Schmittgen, 2001).

Cryo-scanning electron microscopy and light microscopy

Cryo-scanning electron microscopy of dissected *Arabidopsis* seedlings was performed with expert technical assistance from Dr John Shields (Center for Ultrastructural Research, University of Georgia, Athens, GA, USA) as described previously (Scanlon, 2003).

Whole-mounted *Arabidopsis* plants at the two leaf stage were harvested, roots and one cotyledon were removed by dissection, and the remaining shoot was mounted in water and imaged on a Zeiss Axioplan II equipped with a Southern Micro Instruments (Pompano Beach, FL, USA) CCD camera.

Results

The maize genes *narrow sheath1* and *narrow sheath2* map close to duplicated relatives of *PRESSED FLOWER* in *Arabidopsis*

RT-PCR was performed with cDNA prepared from immature maize embryos, using degenerate primers (see Data S1 at http://dev.biologists.org/supplemental/) designed to amplify the conserved homeodomain-encoding sequences of *WUSCHEL* (*WUS*) and *WUS*-related gene products identified in the *Arabidopsis* genome (Mayer et al., 1998). One of these maize amplicons exhibited high sequence conservation with the homeodomain of the At2g28610 open reading frame (Fig. 2A), recently identified to encode the *PRESSED FLOWER* (*PRS*) gene of *Arabidopsis thaliana* (Matsumoto and Okada, 2001). Intermated B73/Mo17 (IBM) recombinant inbred lines

were used to map the *PRS*-homologous maize clone to an interval on chromosome arm 2L that includes the *ns1* locus. Genomic PCR analyses revealed a CACTA transposable element (Kunze and Weil, 2002) inserted in the protein coding region of the *PRS*-homologous maize clone in ns1-R mutants (details described below).

Expression of the narrow sheath (ns) phenotype in lateral organs of maize vegetative and inflorescence shoots is dependent upon homozygosity for mutations at each of two unlinked loci, *narrow sheath1* and *narrow sheath2* (Scanlon et al., 1996). Intriguingly, segregation of a *Dra*I restriction fragment length polymorphism (RFLP), due to the CACTA element insertion in 76 F2 ns mutant plants, revealed that the *PRS*-homologous maize clone hybridizes to more than one genetic locus in maize, each of which co-segregates with the ns mutant phenotype (Fig. 3A). Previous genetic analyses proved that the *ns* loci map to regions of the maize genome that are duplicated (Scanlon et al., 2000). Therefore, we sought to clone the *ns2*-linked sequence through its predicted sequence homology to the *ns1*-linked, *PRS*-related maize clone.

Nested primers homologous to the conserved homeobox region within the *ns1*-linked genomic sequence were employed to amplify homologous maize sequences from a *Dra*I digested genomic DNA library (see Data S1 at http://dev.biologists.org/supplemental/). Two distinct amplicons were identified: one was linked to the *ns1-R* mutation whereas the second, by use of the IBM recombinant inbred population, was mapped to the chromosomal vicinity of *ns2*. Primers unique to duplicated *PRS*-homologous maize clones were used to isolate full-length genomic and cDNA clones of each locus from the maize inbred line B73 (see Materials and methods).

Indeed, the two loci encode highly related homeodomain-containing proteins. The *ns1*-linked transcript encodes a predicted protein of 262 amino acids, which shares 86% identity with the 265 amino acids encoded by the *ns2*-linked gene product (Fig. 2A). Moreover, the position of a 124 bp intron is conserved. Database searches reveal that both predicted proteins bear little overall homology to described plant genes; the highest similarities detected are with two rice homeodomain proteins (OsHD1 and OsHD2; 38% and 37% identity, 99/262 and 97/262 amino acids, respectively) and the *Arabidopsis* protein PRESSED FLOWER (37% identity; 97/262 amino acids). Outside of the conserved homeodomain sequence conservation is low to PRS and OsHD, with the exception of two Q- or H-rich clusters proximal to the homeodomain and a conserved PLKTL E/DLFP motif close to the C terminus (Fig. 2A). Importantly, although any homology is primarily localized to the WUSCHEL-type homeodomain, the *ns1/2*-linked duplicate maize genes are significantly more similar to *PRS* than to *WUSCHEL* or other *Arabidopsis* relatives (Fig. 2B).

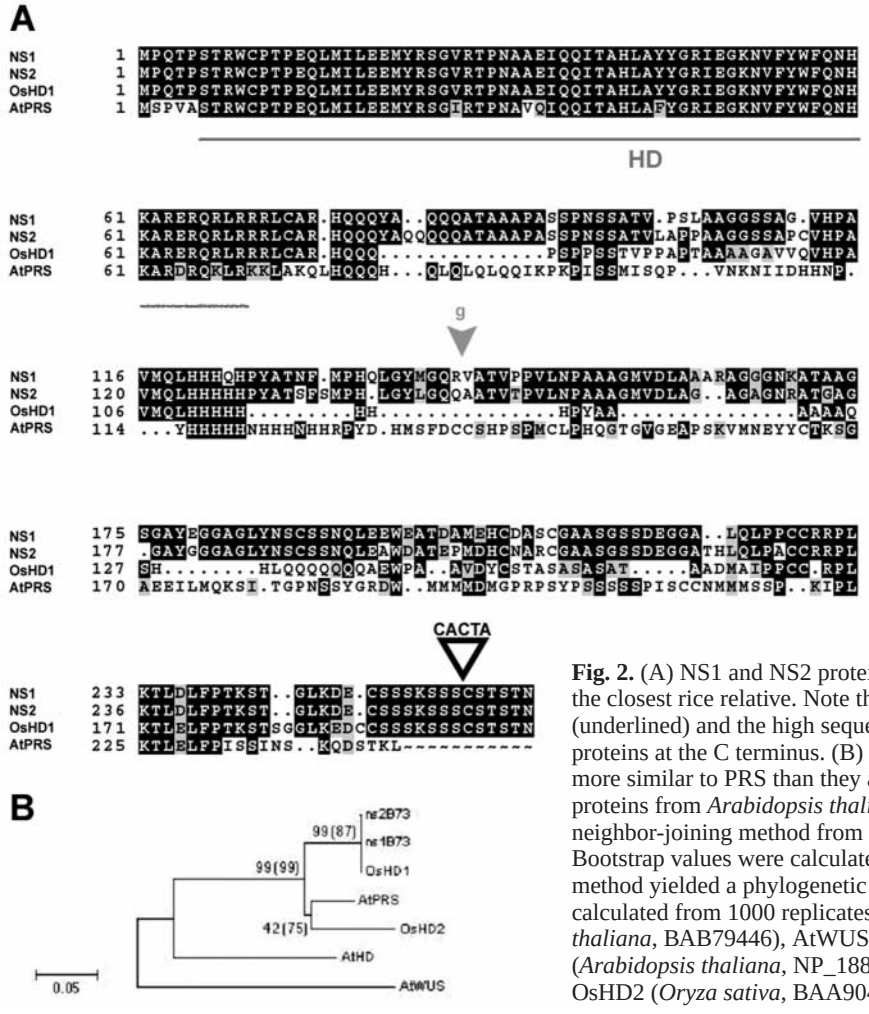

Fig. 2. (A) NS1 and NS2 protein sequence compared with PRS of *Arabidopsis* and the closest rice relative. Note the very high similarity of the homeodomain (underlined) and the high sequence conservation between the maize and rice proteins at the C terminus. (B) Phylogeny of NS/PRS proteins. The NS proteins are more similar to PRS than they are to WUSCHEL, or to other WUSCHEL-like proteins from *Arabidopsis thaliana.* The unrooted tree was generated using the neighbor-joining method from a CLUSTALW alignment of the homeodomain. Bootstrap values were calculated from 1000 replicates. The maximal parsimony method yielded a phylogenetic tree with identical topology (bootstrap values calculated from 1000 replicates are shown in parentheses). AtPRS (*Arabidopsis thaliana*, BAB79446), AtWUS (*Arabidopsis thaliana*, CAA09986), AtHD (*Arabidopsis thaliana*, NP_188428), OsHD1 (*Oryza sativa*, CL042143.26.34) and OsHD2 (*Oryza sativa*, BAA90492).

Structure of *ns1* and *ns2* mutant alleles

The chromosomal map positions, the sequence similarity and the RFLP co-segregation with the ns mutant phenotype suggested that *ns1* and *ns2* might encode PRS orthologous functions in maize. Therefore homologous clones were isolated from various maize genetic stocks, including the reference mutations *ns1-R* and *ns2-R*, non-mutant *Mutator* (*Mu*) transposon lines, and several *ns* mutant alleles derived from *Mu* lines (described in Materials and methods). Sequence analyses of these *ns*-linked clones reveal molecular lesions or chromosomal deletions in nine independent *ns1* mutations and two *ns2* mutant alleles, as described below.

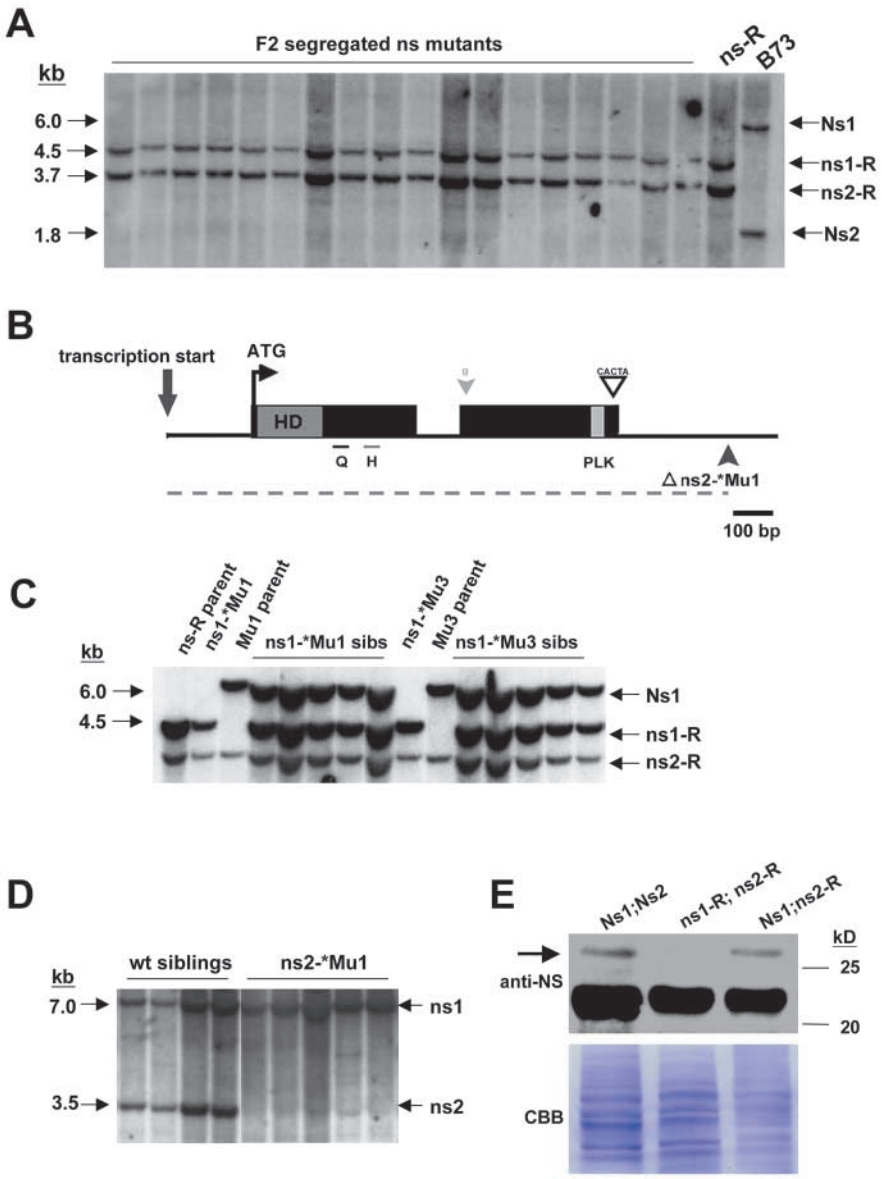

Fig. 3. Analyses of *ns* alleles. (A) A hybridization probe (probe 1; Materials and methods) derived from a *PRS*-homologous, maize genomic clone identifies two distinct *Dra*I restriction fragments linked to the ns mutant phenotype in F2 (ns-R×B73) segregating progeny. Note that no internal *Dra*I restriction sites are present within the sequence of probe 1 obtained from either ns mutant or B73 individuals. (B) Composite gene map of the *ns* duplicate genes, each of which comprises two exons and a single intron. Exons are boxed, the positions of the homeodomain (HD), the glutamine-rich region (Q), the histidine-rich region (H) and the PLK domain are indicated. Solid lines indicate introns and untranslated regions. The position of the extra G in *ns2-R* and of the CACTA element insertion in *ns1-R* are indicated above the drawing. The dashed line below the drawing indicates regions of the *ns2* locus that are deleted in the *ns2-Mu1* allele. (C) Newly identified *ns1-Mu** alleles are deletions. Active mutator lines *Ns1/Ns1*; *ns2-R/ns2-R* (*ns1-Mu6/7* Mu parent) were pollinated with *ns1-R/ns1-R; ns2-R/ns2-R* (ns-R parent) pollen and analyzed for phenotypic progeny in the M1 generation. Southern analysis of ns mutant progeny (shown are *ns1-Mu1* or *ns1-Mu3*) showed lack of the *ns1* wild-type (6 kb) band but presence of the ns1-R (4.5 kb) fragment when hybridized to probe 2 (Materials and methods), whereas wild-type siblings (*ns1-Mu1/3* sibs) always contained both bands (6 kb + 4.5 kb). Note that probe 2 hybridizes more weakly to *ns2* because it includes 5′ UTR sequence specific to the *ns1* locus. (D) Plants homozygous for the *ns2-*Mu1* mutant allele exhibit no hybridizing restriction fragment corresponding to the *ns2* locus, whereas non-mutant siblings exhibit a 3.5 kb band linked to *ns2*. (E) NS protein does not accumulate in young ears obtained from *ns1-R, ns2-R* mutant plants. Polyclonal antibodies raised against an oligopeptide that is conserved in the predicted NS1 and NS2 proteins recognize a protein (arrow) in homozygous non-mutant ears, as well as in non-mutant ears from plants that contain a single non-mutant allele of *Ns1* but are homozygous for the *ns2-R* mutant allele. This ~29 kDa band is absent in ears from ns mutant plants, and corresponds to the predicted molecular weight of the NS proteins. Note that the NS polyclonal antibody is not specific for NS proteins.

Compared with non-mutant alleles of the *ns1*-linked clone, the *ns1-R* allele contains a transposable element insertion close to the C terminus of the protein-coding region (amino acid 256, see Fig. 2A, Fig. 3B). The 1.2 kb element belongs to the CACTA transposable element superfamily (Kunze and Weil, 2002); a characteristic 3 bp (CCT) duplication is identified at the transposon insertion site. Transcript analyses reveal that the transposon-inserted *ns1-R* allele is transcribed and polyadenylated, although the mutant transcript terminates prematurely within the CACTA element. Thus, a potential translation product of the *ns1-R* allele would be truncated at the C terminus.

When compared with non-mutant genomic and cDNA sequences of *ns2*-linked alleles, the *ns2-R* mutant allele contains an extra G nucleotide in the second exon, corresponding to position 779 of the transcript (Fig. 2A). Insertion of this extra nucleotide alters the open reading frame and introduces a premature stop codon at nucleotide 855. Thus, the amino acid sequence of the truncated NS2-R translation product is predicted to diverge from the non-mutant polypeptide after residue 146, and terminates after just 170 total residues. These data reveal that the *ns2-R* mutation is tightly linked to a maize gene that is a duplicate of the *ns1* sequence, and which harbors a predicted frameshift mutation.

In addition, Southern blot analyses of eight, independent *ns1-*Mu* linked alleles recovered from separate transposon-tagging experiments (see Materials and methods) (see also Scanlon et al., 2000) reveal that all *ns1-*Mu* plants contain

deletions of the *ns1*-linked allele contributed by the non-mutant, *Mu*-transposon parent (Fig. 3C and data not shown). Likewise, three independent alleles of the *ns2* mutation identified by *Mu* transposon-tagging harbor deletions of the *ns2*-linked allele contributed by the *Mu* parent. The extent of the deletion within one such allele, *ns2-*Mu1*, was investigated. A 5′-directed chromosome walk used nested primers located 547 bp downstream of the 3′ untranslated region in *ns2-*Mu1* homozygous individuals (see Data S1 at http://dev.biologists.org/supplemental/) and generated an approximately 3 kb genomic clone. For the first 226 bp from the 3′ primer sites the nucleotide sequence of this *ns2-*Mu1* clone is 94% identical to clones obtained (using the same primers and chromosome walking strategy) from B73 and non-mutant *Mutator* lines. However, after the 226 bp 3′ homologous region, the *ns2-*Mu1* clone is completely non-homologous to any sequence contained within a total of 4,548 bp of *ns2*-linked DNA derived from non-mutant clones. These data suggest that the *ns2* gene is entirely deleted in the *ns2-*Mu1* allele (Fig. 3B). This conclusion is supported by Southern blot comparisons of *ns2-*Mu1* homozygous plants and non-mutant siblings, in which no *ns2*-linked hybridizing band is detected in ns2-*Mu1 plants (Fig. 3D). Significantly, the non-mutant *Ns1-Mu*$^{+}$ allele can be amplified from non-mutant siblings of the newly tagged ns1-*Mu1 plants, as well as from the non-mutant *Mu* parental stock. Finally, all thirteen *Mu*-derived *ns1* and/or *ns2* mutant alleles were identified as single ns mutant phenotypes within thirteen separate populations of more than 5,000 siblings each, indicating that these deletion mutations each occurred spontaneously in single, maternal gametes. These accumulated data suggest that the two *PRS*-homologous maize clones identify the *ns1* and *ns2* duplicated loci.

Immunoblot analyses of null *ns-R* mutant alleles

Gene dosage analyses indicated that the recessive *ns-R* mutations are null alleles (Scanlon et al., 2000). In order to test this prediction, polyclonal antibodies were raised against a peptide antigen that is completely conserved in the predicted NS1 and NS2 proteins (see Materials and methods), and used in immunoblot assays of proteins extracted from maize tissues (Fig. 3E). The anti-NS antibodies identify a protein of the approximate molecular weight (29 kDa) predicted for *ns*-encoded proteins in 4-5 cm immature ears obtained from non-mutant plants of the genotypes *Ns1+; Ns2+* and *Ns1+/ns1-R; ns2-R*. At this stage of development, maize ears contain abundant spikelet and floret meristems, as well as immature lateral organs (Kiesselbach, 1949). By contrast, no immunoreactive protein of this predicted molecular weight was detected in mature, non-mutant seedling leaves (data not shown) nor in immature ears of the genotype *ns1-R; ns2-R* (Fig. 3E). These data reveal that the ns-R mutant inflorescences do not accumulate NS proteins; however, NS protein(s) accumulate in non-mutant immature ears. Unfortunately, the anti-NS polyclonal antibody identifies additional protein(s) of a different molecular weight to the predicted NS protein (lower band in Fig. 3E). Although the polyclonal antiserum therefore is not suitable for use in immunohistolocalization analyses, the absence of NS-predicted molecular weight proteins in ns double mutants confirms that on the protein level *ns1-R* and *ns2-R* provide null alleles.

NARROW SHEATH transcripts are detected in meristematic foci and in the margins of lateral organ primordia

The similarities in amino acid sequence raise the question of whether *ns1/ns2* and *PRS* exhibit similar expression patterns and encode orthologous functions in maize and *Arabidopsis*. A probe predicted to hybridize to both *ns1* and *ns2* was used for in situ analyses of *ns* transcription throughout embryonic, vegetative and reproductive stages of maize development. As predicted from analyses of the ns mutant phenotypes in vegetative and floral organs (Scanlon and Freeling, 1998), *ns1* and *ns2* are expressed predominately in tissues enriched for shoot meristems and young lateral organ primordia (Fig. 4). Overall, the pattern of *ns* gene expression is two-staged, similar to that reported for *PRS* (Matsumoto and Okada, 2001). An early-staged *ns* expression is observed at two foci in lateral domains of shoot meristems, whereas later-staged expression appears in the margins of young lateral organ primordia.

Early events in maize embryogenesis establish the apical and basal poles, from which the embryo proper and the suspensor are formed, respectively. Subsequently, morphogenesis in the embryo proper produces the scutellum, a specialized structure adapted for endosperm absorption, and the shoot/root axes. The first histological evidence of the future shoot apical meristem (SAM) is the appearance of a group of densely packed cells on the lateral surface of the transition stage embryo (Randolph, 1936). Subsequently, a bulge of tissue above the developing SAM precedes the emergence of the coleoptile, which forms a collar of tissue that ultimately encloses the shoot meristem (Fig. 4A). In transverse sections through the coleoptilar-staged embryo, *ns* transcripts are first detected in the lateral margins of the emerging coleoptile (Fig. 4B). *ns* transcripts are undetectable after the coleoptile encloses the apex. Therefore, *ns* expression marks marginal cells in the primordial coleoptile but is not detected in the scutellum nor in the pre-coleoptilar staged SAM.

Maize leaves exhibit alternate phyllotaxy, such that successive primordia initiate from the SAM approximately 180° apart and in two ranks (Fig. 4C). Fate-mapping analyses demonstrate that founder cells that form the eventual midrib of the maize leaf are recruited from one SAM flank, whereas margin founder cells occupy the opposing flank (Scanlon and Freeling, 1997). *ns* transcripts accumulate in founder cells of the P0/1 primordium in two foci, located at opposite lateral domains of the shoot apex (Fig. 4D,G). This early *ns* expression focus is limited to a series of adjacent cells in the L1 tissue layer of the apex. No *ns* activity is detected in the founder-cell domains that give rise to the future midrib. Later, *ns* transcripts mark the lateral margins of young leaf primordia (Fig. 4E,F,H,I), and are restricted to single epidermal cells forming the boundary between the abaxial and adaxial leaf surfaces.

The accumulation of *ns* transcripts in male and female maize flowers was also investigated. The data presented in Fig. 4K-Q show *ns* expression in the female inflorescence (ear); equivalent patterns are observed in the male inflorescence (data not shown). Early developmental programs are very similar in the male and female inflorescences of maize; gender-specific differences occur during later stages. An overview of maize inflorescence development is presented in Fig. 4K. In summary, the inflorescence meristem initiates spikelet-pair

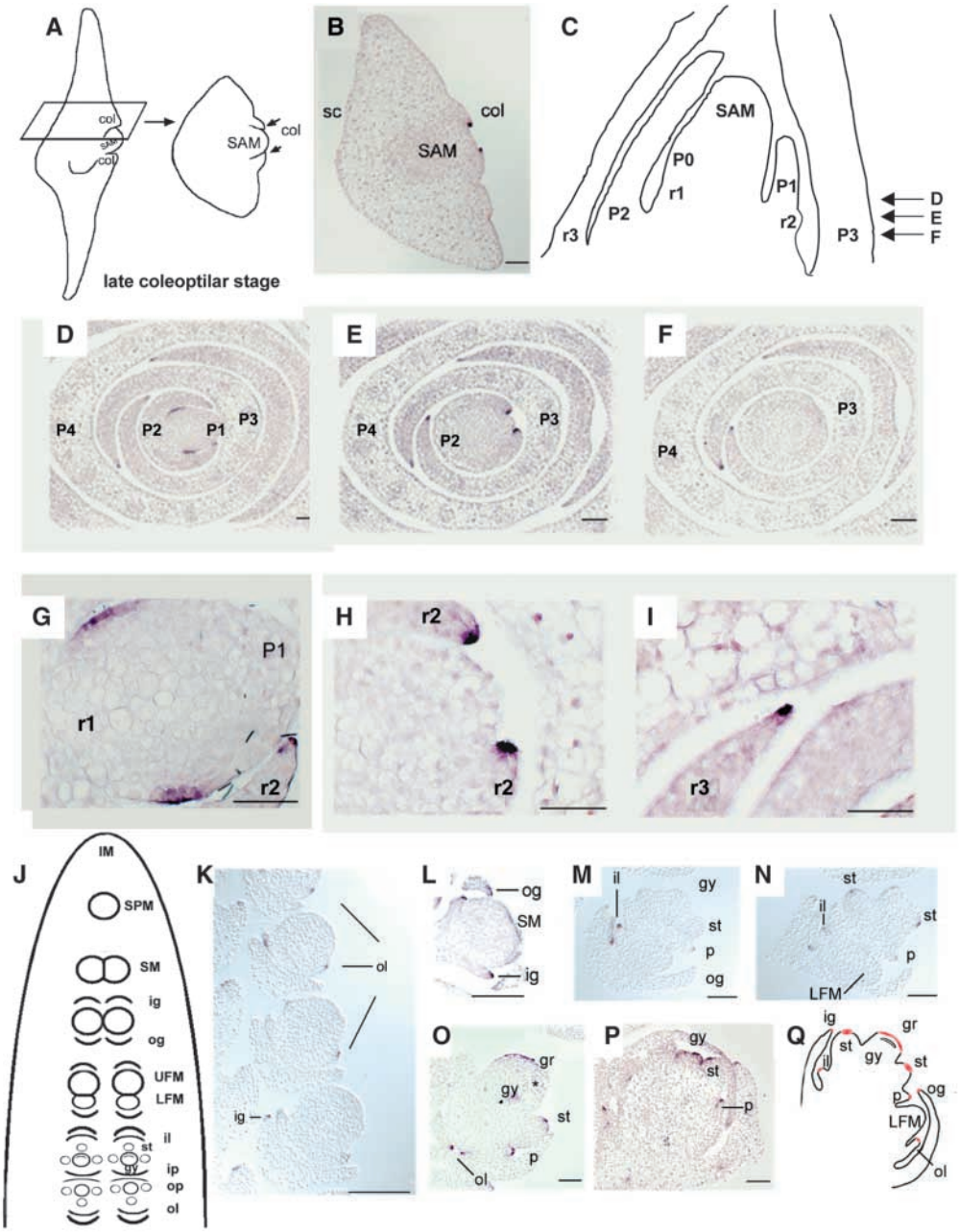

Fig. 4. *ns* transcripts predominately accumulate in tissues enriched for shoot meristems and young primordia. (A) Schematic drawing of coleoptile development. The coleoptile (col) emerges from the periphery of the SAM and grows to form a coleoptilar ring that eventually encloses the SAM. The frame in the cartoon of the coleoptilar stage embryo on the left indicates the plane of the transverse section depicted to the right. (B) In situ hybridization of transverse sections of developing maize embryos reveal that *ns* transcripts accumulate in the tips of the emerging coleoptile. (C) Cartoon depicting a median longitudinal section through the shoot apex of a maize seedling 14 days after germination. The midrib regions of leaf primordia (C-H) are designated by plastochron (P) number, such that the incipient primordium (on the SAM flank) is labeled P0, the next oldest leaf is labeled P1, and so on. The margins (r) of the corresponding leaf primordia are found on the flank of the SAM opposing the midrib. Arrows indicate the position of transverse sections shown in D-F; corresponding close-up images are shown in G-I. (D-I) In situ hybridization of serial transverse sections reveal that *ns* transcripts accumulate in the marginal edges of leaf primordia (r in G-I) and also in two lateral foci in the founder cells of the new leaf primordium (P1 in D and G). (J) Schematic drawing of maize flower development [based on Cheng et al. (Cheng et al., 1983)]. Longitudinal (K,M-P) and transverse (L) sections through female inflorescences or florets at different developmental stages show *ns* activity in marginal cells of all floral organs: glumes (K,L), lemmas (M-O), paleas (K-M), stamens (N-P) and gynoecium (O,P). Expression of *ns* in the gynoecium is detected in the gynoecial ridge (O), a small cleft to the ovule primordium is marked by an asterisk. (K) Longitudinal section of a female inflorescence. (L) Transverse section of a spikelet meristem. (M) Longitudinal section of an upper and lower floret meristem. (N) Longitudinal section of a slightly older flower than that shown in M. (O) Longitudinal section of an upper flower. (P) Longitudinal section of a slightly older upper flower than that shown in O. (Q) Schematic drawing of a longitudinal section through a maize flower, with *ns1* expression domains depicted in red. IM, inflorescence meristem; SPM, spikelet pair meristem; ig, inner glume; og, outer glume; UFM, upper floret meristem; LFM, lower floret meristem; il, inner lemma; ol, outer lemma; ip, inner palea; op, outer palea; gy, gynoecium; st, stamen. Scale bars: 50 μm.

primordia, which give rise to the two spikelet primordia. Each spikelet primordium subsequently develops into an upper and a lower floret. Both florets develop in the tassel, whereas development of the lower floret is aborted in the ear. A non-mutant maize floret comprises leaf-like organs (glumes, lemma and palea), three stamens and the gynoecium. During the formation of monoecious maize flowers, either the stamens or the gynoecium abort during female or male sexual differentiation, respectively. As observed in developing leaves, *ns* transcripts are detected in the margins of all floral organ primordia (Fig. 4L-Q). The specification of marginal identity, as indicated by *ns* activity, is therefore characteristic of all determinate lateral organs of the maize shoot. Conversely, *ns* expression is not detected in indeterminate organs such as the spikelet-pair and spikelet meristems.

Real-time RT-PCR used gene-specific primers (see Table S1 at http://dev.biologists.org/supplemental/) to investigate quantitative expression patterns of the *ns* duplicate genes during development (Fig. 5). All values are normalized to expression levels of the control maize gene *ubiquitin*, as described (Livark and Schmittgen, 2001). No *ns1* or *ns2* expression is detected in roots, seedling leaves or fully expanded coleoptiles. Interestingly, although the levels of *ns1* and *ns2* transcripts are virtually equivalent in vegetative shoot apices (five leaf primordia and the SAM) and in the male inflorescence, *ns2* transcripts are more abundant than *ns1* in the female inflorescence (Fig. 5). In addition, no significant difference in non-mutant *ns1* or *ns2* transcript abundance is detected in inflorescence or vegetative apices isolated from plants in which one *ns* locus was homozygous non-mutant and the other was homozygous mutant (data not shown). Thus, the *ns-R* mutations each failed to induce any compensatory transcript over-accumulation from the corresponding non-mutant locus in either the vegetative or inflorescence/reproductive shoot meristems or primordia.

Previously undescribed phenotypes in *PRESSED FLOWER* mutants, the predicted *narrow sheath* ortholog in *Arabidopsis thaliana*

The first *ns* homologous gene cloned in plants is *PRS* of *Arabidopsis thaliana* (Matsumoto and Okada, 2001). Although *PRS* is a single copy gene in *Arabidopsis* that is expressed in vegetative as well as floral meristems, no mutant phenotype was reported in *Arabidopsis* leaves. Instead, the known Prs$^-$ mutant phenotype is restricted to the flower; lateral sepals are vestigial or completely absent, whereas specialized margin cell types are deleted from the adaxial and abaxial sepals.

However, close inspection of developing Prs$^-$ mutant leaf primordia (Fig. 6B-D,F-H) reveals a previously unreported deletion of the stipules, located at the lateral-most domain of the lower *Arabidopsis* leaf (Medford et al., 1992). Aside from this stipule deletion, mature mutant rosette leaves reveal no obvious phenotype affecting the size, shape or epidermal cell morphology of either the leaf blade or petiole (Fig. 6A,E; data not shown). Likewise, whole-mount and scanning electron microscopic analyses of Prs$^-$ mutant cotyledons reveal no distinguishable phenotype in the blade or petiole (Fig. 6I,J; data not shown). Specifically, Prs$^-$ mutant and non-mutant rosette leaves and cotyledons exhibit equivalent width, length and cellular morphology in the lamina and petiole. Thus, although *PRS* transcripts are detected in the epidermal cells located at the lamina and petiole margins of leaf and cotyledon primordia (Matsumoto and Okada, 2001) (Fig. 6K-L), no obvious phenotype is correlated with this later, primordial-staged expression.

Furthermore, examination of Prs$^-$ mutant flowers reveals the phenotypic deletion of lateral stamens, in addition to the previously reported deletion of the lateral sepals. As shown in Table 1, Prs$^-$ mutants averaged slightly more than four stamens per flower, instead of the normal six stamens found in wild-type plants. Without exception, the deleted or vestigial stamens (as well as the deleted sepals) were located in the lateral position of the whorl.

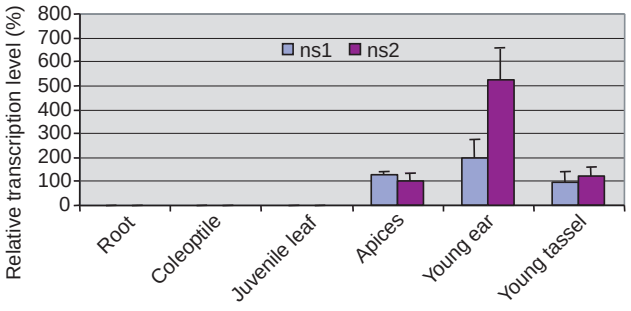

Fig. 5. Quantitative real-time RT-PCR data of transcript abundance (relative to the level of *ns2* transcript in vegetative apices and normalized to ubiquitin transcript levels) in maize tissues employing primers specific for *ns1* or *ns2* transcripts (see Materials and methods). *ns* transcripts accumulate in tissues enriched for vegetative shoot meristems and inflorescence meristems (apices, ears and tassels), but are not detected in mature lateral organs (coleoptile, juvenile leaves) or roots.

Discussion

The *ns1* and *ns2* mutations are null alleles in duplicated maize genes

The *ns1* and *ns2* genes map to regions of the maize genome that are known to be duplicated, and previous analyses suggested that they encode duplicate gene functions (Scanlon et al., 2000). In this report, the *ns* genes are identified by homology to the *PRS* gene of *Arabidopsis*; the predicted amino acid sequences of the *ns* duplicate genes are 86% identical (Fig. 2A). Immunoblot analyses reveal that ns-R mutant inflorescences accumulate virtually no NS protein (Fig. 3D), which is in agreement with previous genetic analyses predicting that the *ns-R* mutations are null alleles. The duplicate genes *ns1* and *ns2* are expressed in nearly identical patterns and at similar developmental stages (Fig. 4), although *ns2* transcripts are more prevalent in reproductive tissues (Fig. 5). However, morphogenetic analyses illustrate the absence of ns phenotypes in plants that are mutated at one *ns* locus, but are non-mutant for the duplicate gene (Fig. 1E-H) (Scanlon and Freeling, 1998). Thus, although the relative transcriptional abundance of each *ns* gene is not equivalent in all maize tissues, the genetic data suggest that redundancy of gene function is still maintained. We speculate that the *ns* duplicate genes may be at an early stage in the evolutionary process of separating their tissue-specific patterns of gene expression, perhaps in a manner exhibited by the anthocyanin-biosynthetic

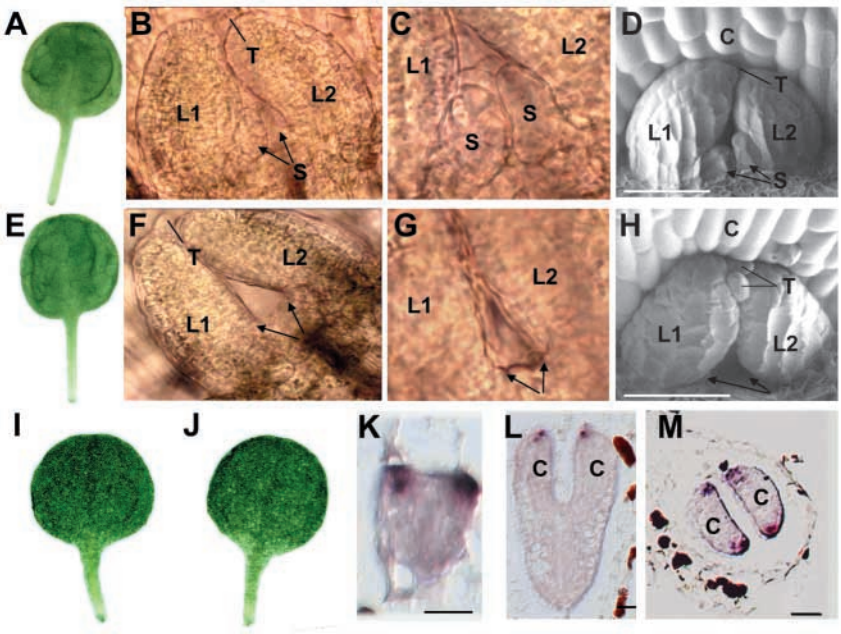

Fig. 6. Phenotypes of Prs⁻ mutant *Arabidopsis* leaves and cotyledons. At maturity non-mutant (A) and Prs⁻ mutant (E) rosette leaves have equivalent phenotypes. Whole-mount (B,C,F,G) and scanning electron microscopic (D,H) analyses of non-mutant (B-D) and Prs⁻ mutant (F-H) leaf primordia (L1, L2) reveal that the lateral stipules (S) are deleted from mutant leaves. Note that the size of the emerging trichomes (T) indicates that the lack of stipule development in Prs⁻ mutant primordia is not due to differences in the developmental age of these samples. No obvious Prs⁻ mutant phenotype is noted in comparisons of mature (I,J) or primordial (not shown) non-mutant (I) and Prs⁻ mutant (J) cotyledons. (K-M) Expression of *PRS* during *Arabidopsis* embryogenesis. (K) *PRS* expression is localized to the tips of the prospective cotyledons in the heart stage embryo. In torpedo stage embryos, *PRS* is expressed at the apices (L) and the lateral margins (M) of the cotyledons (c). *PRS*-expressing cell layers therefore define a border between adaxial and abaxial side of the cotyledons, similar to the maize coleoptile (Fig. 4B). Scale bars: 50 μm.

genes *white pollen1* (*whp1*) and *colorless2* (*c2*). *whp1* and *c2* are maize duplicate genes that map to the same regions of the maize genome as *ns1* and *ns2*, respectively (Gaut and Doebley, 1997). Intriguingly, although an anthocyanin-defective mutant phenotype in maize pollen requires mutations at both *whp1* and *c2*, a single mutation at *c2* is sufficient to condition colorless seed (Franken et al., 1991). Thus, although *whp1* and *c2* have retained overlapping expression in flowers, *c2* functions non-redundantly in vegetative tissues.

ns and Prs⁻ leaf phenotypes: molecular genetic support for a model describing the evolution of leaf morphology

Mutations in *ns1* and *ns2* together confer the deletion of a lateral domain in maize leaves that extends from the midpoint of the distal blade and includes the entire length of the proximal sheath (Fig. 1) (Scanlon et al., 1996). By contrast, the leaf phenotype of the orthologous *Arabidopsis* mutant Prs⁻ is extremely subtle, and limited to the deletion of the proximal, lateral stipules (Fig. 6). No additional phenotype is detected in either the petiole or the lamina of Prs⁻ mutant leaves. A survey of the *Arabidopsis* genome reveals that, unlike the duplicated *ns* sequences of maize, *PRS* is a single copy gene. Therefore, there is no evidence to suggest that additional leaf mutant phenotypes of PRS function are obscured by redundant, *PRS*-homologous gene sequences. Instead, the differential leaf phenotypes conditioned by the *ns* and *prs* mutations are consistent with an existing model describing the evolution of monocot and eudicot leaf morphology.

A model of leaf zonation predicts that bifacial (i.e. dorsoventrally flattened) eudicot leaves are subdivided into a large upper leaf zone comprised of the lamina and petiole, and a greatly diminished lower leaf zone comprising the leaf base and the lateral stipules (Fig. 7A) (Troll, 1955; Kaplan, 1973). By contrast, the model suggests that bifacial monocot leaves have evolved an extended and highly elaborated lower leaf zone and an extremely abbreviated upper leaf zone. In this model, the upper leaf zone of the monocot maize comprises just a short, unifacial fore-runner tip, whereas the majority of the leaf blade and the entire sheath are derived from the lower leaf zone (Fig. 7B). When interpreted in terms of this model, the ns mutant leaf phenotype is localized to a lateral domain in the lower region of the lower leaf zone (i.e. the lower blade

Table 1. Floral organs in Pressed flower⁻ mutants

Floral organ*	Organ number (mean)†	Vestigial organs‡
Sepals (4)	2.41±0.62	14::58
Petals (4)	3.79±0.49	6::58
Stamens (6)	4.54±0.51	7::58

*The normal number of organs in non-mutant *Arabidopsis* is given in parentheses.

†The mean number of sepals, petals and stamens contained within 58 individual flowers of Prs⁻ mutant homozygous plants.

‡Small, underdeveloped (vestigial) organs were counted and included in the tabulations of organ number shown in the second column.

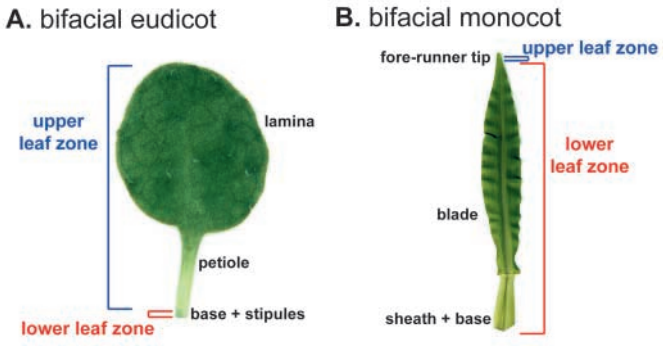

Fig. 7. The domain deletions observed in Prs⁻ mutant and ns mutant leaves are consistent with a model describing the differential elaboration of upper versus lower leaf zones during the morphological evolution of monocot and eudicot bifacial leaves. Details are provided in the text; models are adapted from Troll (Troll, 1955), as elaborated by Kaplan (Kaplan, 1973).

and entire sheath). Extending this model to the dicot *Arabidopsis* leaf, deletion of a lateral domain in the lower part of the greatly diminished, lower leaf zone in Prs$^-$ mutant plants would predict a minor phenotype affecting the stipules. This subtle, stipule-deletion phenotype is precisely that observed in Prs$^-$ mutant leaves. Therefore, the apparent incongruity in the leaf phenotypes observed in maize ns and *Arabidopsis* Prs$^-$ mutants is a predicted outcome of Troll's model of leaf zonation, and supports the hypothesis that the differential elaboration of the upper and lower leaf zones has contributed to the morphological diversity of maize and *Arabidopsis* leaves. Another popular model of leaf morphology suggested that the sheath region of monocot grass leaves evolved via flattening of the petioles of eudicot progenitors (Arber, 1934). However, the lateral domain deletion in ns mutant sheaths, together with the lack of a petiole phenotype in the Prs$^-$ mutant leaf, fails to support this interpretation.

A more controversial topic is the homology of the grass cotyledon (Weatherwax, 1920; Boyd, 1931; Kiesselbach, 1949). Some authors purport that the maize cotyledon is composed wholly of the leaf-like coleoptile. In this view, the scutellum is a grass-specific organ with no obvious counterpart in eudicot embryos. Others argue that the scutellum is the sole grass cotyledon, whereas the coleoptile is the first true leaf. Still others suggest that the scutellum and coleoptile together comprise the apical and basal components of the single grass cotyledon, in which the scutellum represents the highly modified upper leaf component and the coleoptile represents the sheathing base of the cotyledon. Interesting maize mutants exist that lack a coleoptile, but show normal development of embryonic leaves (Elster et al., 2000). In addition, expression of the *ltp2* (*lipid transfer protein 2*) gene is found in the outer cell layer of the scutellum and coleoptile, but is excluded from the L1 layer of the SAM and the epidermis of true leaf primordia (Sossountzov et al., 1991; Bommert and Werr, 2001). These observations suggest that the coleoptile originates laterally from scutellum tissue. Although no ns/Prs$^-$ phenotype is observed in the maize coleoptile (Scanlon and Freeling, 1998) or the *Arabidopsis* cotyledons (Matsumoto and Okada, 2001) (Fig. 6), the conserved marginal pattern of *ns1/PRS* expression (Figs 4, 6) suggests that homologous morphogenetic programs are shared in the coleoptile and cotyledon. By contrast, *ns1* transcripts are never found in the scutellum. These data are compatible with models suggesting that the scutellum is a Gramineae-specific organ that is not homologous to the cotyledon. However, *ns/PRS* expression in lateral organ primordia is confined to the basal, marginal regions; no expression is noted in the upper leaf domains. Thus, the *ns/PRS* expression profiles are equally consistent with models purporting that the maize cotyledon comprises both the apical scutellum (no *ns* expression) and the basal coleoptile (*ns* expression). Moreover, the two-component model of grass cotyledon evolution supports the modification of an existing organ rather than the de novo formation of a Gramineae-specific scutellum, and is thereby more compatible with the conservative mechanism of evolution.

NARROW SHEATH performs a conserved function in maize and *Arabidopsis*

Sequence homology, similarity of gene expression profiles and comparative mutant phenotypes together identify the maize *ns* duplicate genes and *PRS* of *Arabidopsis* as orthologous sequences. Two distinct developmental time points of *ns/PRS* expression are conserved among maize and *Arabidopsis*, defined as an early expression within two lateral, meristematic foci and a later-staged expression in the margins of young lateral organ primordia (Fig. 4) (Matsumoto and Okada, 2001). When considered in the context of ns and Prs$^-$ mutant phenotypes, these expression domains suggest that the essential function(s) of the *ns/PRS* gene product is limited to the early, meristematic expression domains, as predicted in previous clonal analyses of NS function (Scanlon, 2000).

The later, primordial expression pattern of *ns/PRS* is restricted to a few cells at the margins of developing lateral organ primordia (Fig. 4E,F) (Matsumoto and Okada, 2001). However, no maize or *Arabidopsis* mutant phenotype correlates with loss of NS/PRS function in the margins of lateral organ primordia. For example, primordial-staged *PRS* expression is detected in epidermal cells that will eventually form the margins of the *Arabidopsis* leaf lamina, and also in the primordial margins of the cotyledon (Fig. 6K-M). No Prs$^-$ mutant phenotypes are detected in these lateral organ domains (Fig. 6I,J) (Matsumoto and Okada, 2001). Likewise, *ns* is expressed at the edges of the developing coleoptile and leaf primordia (Fig. 4B,D-I), although no ns mutant phenotype is detected in the coleoptile (Scanlon and Freeling, 1998). Moreover, clonal analyses (Scanlon, 2000) uncovered multiple cases wherein *ns1* function was present at the *ns* meristematic focus (i.e. early *ns* expression pattern) but was absent from the margins of developing maize leaf primordia (i.e. late *ns* expression pattern). In all such cases, loss of late-staged NS function in the L1-derived primordial margins was non-phenotypic, whereas loss of early staged function from the *ns* meristematic focus always correlated with the ns mutant phenotype. As discussed below, we suggest that the Prs$^-$ stipule, lateral sepal and lateral stamen deletion phenotypes of *Arabidopsis*, as well as the ns mutant phenotypes in maize lateral organs, all arise from the loss of a conserved, early NS/PRS function in a lateral domain of the vegetative and/or reproductive shoot apical meristem.

Fate-mapping analyses of maize leaf founder cells (Poethig, 1984), as well as clonal sector analysis of NS function (Scanlon, 2000), reveal that maize leaf anlagen are recruited from the circumference of the meristem, such that the midrib founder cells occupy one flank of the SAM and the margin founder cells of the lower leaf (sheath) are recruited in close proximity. In this way midrib sectors may be clonal to sectors on the marginal flank of subtending leaves, with such sectors intersecting both the right and left margins of the leaf sheath (Scanlon and Freeling, 1997). The series of transverse sections through the maize shoot apex shows a first stripe of NS transcriptional activity at the lateral flank of the SAM, where clonal analyses indicated two foci of functional NS activity. However, *ns* transcripts are localized to the tunica of the shoot apex. This L1 pattern conflicts with clonal analyses of NS1 function, which concluded that NS1 function in the L1 layer of the SAM cannot compensate for loss of function in the L2 meristematic layer (Scanlon, 2000). It is possible that mericlinal L1-L2 mutant sectors present at one meristematic focus in these sample plants conditioned a mutant leaf phenotype. Subsequently, post-meristematic invasion of non-mutant L1 clones may have generated ns mutant leaves

containing L2-derived *ns1*-null sectors but a non-mutant epidermis. Additional speculative explanations are possible, although resolution of this apparent discrepancy will await localization analyses of PRS/NS protein within the shoot apex.

Intriguingly, this stripe of NS activity in the SAM appears between the P0 and the P1 primordia, where recruitment of cells into opposing primordial founder-cell domains converges at the lateral flanks of the SAM (see Fig. 8A). NS activity might be required for recruitment of cells at the P0 face of the SAM into the horseshoe shaped P1 primordium, thereby allowing the intermingling of subtending primordial founder-cell domains in the shoot apex. By contrast, the ns mutant phenotype could occur if cells at the P0 face, which in wild type are recruited for the basal lateral leaf domain (mainly sheath) of the P1 primordium, are mutually exclusively recruited for the next primordium (P0) in absence of NS activity (see Fig. 8B). In the absence of NS activity, founder-cell recruitment into the P0 and P1 primordia may compete for cells at the lateral flanks of the SAM in ns mutants, whereas in wild type NS activity allows overlapping founder-cell domains. Thus, one possible NS function as a WUS-type transcription factor may be to maintain developmental competence in a stripe of cells in two lateral SAM domains.

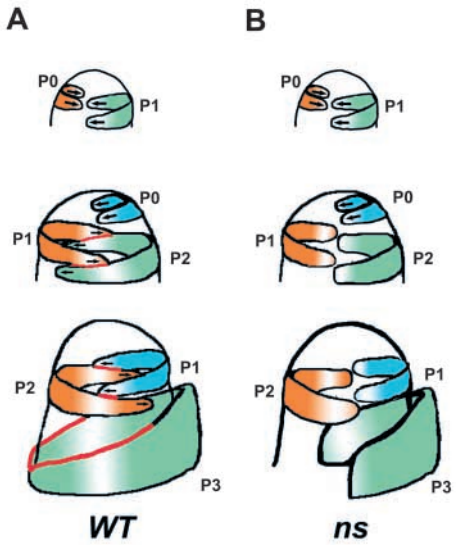

Fig. 8. (A) NS activity (red line) in the maize shoot apex and at the margins of emerging leaf primordia (P0-P3) in wild type. (B) Model of competing founder-cell domains in ns mutants.

NS/PRS functions to establish competence in a lateral shoot meristem domain, not a lateral organ domain

Initial descriptions of the ns mutant phenotypes provided evidence that the NS recruitment signal is not specific for any particular leaf domain, as the deleted leaf domain in ns mutants is not consistent throughout vegetative development. Specifically, the mutant phenotype is most severe in juvenile leaves and is increasingly alleviated in adult leaves (Scanlon et al., 1996). These observations are explained by a model in which NS functions in a specific lateral domain of the SAM to initiate meristematic cells to become organ founder cells. This model suggests that founder cells are recruited from the central and lateral meristematic domains through different gene functions. Whereas as yet undescribed function(s) recruit founder cells from the central domain (green in Fig. 1; Fig. 9), NS functions to recruit founder cells from the lateral meristem domain. After founder cells are initialized from meristem domains, specific organ domain identities (i.e. midrib, margin, etc.) are assigned as the primordium develops. As the circumference of the maize meristem increases with each successive leaf initiation (Bassiri et al., 1992), the percentage of the lateral meristematic domain that is used to form a new organ diminishes. Consequently, loss of function at the NS foci causes a smaller leaf domain deletion in later leaves that form from the larger SAM. In this view, NS does not specify any particular leaf domain, but instead initiates a lateral domain within the shoot meristem.

Likewise, *Arabidopsis* leaves initiate one leaf per node, although recruitment of these eudicot leaves does not include cells from the circumference of the SAM (Irish and Sussex, 1992) (Fig. 9B). Consequently, the lateral extent of the founder-cell domain recruited by PRS function is relatively

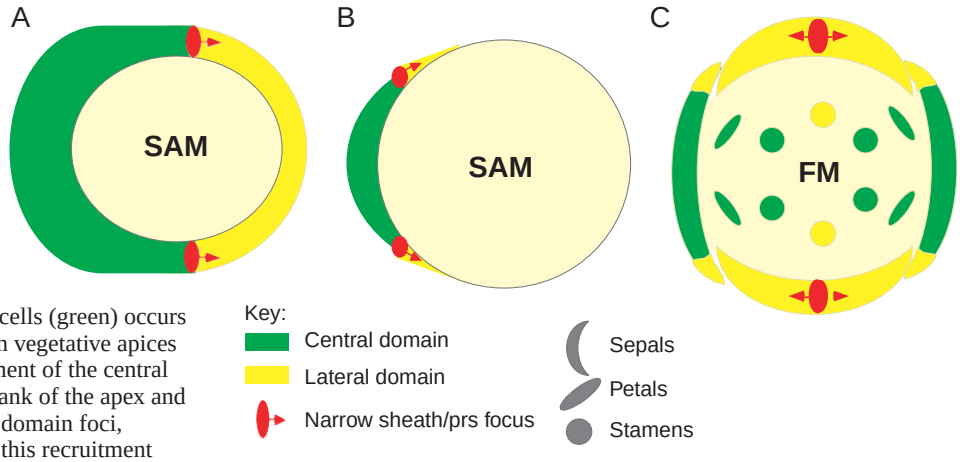

Fig. 9. Model for a conserved NS/PRS function during the recruitment of a lateral founder-cell domain in vegetative (A,B) and reproductive (C) shoot meristems of maize (A) and *Arabidopsis* (B,C). NS/PRS function initiates from lateral foci (red arrows) and recruits founder cells in a lateral meristematic domain (yellow). Recruitment of the central domain founder cells (green) occurs prior to recruitment of the lateral domain. In vegetative apices that produce a single leaf per node, recruitment of the central founder-cell domain occurs from a single flank of the apex and proceeds bi-directionally toward the lateral domain foci, whereas in the *Arabidopsis* floral meristem this recruitment commences simultaneously from both the adaxial and abaxial flanks of the apex. Loss of NS/PRS function causes failure to develop any and all organ domains that are normally derived from the lateral founder-cell domain of the meristem. Further details of this organ domain-blind model of NS/PRS are explained in the text.

small, such that just a small number of founder cells that give rise to basal, lateral leaf domains are deleted in Prs⁻ mutants. Thus, loss of PRS function leads to the deletion of a relatively small leaf region (i.e. the lateral stipules) that normally develops from this lateral domain of the SAM (Fig. 9B).

Strong support for this evolutionarily conserved, and organ domain-blind, model of NS/PRS function is provided by the sepal and stamen deletion phenotypes in *Arabidopsis* Prs⁻ mutants (Fig. 9C). Mutations at *prs* typically condition the deletion of just the margins of the adaxial and abaxial sepals, whereas the two lateral sepals fail to initiate or are vestigial. This phenotype indicates that PRS does not specify a specific marginal domain in these lateral organs, as both the central as well *as* the marginal domains of the lateral sepals are deleted in Prs⁻ mutants. In this view, the expression domain of *PRS* in two lateral points on the *Arabidopsis* floral meristem (Matsumoto and Okada, 2001) correlates to foci from which founder cells contributing to the entire lateral sepals, as well as to the margins of the adaxial and abaxial sepals, are recruited. Moreover, the stamen deletion phenotype of Prs⁻ mutants affects only lateral stamens (Table 1); the adaxial and abaxial stamens are intact. This model predicts that lack of PRS function blocks recruitment of lateral founder-cell domains of the *Arabidopsis* floral meristem, causing the deletion of lateral sepals and stamens. These observations support a model whereby NS/PRS function is conserved to initiate founder cells from a lateral domain of plant meristems.

Intriguingly, no phenotype is observed in Prs⁻ mutant petals, except in cases wherein these second whorl organs are homeotically transformed into sepalloid organ identity (Matsumoto and Okada, 2001). Therefore, although PRS functions in the second whorl of *Arabidopsis* floral meristems, recruitment of petal founder cells may not extend into the PRS functional domain. We speculate that the much smaller size (Smyth et al., 1990; Bossinger and Smyth, 1996) and phyllotactic arrangement of petal primordia (i.e. offset from the lateral meristematic domain) is such that these organs do not develop from founder cells that encroach into the lateral meristematic domain recruited by PRS (Fig. 9C). Currently, efforts are underway to investigate the ns/Prs⁻ mutant phenotype in the compound leaves of tomato and pea. These experiments will further expand our understanding of the role of this gene during the evolution of diverse angiosperm leaf morphology.

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