COMPARATIVE GENETIC ANALYSES OF WOX3 AND WOX4 FUNCTIONS DURING PLANT DEVELOPMENT

by

JIABING JI

(Under the Direction of Michael J. Scanlon)

ABSTRACT

Comprising a deletion of a lateral compartment in maize leaves and leaf homologs, the narrow sheath (ns) phenotype is a duplicate factor trait conferred by recessive mutations at the unlinked loci *ns1* and *ns2*. *ns1* and *ns2* were isolated by homology to the *WUSCHEL-related homeobox gene PRESSED FLOWER* (*PRS/WOX3*), which is required for lateral sepal development in *Arabidopsis*. Analyses of NS protein accumulation verified that the *ns-R* mutations are null alleles. *ns* transcripts are detected early in two lateral foci within maize meristems, and late in the margins of lateral organ primordia. Previously undiscovered leaf phenotypes in the pressed flower mutant support a model whereby the morphology of eudicot leaves and monocot grass leaves has evolved from the differential contribution of upper versus lower leaf zones.

LeWOX4, an ortholog of *AtWOX4*, was cloned from tomato. RT-PCR and *in situ* hybridizations reveal that the WOX4 orthologues from both *Arabidopsis* and tomato are expressed in the stem cells of the vascular procambium, although these species differ in the arrangement of vascular bundles. RNAi-induced knockdown of WOX4 generated no mutant phenotype in tomato or *Arabidopsis*, suggesting that WOX4 may perform redundant functions in these organisms. Constitutive 35S-induced expression of WOX4 in *Arabidopsis* and tomato indicates that WOX4 overexpression may promote procambial activity, leaf complexity and lateral organ development. This represents the first-ever analyses of WOX4 expression and function in any plant system.

INDEX WORDS: *narrow sheath*, *pressed flower*, *WOX4*, maize, tomato, *Arabidopsis*, meristem, leaf development, vascular development, founder cells

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DEDICATION

To my grandmother, mom, aunts, uncles, brother, wife and daughter for their love and support

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Literature review

During plant embryogenesis, the root and shoot axes and their respective meristems are established, and the genes defining vegetative morphogenesis are first expressed and then later resolve to their characteristc patterns. Soon after, the first lateral organs are formed from these meristems. At some point in this process the embryo may or may not enter a period of quiescence, after which the same patterns of gene expression and lateral organ development are resumed upon germination; all subsequent organs of the seedling and mature plant are made via mechanismsas used during embryogenesis. In other words, post-embryonic development of the plant depends on the maintenance of meristems, which are composed of relatively undifferentiated and self-renewable stem cells. All plant organs including leaves, flowers, branches, roots, and vascular tissues are continuously formed from these stem cells (Bäurle and Laux 2003; Bhalla and Singh 2006). The adult plant morphology is determined by post-embryonic developmental patterning activities of these meristems (Clark 2001). Therefore, understanding how meristems are organized and how meristems function is a major goal of plant development biology.

Shoot Apical Meristem and Leaf Development

1. WUSCHEL (WUS) specifies stem cell identity in shoot meristems and is regulated through different pathways

Much of our knowledge about plant meristem biology comes from intensive molecular genetic research in the *Arabidopsis* model system (see Fig.1.1). Histologically, the SAM can be divided into the apical central zone (CZ), the lateral peripheral zone (PZ),

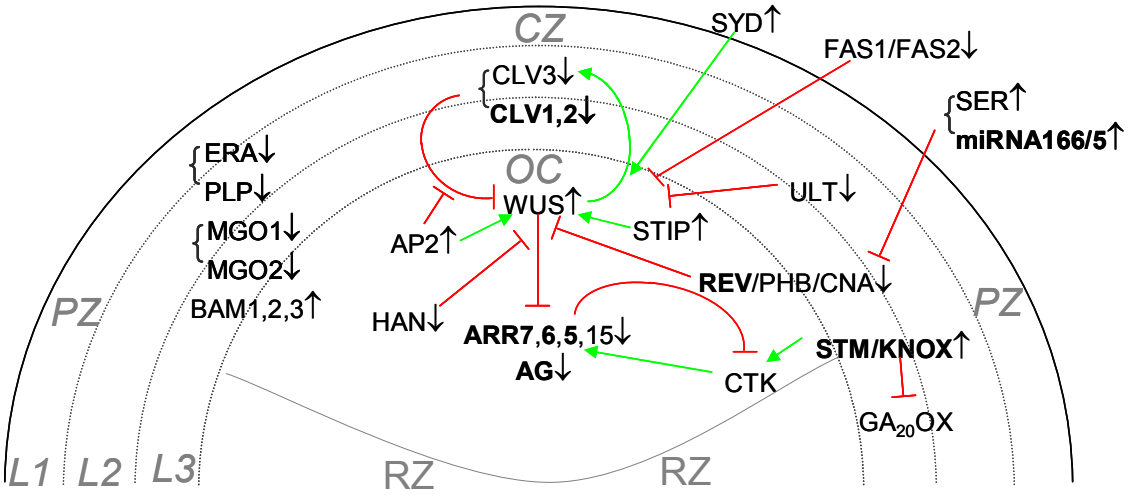

Figure 1.1 Genetics of meristem function. Plant shoot meristems exhibit characteristic layering (L1-L2-L3) and zonation (CZ = central zone; PZ = peripheral zone; OC = organizing center; RZ = rib zone) that is under genetic control. Genes functioning to increase meristem size are designated by an up arrow ↑; those decreasing meristem size are marked with a down arrow ↓. Multiple genetic/biochemical pathways converge or act independently to regulate meristem function. Positive regulators (green arrows) and negative regulators (red bars) are shown, and described in the text. Genes for which functional orthologues are known in maize as well as *Arabidopsis* are shown in **bold**. [Drawing modified from Williams and Fletcher, 2005]

and the central rib zone (RZ) (Lenhard and Laux 1999). The stem cells are renewed in the CZ and replenish cells lost from the PZ during organogenesis. The Rib zone provides cells to the growing stem and central pith. Previous studies have shown that *WUSCHEL* (*WUS*), a founding member of the WOX (WUSCHEL-LIKE HOMEOBOX) family of

putative homeodomain transcription factors, is expressed in the lower part of the CZ called the organizing center (OC) underneath the stem cells. WUS functions non-cell-autonomously to specify the above stem cell identity through activation of the expression of CLAVATA3 (CLV3) in the stem cells (Fig.1.1; Gallois et al., 2004; Laux et al., 1996; Mayer et al., 1998); whereas the stem cells emanate negative signals to the OC through the *CLAVATA* (*CLV*) signaling pathway (Fig.1.1; Brand et al. 2000; Lenhard and Laux 2003; Schoof et al., 2000). The CLV signaling pathway includes CLV1, CLV2, and CLV3. CLV3 is a small polypeptide secreted by the stem cells in the SAM, which moves through the apoplast of the SAM and interacts with CLV1-CLV2 heterodimer receptor complex in the underlying cells. Binding of the CLV3 ligand to the CLV1-CLV2 receptor activates an undefined signal transduction pathway that limits the size of the *WUS* expression domain (Clark et al. 1997; Fletcher et al. 1999; Lenhard and Laux 2003; Rojo et al. 2002; Trotochaud et al.1999; Williams et al. 1997); Thus, WUS functions in a negative feedback loop to delimit its expression domain via activating expression of CLV3 (Lenhard and Laux 2003). Interestingly, CLV1-related receptor-like kinases BAM1 (barely any meristem 1), BAM2 and BAM3 play opposite roles than CLV1and positively regulate SAM development (Fig.1.1; DeYoung et al., 2006). Furthermore, WUS directly suppresses the expression of the two-component *ARABIDOPSIS RESPONSE REGULATOR* 5 (*ARR5*)*, ARR6, ARR7* and *ARR15* genes, which are negative feedback regulators of cytokinin signaling pathway and SAM size (Fig.1.1; Leibfried et al., 2005). Mutations in the maize ARR homologue ABPHYLL1 leads to an enlarged meristem (Giulini et al., 2004), and overexpression of a constitutively active ARR7 allele in *Arabidopsis* reduces the SAM size (Leibfried et al., 2005). Finally, comparative

analysis of a conserved C-terminal domain of WUS orthologues in *Arabidopsis* and *Antirrhinum* suggests that WUS might recruit transcriptional co-repressors via this domain to suppress downstream genes that promote differentiation of stem cell domains (Kieffer et al., 2006). Therefore the stem cell homeostasis is tightly controlled by multiple levels of feedback regulation and hormonal signaling.

Although the signaling mechanism whereby CLV proteins suppress WUS expression is still not clear, recent work has revealed multiple additional molecules involved in the regulation of WUS expression. Mutations in *FASCIATA1* (*FAS1*) and *FASCIATA2* (*FAS2*), subunits of the chromatin assembly factor-1 (CAF-1) in *Arabidopsis*, are defective in the maintenance of *WUS* expression in the SAM (Fig.1.1; Kaya et al., 2001). Similarly, the novel nuclear protein BRUSHY1 (BRU1) functions cooperatively in the replication and stabilization of chromatin structure, and is also required for proper *WUS* expression in SAM (Takeda et al., 2004). Furthermore, the chromatin remodeling factor SPLAYED (SYD), a SNF2 class ATPase, is genetically and biochemically shown to be a direct, specific and positive upstream regulator of WUS expression (Fig.1.1; Kwon et al., 2005). Bäurle and Laux (2005) found that a 57-bp regulatory fragment located 529 bp upstream of the *WUS* start codon is sufficient to mimic the expression pattern of *WUS* mRNA in the SAM, and two distinct regulatory elements named RE1 and RE2 within this 57-bp region are essential for this promoter activity. Interestingly, whereas the effectiveness of RE1 and RE2 function as a promoter seems to depend on their integration site in the genome, the full-length *WUS* promoter showed no integration site preference. This suggests that additional cis-regulatory elements are required for correct chromatin organization at the *WUS* locus.

Several transcription factors have been identified in controlling *WUS* transcription. The GATA transcription factor HANABA TARARU (HAN) and the SAND domain putative transcription factor ULTRAPETALA1 (UTL1) act as negative regulators during the very early and later stages in *Arabidopsis* development respectively (Fig.1.1; Zhao et al., 2004; Carles et al., 2005). In addition, UTL1 acts in a separate genetic pathway than CLV, in spite of their overlapping functions (Carles et al., 2004). The WUS paralogue STIMPY/WOX9 (STIP) is a positive regulator of WUS expression (Fig.1.1; Wu et al., 2005). Interestingly, sucrose can fully rescue the *stip* mutant meristem-depletion phenotype (Wu et al., 2005), suggesting that sucrose-stimulated entry into the cell cycle may sidestep the requirement for STIP. AP2 (APETALA2) modifies the WUS-CLV3 pathway to act in stem cell maintenance (Fig.1.1; Würschum et al., 2006). Furthermore, microRNA 166/165 regulation of the class III HD-ZIP genes PHB, PHV and CAN negatively control *WUS* expression levels and thus restricts SAM activity (Fig.1.1; Green et al., 2005; Prigge et al., 2005; Williams et al., 2005). This microRNA-dependent process may require the function of the zinc-finger protein SER (SERRATE) (Fig.1.1; Grigg et al., 2005).

2. WUS and KNOX genes act in complementary pathways to promote meristem indeterminacy.

In addition to the WUS pathway, several class 1 *KNOTTED1-LIKE HOMEBOX* (*KNOX1*) genes are required for SAM activity. The maize gene *KNOTTED1* (*KN1*) was the first plant homeobox gene cloned from plants (Vollbrecht et al., 1991). SHOOTMERISTEMLESS (STM) (Figure1.1; Barton & Poethig 1993; Long et al., 1996), the *Arabidopsis* KN1 orthologue, acts independently of the CLV3 and WUS pathway and

is necessary to suppress cell differentiation and maintain indeterminate stem cell identity in the SAM (Clark et al., 1996; Gallois et al., 2002; Lenhard et al., 2002). However, STM alone fails to induce stem cell identity (Lenhard et al., 2002) and is not required for SAM formation in the *asymmetric leaf1* (*as1*) mutant background (Byrne et al., 2000). STM and the R2-R3 MYB protein AS1 are mutually inhibitory, and STM negatively regulates *AS1* in stem cells (Byrne et al., 2000). AS1 may physically associate with ASYMMETRIC LEAVES2 (AS2), a plant specific LOB domain protein (Iwakawa et al., 2002; Xu et al., 2002, 2003) to suppress KNOX expression in Arabidopsis leaves. In *as1* and *as2* mutants, three other *KNOX* genes (*BREVIPEDICELLUS* (*BP*)/*KNAT1, KNAT2* and *KNAT6*) are ectopically expressed or upregulated in lateral organs, suggesting that AS1 and AS2 negatively regulate the expression of these *KNAT* genes and exclude them from organ primordia. Normally these *KNAT* genes are expressed in the SAM and may act redundantly with *STM* (Byrne et al., 2000, 2002; Iwakawa et al., 2002; Lin et al., 2003; Ori et al. 2000; Semiarti et al., 2001; Venglat et al., 2002). Furthermore, KNOX proteins function as heterodimers with BEL-like homeodomain proteins (Byrne et al., 2003, Smith & Hake, 2003), induce cytokinin biosynthesis (Fig.1.1; Ori et al., 1999; Frugis et al., 2001) and negatively regulate gibberellic acid biosynthesis (Fig.1.1; Sakamoto et al., 2001; Hay et al., 2002).

In addition, other genes comprising *MGOUN1* (*MGO1*), *MGOUN2* (*MGO2*), *ENHANCED RESPONSE TO ABSCISSIC ACID* (*ERA1*)/*WIGGUM* and *PLURIPETALA* (*PLP*) negatively regulate apical meristem size either through promoting primordia initiation or repressing the expression of *KNAT* genes; these gene products also appear to

work outside of the WUS/CLV signalling pathway (Fig.1.1; Laufs, 1998; Running, 2004; Ziegelhoffer et. al., 2000).

3. Leaf development

Leaf development can be divided into three major steps: leaf founder cell recruitment, establishment of three leaf axes, and leaf primordial growth and differentiation. Leaf primordia are sequentially generated from founder cells present in the PZ of the SAM. The founder cells span all the histogenic layers in PZ, i.e. the tunica layer including L1 in monocots and both L1 and L2 in dicots, and the corpus comprising L2 in monocots and L3 in dicots (Smith & Hake 1992). The mechanisms regulating the timing, position and size of the leaf primordial initials is gradually becoming unraveled. In most simple-leafed plants like maize, *Arabidopsis*, and *Antirrhinum*, the acquisition of founder cell identity is correlated with the down-regulated accumulation of KNOX proteins in the PZ of the meristem. The down-regulation of KNOX expression in leaf primordia is maintained by the MYB-like proteins PHAN/RS2/AS1 (Jackson et al., 1994; Schneeberger et al.1998; Timmermans et al. 1999; Tsiantis et al. 1999; Byrne et al. 2000). Furthermore, KNOX down-regulation is gradual, such that central-midrib compartment of the leaf develops well before the margins of the same leaf (Scanlon et al. 1996; Scanlon 2003). In compound-leafed plants such as tomato, persistence of *KNOX* expression in the margins of leaf primordia (blastozones) is associated with the formation of dissected leaves (Chen et al. 1997; Hareven et al. 1996; Janssen et al. 1997; 1998; Bharathan et al. 2002), and overexpression of *knox* genes results in super compound, highly dissected leaves. Furthermore, activity of the tomato *KNOX* gene LeT6 (KNOX1) appears to require *LePHAN* for its function during formation of dissected leaves (Kim et al. 2003).

However, persistence of *knox* gene expression during primordial stages does not describe the development of all compound leaves plants. For instance, *KNOX1* expression is excluded from developing leaf primordia in pea (Hofer et al. 2001), in which the LEAFY(LFY)/UNUSUAL FLORAL ORGANS(UFO) pathway is shown to regulate the formation of leaflets of these compound leaves (Ingram et al. 1995; Hofer et al.1997; Lee et al. 1997; Gourlay et al. 2000; Taylor et al. 2001; Tsiantis & Hay 2003).

Down-regulation of KNOX during the initiation of leaf primordia may also require auxin transport (Scanlon 2003). Likewise, mutations in *PINFORMED1* (*PIN1*), a polar auxin transport (PAT) efflux carrier, generate plants that fail to initiate lateral organs; this condition can be phenocopied by treatment with PAT inhibitors. However, leaf formation and the corresponding downregulation of KNOX gene expression are both restored through the microapplication of the natural auxin IAA to arrested SAMs (Reinhardt et al., 2000, 2003; Scanlon, 2003). Furthermore, phyllotaxis is regulated by proteins involved in auxin transport such as AUXIN RESISTANT1 (AUX1) and PIN1 (Reinhardt et al., 2003) and the dynamics of auxin flux may in turn be sensitive to SAM size, which is controlled by the antagonisic activities of cytokinin and the putative two-component response regulator ABPHYLL in maize (Jackson & Hake, 1999; Giulini et al., 2004).

After the recruitment of founder cells, leaf primordia reqire further information for correct patterning along three axes of asymmetry. The establishment of the proximal-distal axis requires LIGULELESS 1 (LG1) and LIGULELESS 2 (LG2) in maize, AURICLELESS (AUL) ,LIGULELESS (LG), and COLLARLESS (COL) in rice, and LIGULELESS (LIG) in barley (Becraft et al., 1990; Becraft & Freeling, 1991; Harper &

Freeling, 1996; Kurata et al., 2005; Moreno et al., 1997; Pratchett & Laurie, 1994; Sylvester et al., 1990; Walsh et al., 1998). The maize *lg1*, rice *lg* and barley *lig* loci are colinear and may be orthologous, but there seems to be no *lg1* ortholog in *Arabidopsis* (Reviewed by Piazza et al., 2005). In addition, all gain of function KNOX mutations in maize display transformation of blade tissue identity into sheath. These data implicate the importance of regulated KNOX expression during proximal-distal patterning, although a precise developmental mechanism is unknown. (Becraft & Freeling, 1994; Fowler & Freeling, 1996; Fowler et al., 1996; Freeling & Hake, 1985; Muehlbauer et al., 1997, 1999).

For the elaboration of the medio-lateral axis, the *narrow sheath 1* (*ns1*) and *narrow sheath 2* (*ns2*) genes in maize, and the orthologous *PRESSED FLOWER/ATWOX3* (*PRS/AtWOX3*) gene in *Arabidopsis,* are required for the recruitment of the marginal founder cell domain in the meristem. Loss of NS/PRS function ultimately leads to the deletion of a marginal domain from the leaves and leaf homologous organs (Matsumoto & Okada, 2001; Scanlon, 2000; Scanlon & Freeling, 1997, 1998; Scanlon et al., 1996, 2000). PRS encodes a WUSCHEL-like homeodomain protein (Matsumoto & Okada, 2001), and work presented in Chapter II showed that NS is homologous to PRS/AtWOX3. Moreover, comparative morphogenetic analysis supports a traditional model (Troll, 1995) suggesting that the differing leaf morphology of the eudicot *Arabidopsis* and monocot maize may result from differential contribution of the upper and lower leaf zones during postprimordial stages of leaf development (Troll, 1955; Kaplan, 1973).

How the leaf primordium establishes and maintains the dorsal-ventral axis (i.e. adaxial and abaxial identity) has been studied more extensively. *PHAN* was the first gene found

to affect adaxial-abaxial polarity in *Antirrhinum* (Waites & Hudson, 1995; Waites et al., 1998). Curiously, the PHAN homologues *rs2* in maize and *AS1* in *Arabidopsis* have no such adaxial-abaxial defects (Schneeberger et al.1998; Timmermans et al. 1999; Tsiantis et al. 1999; Byrne et al. 2000), suggesting that PHAN may have acquired a specific function that is not shared by orthologous genes. In *Arabidopsis*, the microRNA regulated *HOMEODOMAIN-LEUCINE ZIPPER III* (*HD-ZIPIII*) genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *INTERFACICULAR FIBERLESS*(*IFL*) /*REVOLUTA* (*REV*) promote adaxial leaf identity through their expression in adaxial domains (Fig.1.2; Emery et al., 2003; McConnell & Barton, 1998; McConnell et al., 2001; Zhong et al., 1997,1999; Zhong &Ye, 1999, 2001, 2004). By contrast, *KANADI* (*KAN*) including *KAN1,2*, and *3*, and *YABBY* (*YAB*) genes such as *FILAMENTOUS FLOWER (FIL), YABBY2 AND YABBY3*, which are GARP and HMG transcription factors respectively, function redundantly to specify leaf abaxial identity (Fig.1.2; Eshed et al., 2001; Kerstetter et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). HD-ZIPIII and KAN may mutually suppress each other (Fig.1.2; Eshed et al., 2001, 2004). HD-ZIPIII function in adaxial specification is conserved in maize as demonstrated (Juarez et al., 2004), and microRNA regulation of *HD-ZIPIII* genes is conserved among all lineages of land plants (Floyed & Bowman 2004). However, *YABBY* genes may function to direct lateral outgrowth of leaf primordia in maize (Juarez et al., 2004), and promote both abaxial and adaxial cell fates through a different developmental pathway in *Antirrinum* (Golz et al., 2004; Navarro et al., 2004). Therefore, HD-ZIPIII and KAN genes may be tightly related to leaf adaxial-abaxial patterning, while YABBY may primarily promote primordial leaf growth (reviewed by Piazza et al., 2005). In addition, AS2 may form a

complex with AS1 in the adaxial domain to suppress KAN expression and promote the differentiation of adaxial cell fate (Fig.1.2; reviewed by Engstrom et al., 2004, Lin et al., 2003).

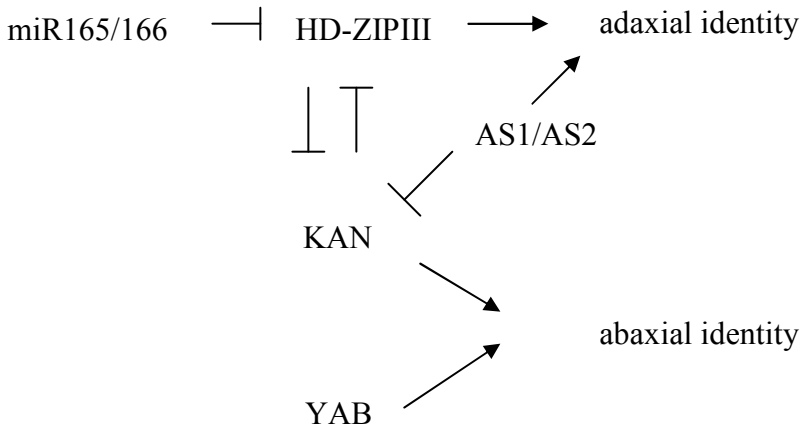

Figure 1.2 Genetic factors invovled in the establishment of leaf adaxial-abaxial polarity in *Arabidopsis*. Positive (arrows) and negative regulators (bars) are shown and described in the text.

Leaf laminar growth requires the proper juxtaposition of adaxial and abaxial compartments (Waites & Hudson, 1995), and is also correlated with cell division regulators and several transcriptional factors including AINTEGUMENTA (ANT), CINCINNATIA (CIN), and JAGGED (JAG) (Dinneny et al., 2004; Hu et al., 2003; Mizukami & Fischer, 2000; Nath et al., 2003; Ohno et al., 2004; Palatnik et al., 2003; Weir et al., 2004). The APETALA2-domain protein ANT functions partly through

promoting *CyclinD3* expression and may be regulated by the auxin inducible gene *Auxin-Responsive Gene Inducing Organ Size* (*AGROS*), which functions downstream of the auxin influx protein AUXIN RESISTANT1 (AXR1) (Hu et al., 2003; Mizukami & Fischer, 2000). CIN is a TCP (TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL FACTOR) transcription factor and may promote leaf marginal tissue sensitivity to cell-cycle arrest signal in *Antirrhinum* (Nath et al., 2003). Similarly, over-expression of *MiR-jaw* in *jaw-D* mutants displays reduced expression of several TCP genes and an uneven leaf surface in *Arabidopsis* (Palatnik et al., 2003). The zinc-finger protein JAG may maintain cells in an active cell-cycle state and promote lateral organ development in *Arabidopsis* (Dinneny et al., 2004; Ohno et al., 2004). Ultimately, the final leaf morphology may be mediated through cell division machinery acting downstream of hormone- and microRNA- dependent pathways.

Root Development

Our recent progress in root development biology largely comes from *Arabidopsis* system. The primary root tissues are generated from the root apical meristem (RAM) and comprise four concentric single-cell layers: epidermis; cortex; endodermis and pericycle; vascular tissues; and root caps (columella and lateral root caps). The RAM is composed of a quiescent center (QC) and organizes the surrounding stem cells, which produce all root tissues. Mature pericycle cells in the basal region of the primary root form lateral root primordia through reactivation of the cell cycle (reviewed by Hardtke, 2006).

The initiation and maintenance of the RAM of the primary root requires auxin and the GRAS family transcription factor SCR (SCARECROW) (Sabatini et al., 1999, 2003).

The highest auxin response in the RAM is regulated through directional auxin transport by PIN (PIN-FORMED) auxin efflux carriers (Blilou et al., 2005; Friml et al., 2002; Petrasek et al., 2006), and requires the activity of AP2 domain transcription factors PLT1 (PLETHORA1) and PLT2 (Aida et al., 2004). PLT transcription is auxin inducible, depends on ARF (AUXIN RESPONSE FACTOR) and Aux/IAA transcription factors, and can feed back to control PIN transcription (Aida et al., 2004; Blilou et al., 2005). Five ARF factors were found to positively regulate primary root development; MONOPTEROS (MP)/ARF5 and NON-PHOTOTROPIC HYPOCOTYL4 (NPH4)/ARF7 are negatively regulated by BODENLOS(BDL)/IAA12 and IAA13, and ARF10, ARF16 and ARF17 are miR160 targets (reviewed by Hardtke, 2006; Hardtke et al. 2004; Mallory et al., 2005; Wang et al., 2005; Weijers et al., 2005). Interestingly, SCR has a role in QC maintenance in addition to promoting asymmetric division in ground tissues (Sabatini et al., 2003). Similar to their role during regulation of SAM cell identity, *CLAVATA-LIKE* (*CLE*) genes such as *CLE19* and *CLE40* are expressed at low abundance in the root and their over-expression negatively regulates RAM activity (Casamitjana-Martinez et al., 2003; Fiers et al., 2004; Hobe et al., 2003). In addition, *WOX5* (*WUSCHEL-LIKE HOMEOBOX*) expression is located in the embryonic QC (Haecker et al., 2004) and a rice *WOX* homologue *QHB* (*QUIESCENT-CENTER-SPECIFIC HOMEOBOX*) is also expressed in the primary and crown root QC (Kamiya et al., 2003). QHB over-expression causes the failure of crown root development (Kamiya et al., 2003), however, the role of the CLE and WOX genes in RAM is not clear up to now.

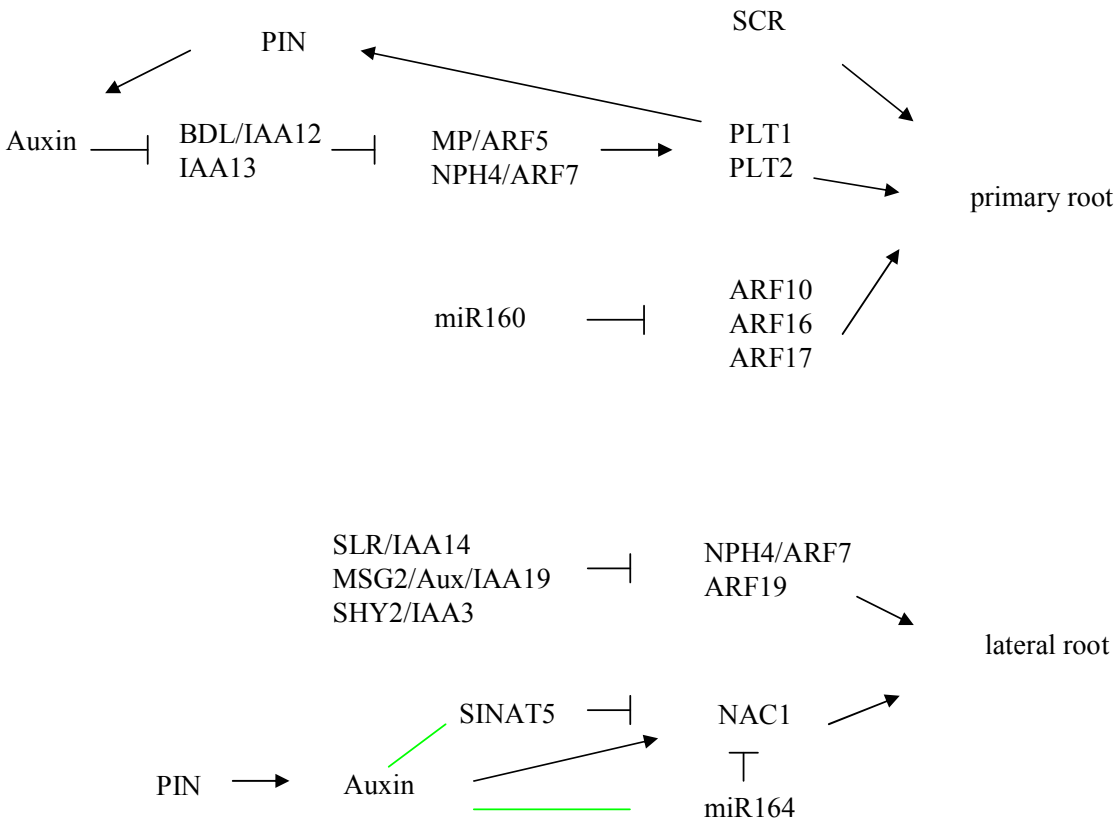

Figure 1.3 Regulators in *Arabidopsis* root development. Positive (arrows) and negative regulators (bars) are shown and described in the text. The green line represents slower induction. Drawing is based on Hardtke, 2006 and Kepinski, 2006.

The initiation of lateral roots is marked by auxin accumulation mediated by PIN family members (Benková et al., 2003), and requires the activity of NAC-domain transcription factor NAC1 (Xie et al., 2000). NAC1 is auxin inducible, and is negatively regulated further by auxin-induced SINAT5 (SINA of Arabidopsis thaliana5) and miR164 (Guo et al., 2005; Mallory et al., 2004; Xie et al., 2002). In addition, AUXIN RESPONSE FACTORS NPH4 (NON-PHOTOTROPIC HYPOCOTYL4)/ARF7 and ARF19 function

redundantly to promote lateral root development (Okushima et al., 2005; Weijers et al., 2005), and might be negatively regulated by SLR (SOLITARY ROOT)/IAA14, MSG2 (MASSUGU2)/Aux/IAA19, and SHY2/IAA3 (Fukaki et al., 2002, 2005; Tatematsu et al., 2004). Together, auxin, ARF proteins, Aux/IAA proteins and microRNAs play key roles in RAM and lateral root development.

Vascular System Development

The vascular system is organized in a continuous network of vascular bundles (strands) (Esau, 1965) that connects all plant organs, and consists of differentiated xylem and phloem tissues and relatively undifferentiated procambial or cambial cells. Vascular patterning begins during embryogenesis and the vascular progenitors (i.e. procambium) differentiate into xylem or phloem in the root, hypocotyl, and cotyledons of young seedlings (Sieburth and Deyholos, 2006). As such, the procambium is commonly referred to as the meristematic tissue, or stem cells, of the vasculature. During subsequent primary growth procambium cells in the root and stem are derived from the progeny of apical meristems, whereas those in lateral organs are initiated in specific positions of young lateral organ primordia (Nieminen et al., 2004; Sieburth and Deyholos, 2006; Ye, 2002). In the radial thickening of gymnosperms and dicots, cambium cells, which originate partly from the procambium within the vascular strands and partly from the parenchyma between the vascular strands, generate secondary vascular tissues (Scarpella and Meijer, 2004).

Vascular patterns are mainly specified by procambium positions (Sieburth and Deyholos, 2006), and polar auxin transport may play a key role in procambial

establishment (Sachs, 1981, 1991). Up to now the transmembrane polar auxin transport protein PIN1 represents the earliest marker for the initials of procambium cells (Scarpella et al., 2006; Scheres B. and Xu J., 2006), and the auxin inducible expression of HD-ZIP gene *AtHD-8* marks the procambium cells in *Arabidopsis* (Baima et al. 1995). Auxin response is also required for the continuous formation of vascular system (Fukuda 2004).

Within the vascular bundles, the procambium differentiates asymmetrically into xylem and phloem tissues. The relative position of xylem versus phloem is closely related to the central-peripheral axis in the stem and the adaxial-abaxial polarity of the lateral organs (reviewed by Carlsbecker and Helariutta, 2005). Correct patterning of vascular bundles in the stem and lateral organs requires HD-ZIPIII family members including PHV,PHB and IFL1/REV, GARP-type KAN genes and AS1/AS2 transcription factors as described above (Emery et al., 2003; Eshed et al. 2004; Juarez et al., 2004; Kerstetter et al., 2001; Lin et al., 2003; McConnell et al., 2001; McHale & Koning 2004; Prigge et al., 2005; Xu et al. 2003; Zhong et al., 1999; Zhong & Ye, 2004). For example, a gain of function mutation in the *IFL1/REV* HD-ZIPIII gene transforms the normal collateral placement of inner xylem and outer phloem into the amphivasal pattern with xylem surrounding phloem (Emery et al., 2003; Zhong et al., 1999; Zhong & Ye, 2004).

Transcription factors APL (ALTERED PHLOEM DEVELOPMENT, a MYB coiled-coil), VND6 and VND7 (VACULAR-RELATED NAC-DOMAIN6 and 7) have been identified to specify vascular cell identity. Specifically, APL is necessary for the differentiation of phloem sieve tube elements and companion cells, and VND6 and VND7 may promote the differentiation of xylem tracheary elements (Bonke et al. 2003; Kubo et al., 2005). In addition, Brassinosteroid signaling may induce xylem proliferation

and simultaneously repress phloem proliferation (Caño-Delgado et al., 2004), while cytokinin may negatively regulate protoxylem specification in *Arabidopsis* roots via negative regulation of the spatial expression domain of AHP6 (ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN6) (Mähönen et al. 2006). ATHB8 and ATHB15/CNA (CORONA) may promote and inhibit xylem proliferation respectively (Kim et al., 2005; Prigge et al., 2005). Furthermore, xylogen may be responsible for the continuity of tracheary element strands (Motose et al., 2001; 2004).

WOX Genes and Plant Development

As described above, WUS is considered as a master regulator of shoot apical meristem development, and functions non-cell autonomously to specify stem cell identity (Gallois et al., 2004; Laux et al., 1996; Mayer et al., 1998). Fourteen WOX (*WUSCHEL RELATED HOMEOBOX*) genes are found in *Arabidopsis* (Haecker et al., 2004); expression patterns of eight members and functional genetic analyses of five members are known. Apical embryo development after fertilization requires the activity of AtWOX2 (Haecker et al., 2004). *PRS/AtWOX3* also functions non-cell autonomously in the PZ of SAM, and plays an important role in the early stage of lateral organ development (Matsumoto & Okada, 2001; Nardmann and Ji, et al., 2004). *AtWOX5* expression marks the identity of the quiescent center, which can be traced back to the hypophyseal cell of the embryo, and suggests that similar developmental mechanisms operate in both the SAM and the RAM (Haecker et al. 2004). PFS2 (PRETTY FEW SEEDS2)/AtWOX6 may regulate ovule patterning, or suppress ovule cell differentiation and thus maintain cell indeterminancy (Park et al., 2004). Furthermore, STP/AtWOX9 is also required for

meristem maintenance and growth in *Arabidopsis* (Wu et al., 2005). As a group, WOX genes are associated with the development of plant meristems, wherein they function non-cell-autonomously to organize various stem cell populations.

Purpose and Outline of this dissertation

Chapter 2 Comparative analysis of WOX3 function

All lateral organs (leaves, flowers and stems) are derived from shoot apical meristem in higher plants, and the leaf is considered to be the default state. Hence, the elucidation of leaf morphogenetic programs is fundamental to our understanding of all plant lateral organ development.

Maize has provided important insights into leaf developmental mechanisms. In maize, founder-cell recruitment begins on one SAM flank, which corresponds to the future midrib region of the leaf, and spreads toward the opposite flank, from where the leaf margins arise (Fig. 2.1A; Poethig, 1984; Poethig and Szymkowiak, 1995). Analyses of many maize leaf mutants have suggested that the leaf is likely composed of developmental domains or compartments and these domains are established through the action of specific genes (Freeling 1992). 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, although the upper portion of the leaf and the leaf length are not changed (Fig. 2.1 D, F; Scanlon et al., 1996). Both vegetative and floral phytomers are affected; whereas no phenotypic alteration is observed in the embryonic coleoptile, shoot meristems or roots (Scanlon and Freeling, 1998). The ns phenotype is a duplicate factor trait conferred by recessive mutations at two unlinked loci, *NS1* and *NS2* (Scanlon et al.,

2000). Heterozygous plants harboring just a single, non-mutant copy of either *NS* gene display the non-mutant phenotype.

If the leaf is really a mosaic of domains, the ns mutant might represent a deleted form of the leaf along the mediolateral axis, from which a domain has been removed. At maturity, ns mutant leaves are extremely narrow and devoid of specific cell and tissue types normally found at the edges of nonmutant maize leaves (Scanlon et al. 1996). Clonal analysis and KNOX immunolocalization revealed that a meristematic domain is not employed in the mutants, which normally forms a large domain including the leaf margins in nonmutant leaves and is patterned in the meristem by *NS* gene activity from two meristematic foci. Both foci could constitute the boundary between the central and lateral domain (Scanlon et al 1996; 1997; 2000; Scanlon 2000). In animals, the boundaries of neighboring compartments serve as organization centers for pattern formation in the development organs such as the *Drosophila* wing. Thus the ns mutant provides an elegant genetic system for investigating the universality of compartmentalization mechanisms across the plant and animal kingdoms, and the functions of meristem.

The molecular identity of NS has long been intriguing. Therefore, Chapter 2 of my dissertation presents cloning and molecular characterization of *ns* genes, as well as a comparative analysis of this conserved gene function in *Arabidopsis*. This work was performed in collaboration with researchers at the University of Köln, Germany.

Chapter 3 Comparative analysis of WOX4 function

Vascular patterns are largely determined by the procambium, meristematic tissue that generates xylem and phloem. (Sieburth and Deyholos, 2006). The genetic control of

procambium identity is not well understood although auxin polar transport and auxin signalling are indispensable to this process (Sachs, 1981; 1991; Scarpella et al., 2006; Scheres B. and Xu J., 2006 (Baima et al. 1995). Previous studies based on transcript profiling in *Arabidopsis* and poplar suggest that the SAM and the cambium are functionally related, and express similar developmental genes (Schrader et al. 2004; Zhao et al., 2005). As described earlier, *WUS* and *STP/AtWOX9* are essential to SAM development (Gallois et al., 2004; Laux et al., 1996; Mayer et al., 1998; Wu et al., 2005), and *NS/PRS/AtWOX3* is expressed in the PZ of SAM and required for normal lateral organ development (Matsumoto & Okada, 2001; Nardmann and Ji, et al., 2004; Scanlon et al 1996; 1997; 2000; Scanlon 2000). We have discovered that WOX4 is expressed in the developing vasculature of tomato, an economically important crop with extensive procambial tissue and bicollateral (xylem between two masses of phloem) vascular bundle organization in stem and leaves. Furthermore, its compound leaves constitute an excellent model for analyses of the relationship between vasculature development and leaf morphology. Finally, comparative analysis with *Arabidopsis,* which has a collateral (phloem toward the exterior and xylem toward the interior) vascular bundle pattern and simple leaf morphology, may generate new insights into vascular development.

To our knowledge, WOX4 has not been characterized in plants; therefore, Chapter 3 of my dissertation presents the cloning and functional analysis of *LeWOX4* in tomato, as well as a comparative analysis of its ortholog *AtWOX4* in *Arabidopsis*. This work was done in collaboration with researchers at the University of California, Davis, and represents the first ever expression and functional analyses of WOX4 in any plant system.

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CHAPTER 2

THE MAIZE DUPLICATE GENES *NARROW SHEATH1* AND *NARROW SHEATH2* ENCODE A CONSERVED HOMEOBOX GENE FUNCTION IN A LATERAL DOMAIN OF SHOOT APICAL MERISTEMS[1]

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[1]Jiabing Ji, Judith Nardmann, Wolfgang Werr, and Michael J. Scanlon. 2004. *Development*. 131 (12): 2827-2839. The material is copyrighted by The Company of Biologists and reprinted with permission of publisher (see APPENDIX B). The contribution from Nardmann J. is noted where applicable.

Abstract

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.

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.2.1). The ns margin deletion phenotype does not extend into the upper portion of the leaf, and

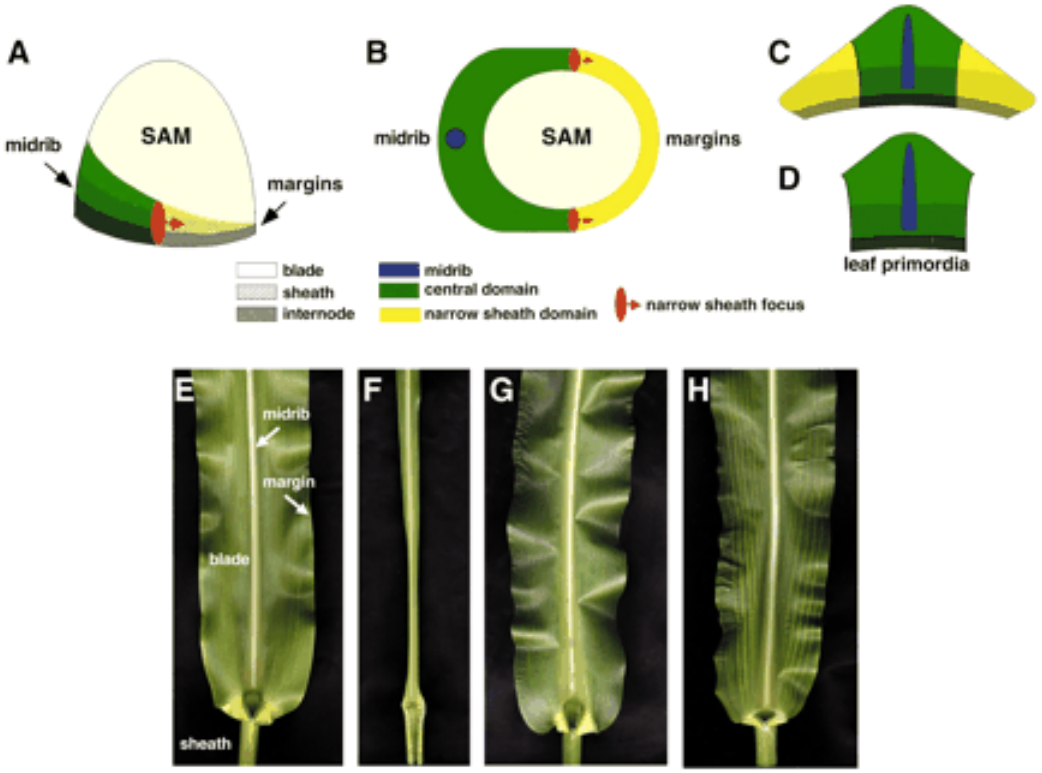

Figure 2.1 The narrow sheath mutant phenotype is a deletion of a lateral compartment that includes the leaf margins. (A) Model depicting the recruitment of maize founder-cells in two distinct compartments, corresponding to the central domain (green) and the ns lateral domain (yellow). (B) Cartoon of 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 narrow sheath mutant (D) and the non-mutant leaf primordium (C). Non-mutant leaves are comprised of 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, 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 narrow sheath mutant phenotype is a

(**Figure 2.1** legend continued) duplicate factor trait, dependent upon mutations at both *narrow sheath1* and *narrrow sheath2.* (E) Mature maize leaves from plants homozygous for non-mutant alleles of *Ns1* and *Ns2;* (F) ns mutant leaves homozygous for mutations at both *ns1* and *ns2;* and non-mutant leaves from plants of the complimentary genotypes *Ns1, ns2* (G), and *ns1, Ns2* (H). SAM = shoot apical meristem.

the leaf length is also unaffected (Fig. 2.1A-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. 2.1G-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. 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. 2.1A-D). The central compartment includes the midrib and distal leaf tip, and does not require NS function for its recruitment (green in Fig. 2.1). 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. 2.1).

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.

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, utilizing degenerate primers (see Supplementary Materials) 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 to the homeodomain of the At2g28610 open reading frame (Fig. 2.2A), 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

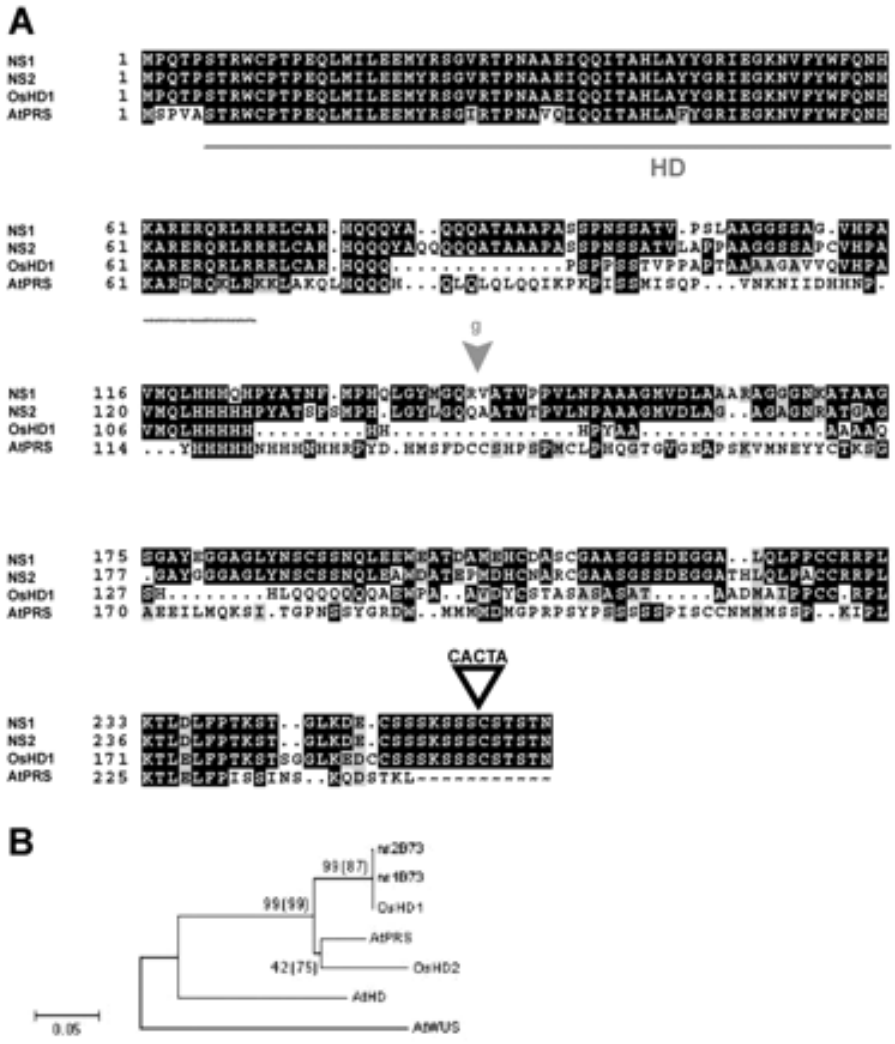

Figure 2.2 (A) NS1 and NS2 protein sequence in comparison to PRS of *Arabidopsis* and the closest rice relative. Note the very high similarity of the homeodomain (underlined) and the high sequence conservation between the maize and rice proteins at the C-terminus. B) Phylogeny of NS/PRS proteins. The 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 neighbor-joining method from a CLUSTALW alignment of the homeodomain. Bootstrap values were calculated from 1,000 replicates. The maximal parsimony method yielded a phylogenetic tree with

(**Figure 2.2** legend continued) identical topology (bootstrap values calculated from 1,000 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). Note: *NS1* was cloned by Nardmann J..

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 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. 2.3A). 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 via 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 *DraI* digested genomic DNA library. Two distinct amplicons were identified; one was linked to the *ns1-R* mutation whereas the second via use of the IBM recombinant inbred population was mapped to the chromosomal vicinity of *ns2*. Primers unique to duplicated *PRS*-homologous maize clones were utilized 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. 2.2A).

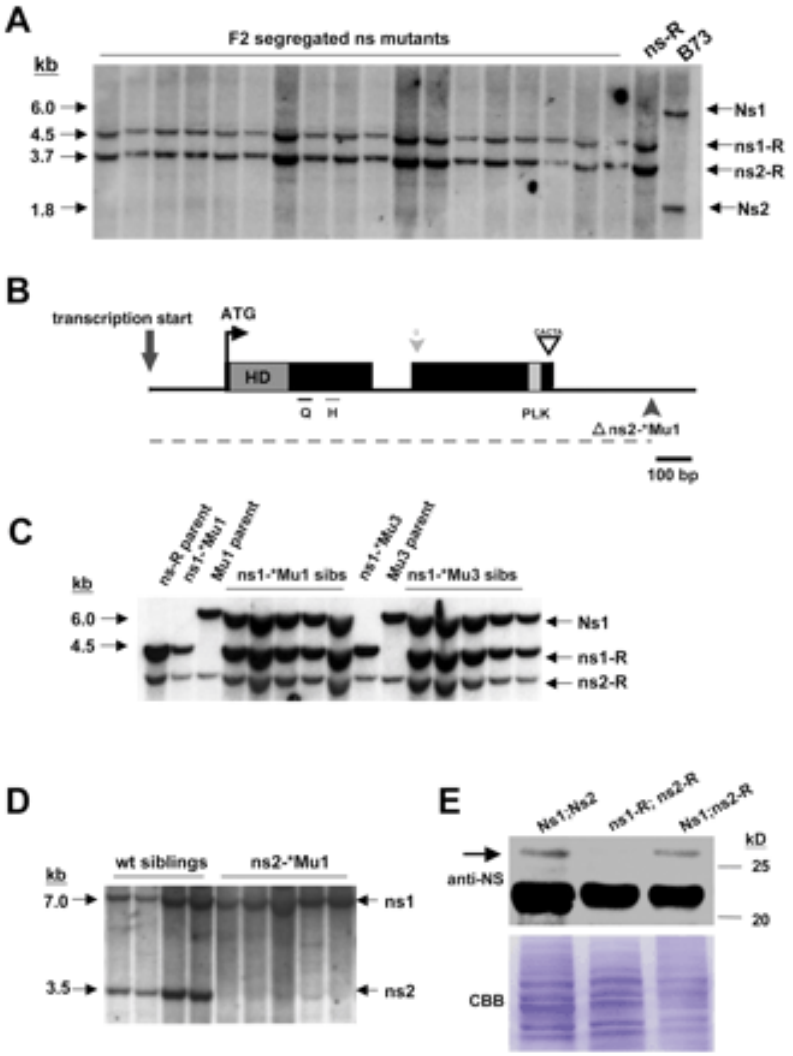

Figure 2.3 Analyses of NS alleles. (A) A hybridization probe (probe1; Materials and Methods) derived from a PRS-homologous, maize genomic clone identifies two distinct *Dra*i restriction fragments linked to the ns mutant phenotype in F2 (ns-R X B73) segregating progeny. Note that no internal *Dra*I restriction 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 is comprised of 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 = introns and untranslated regions. The position of the extra G in *ns2-R* and the

(**Figure 2.3** legend continued) 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-Mu6 or ns1-Mu7) showed lack of the *NS1* wildtype (7 kb) band but presence of the ns1-R fragment (4.8 kb) when hybridized to probe 2 (Materials and Methods), whereas wildtype siblings (ns1-Mu6/7 sibs) always contained both bands (7 kb + 4.8 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 recognizes 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 approximately 29 kDa band is absent in ears from ns mutant plants; and corresponds to the predicted MW of the NS proteins. Note that the NS polyclonal antibody is not specific for NS proteins.

Note: CACTA element insertion in *ns1-R* and an extra G in *ns2-R* were discovered by Nardmann J..

Moreover, a single 124 bp intron is conserved in number and position. Database searches reveal that both predicted proteins bear little overall homology to described plant genes; the highest similarities are detected to 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 PLKTLE/DLFP motif close to the C-terminus (Fig. 2.2A). 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. 2.2B).

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 three *ns2* mutant alleles, as described below.

Compared to 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 (aa 256 Fig. 2.2A, 2.3B). 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 to 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. 2.2A)**.** 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, DNA gel-blot analyses of eight, independent *ns1-*Mu* linked alleles recovered from separate transposon-tagging experiments (Materials and Methods; 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. 2.3C 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 utilized nested primers located 547 bp downstream of the 3' untranslated region in *ns2-*Mu1* homozygous individuals (see Supplementary Materials) 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. After the 226 bp 3' homologous region, however, 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 DNA gel 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. 2.3D). Significantly, the non-mutant *Ns1-Mu* $^{+}$ allele can be amplified from nonmutant 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 utilized in immunoblot assays of proteins extracted from maize tissues (Fig. 2.3E). 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). In contrast, no immuno-reactive protein of this predicted molecular weight is detected in mature, non-mutant seedling leaves (data not shown) nor in immature ears of the genotype *ns1-R; ns2-R* (Fig. 2.3E). 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 apparent molecular weight that is dissimilar to the predicted NS protein (lower band in Fig. 2.3E). Although the polyclonal antiserum therefore is not suitable for use in imunohistolocalization 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 beg the question as to 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 utilized 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. 2.4). 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 emerging of the coleoptile, which forms a collar of tissue that ultimately encloses the shoot meristem (Fig. 2.4A). In transverse sections through the coleoptilar-staged embryo, *NS* transcripts are first detected in the lateral margins of the emerging coleoptile (Fig. 2.4B). *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 or in the pre-coleoptilar staged SAM.

Maize leaves exhibit alternate phyllotaxy, such that successive primordia initiate from the SAM approximately 180 degrees apart and in two ranks (Fig. 2.4C). Fate mapping analyses demonstrate that founder cells that form the eventual midrib of maize leave 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. 2.4D; 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

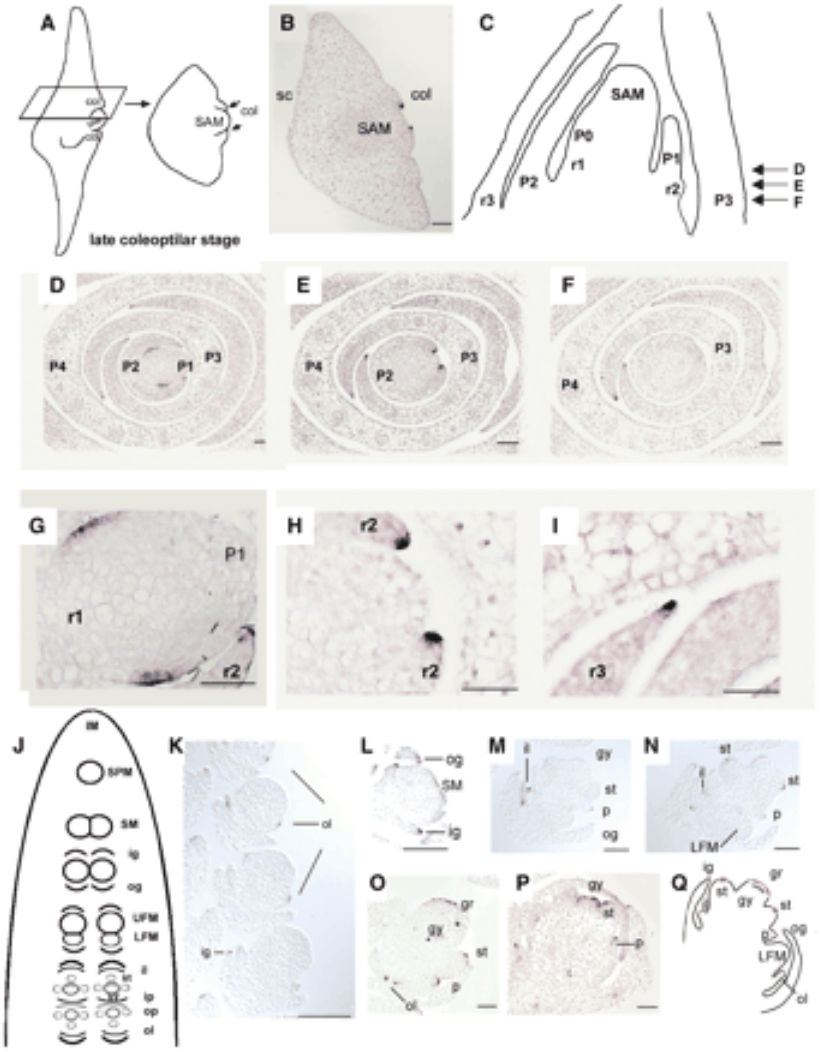

Figure 2.4 *ns* transcripts predominately accumulate in tissues enriched for shoot meristems and young primordia. (A) Schematic drawing of coleoptile development. The coleoptile (col) emerges from the periphery of the SAM and grows to form a coleoptilar ring that eventually encloses the SAM. The frame 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)

(**Figure 2.4** legend continued) 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.

Note: this figure was fully contributed by Nardmann J..

(Fig. 2.4E-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. 2.4K-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. 2.4K. In summary, the inflorescence meristem initiates spikelet-pair 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 is comprised of leaf-like organs (glumes, lemma and palea), three stamens and the gynoecium. During the formation of monoecious maze 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. 2.4L-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 utilized gene-specific primers (see Table 2.1) to investigate quantitative expression patterns of the *ns* duplicate genes during development (Fig. 2.5). 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* transcript 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. 2.5). In addition, no significant difference in nonmutant *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 orthologue in *Arabidopsis thaliana*

The first *narrow sheath* 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. 2.6 B-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. 2.6A and E and data not shown). Likewise, whole mount and scanning electron microscopic analyses

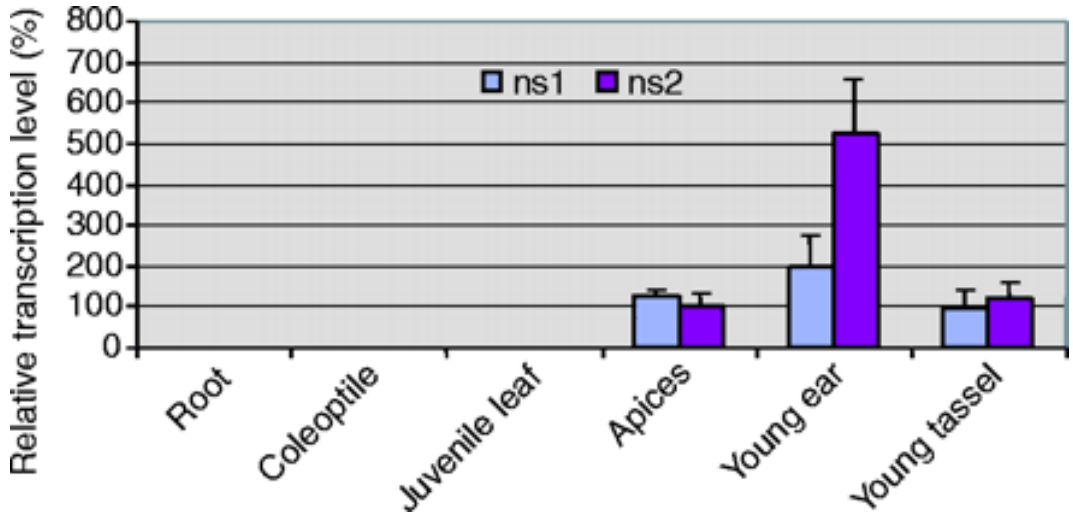

Figure 2.5 Quantitative real time RT-PCR data of transcript abundance (relative to the level of *ns2* transcript in vegetative apices and normalized to ubiquitin transcript levels) in maize tissues employing primers specific for *ns1* or *ns2* transcripts (see Materials and Methods). NS transcripts accumulate 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.

of *prs* mutant cotyledons reveal no distinguishable phenotype in the blade or petiole (Fig. 2.6I-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. 2.6K-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 2.1, *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.

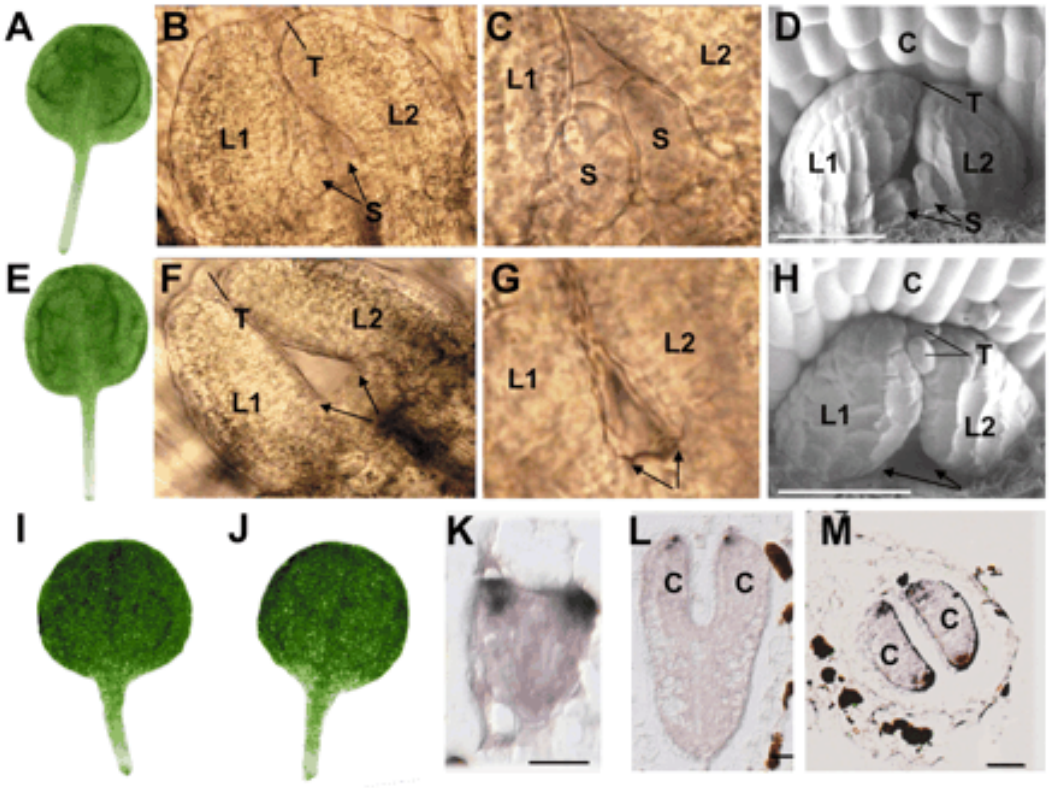

Figure 2.6 Phenotypes of *prs* mutant *Arabidopsis* leaves and cotyledons. At maturity non-mutant (A) and *prs* mutant (E) rosette leaves have equivalent phenotypes. Whole mount (B-C; F-G) and scanning electron microscopic (D, H) analyses of non-mutant (B-D) and *prs* mutant (F-H) leaf primordia (L1, 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 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. *PRS* expressing cell layers therefore define a border between adaxial

(**Figure 2.6** legend continued) and abaxial side of the cotyledons, similar to the maize coleoptile (Fig. 4B). All scale bars = 50 μm.

Note: Figure 2.6 K-M was done by Nardmann J.

Table 2.1 Floral organs in pressed flower mutants

Floral organ [a]	Organ number (mean)[b]	Vestigial organs [c]
Sepals (4)	2.41 ± 0.62	14::58
Petals (4)	3.79 ± 0.49	6::58
Stamens (6)	4.54 ± 0.51	7::58

[a] The normal number of organs in non-mutant Arabidopsis is given in parentheses

[b] The mean number of sepals, petals and stamens contained within 58 individual flowers of *prs* mutant homozygous plants.

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

DISCUSSION

The *narrow sheath1* and *narrow sheath2* mutations are null alleles in duplicated maize genes

The *narrow sheath1* and *narrow sheath2* 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. 2.2A). Immunoblot analyses reveal that *ns-R* mutant inflorescences accumulate virtually no NS protein (Fig. 2.3D), 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. 2.4), although *ns2* transcripts are more prevalent in reproductive tissues (Fig. 2.5). However, morphogenetic analyses illustrate the absence of *ns* mutant phenotypes in plants that are mutated at one *ns* locus, but non-mutant for the duplicate gene (Fig. 2.1E-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 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.

narrow sheath **and** ***pressed flower*** **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. 2.1; Scanlon et al, 1996). In contrast, the leaf phenotype of the orthologous *Arabidopsis* mutant *prs* is extremely subtle, and limited to the deletion of the proximal, lateral stipules (Fig. 2.6). 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. dorsiventrally flattened) eudicot leaves are subdivided into a large upper leaf zone comprised of the lamina and petiole, and a greatly diminished lower leaf zone comprised of the leaf base and the lateral stipules (Fig. 2.7A; Troll, 1955; Kaplan, 1973). In 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

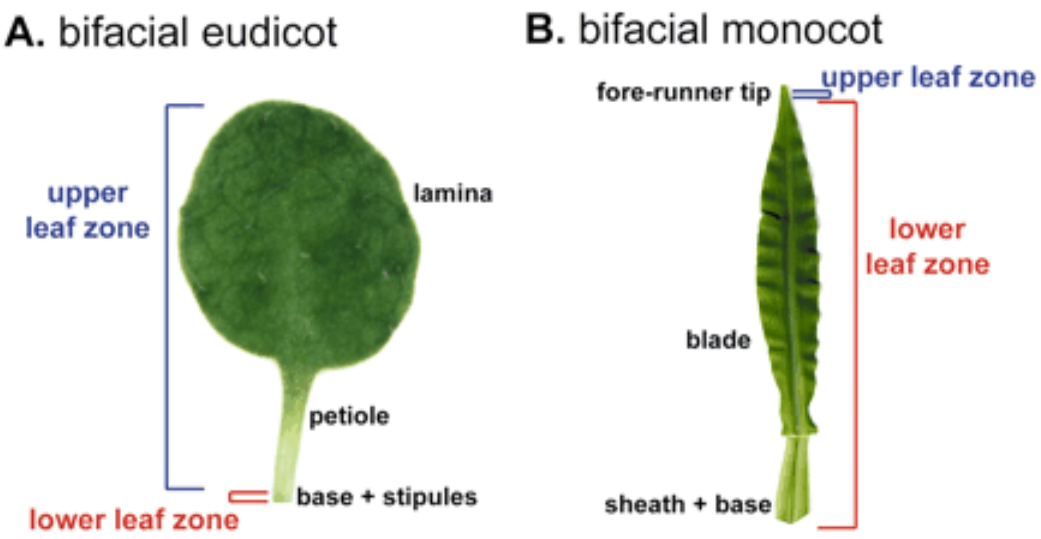

Figure 2.7 The domain deletions observed in *prs* mutant and ns mutant leaves are consistent with a model describing the differential elaboration of upper versus lower leaf zones during the morphological evolution of monocot and eudicot bifacial leaves. Details are provided in the text; models are adapted from Troll (1955) as elaborated in Kaplan (1973).

monocot maize is comprised of 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. 2.7B). 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 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 comprised 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, yet 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 argue that the coleoptile originates laterally from scutellum tissue. Although no ns/prs phenotype is observed in the maize coleoptile (Scanlon et al., 1998) or the *Arabidopsis* cotyledons (Matsumoto and Okada, 2001; Fig. 2.6), the conserved marginal pattern of *NS1/PRS* expression (Fig. 2.4; Fig. 2.6) suggests that homologous morphogenetic programs are shared in the coleoptile and cotyledon. In 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 timepoints 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. 2.4; Matsumoto et al, 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. 2.4E, 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. 2.6K-M). No *prs* mutant phenotypes are detected in these lateral organ domains (Fig. 2.6 I-J; Matsumoto and Okada, 2001). Likewise, *NS* is expressed in the edges of the developing coleoptile and leaf primordia (Fig. 2.4B; 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 vegetative and/or reproductive shoot apical meristems.

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. 2.8A). *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. In contrast, the *ns* mutant phenotype could result if cells at the P0 face, which in wildtype are recruited for the basal lateral leaf domain (mainly sheath) of the P1 primordium, mutually exclusively are recruited for the next primordium (P0) in absence of *NS* activity (see Fig. 2.8B). In 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 wildtype *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.

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, since 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 were explained by a model in

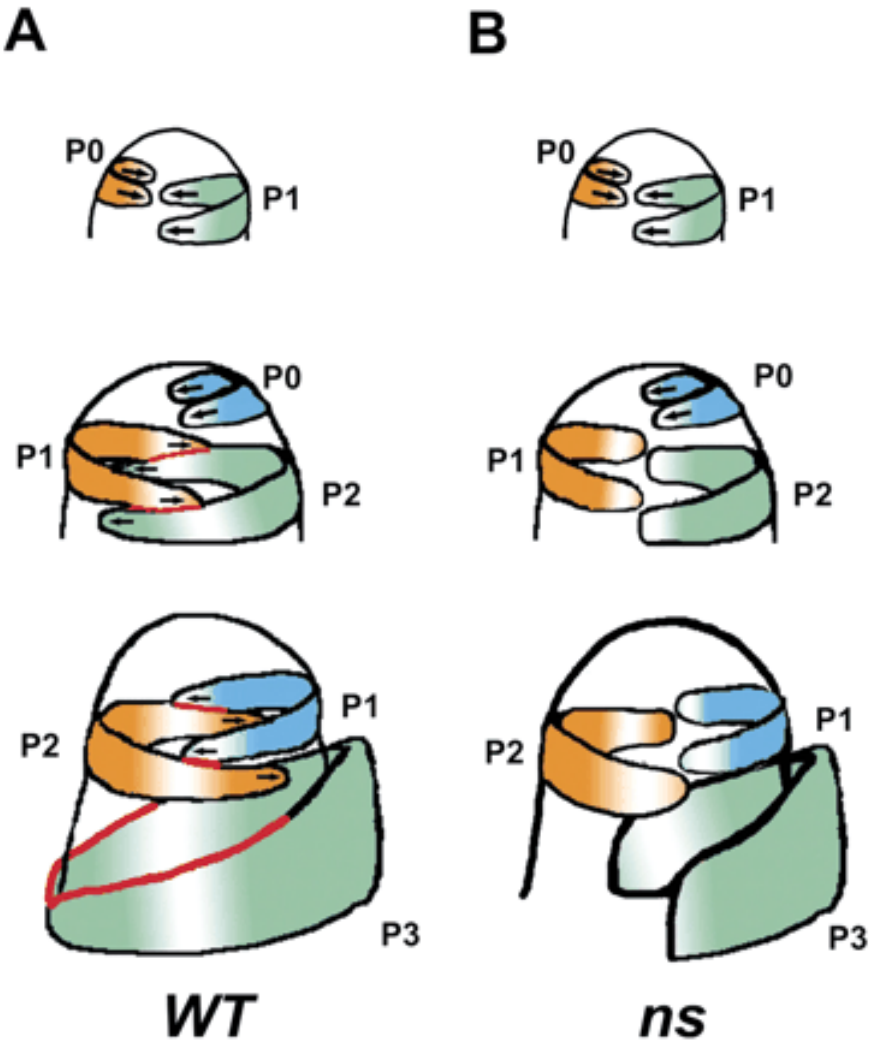

Figure 2.8 (A) NS activity (red line) in the maize shoot apex and at the margins of emerging leaf primordia (P0-P3) in wildtype, and (B) model of competing founder cell domains in ns mutants.

Note: this figure was fully contributed by Nardmann J..

which *NS* functions in a specific lateral domain of the SAM to initialize meristematic cells to become organ founder-cells. This model suggests that founder cells are recruited from the central and lateral meristematic domains via different gene functions. Whereas as yet undescribed function(s) recruit founder cells from the central domain (green in Fig. 2.1; Fig. 2.9), *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 utilized 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 initializes 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. 2.9B). Consequently, the lateral extent of the founder-cell domain recruited by PRS function is relatively 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. 2.9B). 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.

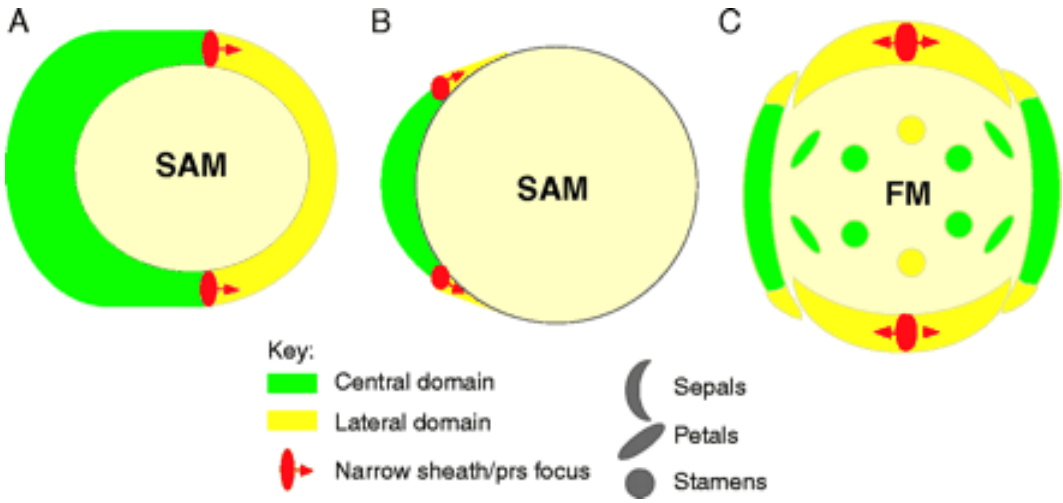

Figure 2.9 Model for a conserved NS/PRS function during recruitment of a lateral founder-cell domain in vegetative (A-B) and reproductive (C) shoot meristems of maize (A) and *Arabidopsis* (B-C). NS/PRS function initiates from lateral foci (red arrows) and recruits founder-cells in a lateral meristematic domain shown in yellow. Recruitment of the central domain founder cells shown in 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.

2.9C). 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, since 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 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, rendering the deletion of lateral sepals and stamens. These observations support a model whereby *NS/PRS* function is conserved to initialize 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. 2.9C). 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 function during the evolution of diverse angiosperm leaf morphology.

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, Intl, Johnston IA. *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* that were derived from *Mutator* transposon stocks were generated using a directed transposon tagging strategy as described in Scanlon et al., (2000). To summarize this strategy, ns-R mutant male plants homozygous for the reference alleles *ns1-R* and *ns2-R* were crossed to non-mutant female plants from *Mutator* active backgrounds into which the *ns2-R* allele had been introgressed. The genotypes of the ns mutant progeny were therefore *ns1-R/ns1-Mu;ns2-R/ns2-R.* The five independent alleles of *ns2* derived from *Mutator* transposon stocks were generated using a similar transposon tagging strategy, in which male plants homozygous for the reference alleles *ns1-R* and *ns2R* were crossed to non-mutant female plants from *Mutator* active backgrounds homozygous for *ns1-R*. RFLP analyses utilizing *ns2*-linked maize clones contained within a paracentric inversion that includes *ns2-R*, indicated that these independently derived progenitor-tagging stocks were also homozygous for the non-mutant *Mutator* allele *Ns2-Mu*$^{+}$. These parental genotypes were also inferred by the appearance of ns mutant plants in the M1 tagging generations in ratios of less than 1/5,000 plants. Ultimately, analyses with *ns1* and *ns2* clones verified the genotypes of all

Mu tagging parental lines. Thus, all thirteen independently derived *ns*-**Mu* alleles arose *de novo* in single gametes generated by the respective maternal, *Mutator* tagging parents.

Cloning of *narrow sheath* genomic DNA and cDNAs

To amplify the homeodomain of the *ns1* gene (AC AJ536578), PCR was performed on genomic DNA of *Rscm2* maize using the primer pair ZmHD1 and ZmHD2 (Table 2.2). Rapid amplification of 5´cDNA ends was then performed on RNA of *Rscm2* coleoptilar stage embryos with primers ZmPRSa and ZmPRSb using the FirstChoice ™ RLM-RACE Kit (Ambion) according to the manufacturer´s protocol; 3´-RACE was performed with primers ZmPRSc and ZmPRSd. The complete genomic sequence of the *ns1* gene was obtained with multiple gene-specific primers (ZmPRS1-ZmPRS4; Supplementary Table) using the Universal Genome Walker ™ Kit (Clontech). All PCR products were cloned into pCRII TOPO (Invitrogen) and sequenced. The ZmPRS clone was mapped to chromosome 2, between markers umc1003 (zpu1) and umc1065 (pbf1) using the DNA Kit of 94 IBM Lines (http://www.maizemap.org/dna_kits.htm), very close to *ns1* (Scanlon et al., 2000).

In order to clone *ns2* (GenBank AC AY472082) by homology to *ns1*, nested primers (Supplementary Table) were designed from the conserved homeobox region of *ns1* and used to identify homologous PCR products from a *DraI* digested genomic DNA library prepared from ns mutant leaf using the Universal Genome Walker™ Kit (Clontech). The *ns* primers used were ns2F1 and ns2F2 (Supplementary Table) and two PCR products were obtained. One product was identical to the *ns1-R* allele and the second product was later mapped to *ns2* (below and RESULTS). Additional Genome Walker™ reactions were performed in order to obtain the complete 5' and 3' regions of

Table 2.2. Oligonucleotide primers used in this study

name	primer sequence	primer use
ZmHD1	TGGTG(C/T)CC(I)AC(I)CC(I)GA(G/A)CA GC(C/T)TT(G/A)TG(G/A)TT(C/T)TG(G/A)AACC	genomic PCR
ZmHD2	A(G/A)TA(G/A)AA	genomic PCR
ZmPRSa	GTTCTTGCCCTCGATGCGG	5' RACE
ZmPRSb	CTGCTGGATCTCCGCCGCGTTG	5" RACE
ZmPRSc	CCTAGGGTAGGCGTTGAATC	3" RACE
ZmPRSd	GAGCGCTAGCAGTAGCAATAG	3" RACE
ZmPRs1	GAACCGCAAAAGGCATAGTCAC	Genome Walker
ZmPRS2	CAGGTAGCGTCACACACAAAC	Genome Walker
ZmPRS3	GTACATCTCCTCCAGGATCATCAGCTG	Genome Walker
ZmPRS4	CTATTGCTACTGCTAGCGCTCGGATTC	Genome Walker
ns2F1	CAACGCGGCGGAGATCCAGCAG	3' Genome Walker
ns2F2	CTAACAGCAGCGCCACCCTTC	3' Genome Walker
ns2F3	GGGTACATGTCATGAAAAACAATGAAAGCG	3' Genome Walker
ns2R1	GCCCCAAGTAGCCCTAATAATAATCAAGTG	5' Genome Walker
ns2R2	GCTCCTGATCATAGTTTTTGGCGGTAGTT	5' Genome Walker
ns2R3	ACTCAAACTTGTTGCGCTCATTTCCT	5 Genome Walker
ns2F2	CTAACAGCAGCGCCACCCTTC	3' RACE
ns2F5	AGGTTGCCTGTTAGCTCATGATA	3' RACE
ns2F6	GCTCATGATAGACTGCTGATTGA	3' RACE
ns2R4	CACAATACAACGCAACGCACCGATATAA	5' RACE
ns2R5	CACTTGAGACGATGACCGAATCCAC	5' RACE
ns2R6	CGATCGACTGACGATGGATTCAACAC	5' RACE
ns2R7	GAAGCTTAGCTGTGTGTGCGCTGACT	5' RACE
ns1F	ATGGAGGTGGAGCTGGGTTA	Real time PCR
ns1R	cacagatCAGTGCTCCATTGCATCTGtG	Real time PCR
ns2F	cacacttATTCGGTCATCGTCTCAAGTGtG	Real time PCR
ns2R	AATACAACGCACCGCAACGA	Real time PCR
probe1F	CTAACAGCAGCGCCACCGT	genomic PCR of probe 1
probe1R	AGCTGCCCGATGCCG3	genomic PCR of probe 1
[a]ubiqF	gacggCCTCCACCTTGTGCTCCGtC	Real time PCR
[a]ubiqR	GGAGAGAGGGCACCAGACGA	Real time PCR
ns2gF7	TTGCTCCACATCCACTAACTAA	Genomic DNA contamination
ns2gR8	GACTGATTTTCACGCTTT	Genomic DNA contamination

the *ns2* alleles from inbred B73, the ns-R mutant and the ns2-*Mu1 mutant.

Full length cDNA copies of *ns2* transcripts (GenBank it584557) were obtained by Rapid Amplification of cDNA Ends (RACE), performed according to recommended protocols (GeneRacer™ Kit, Invitrogen, Life technology) using four gene-specific primers (ns2R4, ns2R5, ns2R6, ns2R7; Supplementary Table). In these assays, starting total RNA was isolated from B73 and *ns-R* apical tissues (maize vegetative apices containing P5 and lower primordia plus the shoot apical meristem). The results obtained with Genome Walker™ and GeneRacer™ were confirmed by standard genomic PCR and RT-PCR using *ns2*-specific primers. All PCR products were cloned into pCRII or pCR4 vectors (Invitrogen, Life Technology) using standard procedures, and were sequenced with Bigdye Terminator version 3.1 Cycle Sequencing Kit™ (Applied Biosystems). Consensus sequences for the *ns2* full-length cDNA and genomic DNA clones were obtained using software found at Multalin™ (http://prodes.toulouse.inra.fr/multalin/multalin.html).

Computational and database analysis

The multiple alignment was performed using CLUSTAL W (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 are 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 are as follows. Probe 1 is a 479 bp 5' genome walker product (upstream of primer ZmPRSb, Supplementary Table) that hybridizes to both *ns* loci. Probe 2 is a 792 bp genomic PCR product (between primers ZmPRSc and ZmPRS2, Supplementary Table) that also includes the 5' UTR of *ns1*.

Antibody production, protein extraction, and immunoblot analyses

Affinity purified NS polyclonal antibodies were generated (Biosource International) against a synthetic peptide comprised of 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 terminal. 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 procedures described in Fu et al. (2002). Approximately 30 µg of soluble protein were used in Western gel blot analyses; protein gel electrophoresis, transfer, and Coomassie Brilliant Blue staining were performed as described (Sambrook et al., 1989). Immunoblot analyses were performed as 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 nonradioactive in situ hybridization, samples were prepared following the protocol of 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 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 sense or antisense orientation to the T7 promotor were used as a template for synthesis of Digoxygenin-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 (2001).

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

Total RNA was extracted from nonmutant 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 subsequently used to prepare first strand cDNA as described in 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 that is within the transcribed region of the gene, and a second

primer, ns2R8, which is from the 3' untranscribed region (Supplementary Table). Real time RT-PCR amplification was performed in a volume of 25 µl using 100ng of cDNA template, 0.2mM of each dNTP, 3mM $MgCl_2$, 250nM of each primer, and 1U of Platinum *Taq* ™ DNA Polymerase (Invitrogen, Life technology). The cycling program was 95 °C, for 2 min and 45 cycles of 95°C, 10s; 57°C, 10s; 72°C, 15s; using a Cepheid Smart Cycler ™. The *LUX*™ primers were designed online (www.invitrogen.com/LUX) and are contained in the Supplementary Table.

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 (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 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) CCD camera.

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CHAPTER 3

COMPARATIVE GENETIC ANALYSES OF WOX4 FUNCTION IN *ARABIDOPSIS* AND TOMATO[1]

[1]Jiabing Ji and Michael J. Scanlon. To be submitted to *The Plant Cell*.

Abstract

Plant shoot lateral organs and vascular tissues arise from founder cells, stem cells that are recruited from meristematic tissues. Previous studies have shown that the members of the WUSCHEL related homeobox (WOX) gene family function to organize various stem cell populations throughout plant development. A *WOX* gene (termed *LeWOX4*) was cloned from tomato that encodes a putative transcription factor of 242 amino acids. Conservation of gene structure and phylogenetic analysis of the homeodomain region suggests that *LeWox4* is orthologous to the *Arabidopsis* gene *WOX4*, whose function is previously undescribed in any species. RT-PCR and *in situ* hybridizations reveal that WOX4 is expressed in the stem cells of the vascular procambium in both *Arabidopsis* and tomato, although these species differ in the arrangement of vascular bundles. Specifically, *Arabidopsis* exhibits collateral vascular bundles (xylem adaxial to phloem), whereas tomato contains bicollateral bundles in which xylem is sandwiched between two patches of phloem. Reverse genetic approaches (RNAi and constituitive overexpression) were used in comparative analyses of the function of the previously uncharacterized *WOX4* homologs during the development of the morphologically distinct shoot lateral organs of *Arabidopsis* and tomato. The response of *WOX4* transcription to various plant hormones is investigated, and the effect of *WOX4* overexpression on accumulation of genes required for vascular morphogenesis and leaf development are presented. These cumulative data suggest that WOX4 may function to promote procambium activity, leaf complexity and lateral organ development in tomato and *Arabidopsis*.

Introduction

The vascular system provides mechanical support for the plant body, transports water, solutes and signaling molecules throughout the plant protoplasm, and may direct plant architecture (Kim et al., 2005; Scarpella and Meijer, 2004; Sieburth and Deyholos, 2006). Organized in a continuous network of vascular bundles (strands) (Esau, 1965) that connects all plant organs, the vasculature consists of differentiated xylem and phloem tissues that are derived from relatively undifferentiated procambial or cambial meristematic cells.

Organization of vascular tissues occurs at two levels. At the organ level, the protostele (in which phloem surrounds the solid xylem at the stele center) and siphonostele (in which individual vascular bundles are formed as a ring in the stele or scattered throughout the ground tissue) patterns can be recognized in primary stems and roots (Ye, 2002), while reticulate and striate (parallel) patterns are typically found in leaf vascular systems (veins) (Scarpella and Meijer, 2004). At the individual vascular bundle level, the most common is the collateral pattern (phloem toward the exterior and xylem toward the interior), followed by the bicollateral pattern (xylem between two masses of phloem) and the less common amphivasal (phloem completely surrounded by xylem) and amphicribral patterns (xylem completely surrounded by phloem) (Ye, 2002).

Vascular patterning begins during embryogenesis and the vascular progenitors, called procambium, differentiate into xylem or phloem in the root, hypocotyl, and cotyledons of young seedlings (Sieburth and Deyholos, 2006). During subsequent primary growth, procambium cells in the root and stem are derived from the progeny of apical meristems, and those in lateral organs like leaves are formed de novo in young primordia (Nieminen

et al., 2004; Sieburth and Deyholos, 2006; Ye, 2002). Secondary vasculature is responsible for the radial thickening of plant tissues, and is formed from cambium cells that originate from both the procambium within the vascular strands and from the parenchyma between the vascular strands (Scarpella and Meijer, 2004).

Vascular patterns are specified mainly by procambium position (Sieburth and Deyholos, 2006), and polar auxin transport may play a key role in procambial development (Sachs, 1981, 1991). The transmembrane polar auxin transport protein PIN1 may represent the earliest marker for the procambial initials (Scarpella et al., 2006; Scheres B. and Xu J., 2006), and the auxin inducible expression of the HD-ZIP gene *AtHD-8* marks the procambium cells in *Arabidopsis* (Baima et al. 1995). Auxin response is also required for the continuous formation of vascular system (Fukuda 2004).

Within the vascular bundles, the procambium differentiates asymmetrically into xylem and phloem tissues. The relative position of xylem versus phloem is closely correlated to the central-peripheral axis in the stem and the adaxial-abaxial polarity of lateral organs (reviewed by Carlsbecker and Helariutta, 2005). Correct patterning of vascular bundles in the stem and lateral organs requires HD-ZIPIII family members including PHV,PHB and IFL1/REV, GARP-type KAN genes and AS1/AS2 transcription factors (Emery et al., 2003; Eshed et al. 2004; Juarez et al., 2004; Kerstetter et al., 2001; Lin et al., 2003; McConnell et al., 2001; McHale & Koning 2004; Prigge et al., 2005; Xu et al. 2003; Zhong et al., 1999; Zhong & Ye, 1999, 2004). For example, a gain of function mutation in the *IFL1/REV* HD-ZIPIII gene transforms the normal collateral placement of inner xylem and outer phloem into the amphivasal pattern with xylem surrounding phloem (Emery et al., 2003; Zhong et al., 1999; Zhong & Ye, 2004).

The transcription factors APL (ALTERED PHLOEM DEVELOPMENT, a MYB coiled-coil protein), VND6 and VND7 (VACULAR-RELATED NAC-DOMAIN6 and 7) specify vascular cell identity. Specifically, APL is necessary for the differentiation of phloem sieve tube elements and companion cells, and VND6 and VND7 may promote the differentiation of xylem tracheary elements (Bonke et al. 2003; Kubo et al., 2005). In addition, Brassinosteroid signaling may induce xylem proliferation and simultaneously repress phloem proliferation (Caño-Delgado et al., 2004), while cytokinin may negatively regulate protoxylem specification in *Arabidopsis* roots via negative regulation of the spatial expression domain of AHP6 (ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN6) (Mähönen et al. 2006). ATHB8 and ATHB15/CNA (CORONA) may promote and inhibit xylem proliferation respectively (Kim et al., 2005; Prigge et al., 2005). Furthermore, xylogen (a secreted arabinogalactan protein) may be responsible for the continuity of tracheary element strands (Motose et al., 2001; 2004).

Transcript profiling suggests that the SAM and cambium are functionally related meristematic tissues. We are interested in comparative analyses of the role of *WOX* genes during plant meristem function. Here we report the cloning of *WOX4* orthologs from *Arabidopsis* and tomato, plant species that differ markedly in leaf complexity and vasculature anatomy. Specifically, *Arabidopsis* leaves are simple and tomato leaves are compound, whereas *Arabidopsis* vasculature is collateral and tomato veins are bicollateral. Comparative functional analyses of the previously uncharacterized *WOX4* genes reveal a role during procambial patterning and lateral organ development. In

addition, the response of *WOX4* transcription to various plant growth regulators, and interactions between *WOX4* and other vascular or leaf patterning genes are investigated.

Results and Discussion

Cloning of *LeWOX4*

To further our understanding of the functional evolution of *WOX* genes in shoot development, we first isolated *WOX* genes from the tomato genome. Degenerate genomic PCR and RT-PCR were performed to specifically amplify the conserved homeobox-containing sequences of *WOX* (*WUSCHEL related homeobox*) from the tomato cultivar Ailsa Craig. Phylogenetic analysis with all WOX genes in *Arabidopsis* (Haecker et al. 2004) showed that one of the PCR products exhibited high sequence similarity to the homeobox region of *AtWOX4* gene of *Arabidopsis thaliana* (Fig. 3.1B), which to date has not been genetically characterized. Based on these sequence alignments, this *WOX* gene was designated as *LeWOX4*. Geneomic DNA and cDNA sequences upstream and downstream from the homeobox were obtained via Genome Walker and RACE, respectively. The putative full-length cDNA of *LeWOX4* is 1069 bp, and contains a 5' UTR of 22 bp and a 3' UTR of 318 bp. *LeWOX4* is predicted to encode a protein of 242 amino acids. Alignment of the *LeWOX4* cDNA and genomic DNA sequences suggests the presence of two introns, as shown in Fig. 3.1A. Southern hybridization of genomic DNA with a probe from *LeWOX4* indicated that *LeWOX4* is most likely a single copy gene in tomato (Fig. 3.2).

Likewise, RT-PCR, and 5' and 3' RACE were employed to obtain *AtWOX4* cDNA from shoot apex-enriched tissues of *Arabidopsis thaliana* ecotype Columbia. *AtWOX4*

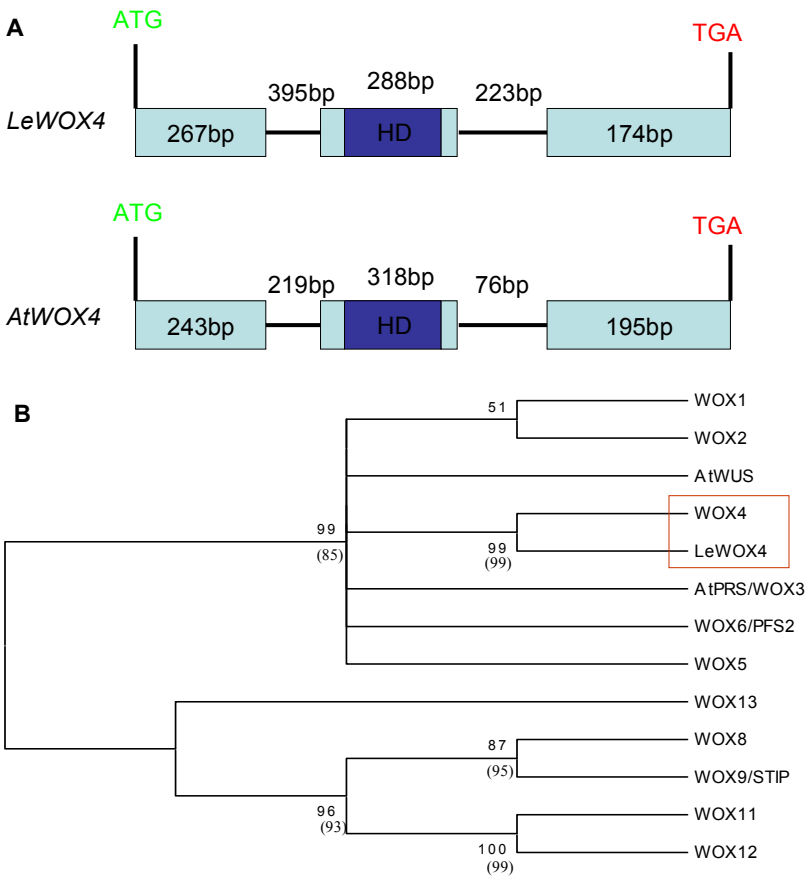


Figure 3.1 Genic structure, phylogeny and sequence comparison of *LeWOX4* and *AtWOX4*. (A) Genic structure of WOX4. Exons are boxed and introns solid lined. The size of individual exons and introns is shown in the box or above the line. Note that UTR is not included. (B) Phylogeny of WOX proteins. LeWOX4 is more similar to WOX4 than to other WOX proteins from *Arabidopsis*. The unrooted tree was constructed using

(Figure 3.1 legend continued) Neighbor-Joining method from a CLUSTALW alignment of the homeodomain with bootstrap values calculated from 1000 replicates. The Maximal Parsimony method generated an identical phylogenetic tree and bootstrap values calculated from 1000 replicates are indicated in parentheses. (C) LeWOX4 and AtWOX4 protein sequence comparison. Note the sequence conservation at the N and C termini besides the internal homeodomain.

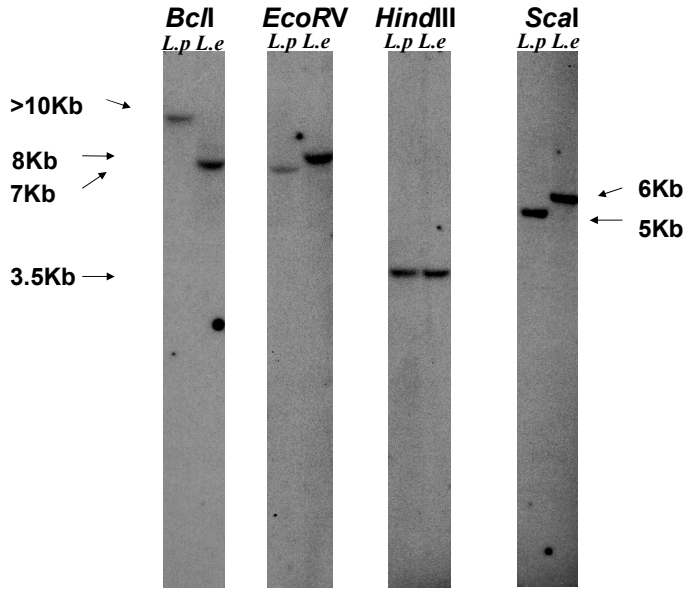


Figure 3.2 Tomato genomic DNA gel blot hybridization with *LeWOX4*. A hybridization probe of 262 bp derived from the 5' end of *LeWOX4* ORF identifies only one restriction fragment in *Lycopersicon pennellii* cv.TA56 and *Lycopersicon esculentum* cv.TA209 genomic DNA digested with various restriction enzymes.

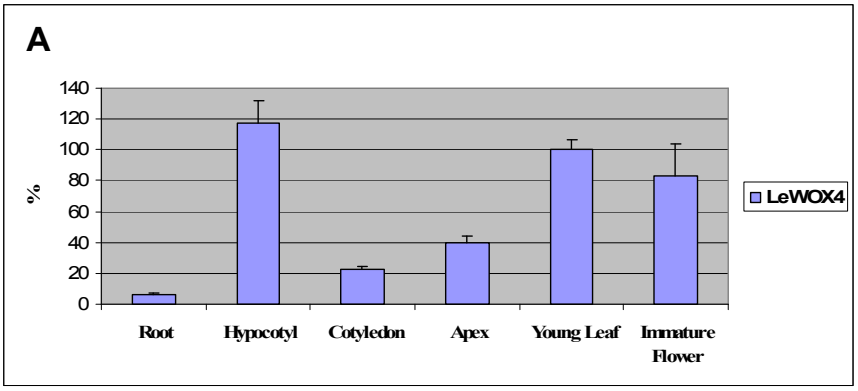
cDNA is 1014 bp, and its 5' and 3' UTR are 33 and 225 bp respectively. The single copy *AtWOX4* gene is predicted to encode a 251 amino acid protein and also contains two introns, as described above for *LeWOX4* (Fig. 3.1A).

Mapping of *LeWOX4*

In order to identify any candidate tomato mutations that are linked to *LeWOX4,* the gene was genetically mapped by standard techniques. A mapping population comprising 50 introgression lines (Eshed et al., 1992) was analyzed with CAPS genetic markers, which anchored *LeWOX4* to chromosome 4L between markers TG182 and CP57. Interestingly, the *entire* mutation that produces simplified leaves also maps to this chromosomal region. However, no major molecular lesions were identified in *WOX4* alleles in *entire* mutant plants after sequencing 2116bp upstream of the *WOX4* 5' UTR, 1688bp of the transcribed region, or 618bp of the 3'UTR. In addition, Northern hybridization revealed that *entire* mutant seedlings accumulate normal levels of *LeWOX4* transcript (Data not shown). These data suggest that *LeWOX4* is not allelic to the *entire* mutation. As observed for *LeWOX4* in tomato, there are no known mutations of *AtWOX4* available in the *Arabidopsis* databases.

***WOX4* expression is developmentally regulated**

In order to predict the potential functions of *WOX4*, we investigated the expression patterns of *WOX4* in various tissues. Quantitative RT-PCR reveals that *LeWOX4* is expressed in multiple tomato tissues, although it is relatively more abundant in the hypocotyls, young leaves and flowers (Fig. 3.3 A). To examine *LeWOX4* expression more specifically, we performed in-situ hybridization in various tomato tissues. *LeWOX4* transcripts accumulate in the procambium tissues of the stem (Fig. 3.3 B, C, D) and leaf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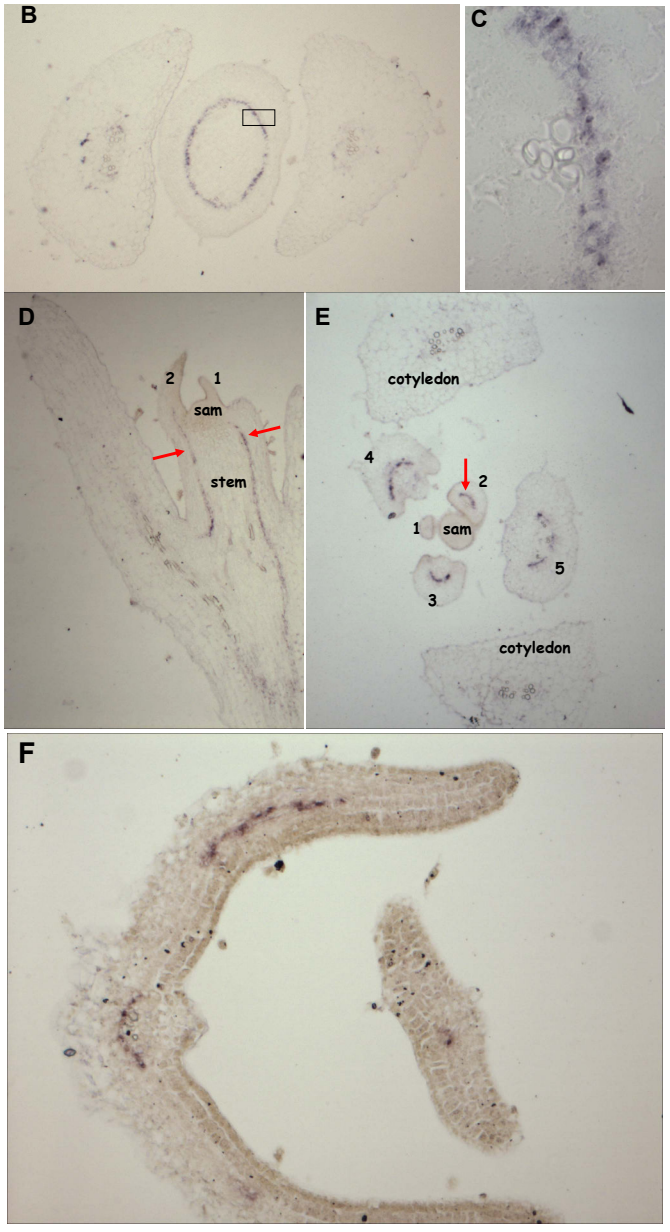
Cotyledon
Apex
Young Leaf
Immature Flower
LeWOX4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cotyledon
F

Figure 3.3 Expression pattern of *LeWOX4*. (A) Quantitative real-time RT-PCR data of *LeWOX4* transcript abundance in tomato tissues relative to the level of *LeWOX4* transcript in young leaf after normalization to *LeACTIN* transcript level. Tomato cv. Ailsa Craig was used. Roots, hypocotyls, and cotyledons were collected from 7 days old seedlings, apex-enriched tissues from 12 days old seedlings, and young leaves and flowers from 60 days old plants. (B-F) In situ hybridization of transverse (B, C, E and F) and longitudinal (D) sections of 12days old seedlings of tomato cv. Ailsa Craig reveal that *LeWOX4* transcripts accumulate in the procambium tissues of the stem (B-D), petiole (E) and leaf blade (F). C is the close-up image of the inset in B. The youngest leaf primordium is labeled as 1, and the next oldest leaf as 2, and so on.

primordia (Fig. 3.3 E, F), suggesting a possible role of *LeWOX4* in vascular development. This *in situ* result was further supported by GUS staining of multiple tissues from tomato plants transformed with the GUS reporter gene fused to the *LeWOX4* promoter (data not shown). GUS staining also shows that *LeWOX4* is expressed in the abscission regions near the leaf base and pedicel (the stalk of an individual flower) (data not shown).

Quantitative RT-PCR and *in situ* hybridization analyses of *AtWOX4* showed similar expression patterns, although *AtWOX4* transcripts tends to accumulate more in the inflorescence stems and young flowers (Fig. 3.4 A, B). In addition, GUS reporter fused to *AtWOX4* promoter revealed that it is also expressed in the base of the cauline leaf, i.e. leaf trace (A strand of vascular tissue extends between the vascular bundle of a stem and a leaf) and lateral shoot. These data suggest that the expression patterns of *LeWOX4* and *AtWOX4* are largely conserved, despite the divergence of tomato and *Arabidopsis* from a common ancestor approximately 150 million years ago (Van der Hoeven et al., 2002). Collectively, *LeWOX4* and *AtWOX4* show similar genic structure, sequence conservation, expression patterns, and copy number in tomato and *Arabidopsis*. Therefore, it is likely that *LeWOX4* and *AtWOX4* are orthologous genes.

***WOX4* expression is physiologically regulated**

Plant hormones are proposed to play important roles in vascular development and *WOX4* genes are expressed in vascular tissues. Therefore we investigated whether *WOX4* transcript accumulation is regulated by various plant hormones. Both *LeWOX4* and *AtWOX4* are down-regulated by the cytokinin 6-BA, and up-regulated by the gibberrelin GA_3 and the abscisic acid ABA (Fig.3.5 A, B). However, *LeWOX4* is down-regulated by the auxin 1-NAA, whereas *AtWOX4* is up-regulated, indicating differential auxin

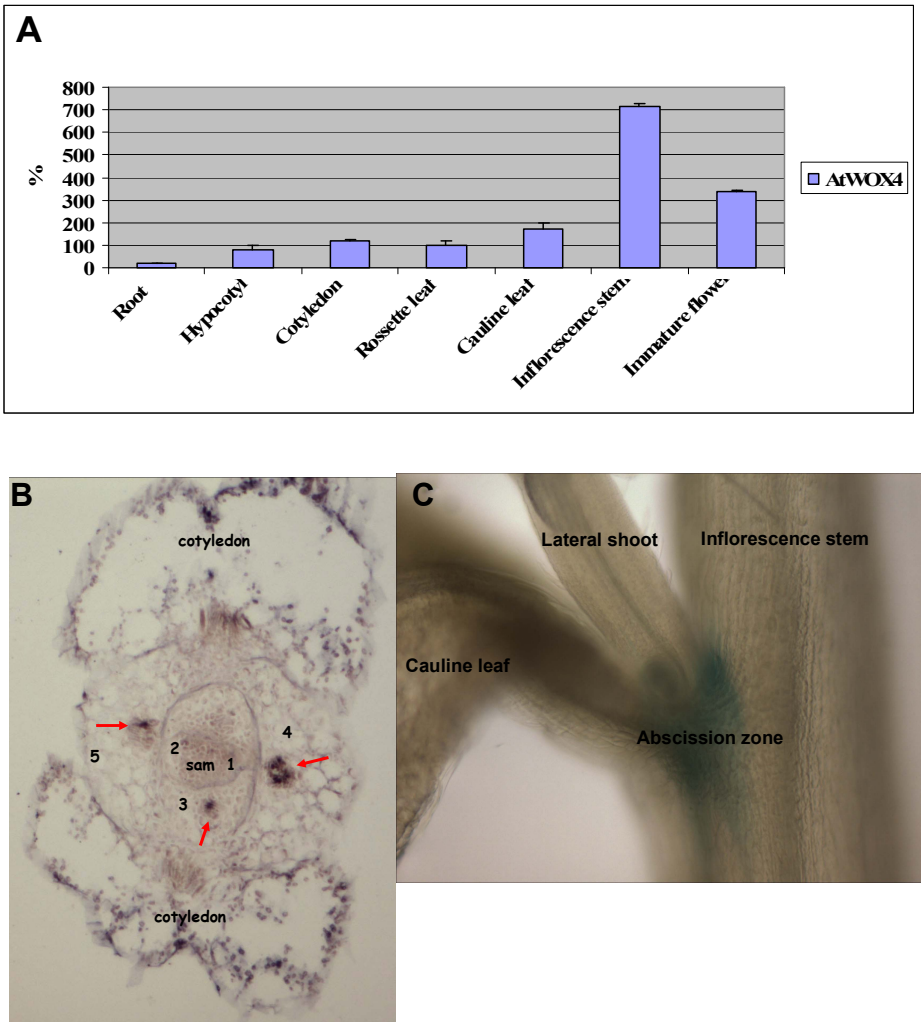

Figure 3.4 Expression pattern of *AtWOX4*. (A) Quantitative real-time RT-PCR data of *AtWOX4* transcript abundance in *Arabidopsis* tissues relative to the level of *AtWOX4* transcript in young rosette leaf after normalization to *AtACTIN2* transcript level. *Arabidopsis* ecotype Columbia was used. Roots, hypocotyls, and cotyledons were collected from 8 days old seedlings, and rosette leaves, cauline leaves, inflorescence stems and immature flowers from 4 weeks old plants. (B) In situ hybridization of a transverse section of 7 days old seedlings of Columbia reveal that *AtWOX4* transcripts accumulate in the vascular tissues of leaves. The youngest leaf primordium is labeled as

(Figure 3.4 legend continued) 1, and the next oldest leaf as 2, and so on. (C) GUS staining of the inflorescence stem segments from an 18 days old Columbia plant transformed with AtWOX4::GUS reporter construct. Note GUS staining in the base of cauline leaf and lateral shoot.

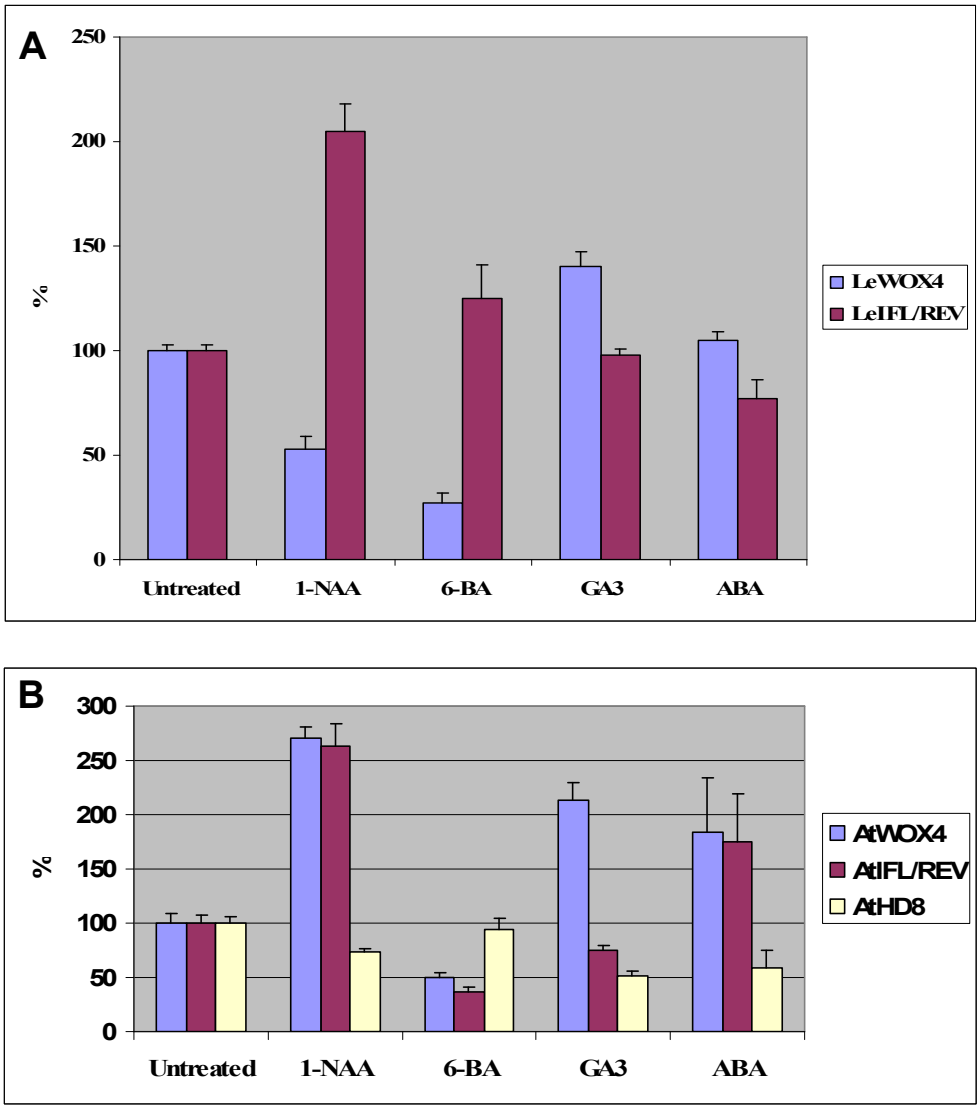

Figure 3.5 Expression regulation of *WOX4* and other patterning genes by plant hormones. (A) Quantitative real-time RT-PCR data of *LeWOX4* and *LeIFL/REV* transcripts relative to the level of their corresponding transcripts in the untreated young seedlings of Ailsa Craig after normalization to *LeACTIN* transcript level. (B) Quantitative real-time RT-PCR data of *AtWOX4*, *AtIFL1/REV* and *AtHD8* transcripts relative to the level of their corresponding transcripts in the untreated young seedlings of Columbia after normalization to *AtACTIN2* transcript level.

responses for these *WOX4* orthologues.

AtIFLl/REV and *AtHD-8* are transcription factors involved in vascular development of *Arabidopsis* (Baima et al. 1995, 2001; Emery et al., 2003; Zhong et al., 1999; Zhong & Ye, 1999, 2004). As with *WOX4*, the transcript accumulation of these vascular genes in response to specific plant hormones was examined. Although *LeIFL/REV* expression is likewise upregulated by 1-NAA (Fig.3.5A), 6-BA, GA_3 and ABA had no noticeable effect. These responses of *ILeIFL/REV* to plant hormones are quite different from that observed for *LeWOX4*. In *Arabidopsis*, *AtIFL1/REV* shows similar response to 1-NAA, 6-BA and ABA as *AtWOX4* (Fig.3.5B), yet opposite response to GA_3. Furthermore, *AtHD8* is down-regulated by 1-NAA, GA_3 and ABA, but it is not influenced by 6-BA treatment (Fig.3.5B). The response of *AtHD8* to various hormonal treatments is dramatically different from that of *AtWOX4* and *AtIFL1/REV*, suggesting that these three gene products may act in different levels or different pathways, even though they may all play roles in vascular development. This idea is consistent with previous genetic characterization on *AtIFL1/REV* and *AtHD8* in vascular development, i.e., *AtIFL1/REV* is required for interfacicular fiber development while *AtHD8* may promote xylem proliferation (Baima et al. 1995, 2001; Emery et al., 2003; Zhong et al., 1999; Zhong & Ye, 1999, 2004).

Functional analyses of *LeWOX4* and *AtWOX4*

There are no known mutations in *LeWOX4* and *AtWOX4* genes. In order to understand the developmental roles of *WOX4* we generated both constitutive over-expression constructs and RNAi constructs for both *LeWOX4* and *AtWOX4*, and sought to analyze the developmental phenotypes in plants harboring these mutant constructs. In total, 19

putative *LeWOX4* over-expression T1 plants were obtained in VF36 tomato, 8 of which were verified by PCR. Four PCR-positive T1 plants (including LT2-2, LT2-9, and LT2-18) showed similar phenotypes and accumulated 12-57 times more *LeWOX4* transcripts as compared to wild type VF36 (Fig.3.8A), which verified these plants as bona-fide LeWOX4overexpression mutants. LeWOX4 overexpression plants tend to exhibit reduce apical dominance (i.e. highly branched), and are shorter in statue owing to reduced internode length (Fig. 3.6A, B). LeWOX4 overexpression (o/e) leaves are yellowish, hyponastic (i.e. curled upwards), and highly compound; the numbers of primary, intercalary, secondary and tertiary leaflets are all increased (Fig. 3.6C). In addition, leaflets are produced from the middle of rachis, a phenotype that is not observed in wild type VF36 (Fig. 3.6D). Cross sections from the stem, petiole and leaflet midvein revealed that supernumery phloem and especially xylem tissues are produced in *LeWOX4* over-expression plants, and these vascular elements tend to cluster together (Fig. 3.7A-F). Although more numerous, the LeWOX4 o/e xylem and phloem elements are smaller compared with those in wild type VF36. In addition, the size, shape and alignment of non vascular cells (i.e. the epidermis, parenchyma, and cortex cells) are also severely affected in these organs (Fig. 3.7A-F). When considered in light of the LeWOX4 expression data (Fig.3.3), these LeWOX4 o/e phenotypic data suggest that LeWOX4 may function to promote procambial activity. Furthermore, overly-abundant LeWOX4 activity may interfere with vascular bundle patterning by producing excessive xylem and phloem elements, which may disrupt intercellular communication between vasculature and neighboring nonvascular cells resulting in abnormal anatomical patterning.

Nine putative *AtWOX4* over-expression T1 plants were generated in the *Arabidopsis*

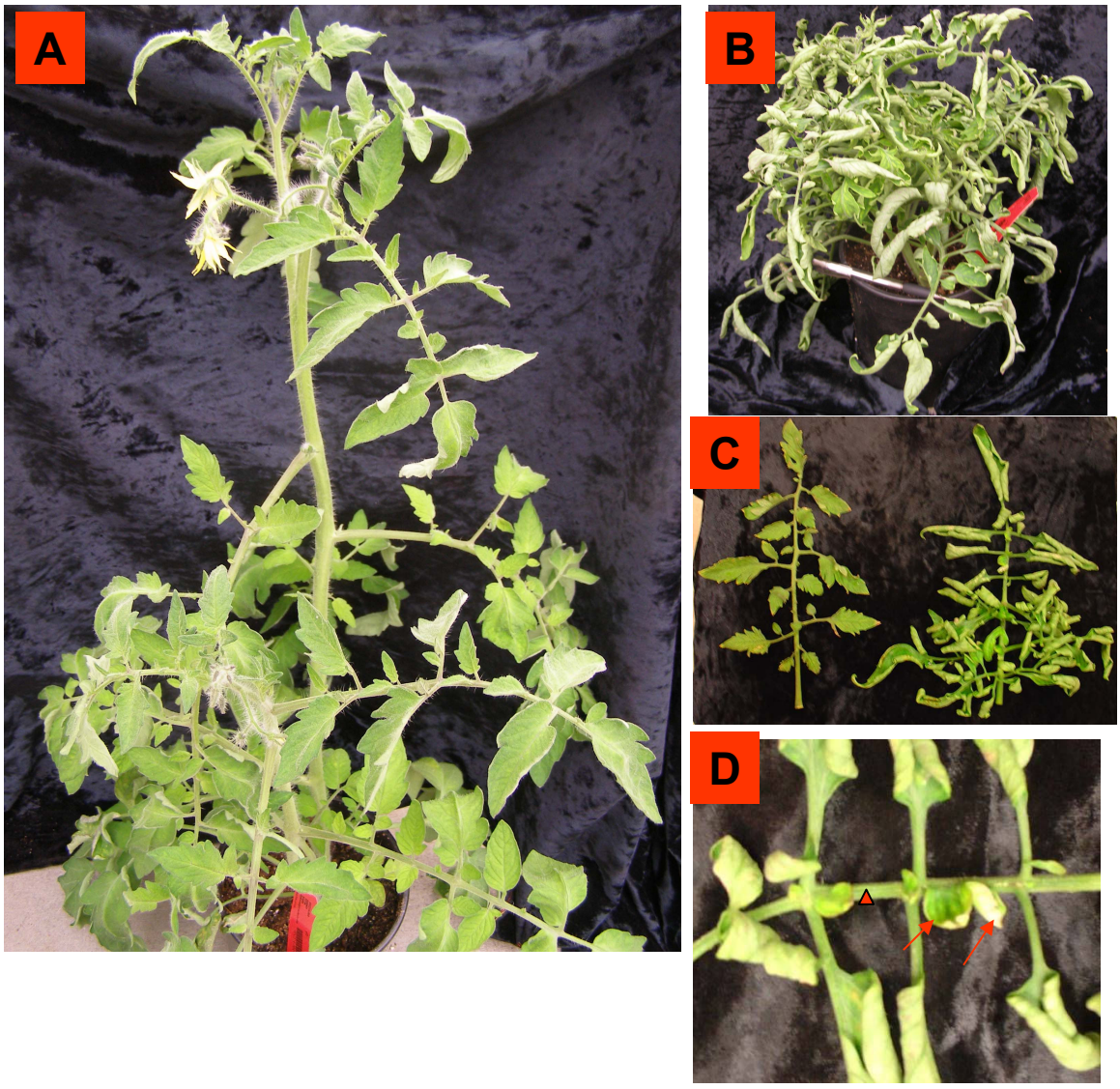

Figure 3.6 Phenotype of LeWOX4 overexpression. (A) Wild type VF36 plant. (B) LeWOX4 overexpressed VF36 at the same stage. (C) Mature leaves from wild type VF36 (left) and LeWOX4 overexpressed VF36 (right). (D) Leaflets (arrows) come out from the middle of rachis (arrowhead) in LeWOX4 overexpressed VF36, which is not present in wild type VF36.

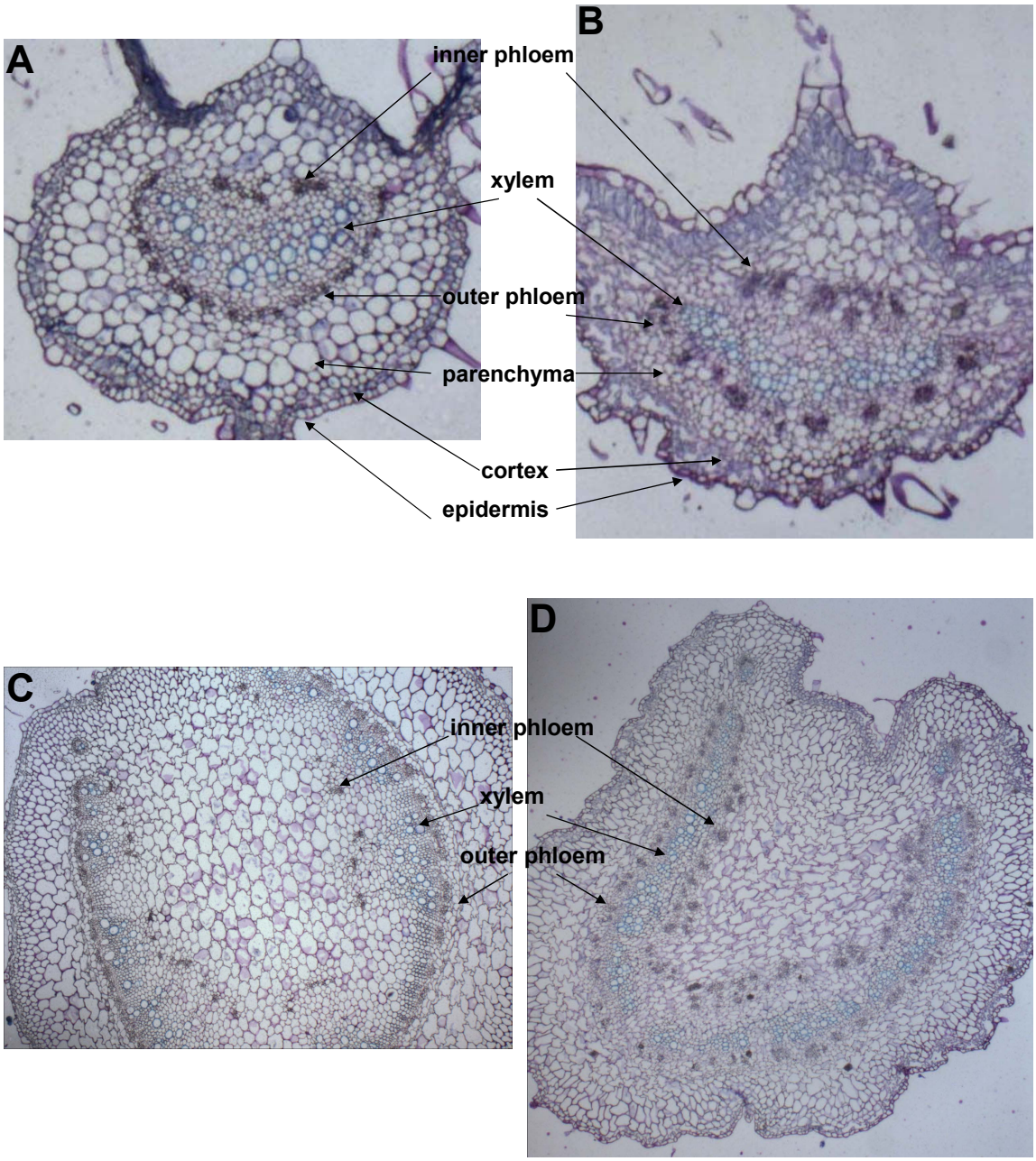
A
inner phloem
xylem
outer phloem
parenchyma
cortex
epidermis
B
C
inner phloem
xylem
outer phloem
D
E
outer phloem
xylem
inner phloem
F

Figure 3.7 LeWOX4 overexpression alters vascular patterning. Transverse sections are from the leaf blade (A-B), petiole (C-D) and stem (E-F) from wild type VF36 (A, C, E) and LeWOX4 overexpressed VF36 (B, D, E) at the same stages. Details are provided in the text.

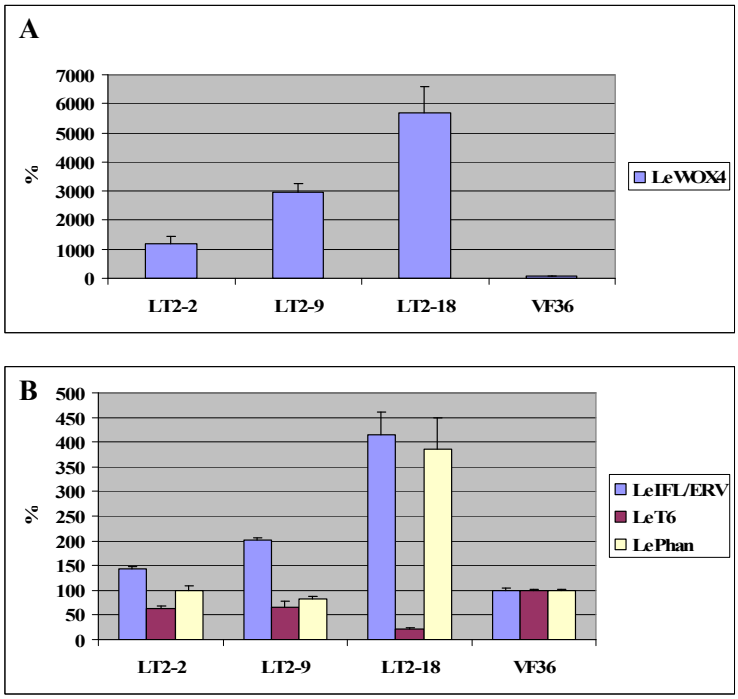

Figure 3.8 Gene expression levels in *35S::LeWOX4* transformed tomato leaves. Young leaves at the same stages from independent transformants and non-transformant VF36 were collected. (A) Quantitative real-time RT-PCR data of *LeWOX4* transcript relative to the level of *LeWOX4* transcript in the leaf of VF36 after normalization to *LeACTIN* transcript level. (B) Quantitative real-time RT-PCR data of *LeIFL/REV, LeT6* and *LePHAN* transcripts relative to the level of their corresponding transcripts in the leaf of VF36 after normalization to *LeACTIN* transcript level.

Columbia background, all of which were verified by PCR. Further detailed phenotypic analyses were performed on lines LA2-1 and LA2-4. The segregation of kanamycin resistant and phenotypic seedlings in the T2 progeny indicates that these two lines each harbor a single transgene insertion. Plants LA2-1 and LA2-4 accumulated 206-220 times more *AtWOX4* transcripts than wild type Columbia (Fig.3.10A), which verifies the efficiency of the over-expression constructs utilized in this study. The AtWOX4 o/e plants each displayed equivalent mutant phenotypes, including: loss of root gravitropism (Fig.3.9 A, C); reduced root growth (Fig.3.9 B); and subsequently delayed shoot growth (Fig.3.9 B). Frequently, the primary root in AtWOX4 o/e plants forms a broad, blunted tip and fails to develop or retain a root cap (Fig.3.9E). Subsequently, the primary root aborts further growth and generates a new lateral root from the elongation zone of the primary root (Fig.3.9D). Normally, lateral roots are formed from the differentiation zone of the primary root, which is located far far away from the elongation zone along the longitudinal axis of the developing root. The root phenotype is reiteratedin the lateral and secondary lateral roots, producing a short, bushy root architecture in AtWOX4 overexpressing plants (Fig.3.9B). It will be interesting to investigate whether there is similar root phenotype in *LeWOX4* over-expressors. As described above, the shoot system becomes more branchy and stunted in *LeWOX4*, a phenotype that appears to mimic the root phenotype in AtWOX4 overexpression plants. When considered together, over-expression of WOX4 seems to reduce apical dominance in tomato shoots and promote the initiation of branch roots in *Arabidopsis*. It remains to be seen whether auxin transport, accumulation or response has been changed in these mutants because auxin plays important roles in vascular tissue development, gravitropism, root initiation,

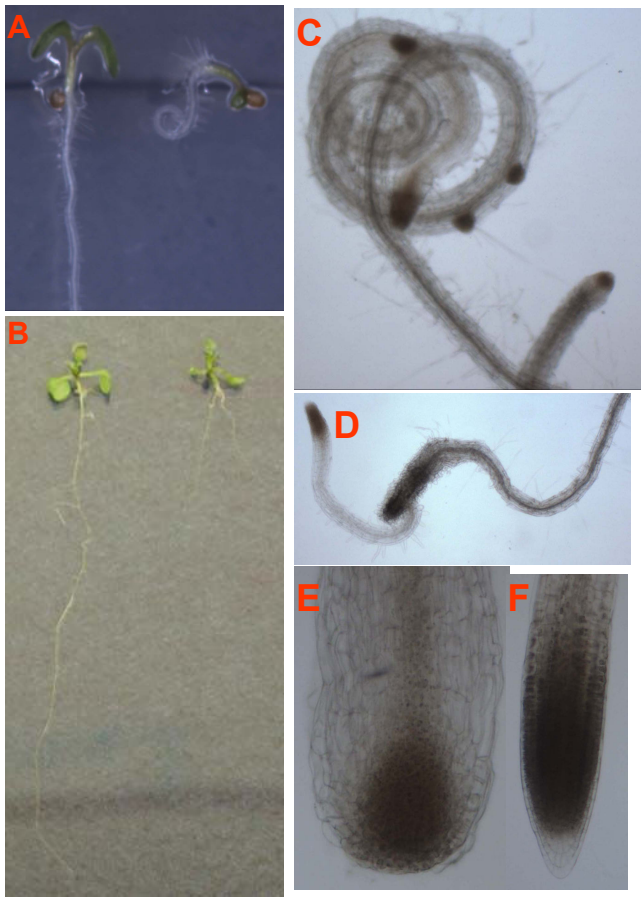

Figure 3.9 Phenotype of *AtWOX4* overexpression. (A-B) 4 days (A) and 10 days (B) old seedlings grown vertically in solid ½ MS medium. The left and right seedlings are wild type Columbia and AtWOX4 overexpressor respectively in A and B. Loss of gravitropism (C) and formation of lateral root from the elongation zone of primary root (D) in AtWOX4 overexpressor. Root tip in AtWOX4 overexpressor (E) and wild type Columbia (F).

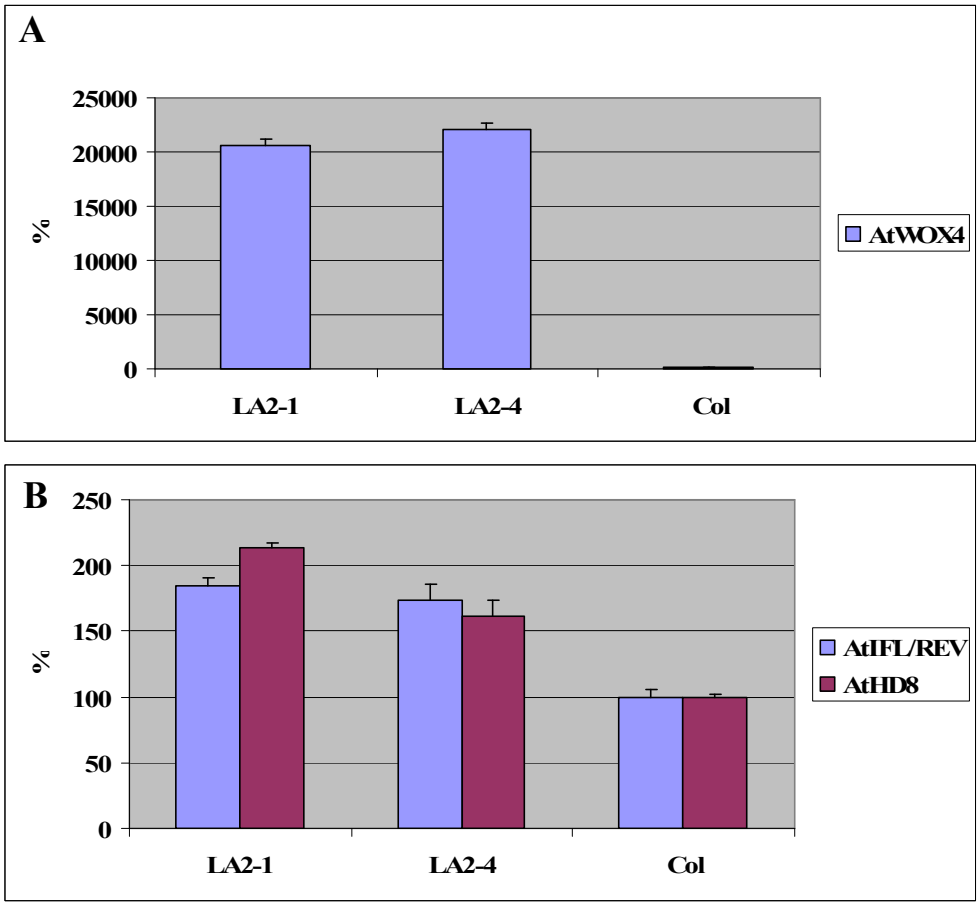

Figure 3.10 Gene expression levels in *35S::AtWOX4* transformed *Arabidopsis* rosette leaves. Young leaves at the same stages from independent T2 transformants and non-transformant Columbia were collected. (A) Quantitative real-time RT-PCR data of *AtWOX4* transcript relative to the level of *AtWOX4* transcript in the leaf of Columbia after normalization to *AtACTIN2* transcript level. (B) Quantitative real-time RT-PCR data of *AtIFL1/REV*and *AtHD8* transcripts relative to the level of their corresponding transcripts in the leaf of Columbia after normalization to *AtACTIN2* transcript level.

and apical dominance (Davies, 2004).

Twenty-three putative RNAi T1 plants in the tomato VF36 background were produced; seven were verified by PCR but none showed an obvious shoot phenotype. Similarly, twelve putative RNAi T1 plants in *Arabidopsis* Columbia background were analyzed; T2 progenies from 10 of AtWOX4 RNAi plants were verified by PCR, but none showed any obvious shoot, root or vascular phenotypes. This suggests that our knock down RNAi constructs may not work efficiently, or perhaps more likely, genetic redundancy in both tomato and *Arabidopsis* is responsible for the absence of phenotype. Another possibility is that specific growth conditions may be required for a mutant phenotype.

Interactions between *WOX4* and other vascular and leaf patterning genes

Since LeWOX4 overexpression affects vascular development and generates highly compound leaves, we investigated whether the expression of other vascular and leaf patterning genes is altered in LeWOX4 overexpressors. In all three independent 35S~*LeWOX4* transformants, LeIFL/REV expression is upregulated from 1.4 to 4.1 times as compared to wild type (Fig. 3.8B). The expression of additional vascular patterning genes in AtWOX4 overexpressors was similarly investigated. AtIFL1/REV and AtHD8 are both upregulated to 1.6 to 2.1 times in the T2 progeny of two independent *Arabidopsis* WOX4 overexpression lines (Fig. 3.10B). In *Arabidopsis*, loss of function in AtIFL1/REV mutants has reduced xylem development (Zhong & Ye., 1999), and overexpression of AtHD8 produces more xylem (Baima et al., 2001).

Interestingly, the expression of *LeT6* is downregulated to 23-65% of wild type levels whereas accumulation of *LePHAN* transcript is unaltered in two transformants and upregulated to 3.9 times in one transformant (Fig. 3.8B). In tomato, it was shown that

LeT6 promotes leaf complexity and negatively regulates LePHAN. Furthermore, LeT6 requires LePHAN activity in order to promote leaflet formation (Chen et al., 1997; Janssen et al., 1998; Kim et al., 2003; Koltai and Bird, 2000). Our data suggest that there may be a close connection between vascular patterning and leaf compoundness, and *LeWOX4* overexpression may activate a different pathway than the KNOX-PHAN pathway to regulate leaf complexity in tomato. Another possibility is that LeWOX4 overexpression activates the LeT6/LePHAN genetic pathway but acts downstream of of these two genes. Further characterization expression of LeWOX4 in the mutants of *LeT6* and *LePHAN* may distinguish between these possibilities.

Materials and Methods

Plant growth conditions

Tomato plants were grown in a greenhouse at 25±2^{o}C under natural daylight. *Arabidopsis thaliana* plants were sown in solid (0.7% agar) or liquid medium containing ½ MS basal medium (Sigma-Aldrich) plus 1% sucrose (pH 5.7), stratified at 4^{o}C for 3 days, and grown in a laboratory at 23^{o}C under continuous illumination. Otherwise, *Arabidopsis* plants were cultivated in RediEarth medium in the greenhouse of Plant Biology Department, UGA.

DNA isolation and gene cloning

Genomic DNA was isolated from tomato leaves or *Arabidopsis* whole seedlings as described (Dellaporta et al., 1983), or from young leaves with Extract-N-Amp™ Plant PCR Kits (Sigma-Aldrich). A degenerate primer was designed from the most conserved C terminal of homeodomain region from WOX proteins in *Arabidopsis* and WUS

proteins from different species including tomato, petunia and *Antirrhinum* (see Table 3.1). Then degenerate Genome walker was employed to clone the 5' end of *LeWOX4* genes from tomato. Further genome walker was performed to get the 5' and 3' end of genomic DNA. The promoters of *LeWOX4* and *AtWOX4* were amplified respectively from the diluted Ailsa Craig and Columbia genomic DNA preparation. All PCR products were generated with high-fidelity DNA Polymerase (invitrogen), cloned into TOPO vectors, and sequenced (Applied Biosystems). The primers contained corresponding restriction sites at the 5' ends and were summarized in Table 3.1.

DNA gel blot hybridization

Genomic DNA was isolated from tomato leaves and analyzed by gel blot hybridization analysis as described before (James et al., 1995). Hybridization probes were radioactively prepared as described (Fu et al., 2002).

Mapping of *LeWOX4*

50 *Lycopersicon pennellii*-derived introgression lines (ILs), which together cover the entire genome in the background of *L. esculentum* Var. M82 (Eshed et al., 1992), were used to map *LeWOX4* with a CAPS marker.

RNA isolation, gene cloning and RT-PCR

Total RNA was isolated, DNase I treated, and reverse transcribed into 1st strand cDNA following the same protocol as described (Nardmann and Ji et al., 2004). Degenerate 5'RACE was employed to clone the 5' end of the *LeWOX4* gene from tomato, whereas 3'RACE was used to obtain the rest of the full-length cDNA of *LeWOX4*. 5' and 3' RACE were also performed to obtain 5' and 3' UTR of *AtWOX4*. The encoding regions of *LeWOX4* and *AtWOX4* were amplified correspondingly from Ailsa Craig and

Table 3.1 Summary of oligonucleotide primers used in this project

name	primer sequence	primer use
TR6	CTT(A/G)TGGTT(T/C)TGGAACCAGTA(G/A)AAGA	5' Genome Walker and 5'RACE for tomato WOX genes
TR18	ACGCATCCCACCTCTATACAAC	5' Genome Walker for LeWOX4
TR19	TTCCTCCTGGGTGTGATTCC	5' Genome Walker for LeWOX4
TR24	ATTGGTTGTGTTGAGCTTAGGAG	5' Genome Walker for LeWOX4
TR25	GCAACCAAGTGTAAGTGAAGGAG	5' Genome Walker for LeWOX4
TR29	TTCTTGAAGAGCATTAAATGAAACA	5' Genome Walker for LeWOX4
TR30	TTTTAAAAATGCATTGACAAACAAC	5' Genome Walker for LeWOX4
TF21	CCCAACGCACAACAAATAGA	3' Genome Walker for LeWOX4
TF24	CGCAACAAATAGAGCAAATCAC	3' Genome Walker for LeWOX4
TF28	TGATATGAACCCTTTGCACT	3' Genome Walker for LeWOX4
TF32	TTCAACTCTTCCCATTGCATC	3' Genome Walker for LeWOX4
TF26	TGGGATCATCATCAGGAA	Genomic RCR for LeWOX4
TR22	CAAAGCATGCAAATGAAGA	Genomic RCR for LeWOX4
TR16	TCACG(A/G)GCTTTATGGTT(C/T)TGAA	5'RACE for LeWOX4
TF30	CA(A/G)AACCATAAAGC(C/T)CGTGAA	3'RACE for LeWOX4
TF31	CCATAAAGC(C/T)CGTGAAAGA	3'RACE for LeWOX4
TF40B	GTCGACAAAAAGGGGATATTTGGGTTAT	Cloning LeWOX4 promoter
TR32	GGATCCGGAGCTAGACAGCTTTTAGTAT	cloning LeWOX4 promoter
TF39	GGATCCATGTACATGGGATCAT	Cloning LeWOX4 ORF with BamHI site
TR31	GAGCTCTCATCTCATGCCTTCT	Cloning LeWOX4 ORF with SacI site
TF46	GGATCCGGCGCGCCTCTAGAAAAGACTCCTCTCAGGTAAT	Cloning 1st intron of LeWOX4 BamHI,AscI and XbaI site
TR38	CCGCGGAGATCTCCTAGGTGGGTGTGATTCCACCTACA	Cloning 1st intron of LeWOX4 SacII,BglII and AvrII site
TF43	GGATCCTGAATTTATTATAAGAATA	Cloning sense 3'UTR of LeWOX4 with BamHI site
TR35	TCTAGAGGCGCGCCAAGAAGAAAATAATAGATT	Cloning sense 3'UTR of LeWOX4 with XbaI and AscI sites
TF44	GAGCTCTGAATTTATTATAAGAATA	Cloning antisense 3'UTR of LeWOX4 with SacI site
TR36	CGGCCGCCTAGGAAGAAGAAAATAATAGATT	Cloning antisense 3'UTR of LeWOX4 with EagI AND AvrII sites
PBI35S	CTATCCTTCGCAAGACCCTTCCTC	PCR verification of 35S::LeWOX4
TR31	GAGCTCTCATCTCATGCCTTCT	PCR verification of 35S::LeWOX4
TF46	GGATCCGGCGCGCCTCTAGAAAAGACTCCTCTCAGGTAAT	PCR verification of LeWOX4 RNAi
NOSTR	ATCGCAAGACCGGCAACAGGATTC	PCR verification of LeWOX4 RNAi
TF47	CCACCTCATTTCCCACTTTCT	PCR verification of LeWOX4::GUS
GUSR	CACCAACGCTGATCAATTCCAC	PCR verification of LeWOX4::GUS
GR5	CGACTGGAGCACGAGGACACTGA	5'RACE for AtWOX4
AtWOX4R8	AAGTTCTCAAATCCCCAGCTCCTAC	5'RACE for AtWOX4

GR5N	GGACACTGACATGGACTGAAGGAGTA	5'RACE for AtWOX4
AtWOX4R11	TCGATTTTCCCGTACTTACCGAGTT	5'RACE for AtWOX4
AtWOX4F3	ATGGTGGAGAAGGAGGAGTTAGTGG	3'RACE for AtWOX4
GR3	GCTGTCAACGATACGCTACGTAACG	3'RACE for AtWOX4
GR3N	CGCTACGTAACGGCATGACAGTG	3'RACE for AtWOX4
AtWOX4F2B	GTCGACTTTTTATGTAAATAAATTTATATA	Cloning AtWOX4 promoter
AtWOX4R2	GGATCCTGCTATATGTTAAAACTAGCAAAT	Cloning AtWOX4 promoter
AtWOX4F1	GGATCCATGAAGGTTCATGAGTTT	Cloning AtWOX4 ORF
AtWOX4R1	GAGCTCTCATCTCCCTTCAGGATG	Cloning AtWOX4 ORF
AtWOX4F4	GGATCCAGTCATGAAGGTGAGGCAGA	Cloning sense 3'UTR of AtWOX4
AtWOX4R3	GGCGCGCCGAGCTAAACTGAGCATTCAT	Cloning sense 3'UTR of AtWOX4
AtWOX4F5	GAGCTCAGTCATGAAGGTGAGGCAGA	Cloning antisense 3'UTR of AtWOX4
AtWOX4R5	CCTAGGGAGCTAAACTGAGCATTCAT	Cloning antisense 3'UTR of AtWOX4
PBI35S	CTATCCTTCGCAAGACCCTTCCTC	PCR verification of 35S::AtWOX4
AtWOX4R1	GAGCTCTCATCTCCCTTCAGGATG	PCR verification of 35S::AtWOX4
TF46	GGATCCGGCGCGCCTCTAGAAAAGACTCCTCTCAGGTAAT	PCR verification of AtWOX4 RNAi
NOSTR	ATCGCAAGACCGGCAACAGGATTC	PCR verification of AtWOX4 RNAi
AtWOX4F	TTTGGTTTCCCGACGTCCCACTTT	PCR verification of AtWOX4::GUS
GUSR	CACCAACGCTGATCAATTCCAC	PCR verification of AtWOX4::GUS
TATNF1	GAGCATCCTGTCCTCCTAACTG	Real time PCR for LeACTIN
TATNR1	GACCATCACCAGAGTCCAACAC	Real time PCR for LeACTIN
TF25	CAAAGTCCAAGAACACCATCA	Real time PCR for LeWOX4
TR21	CCATCCCCTACACTTTCTCT	Real time PCR for LeWOX4
LeIFLF1	CGTCTGAAGAAAATGCTGTCC	Real time PCR for LeIFL1/REV
LeIFLR1	ATTTCGTTTGTCCCCTCATTC	Real time PCR for LeIFL1/REV
LeT6F3	GGACCAGAAGCAAATAAACAAC	Real time PCR for LeT6
LeT6R2	ATTGGGAAATGGTTAGCAAGAA	Real time PCR for LeT6
LePHANF2	AAAAGAGCAGAAATTGGCAGAG	Real time PCR for LePHAN
LePHANR2	AGGAGCAAGGAAACACAAAAGA	Real time PCR for LePHAN
ACT2F	TCGTTTCTATGATGCACTTGTGTG	Real time PCR for AtACTIN2
ACT2R	ATGGGACTAAAACGCAAAACGA	Real time PCR for AtACTIN2
AtWOX4F6	GCGAGAGACAGAAGCAGAAGA	Real time PCR for AtWOX4
AtWOX4R12	TCCACTAACTCCTCCTTCTCCA	Real time PCR for AtWOX4
AtIFLF1	GCCTTCTCCTTTGTAAACTGG	Real time PCR for AtIFL1/REV
AtIFLR1	CAGAGACACCTAAACAACAACCA	Real time PCR for AtIFL1/REV
AtHD8F	AGGGGTTTATGTGTATGGATGG	Real time PCR for AtHD8
AtHD8R	CAGTTGAGGAACATGAAGCAGA	Real time PCR for AtHD8

Columbia cDNA preparations with high-fidelity DNA Polymerase (invitrogen), cloned into TOPO vectors, verified by sequencing (Applied Biosystems), and ligated into pre-restricted pBI101 vectors. Compatible restriction sites were incorporated into the 5' ends of the primers, which were also summarized in Table 3.1.

Quantitative real-time RT-PCR was performed in a volume of 20 µl containing 2 µl cDNA template (50 ng/µl), 200 nM of each primer (see Table 3.1), and 10µl 2x iQ SYBR Green Supermix (Bio-Rad). PTC 200 Peltier Thermal Cycler (MJ Research) was run under the program of 95^{o}C for 3 minutes; and 40 cycles of 94^{o}C for 15 seconds, X ^{o}C (X value depends on the primers used) for 15 seconds and 72 ^{o}C for 15 seconds. Each sample was assayed in three replicates. Analysis of relative transcription for genes in study normalized to control *LeACTIN* or *AtACTIN2* expression was performed as published (Livak & Schmittgen, 2001).

In-situ hybridization

In situ hybridization was performed following the protocol of Jackson, 1991. The probes for tomato were from the 5' end and 3'UTR of *LeWOX4*; *Arabidopsis* probes were obtained from the 3'UTR of *AtWOX4*.

DNA constructs and plant transformation

All constructs (Fig.3.11) were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The cotyledons of tomato cultivar VF36 were transformed by the leaf-disc method (McCormick, 1991). The wild type *Arabidopsis thaliana* ecotype Columbia was transformed by the floral dip method (Clough & Bent 1998). Transformants were selected on ½ MS medium containing 50mg/L kanamycin (pH5.7) and then transplanted into water saturated RediEarth medium.

Plant hormonal treatment

Tomato Ailsa Craig seeds were sterilized and grown in Petri dishes containing the above described liquid MS medium (20ml) in dark at 23 ^{o}C for 7 days. Young seedlings were transferred to the same fresh liquid medium (20ml) supplemented with 50 μM 1-NAA (1-Naphthylacetic acid), 50 μM GA_3 (Gibberellic acid), 50 μM ABA (Abscisic acid), 50 μM 6-BA (6-benzylaminopurine, cytokinin) (Sigma-Aldrich), or 0.5% Ethanol or 0.5% DMSO (hormone solvent controls). After 24 hours of incubation in the dark with gentle shaking, whole seedlings were harvested, immediately frozen in liquid nitrogen and subject to RNA isolation. *Arabidopsis* Columbia was treated similarly except that the plants were kept in dark at 23 ^{o}C for 5 days after stratification.

GUS staining

Roots, leaves and inflorescence stem segments of the transgenic plants were stained for GUS activity according to Jefferson et al. (1987). The stained tissues were fixed, dehydrated, cleared, embedded and sectioned as described (Kim et al., 2003).

Phylogenetic analysis

Phylogenetic trees were generated using MEGA3 with default parameters (Kumar et al., 2004) through the Neighbor-Joining and Maximal Parsimony methods from a ClustalW (http://www.ebi.ac.uk.clustalw) alignment of the homeodomain region among LeWOX4 and *Arabidopsis* WOX proteins. Sequence data is available in the GenBank database as shown with the accession numbers.

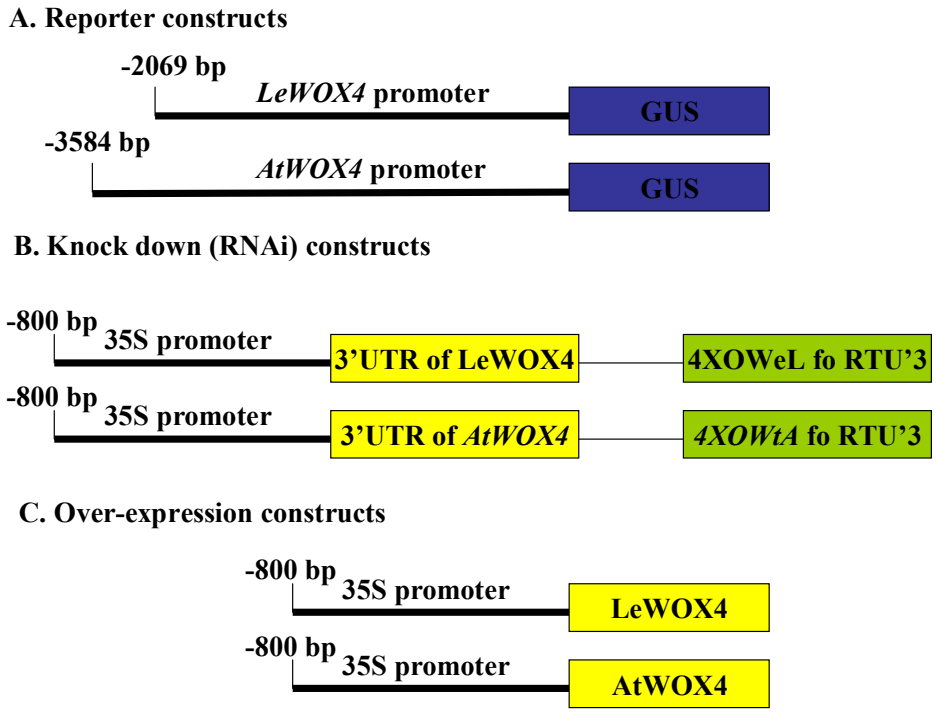

Figure 3.11 Schematic diagram of *WOX4* constructs used in this study. Note that the spacer sequence used in knock down constructs is from the first intron of *LeWOX4*, and all the constructs are in the background of pBI101 vector.

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CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

Comparative genetic analysis is a powerful tool for studying the evolution of plant development.

The *Mutator* (*Mu*) transposon tagging system has been widely employed in maize forward genetics as a tool for the generation and cloning of mutant alleles. However, in the case of *ns*, *Mu* transposon tagging was not successful, most likely due to some unknown factor(s) present in our *Mutator* lines that generates chromosomal deletions at an unusually high frequency. Therefore, a comparative genetic approach was utilized in order to isolate the *ns* genes. Based upon their meristem domain-specific expression patterns and the homology of leaves and sepals, we hypothesized that *ns* may be orthologous to the *Arabidopsis* homeobox gene *prs* (Matsumoto & Okada, 2001). In fact, molecular and genetic experiments confirmed this hypothesis, and identified the *ns1* and *ns2* genes as duplicated maize WOX genes (Nardmann and Ji et al., 2004). In turn, a comparative genetic approach was utilized to investigate unknown functions of *prs* during leaf development in *Arabidopsis*. These studies found that *prs* is required for recruitment of the founder cells that form the lateral stipules of the *Arabidopsis* leaf (Nardmann and Ji et al., 2004). Importantly, these comparative molecular and morphogenetic analyses support a traditional model (Troll, 1955) suggesting that the disparate morphologies of the eudicot *Arabidopsis* and monocot maize leaves results from the differential contribution of the upper and lower leaf zones during postprimordial stages of leaf development in these species (Troll, 1955; Kaplan, 1973). Furthermore, comparative analysis of WOX4 in tomato and *Arabidopsis* reveals that WOX4 overexpression may promote procambial activity, leaf complexity and lateral organ development. In the future, this comparative approach can be extended to analyses of

non-model, basal angiosperm species, in order to discern fundamental developmental strategies in the evolution of leaf morphology.

NS/PRS promises to generate new insights into early events in lateral organ development

NS/PRS encodes a WUSCHEL-like homeodomain transcription factor whose expression is conserved in maize and *Arabidopsis* (Matsumoto & Okada, 2001; Nardmann and Ji et al., 2004). Although expressed in only a few cells in the tunica layer of the SAM (Matsumoto & Okada, 2001; Nardmann and Ji et al., 2004), NS/PRS functions non-cell autonomously to direct founder cell recruitment throughout a larger, lateral domain of the SAM (Scanlon, 2000; Scanlon & Freeling, 1997; Scanlon et al., 1996, 2000). Furthermore, NS/PRS affects not only leaf development but also development of leaf homologous organs (Scanlon & Freeling, 1998). Hence, analyses of the mechanisms of NS/PRS function will generate novel insights into understanding the coordination and propagation of founder cell recruitment signals in both vegetative and reproductive shoot apical meristems.

***WOX4* functional analyses suggest novel important roles of vascular patterning in plant development**

LeWOX4 is expressed in the procambium tissues in stems and leaves, and its overexpression alters the vascular development of leaves and stems and generates highly compound leaves in tomato. Surprisingly, this super-compound leaf phenotype is correlated with the downregulation of *LeT6* whereas that of *LePHAN* is not reduced (Kim et al., 2003, 2003). This suggests that there is an intimate connection between vascular

patterning and leaf compoundness that may be unrelated to the LeT6/LePHAN pathways of compound leaf development.

AtWOX4 shows similar expression patterns as *LeWOX4*. AtWOX4 overexpression results in loss of root gravitropism and arrested root tips. New lateral roots are formed ecotopically from the elongation zone rathern than the differentiation zone of the primary root. This root phenotype is reiterated in the lateral and secondary lateral roots, producing a short, bushy root architechture in *AtWOX4* overexpression mutants. Further detailed analysis of root anatomy in *Arabidopsis* may reveal whether there is any defect in vascular system and whether these phenotypes reflect only secondary effects. It will be also interesting to investigate whether there is similar root phenotype in tomato *LeWOX4* over-expressors.

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APPENDIX A

PRELIMINAY MOLECULAR ANALYSES OF PRESSED FLOWER FUNCTIONS IN *ARABIDOPSIS* SHOOT MERISTEM DEVELOPMENT

[1]Jiabing Ji and Michael J. Scanlon. To be submitted to *Development*.

Abstract

The duplicate factor *NARROW SHEATH (NS)* genes of maize (*ns1* and *ns2*) and the *Arabidopsis PRESSED FLOWER (PRS)* gene are functional orthologs involved in lateral organ development. Previous studies revealed contradictions between the expression patterns and functional requirements of NS/PRS. To understand the molecular mechanism of NS/PRS functions, the *Arabidopsis* system was used owing to its facility in transgenic experiments. A 2742 bp long *PRS* promoter was isolated and fused in frame with the coding part of its cDNA clone. This construct successfully rescued the *prs* mutant phenotype. *prs* mutant was also transformed with PRS-GFP and/or PRS-GUS fusion proteins under the control of its own promoter and three different meristem-layer specific promoters from *Arabidopsis*. *In planta* assays will correlate the PRS protein accumulation pattern with the domain of PRS function. Constructs representing serial deletions of the *PRS* promoter were generated to investigate how the expression of this gene is cis-regulated. Furthermore, to reveal how *WOX* (*WUSCHEL-RELATED HOMEOBOX*) gene functions have evolved, the *WUS* (*WUSCHEL*) and *AtWOX4* genes, paralogs of *PRS*, were fused to the promoter of *PRS* and transformed into the *prs* mutants. Therefore, the contribution of the promoter and coding region to their diverse functions is testable. Finally, sucrose failed to rescue the *prs* phenotype, suggesting that *PRS* may act through a different pathway than *STIMPY/AtWOX9*.

Introduction

One of the essential functions of the shoot apical meristem (SAM) is organogenesis. Plant lateral organs are coordinately formed from founder cells present in the peripheral zone (PZ) of the SAM. The founder cells comprise all histogenic layers of the PZ, including the tunica layer (L1 in monocots and both L1 and L2 in dicots) and the internal corpus (L2 in monocots and L3 in dicots) (Smith & Hake 1992). Polar auxin transport (PAT), auxin signalling and *knox* gene down-regulation play important roles in this process (Jackson et al., 1994; Reinhardt et al., 2000, 2003), which may also involve the activities of phyllotaxy determinants such as ABPHYLL1 and TERMINAL EAR1 (Jackson & Hake, 1999; Giulini et al., 2004; Veit et al., 1998). Moreover, research on NS1 (NARROW SHEATH1) and NS2 suggests that recruitment of leaf founder cells from the shoot apical meristem is performed in at least two steps (Matsumoto & Okada, 2001; Scanlon, 2000; Scanlon & Freeling, 1997, 1998; Scanlon et al., 1996, 2000), such that the central leaf zone and the lateral domain are recruited by different gene functions (Scanlon 2003). Therefore, shoot organogenesis requires proper positional information and intercellular communication.

Two types of intercellular communication are described in plant development: (1) ligand-receptor processes and (2) symplastic (through membranes and living cells) transport. The ligand-receptor mediated process employs the apoplastic (through cell walls and intercellular spaces) transport of small peptides or plant growth regulators, which bind to specific membrane bound receptors. Other signalling molecules such as mRNAs, siRNA (small interfering RNA) and proteins are transported via plasmodesmata throughout the symplasm (reviewed by Kurata et al., 2005). Analyses of the maize

meristem identity gene *KN1* (*KNOTTED1*) have pioneered the study of movement of non-cell-autonomous transcription factors (Hake & Freeling 1986; Lucas et al., 1995; Kim et al., 2002; 2003; 2005). Subsequently, in *Arabidopsis* a GRAS family transcription factor SHR (SHORT ROOT), a small Myb-like protein CPC (CAPRICE), the basic-helix-loop-helix protein GL3 (GLABRAS 3) and EGL3 (ENHANCER OF GLABRAS 3) were found to move intercellularly and selectively through plasmodesmata (Bernhardt et al., 2003; 2005; Gallagher et al., 2004; Helariutta et al., 2000; Kurata et al., 2005; Nakajima et al., 2001; Sena et al., 2004; Wada et al., 1997; 2002) . These genes regulate the radial patterning of roots or root epidermal cell development. Similarly, selective trafficking of MADS box genes *DEF* (*DEFICIENCES*) and *GLO* (*GLOBOSA*) in *Antirrhinum* is required for normal petal development (Perbal et al., 1996). In contrast, the floral identity gene *LFY* (*LEAFY*) was shown to move non-selectively through cell layers via diffusion (Sessions et al., 2000; Wu et al., 2003). Furthermore, the APETALA3 (AP3) protein does not move intercellularly, but functions non-cell-autonomously via the regulated production of intercellular signals (Jenik & Irish, 2001).

NS is orthologous to PRS(PRESSED FLOWER) and encodes a WUSCHEL-like homeodomain transcription factor (Matsumoto & Okada, 2001; Nardmann and Ji et al., 2004). *NS/PRS* expression is conserved in maize and *Arabidopsis*, and can be divided into an early stage pattern within two lateral meristematic foci and a later staged pattern in the margins of young lateral organ primordia (Matsumoto and Okada, 2001; Nardmann and Ji et al., 2004). Mutant phenotypes (Scanlon et al., 1996; Matsumoto and Okada, 2001; Nardmann and Ji et al., 2004) and clonal analyses of NS1 function (Scanlon, 2000)

suggest that the evolutionarily conserved, essential function of the *NS/PRS* gene product may be restricted to shoot meristems; no genetic evidence has yet revealed NS/PRS function in primordial stages. *In situ* hybridization of transverse sections through the maize shoot apex shows NS transcripts localized to two foci in the tunica (L1) of the shoot meristem PZ. However, this L1 expression pattern conflicts with clonal analyses of NS1 function, which concluded that NS1 function is required in the corpus (L2 meristematic layer) (Scanlon, 2000). A similar conflict is also revealed in *prs* mutant lateral sepals deletion phenotype, since sepals are derived from alll three tissue layers although PRS expression is confined to the tunica (Matsumoto and Okada, 2001). Furthermore, although expressed in only a few tunica cells, NS/PRS functions non-cell autonomously throughout a larger, lateral domain of founder cells (Matsumoto & Okada, 2001; Nardmann and Ji et al., 2004). Hence, analyses of the mechanisms of NS/PRS function will generate novel insights into understanding the coordination and propagation of founder cell recruitment signals in the SAM.

The primordial expression pattern of *NS/PRS* is restricted to a few cells at the margins of developing lateral organs. However, no maize or *Arabidopsis* mutant phenotype correlates with loss of NS/PRS function in the margins of lateral organ primordia including the leaves and coleoptile in maize and the cotyledons in *Arabidopsis* (Scanlon and Freeling, 1998; Matsumoto and Okada, 2001; Nardmann and Ji. et al. 2004). Moreover, clonal analyses uncovered multiple cases in which 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 (Scanlon, 2000). These data raise questions as to whether the primordial –

staged NS/PRS transcripts are translated at all, or whether higher order functional redundancy is present.

In this report we employed PRS-GFP and PRS-GUS fusion proteins under the control different layer-specific *Arabidopsis* promoters in order to investigate the timing and tissue layer specificity of non cell-autonomous PRS function. These experiments are designed to determine in which meristematic layer PRS protein activity is essential for founder cell recruitment, whether the early stage PRS protein can traffic between meristematic cell layers, and whether the later-staged PRS transcripts observed in the margins of young lateral organ primordia are translated into protein. Furthermore, serial deletions of the *PRS* promoter region were generated to investigate the cis-regulation of *PRS* expression. Lastly, the functional evolution of the *WOX* (*WUSCHEL-RELATED HOMEOBOX*) gene family of *Arabidopsis*, (Haecker et al., 2004; Matsumoto & Okada, 2001; Nardmann and Ji, et al., 2004; Park et al., 2004; Wu et al., 2005) will be investigated by fusing the promoter of *PRS* to the ORFs of *WUS* (*WUSCHEL*) and *WOX4* genes, paralogs of *PRS,* and determining if these transgenes can complement PRS function in a *prs* mutant background. Taken together, these experiments are a beginning step towards understanding the mechanism of non-cell autonomous NS/PRS function.

Results and Discussion

The cloned *PRS* promoter and encoding region fully function

To investigate the molecular mechanism of PRS functions *in planta*, a genomic DNA fragment located 2742 bp upstream of the putative PRS translation start codon was cloned and fused in frame with the PRS ORF to generate the p*PRS*-PRS construct.

Transformation of this construct into the *prs* mutant (Fig.A.1 A; Matsumoto & Okada, 2001) fully rescued *prs* mutant floral phenotypes (Fig.A.1B), suggesting that this fragment of the native PRS promoter contains all necessary cis-elements for the proper function of *PRS*, and that the protein encoded by the cloned PRS cDNA functions correctly *in planta*. PCR confirmed the presence of the transgene in the rescued transformants (Fig.A.1 C).

The spatial requirement for PRS function

To reveal whether the PRS protein can traffic between and/or within meristematic cell layers and whether the primordial-staged PRS transcripts are translated into protein, we fused the GFP and GUS reporters to the C-terminal of PRS protein under the control of the native promoter (described above) and transformed *prs* mutant plants (Fig.A.2 A). The expression pattern of p*PRS*-PRS~GFP and p*PRS*-PRS~GUS fusion reporters will be compared with free GFP and GUS from negative controls that include p*PRS*-GUS and p*PRS*-GFP constructs. The accumulation pattern of cell-autonomous mGFP5-ER reporter will be used to determine whether the cloned PRS promoter fragment directs the native PRS expression pattern in two lateral foci of the SAM tunica (Kim et al., 2003).

To investigate in which meristematic layer PRS functions during founder cell recruitment, we utilized three gene-specific promoters from *Arabidopsis* to express PRS~GUS fusion protein in different meristematic layers. These included the L1 – specific construct p*AtML1*-PRS~GUS *(AtML1 =MERISTEM LAYER 1*), the L1-L2 – specific construct p*AtSCR*-PRS~GUS (*AtSCR =SCARECROW*) and the L2-L3 –specific construct p*AtSTM*-PRS~GUS (*AtSTM =SHOOTMERISTEMLESS*) (Fig.A.2 B) (Sessions et al., 1999; Kim et al., 2002; 2003). All these constructs were transformed into *prs*

mutant plants and ready to be screened for phenotypic rescue and PRS~GFP accumulation patterns.

The essential cis-regulatory elements in *PRS* promoter

The meristematic expression of *NS/PRS* genes is uniquely localized to two specific foci, which prompted us to examine its regulation (reviewed by Kurata et al., 2005; Matsumoto and Okada, 2001; Nardmann and Ji. et al. 2004). Toward this end, six fragments corresponding to serial deletions of the *PRS* promoter were cloned and fused to either PRS~GUS, PRS, or GUS, and transformed into the *prs* mutant. The rescue of *prs* mutant phenotype will identify essential cis-regulatory elements in the *PRS* promoter. These constructs will provide starting materials for subsequent identification of essential short sequence motifs within the *PRS* promoter as was done for *WUS* (Baürle & Laux 2005), and the corresponding transgenics will be useful for genome-wide genetic isolation of upstream regulators of *PRS* through enhancer or suppresser screening.

Plant growth regulators affect the expression of *PRS*

Leaf stipules are heavy sinks of auxin (Avsian-Kretchmer et al., 2002) and *prs* is a stipule deletion mutant. Polar auxin transport (PAT) and auxin accumulation are shown to affect the positioning of young lateral organ primordia (Reinhardt et al., 2000, 2003). This implies that the genes involved in the recruitment of founder cells for lateral organ primordia may be subject to auxin regulation. Since NS/PRS functions during founder cell recruitment (Matsumoto & Okada, 2001; Scanlon, 2000; Scanlon & Freeling, 1997, 1998; Scanlon et al., 1996, 2000) it is possible that auxin may regulate the expression of NS/PRS. To test this hypothesis, we treated *Arabidopsis* Columbia seedlings with the synthetic auxin 1-NAA and monitored the expression level of *PRS* with quantitative

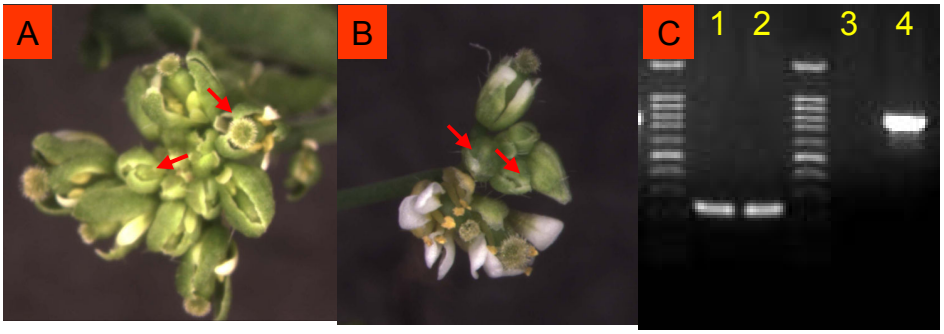

Figure A.1 The cloned *PRS* promoter and ORF can rescue the *prs* mutant floral phenotype. A. Inflorescence of *prs*. Note the absence of lateral sepal development in the mutant flowers (arrow) B. Inflorescence of *prs* transformed with p*PRS*-PRS. Note that the rescued flowers have normal lateral sepal development (arrow). C. PCR detection of *AtWOX4* (Lane 1 and 2) and p*PRS*-PRS (Lane 3 and 4). Lanes 1 and 3 are from *prs* mutant, and lanes 2 and 4 from *prs* transformed with p*PRS*-PRS.

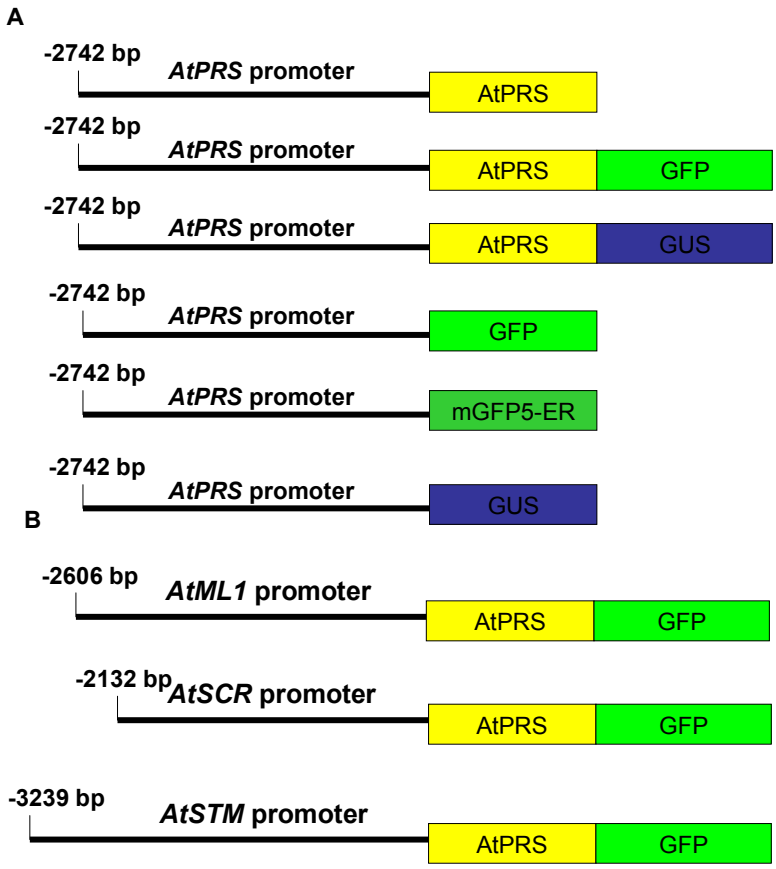

Figure A.2 Constructs of fusion reporter under the control of the native *PRS* promoter (A) and layer-specific promoters (B). See the detailed description in the text.

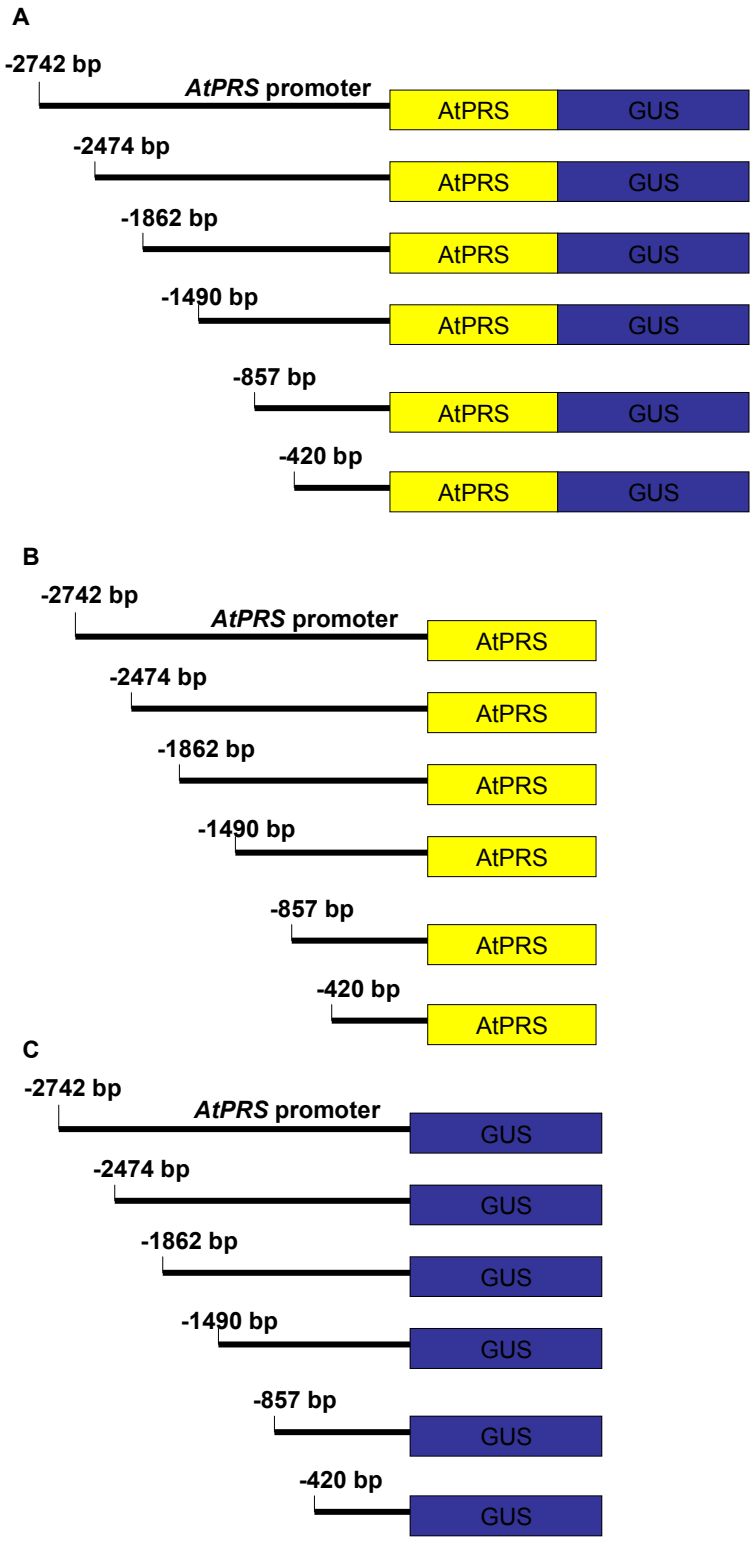

Figure A.3 Constructs containing serial deletions of *PRS* promoter. A. Fused with *PRS*-GUS. B. Fused with PRS ORF alone. C. Fused with GUS alone

RT-PCR. After 24 hours of 1-NAA treatment *PRS* transcripts accumulate to about half the level observed in untreated seedlings (Fig.A.4), suggesting that auxin represses PRS expression in the Arabidopsis shoot. The effects of additional plant growth regulators on PRS transcription were also measured. Whereas GA_3 showed similar effects as 1-NAA, ABA treatment increased the expression of *PRS* to almost three times higher than untreated samples. These data suggest that ABA signaling may play a previously unreported role during the recruitment stage of plant lateral organ development. However, the cytokinin 6-BA seems to have no obvious effect on the expression of *PRS*. It is possible that in the shoot apical meristem the expression of *PRS* is dynamic and regulated by interactions of plant hormones. Transgenic plants harboring p*PRS*-PRS~GFP and p*PRS*-PRS~GUS fusion reporters will be utilized to examine the effect of plant hormone application on the accumulation of PRS protein.

Sucrose failed to rescue the *prs* mutant phenotype

Sucrose can rescue the aborted meristem phenotype of the *stimpy* mutant, presumably by stimulating entry into the cell cycle (Wu et al., 2005). *STIMPY* is a recessive mutation in the *WOX9* gene; we therefore wondered if sucrose can also rescue the *prs* mutant phenotype. When grown in the presence of a wide range of sucrose concentrations (from 5mM up to 90mM) the *prs* mutant phenotype was not rescued. This suggests that *PRS* may act through a different morphogenetic pathway than *STIMPY/AtWOX9*. Alternatively, sucrose induced entry into the cell cycle may not be sufficient to complement the *prs* mutation.

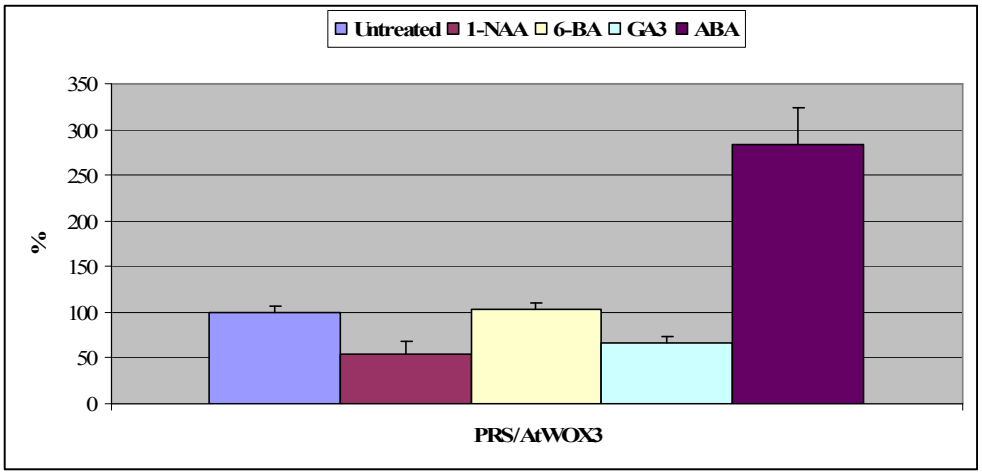

Figure A.4 Relative transcript abundance of PRS after plant hormonal treatments analyzed with quantitative real-time RT-PCR. The PRS RNA level of the untreated sample was set as 100% after normalization with ACTIN2.

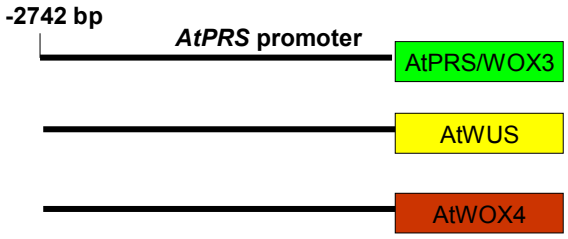

Figure A.5 *WUS* and *WOX* gene expression constructs utilized to study the evolution of WOX gene function. All constructs are driven by the native PRS promoter.

Functional evolution of *WOX* genes

WUS and 14 paralogous *WOX* (*WUSCHEL-RELATED HOMEOBOX*) genes are present in *Arabidopsis*, five of which are shown genetically to function non-cell-autonomously during the organization of various stem cell populations within plant meristems (Haecker et al., 2004; Matsumoto & Okada, 2001; Nardmann and Ji, et al., 2004; Park et al., 2004; Wu et al., 2005). The expression patterns of 8 *WOX* genes are shown to localize to disparate stem cell populations in various plant meristems, which demonstrates the functional divergence of *WOX* gene family members. In addition, WOX gene coding sequences have also diverged, even within the relatively conserved homeodomain region (Haecker et al., 2004). To examine the functional evolution of distinct WOX proteins, the ORFs of *WUS* (*WUSCHEL*) and *AtWOX4* genes were fused to the PRS promoter (Fig.A.5) and transformed into the *PRS* mutants. Rescue of the mutant phenotype may indicate that the diversification of WOX gene family function may be largely attributed to their divergent promoters, instead of distinct protein function.

Materials and Methods

Plant growth conditions

Arabidopsis thaliana plants were sown in solid (0.7% agar) or liquid medium containing ½ MS basal medium (Sigma-Aldrich) plus 1% sucrose (pH 5.7), stratified at 4^{o}C for 3 days, and grown in a laboratory at 23^{o}C under continuous illumination. Otherwise, plants were cultivated in RediEarth medium in the Plant Biology Department greenhouse at Athens, GA.

DNA isolation and gene cloning

Genomic DNA was isolated from *Arabidopsis* whole seedlings as described (Dellaporta et al., 1983), or from young leaves with Extract-N-Amp™ Plant PCR Kits (Sigma-Aldrich). The promoters were amplified from diluted Columbia genomic DNA using high-fidelity DNA Polymerase (invitrogen), verified by sequencing (Applied Biosystems), and ligated in frame with WOX, GUS or GFP reporter gene in pBI101 vector. All primers utilized in PCR and construct building are summarized in Table 4.1.

RNA isolation, gene cloning and RT-PCR

Total RNA isolation and DNase I treatment, and cDNA synthesis were performed as described (Nardmann and Ji et al., 2004). The coding regions of *PRS*, *WUSCHEL* and *AtWOX4* were amplified from Columbia cDNA preparation with high-fidelity DNA Polymerase (invitrogen), verified by sequencing (Applied Biosystems), and ligated into pre-restricted pBI101 vectors. Compatible restriction sites were incorporated into the 5' ends of the PCR primers as summarized in Table A.1.

Quantitative real-time RT-PCR was performed using SYBR green Supermix (Bio-Rad); primers are described in Table A.1. Each sample was assayed in three replicates. Analysis of relative *PRS* normalized to control *ACTIN2* expression was performed as described (Livak & Schmittgen, 2001).

DNA constructs and plant transformation

All constructs were in the background of pBI101 vector and introduced into *Agrobacterium tumefaciens* strain LBA4404 or GV3101 by electroporation. The *Arabidopsis thaliana* wild type Columbia and mutant *prs* (*pressed flower*) were transformed by floral dip (Clough & Bent 1998). Transformants were selected on ½ MS medium containing 50mg/L kanamycin and then transplanted into water saturated

Table A.1 Summary of oligonucleotide primers used in this study

name	primer sequence	primer use
PRSF8	AAGCTTACGCGTACGTGTGTACGTGAATGA	PRS promoter (2742bp)
PRSF11	AAGCTTAAAGGAAATGCAGAGGCAAA	PRS promoter (2474bp)
PRSF13	AAGCTTCCGGAACCAATCAACCTAAAC	PRS promoter (1862bp)
PRSF14	AAGCTTGACAGAGGGGCAGAGACAGG	PRS promoter (1490 bp)
PRSF15	AAGCTTTGGCGTTTCTCTTTGTTTTG	PRS promoter (857bp)
PRSF16	AAGCTTAAAGTGAAAACTCCCGACAAAA	PRS promoter (420bp)
PRSR6	GGATCCGGCGCGCCTCTCCGTTCAGACAAA TATTGATG	PRS promoter
AtML1F1	AAGCTTTCATCACCAATCAACCATCAA	AtML1 promoter
AtML1R1	GGCGCGCCGGTGACTTCTGCGCCGGACTTA GT	AtML1 promoter
AtSCRF3	AAGCTTTGCTGGTGTTGAATGGATAAGG	AtSCR promoter
AtSCRR1	GGCGCGCCGGAGATTGAAGGGTTGTTGGT	AtSCR promoter
AtSTMF1	AAGCTTCTTAAATGAGAGACCCCCGAAA	AtSTM promoter
AtSTMR1	GGCGCGCCCTTCTCTTTCTCTCACTAGTATT AT	AtSTM promoter
PRSF10	GGATCCATGAGTCCTGTGGCTTCAACGA	PRS ORF
PRSR4	GAGCTCTTAAAGTTTGGTACTGTCTTGTT	PRS ORF
PRSF12	GGCGCGCCATGAGTCCTGTGGCTTCAACGA	PRS ORF fused with GFP
PRSR3	GGATCCCAAGTTTGGTACTGTCTTGTT	PRS ORF fused with GFP
PRSF12	GGCGCGCCATGAGTCCTGTGGCTTCAACGA	PRS ORF fused with GUS
PRSR5	GGATCCAAGTTTGGTACTGTCTTGTT	PRS ORF fused with GUS
mgfp5ERF	GGATCCAAGGAGATATAACAATGA	mgfp5-ER ORF
mgfp5ERR	GAGCTCTTAAAGCTCATCATGTTT	mgfp5-ER ORF
AtWUSF1	GGATCCATGGAGCCGCCACAGCATCAGCA	WUS ORF fused with GUS
AtWUSR1	GAGCTCCTAGTTCAGACGTAGCTCAAGAG	WUS ORF fused with GUS
AtWOX4F1	GGATCCATGAAGGTTCATGAGTTT	AtWOX4 ORF fused with GUS
AtWOX4R1	GAGCTCTCATCTCCCTTCAGGATG	AtWOX4 ORF fused with GUS
AtWOX4F6	GCGAGAGACAGAAGCAGAAGA	PCR verification for AtWOX4
AtWOX4R12	TCCACTAACTCCTCCTTCTCCA	PCR verification for AtWOX4
PRSF10	GGATCCATGAGTCCTGTGGCTTCAACGA	PCR verification for PRS
NOSTR	ATCGCAAGACCGGCAACAGGATTC	PCR verification for PRS
PRSF18	GATACCATTGAAAACCCTGGAA	Real time PCR for PRS
PRSR8	AGCCTTTGACACATCATACAG	Real time PCR for PRS
ACT2F	TCGTTTCTATGATGCACTTGTGTG	Real time PCR for ACTIN2
ACT2R	ATGGGACTAAAACGCAAAACGA	Real time PCR for ACTIN2

RediEarth medium. T1 and T2 *Arabidopsis* transgenic seedlings were used for further experiments.

Plant hormonal treatment

Columbia seeds were grown in the above described liquid medium (20ml) in dark at 23 ^{o}C for 5 days after stratification, and then transferred to the same fresh liquid medium (20ml) supplemented with 50 μM 1-NAA (1-Naphthylacetic acid), 50 μM GA_3 (Gibberellic acid), 50 μM ABA (Abscisic acid), 50 μM 6-BA (6-benzylaminopurine) (Sigma-Aldrich), or 0.5% Ethanol or 0.5% DMSO (hormone solvent control). After 24 hours of incubation in dark with gentle shaking, the whole seedlings were harvested, immediately frozen in liquid nitrogen and subject to RNA isolation.

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APPENDIX B

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