Tissue Specificity and Evolution of Meristematic WOX3 Function[1][W][OA]

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The *WUSCHEL*-related homeobox (*WOX*) gene *PRESSED FLOWER1* (*PRS1*) performs a conserved function during lateral organ development in Arabidopsis (*Arabidopsis thaliana*). Expressed in the periphery of the shoot meristem, PRS1 recruits founder cells that form lateral domains of vegetative and floral organs. Null mutations in *PRS1* cause the deletion of lateral stipules from leaves and of lateral sepals and stamens from flowers. Although *PRS1* expression is described in the L1 layer, PRS1 recruits founder cells from all meristem layers. The mechanism of non-cell autonomous PRS1 function and the evolution of disparate *WOX* gene functions are investigated herein. Meristem layer-specific promoters reveal that both L1 and L1-L2 expression of *PRS1* fail to fully rescue PRS1 function, and PRS1 protein does not traffic laterally or transversely between shoot meristem layers. PRS1 protein accumulates within all meristematic cell layers (L1-L2-L3) when expressed from the native promoter, presumably due to low-level transcription in the L2 and L3 layers. When driven from the *PRS1* promoter, full rescue of vegetative and floral prs1 mutant phenotypes is provided by *WUSCHEL1* (*WUS1*), which is normally expressed in the stem cell organizing center of shoot meristems. The data reveal that WUS1 and PRS1 can engage in equivalent protein-protein interactions and direct transcription of conserved target genes, suggesting that their subfunctionalization has evolved primarily via diverse promoter specificity. Unexpectedly, these results also suggest that meristematic stem cells and lateral organ founder cells are intrinsically similar and formed via equivalent processes such that their ultimate fate is dependent upon stage-specific and domain-specific positional signaling.

The *WUSCHEL*-related homeobox (*WOX*) genes of Arabidopsis (*Arabidopsis thaliana*) comprise a 15-member family of plant-specific transcriptional regulators that play fundamental roles in plant development (Haecker et al., 2004). Multiple analyses of *WOX* gene expression combined with more limited genetic analyses in a variety of plant species suggest a conserved *WOX* gene function during the establishment of stem cell or initial cell identity in plant embryos, meristems, or lateral organs (for review, see Chandler et al., 2008; Ten Hove and Heidstra, 2008). For example, WUSCHEL1 (WUS1) is required to organize stem cells in the shoot apical meristem (SAM; Laux et al., 1996; Mayer et al., 1998), WOX5 in root meristems (Gonzali et al., 2005; Sarkar et al., 2007), PRETTY FEW SEEDS2/WOX6 in developing ovules (Park et al., 2005), STIMPY1/WOX9 in embryos and shoots (Wu et al., 2005, 2007), whereas WOX2 and STIMPY-LIKE1/WOX8 (STPL1) regulate cell fate in the apical and basal poles of the early proembryo (Breuninger et al., 2008). Analyses of WOX3 function in Arabidopsis (*PRESSED FLOWER1* [*PRS1*]; Matsumoto and Okada, 2001; Nardmann et al., 2004) and in maize (*Zea mays*; the duplicate genes *narrow sheath1* [*ns1*] and *narrow sheath2* [*ns2*]; Scanlon et al., 1996; Scanlon, 2000; Nardmann et al., 2004) identified a conserved function during recruitment of founder cells from lateral domains of shoot meristems that ultimately form lateral and marginal regions of leaves and leaf orthologs of the flower. Thus, null mutations in *PRS1* prevent the initialization of lateral founder cells, cause brachytic plant phenotypes, and render the preprimordial deletion of lateral stipules from vegetative leaves and lateral sepals/stamens from Arabidopsis flowers (Fig. 1; Matsumoto and Okada, 2001; Nardmann et al., 2004). Likewise, mutations in both *ns1* and *ns2* in maize cause narrow leaf and floral organ phenotypes due to the failure to recruit founder cells that give rise to lateral domains in these organs (Scanlon et al., 1996; Scanlon and Freeling, 1997, 1998).

A second function described for several WOX proteins is non-cell autonomy, wherein phenotypic responses to *WOX* gene function are observed in cells and tissues outside the domain of *WOX* gene expression. For example, WUS1 confers stem cell identity to cells the central zone, a SAM domain located above the region of WUS1 RNA accumulation (Mayer et al., 1998). Similarly, expression of *PRS1* is described in the

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[1] This work was supported by the National Science Foundation (grant nos. IBN–517070, IBN–0649810, and PGRP–032195 to M.J.S.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.130765

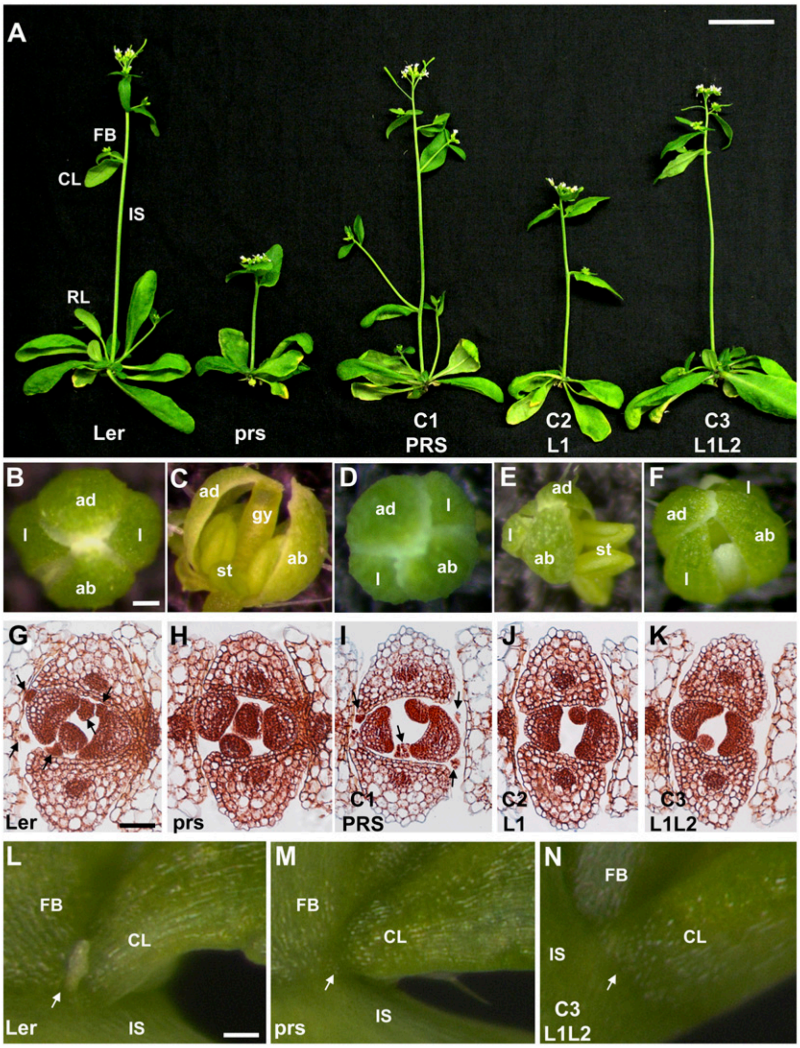

Figure 1. Expression of *PRS1* in the L1 and L1-L2 histological layers fails to complement prs1 mutant phenotypes. A, Whole plant phenotypes of (left to right) prs1 non-mutant (L*er*), prs1 mutant (prs), and transgenic plants expressing PRS~GFP fusion proteins driven by the native *PRS1* promoter (C1 PRS), the L1-specific promoter *ML1* (C2 L1), and the L1-L2-layer-specific promoter *SCR1* (C3 L1L2). Note that the short plant stature phenotype seen in prs mutant plants is complemented in C1 PRS plants; partial complementation is seen in C3 L1-L2 plants, and still weaker complementation is observed in C2 L1 plants. B to F, Stage 12 floral phenotypes (i.e. prior to bud opening; Smyth et al., 1991) of plants shown in A. Whereas L*er* (B) and C1 PRS (D) flowers have four sepals of normal width, prs1 mutant (C) flowers typically fail to develop lateral sepals. E, C2 L1 flowers exhibit incomplete rescue of prs1 mutant floral phenotypes. Although C3 L1-L2 (F) flowers form four sepals, the lateral sepals are narrow and fail to fully enclose the underlying floral organs. G to K, Transverse sections of shoot apices of vegetative seedlings show development of lateral stipules (arrows) in rosette leaves of L*er* (G) and C1 PRS (I) plants, but not in prs1 mutants (H), C2 L1 plants (J), or C3 L1-L2 plants (K). L to N, Lateral stipule (arrow) formation at the base of cauline leaves in L*er* plants; prs1 mutant (M) and C3 L1-L2 plants (N) fail to form stipules on cauline leaves. CL, Cauline leaf; FB, floral bud; ad, adaxial sepal; ab, abaxial sepal; l, lateral sepal; st, stamen; gy, gynoecium; IS, inflorescence stem. Bars in A = 3 cm; B (for B to F) = 100 μm; G (for G to K) = 50 μm; L (for L to N) = 50 μm.

proto-epidermal (i.e. L1) layer of shoot meristems at two lateral foci and in the L1-derived layers of lateral organ primordia (Matsumoto and Okada, 2001). However mutations in *PRS1* render the deletion of lateral sepals and stamens (Fig. 1B; Matsumoto and Okada, 2001), which are derived from all three histological layers (L1-L2-L3) of the floral meristem (Jenik and Irish, 2000). During sepal emergence at stage 3, *PRS1* expression is localized to lateral regions of the floral meristem. In addition to the deletion of lateral sepals, however, the adaxial and abaxial sepals of prs1 mutant flowers are thinner than wild-type sepals owing to the deletion of the marginal, knife-edge cells in these organs (Matsumoto and Okada, 2001). These data suggest that PRS1 functions non-cell autonomously in the lateral dimension of the floral meristem. Maize shoot meristems contain just two tissue layers (L1-L2). Likewise, although transcripts of the maize *PRS1* ortholog *ns1* accumulate in the SAM L1 layer at two lateral foci, NS1 signals the recruitment of leaf founder cells from a much larger lateral domain within the shoot apex and *ns* mutations cause the deletion of lateral domains derived from both L1 and L2 tissue layers (Scanlon et al., 1996; Scanlon, 2000; Nardmann et al., 2004). More curiously, clonal analyses reveal that *ns1* expression in the L1 layer alone is not sufficient to confer NS1 function; expression is required in the L2 layer of the SAM (Scanlon, 2000). The nature of the NS/PRS1 non-cell autonomous signals and their mode of propagation are not yet known.

Experiments were performed to address these apparent discrepancies in the domains of *WOX3* gene expression and function and to investigate potential mechanisms of WOX3 non-cell autonomy during recruitment of lateral organ founder cells. Owing to its relatively small molecule weight, the GFP does not impede intercellular movement of fusion proteins and so is routinely used for investigations of protein trafficking (for review, see Heinlein, 2002). Meristem layer-specific and domain-specific promoters were employed to drive expression of PRS1~GFP fusion proteins and determined that full PRS1 function requires its accumulation in all three meristematic tissue layers. No evidence was found for either transverse or lateral trafficking of PRS1 protein in meristem cell layers. Instead, the data indicate that *PRS1* is expressed in a dynamic, stage-specific manner in all three meristem layers, albeit at low levels in the L2 and L3. These data suggest that PRS1 non-cell autonomy does not involve its intercellular trafficking. Instead, non-cell-autonomous PRS1 function involves either a transducible downstream signal or the release of an upstream inhibitory signal, which is propagated laterally in the shoot meristem. WUS1 is able to fully complement PRS1 function, suggesting that the neofunctionalization of these *WOX* genes has occurred primarily by the evolution of promoter specificities, rather than by divergence of protein functions. Furthermore, these data suggest that the indeterminate stem cells of the SAM central zone and the lateral organ founder cells of the SAM peripheral zone are essentially equivalent at formation, whereupon their fate is determined by position-dependent signaling.

RESULTS

Promoter Specificity of PRS Function

A 2,742-bp fragment of genomic DNA upstream from the predicted start codon of the Arabidopsis *PRS1* gene was isolated from the Columbia ecotype and used to express the 966-bp *PRS1* coding region fused in frame to the GFP. This p*PRS1*-PRS∼GFP construct (designated C1; Fig. 2) complemented all vegetative and floral phenotypes of prs1 null mutant plants. C1-transformed prs1 plants displayed normal plant stature and developed lateral leaf stipules and lateral floral sepals (Fig. 1, A, I, and D). In contrast, prs1 mutants failed to develop lateral leaf stipules (Fig. 1H), and over 75% of prs1 mutant flowers developed fewer than four sepals (Fig. 1C; Fig. 3A). Moreover, of the 24.2% of prs1 mutant flowers that did form four sepals, none developed four sepals of normal, nonmutant width (Fig. 3B). In contrast, over 97% of C1-transformed plants formed four sepals (Fig. 3A), 100% of which were of normal width (Fig. 3B). These data reveal that both the cloned *PRS1* promoter fragment and the PRS1∼GFP translational fusion protein can confer all known PRS1 functions.

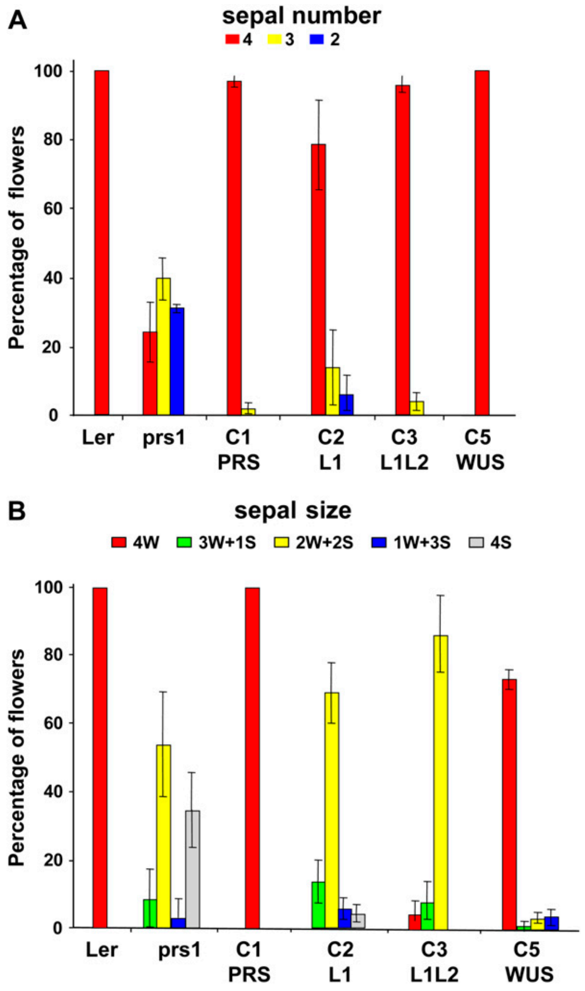

Figure 3. Sepal initiation and morphology are disrupted by loss of PRS1 function. Total number of sepals initiated (A) and sepal size relative to L*er* floral buds (B) are reduced in prs1 mutant flowers, but are restored to near normal levels in C1 PRS1 and C5 WUS1 transgenic mutant plants. Although sepal number is improved in C2 L1 and C3 L2L3 transgenics (A), the sepal size phenotype is not complemented (B).

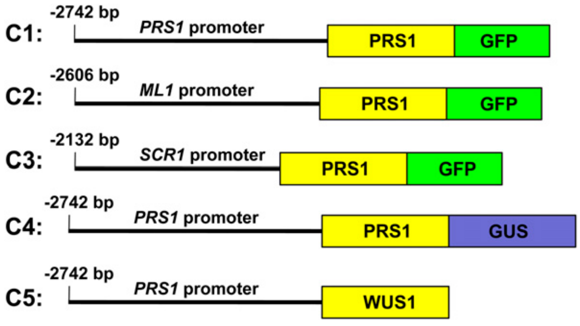

Figure 2. Expression constructs used in this study. Promoter regions are depicted as black bars; open reading frames are shown as colored rectangles. Not to scale. Plasmid details are provided in "Materials and Methods."

To investigate the layer specificity of PRS1 function in shoot meristems, the L1-specific promoter *MERISTEM LAYER1* (*ML1*) and the L1-L2-specific *SCARECROW1* (*SCR1*) promoter were used to drive expression of PRS1∼GFP fusion constructs in prs1 null mutant plants. The tissue layer specificities of the *ML1* and *SCR1* promoters are well documented (Sessions et al., 1999; Wysocka-Diller et al., 2000; Kim et al., 2002, 2003). p*ML1*-PRS1∼GFP constructs (designated C2) provided weak complementation of prs1 mutant phenotypes. C2 plants were intermediate in stature between prs1 mutant and non-mutant *Ler* (Fig. 1A). Although over 78% of C2 flowers developed four sepals (Fig. 3A), none contained four sepals of normal width (Fig. 3B). Instead, the majority of C2 plants developed very narrow lateral sepals (Fig. 1E), whereas no plants were identified that contained lateral leaf stipules (Fig. 1J). These data demonstrate that transcription of *PRS1∼GFP* in the L1 meristematic layer fails to fully complement PRS1 function during vegetative and floral development.

In comparison, p*SCR*-PRS1∼GFP constructs (designated C3) conferred improved complementation of prs1 mutant plant stature and floral phenotypes, although full rescue was not achieved by L1-L2 expression of *PRS1*. Specifically, C3 plants grew to nearly normal height (Fig. 1A), and over 95% of C3 flowers formed four lateral sepals (Fig. 3A). Although improved over C2 plants, nonetheless, over 86% of C3 flowers that developed four sepals formed two wide sepals (i.e. adaxial and abaxial) and two abnormally narrow lateral sepals (Figs. 1F and 3B). Furthermore, analyses of seedling shoot apices revealed that C3 rosette leaves failed to develop lateral stipules. Although the *SCR1* promoter utilized in C3 constructs drives expression in the L1-L2 layers of inflorescence and floral meristems, *SCR1* expression within the vegetative SAM is primarily localized to the L1 cell layer (Wysocka-Diller et al., 2000; Kim et al., 2002). Therefore, in addition to analyses of rosette leaves, lateral stipule development in C3 plants was also monitored in cauline leaves, which are derived from the inflorescence meristem. As shown in Figure 1N, lateral stipules also fail to develop at the base of C3 cauline leaves, despite *PRS1* expression in the L1-L2 meristematic layers. Thus, expression of *PRS1∼GFP* in the L1-L2 layers also fails to render complete complementation of prs1 mutant floral and vegetative phenotypes.

Tissue Domain Specificity of PRS1 Function

Previous analyses of stage 3 floral meristems described *PRS1* transcript accumulation at two lateral foci within the outer meristem layer (Matsumoto and Okada, 2001). Comprised of 2742 bp of the *PRS1* promoter driving the PRS1∼GFP fusion protein (Fig. 2), construct C1 complements the prs1 mutant phenotype and thereby seemingly confers full PRS1 function (Fig. 1). Confocal imaging from above the stage 3 floral meristem (i.e. during sepal emergence; Smyth et al., 1991) of C1 plants revealed the accumulation of PRS1∼GFP fusion protein in two lateral foci, in agreement with previous descriptions of transcript accumulation. In contrast to the accumulation of *PRS1* transcripts, however, PRS1∼GFP signal is not confined to the L1 layer but is observed in multiple meristematic layers (Fig. 4, A and B). Imaging of C1 floral meristems identified a punctate, presumably nuclear-localized pattern of PRS1∼GFP accumulation within the L1-L3 layers.

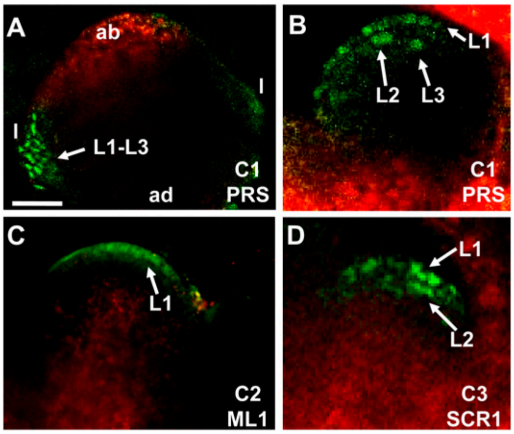

Figure 4. Layer-specific accumulation of PRS∼GFP fusion proteins. Confocal imaging of top view (A) of stage 3 floral meristem in C1 PRS transgenic plants reveals accumulation of PRS1∼GFP fusion protein (arrow) in two lateral foci and in multiple cell layers. B, Side image of lateral domain of C1 PRS floral meristem. C, When expressed from the *ML1* promoter, PRS1∼GFP accumulation is observed only in the L1 layer of C2 L1 transgenic plants. D, PRS1∼GFP fusions expressed from the *SCR1* promoter are observed in L1-L2 layers of the C3 L1-L2 floral meristem. l, Lateral domain; ab, abaxial domain; ad, adaxial domain. Bar = 25 μm.

GFP-tagged PRS1 protein is restricted to the L1 layer when expressed from the L1-specific *ML1* promoter in C2 floral meristems (Fig. 4C). Cell autonomy of the PRS1∼GFP fusion protein is likewise indicated in C3 plants; punctate GFP labeling is confined to the L1-L2 cell layers of the floral meristem when driven from the *SCR1* promoter (Fig. 4D). In lieu of previous reports detecting native *PRS1* transcript accumulation only in the L1 (Matsumoto and Okada, 2001), the inability of PRS1 to traffic transversely from the L1 or L2 meristematic cell layers raises questions as to the mechanism of PRS1∼GFP protein accumulation in the L1-L3 layers when driven by the *PRS1* promoter. To address this question, we analyzed the accumulation patterns of the cell-autonomous reporter protein β-glucuronidase (GUS; Jefferson et al., 1987) when fused to PRS1 and expressed from the *PRS1* promoter. Due to the large M_r of the GUS protein, GUS fusion proteins are unable to traffic between meristematic cell layers (Kim et al., 2002, 2003) and serve as useful markers of plant promoter specificity.

Construct p*PRS1*-PRS1∼GUS (designated C4; Fig. 2) fully complemented the prs1 mutant phenotypes, including plant height, sepal number, sepal size, and formation of lateral stipules (Fig. 5, A–D; data not shown). Owing to the inability of GUS fusion proteins to traffic intercellularly (Kim et al., 2003), these data suggest that PRS1 function does not require its intercellular trafficking. Histological analyses of C4 plants revealed that PRS1-GUS fusion protein accumulated in a dynamic pattern during floral meristem development (Fig. 5, E–J). GUS signal is observed in the L1-L3 layers during emergence of the stage 1 floral meristem from the inflorescence meristem (Fig. 5, E and F). No GUS accumulation was noted in later stage 2 floral meristems (Fig. 5E); however, GUS accumulation reappeared within the L1-L3 layers at two lateral

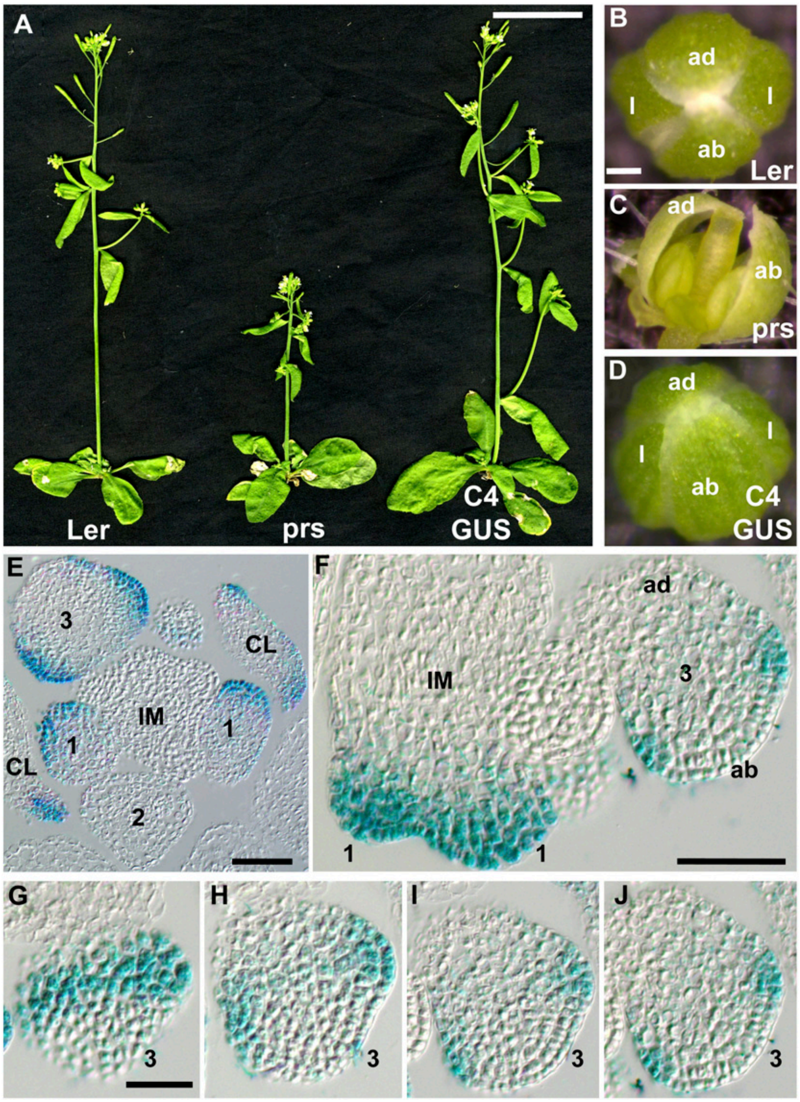

Figure 5. Functional analyses and accumulation of PRS1∼GUS fusion proteins expressed from the native *PRS1* promoter. C4 GUS transgenic prs1 mutant plants exhibit normal plant height (A) and normal sepal development (D) compared to prs1 mutant (C) and L*er* (B) flowers. E and F, GUS staining of C4 GUS inflorescences reveal accumulation of PRS1∼GUS fusion proteins in the margins of cauline leaves, in stage 1 floral meristems, and in stage 3 floral meristems. G to J, Serial sections from the crown (G) of a stage 3 floral meristem in C4 GUS transgenic plant into medial sections (J) reveal a stripe of PRS1∼GUS accumulation across the meristem dome that bypasses the meristem median region at two lateral foci. CL, Cauline leaf; IM, inflorescence meristem; ad, adaxial domain; ab, abaxial domain; l, lateral domain; 1, stage 1 floral meristem; 2, stage 2 floral meristem; 3, stage 3 floral meristem. Bars in A = 3 cm; B (for B to D) = 100 μm; E and F = 50 μm; G (for G to J) = 25 μm.

foci during sepal initiation in stage 3 floral meristems (Fig. 5, E and F). Unexpectedly, serial sections from the apical to basal poles of the stage 3 meristems revealed GUS accumulation not just at two lateral foci but in an apical arc that traverses and surrounds the upper dome of the floral meristem (Fig. 5, G–J). Considering the cell autonomy of GUS fusion proteins, these data reveal that *PRS1* is in fact transcribed in all three layers of the Arabidopsis floral meristem.

WUS1 Can Complement PRS1 Function

To analyze the subfunctionalization of the WOX proteins PRS1 and WUS1, the p*PRS1* promoter was used to drive expression of the WUS1 coding sequence in prs1 mutant plants (construct C5: Fig. 2). The founding member of the WOX homeodomain family, *WUS1*, is normally expressed in the organizing center of shoot meristems, immediately below the central zone (Mayer et al., 1998). WUS1 provided full complementation of PRS1 function during floral and vegetative development (Fig. 6, A, D, and G). C5 plants were of normal height and developed lateral leaf stipules; over 99% of C5 flowers developed four sepals of non-mutant width (Fig. 3).

NS1 Protein Localizes to the L1 and L2 Layers of the Maize Apex

A polyclonal antibody was raised against full-length maize NS1 protein (see “Materials and Methods”) and used to investigate the accumulation of NS protein in maize vegetative shoot apices. Compared to previous in situ hybridization analyses wherein *NS* mRNA was detected in the L1 layer at two lateral foci in the maize SAM (Nardmann et al., 2004; Henderson et al., 2005; Fig. 7A), immunohistolocalization assays also identified NS protein in two lateral foci, although accumulation is observed in both the L1 and L2 apical layers (Fig. 7B). These data reveal that, as in Arabidopsis, accumulation of maize PRS1 orthologous proteins is detected in all histological layers of shoot meristems,

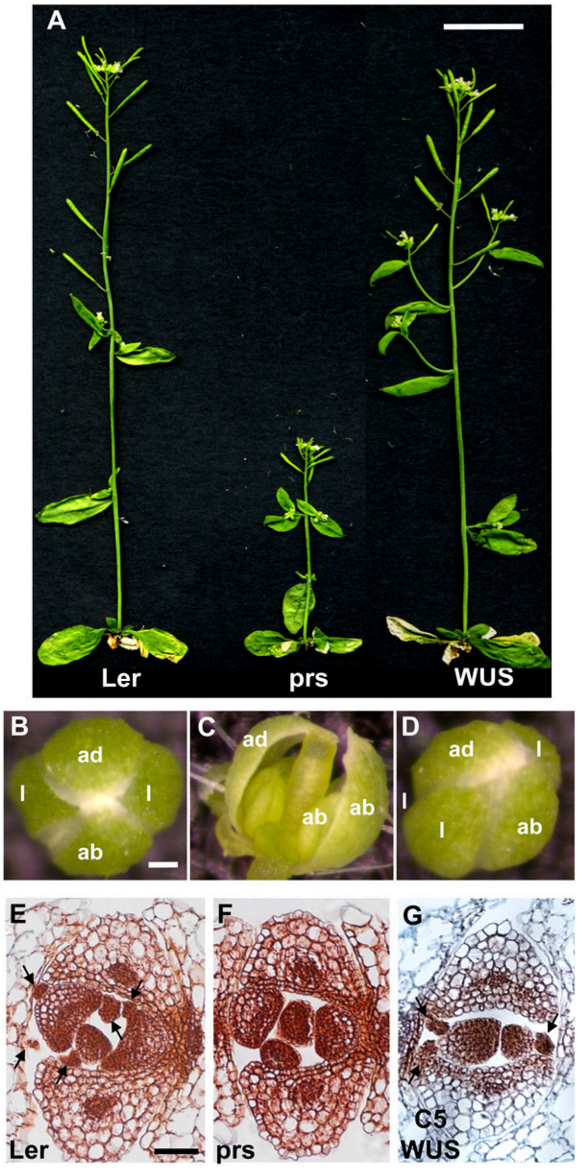

Figure 6. WUS1 fully complements PRS1 function. When expressed from the native *PRS1* promoter, WUS1 (C5 WUS) fully complements all phenotypes (A, E, and I, respectively). l, Lateral sepals; ad, adaxial sepals; ab, abaxial sepals; arrows, lateral stipules. Bars in A = 3 cm; B (for B to D) = 100 μm; E (for E to G) = 50 μm.

in contrast to previous analyses reporting expression of *NS* transcripts exclusively in the L1 layer (Nardmann et al., 2004).

DISCUSSION

PRS1 Is Required in All Meristem Layers and Does Not Traffic Intercellularly

Both L1-specific and L1-L2-specific expression of *PRS1* fail to confer full PRS1 function, and PRS1-GFP fusion proteins fail to traffic from the L1 or the L2 into underlying meristematic cell layers (Figs. 1, A, E–F, and J–K, and 4, C and D). In contrast, both the PRS1∼GFP fusion protein and the cell-autonomous PRS1∼GUS fusion protein provide full complementation of prs1 mutant phenotypes and accumulate in all three meristematic cell layers when driven from the *PRS1* promoter. Taken together, these data suggest that PRS1 function during recruitment of lateral organ founder cells does not require transverse trafficking of PRS1 protein in Arabidopsis shoot meristems.

In lieu of the inability of PRS1 to traffic transversely from the L1 into the L2 or from the L2 into the L3 (Fig. 4, C and D), the observed accumulation of PRS1∼GFP protein in the L1-L2-L3 meristematic layers of fully complemented prs1 mutant plants (Figs. 4, A and B, and 5, E–J) suggests strongly that the *PRS1* promoter drives transcription in all histological layers of the floral meristem. GUS fusion proteins are cell autonomous (Kim et al., 2002, 2003), and the accumulation of PRS1∼GUS in all three meristematic layers also supports the interpretation that *PRS1* is transcribed within the L2 and L3 layers as well as in the L1. Furthermore, the data suggest that *PRS1* transcription in the L2 and L3 layers is of such low abundance that it is undetectable by in situ hybridization analyses.

Comparisons of mRNA localization and protein accumulation of the duplicate maize *PRS1* orthologs *NS1* and *NS2* suggest an analogous scenario. Whereas in situ hybridization analyses detected *NS* transcripts in only the L1 meristem layer (Nardmann et al., 2004; Henderson et al., 2006; Fig. 7A), NS proteins accumulate in both the L1 and L2 layers. These data offer an explanation for results obtained in previous clonal analyses of NS1 function, which revealed that *NS1* expression is required in the L2 layer of the SAM. Despite the detection of *NS1* mRNA in the L1 layer only, meristematic sectors in which NS1 function was present in the L1 but lost in the adjoining L2 layer conditioned ns mutant phenotypes (Scanlon, 2000).

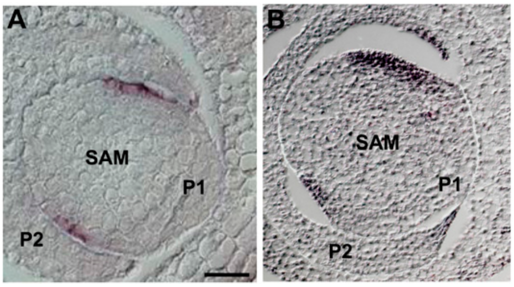

Figure 7. NS proteins accumulate in the L1 and L2 layers of the maize SAM. A, In situ hybridization analysis of a transverse section through the maize shoot apex reveals *ns* mRNA expression (purple) in two lateral foci in the L1 layer of the SAM. B, Immunohistolocalization analyses of slightly oblique, non-median section through the maize shoot apex reveals NS protein accumulation (purple) in two lateral foci, but in the L1-L2 layers. The image shown in A was generated by J. Nardmann and W. Werr and reproduced from Nardmann et al. (2004). Bar = 25 μm.

Likewise, in Arabidopsis, the accumulation of PRS1 protein in all meristematic cell layers correlates with PRS1 function during the initiation of lateral sepals, which are derived from the L1-L2-L3 layers of floral meristems (Jenik and Irish, 2000). Therefore, as in Arabidopsis, we propose that *NS* transcripts accumulate in both the L1 and L2 meristem layers (Fig. 7B), although expression in the L2 layer is below the detection level of our in situ hybridization protocol. Indeed, comparative massively parallel sequencing of cDNA prepared from tissues laser-microdissected from the L1 versus the L2 layer of the maize SAM reveals the presence of *NS* transcripts in both SAM layers (nine total transcripts identified in microdissected L1 tissue versus four transcripts identified in the L2; K. Ohtsu and P. Schnable, unpublished data), as predicted from the NS immunohistolocalization data presented herein (Fig. 7B).

Figures 5 and 7 also provide no evidence of WOX3 lateral trafficking within shoot meristems. In both Arabidopsis and maize, the lateral localization of WOX3 protein is equivalent to the lateral accumulation of *WOX3* transcript. Despite the restricted accumulation of NS RNA and protein to two lateral foci during leaf initiation (Fig. 7), NS functions non-cell autonomously to recruit founder cells in a larger domain that extends laterally to include the leaf margins (modeled in Fig. 8A; Scanlon et al., 1996; Scanlon, 2000; Nardmann et al., 2004). Similarly, although PRS1 accumulates in two lateral foci of the stage3 floral meristem, genetic analyses revealed that PRS is required for the development of lateral sepals as well as the margins of the adaxial and abaxial sepals (Matsumoto and Okada, 2001). As modeled in Figure 8B, PRS1 functions non-cell autonomously in lateral domains of the floral meristem that extend beyond the region of *PRS1* transcription and protein accumulation. Thus, our results suggest that WOX3 non-cell autonomy in maize and Arabidopsis meristems is not accomplished by lateral trafficking of WOX3 protein but via the induction of a transducible signal or the release of an upstream inhibitory signal that extends laterally from the site of WOX3 accumulation.

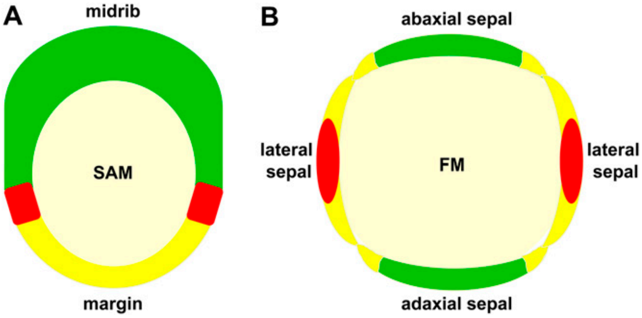

Figure 8. Model for WOX3 accumulation and lateral non-cell autonomy in maize and Arabidopsis. A, Leaf development from the maize SAM. Polar auxin transport is required to initiate maize leaf development at the midrib region and recruit founder cells for the central domain (green). Accumulation of NS protein localizes to two lateral foci (red), although NS functions non-cell autonomously to recruit founder cells in the much larger margin domain shown in yellow (Scanlon, 2000). Loss of NS function generates mutant half leaves in which the entire margin domain is deleted (Scanlon et al., 1996). B, Sepal development in stage 3 floral meristem (FM) in Arabidopsis. The adaxial and abaxial sepals initiate first (green). PRS1 accumulates at two lateral foci (red) to initiate recruitment of lateral sepal founder. Genetic analyses (Matsumoto and Okada, 2001) reveal that PRS1 functions non-cell autonomously in a larger lateral domain (yellow) to effect recruitment of founder cells that give rice to the entire lateral sepals in addition to the margins of the adaxial and abaxial sepals.

PRS1 Accumulation Pattern in Floral Meristems Is Dynamic

Analyses of GUS activity in transgenic plants expressing PRS1-GUS fusion proteins from the *PRS1* promoter (construct C4, Fig. 2) demonstrate a dynamic pattern of PRS1 accumulation during floral meristem emergence and sepal initiation (Fig. 5, E–J). No PRS1 accumulation is observed in the inflorescence meristem proper; however, PRS1∼GUS staining is observed at the meristem periphery in a broad domain that encompasses all three tissue layers of the initiating floral meristem at stage 1 (Fig. 5, E and F). Notably, loss of PRS1 function during stage 1 has no apparent effect on floral meristem initiation; prs1 mutants show no defects in floral meristem number or morphology (Matsumoto and Okada, 2001).

Intriguingly, apical-to-basal serial sections through the floral meristem revealed that stage 3 PRS1 accumulation is not limited to two lateral foci but actually forms a stripe of expression that encircles the upper meristem dome and passes through the two lateral foci at opposite sides of the floral apex (Fig. 5, G–J). Thus, during floral meristem ontogeny, PRS1 accumulation initiates as a rather broad and nonspecific domain at stage 1 but focuses to a lateral stripe of expression during stage 3. Previous analyses proposed that PRS1 expression at two lateral foci during stage 3 floral meristems is required to initialize founder cells that give rise to lateral sepals (Matsumoto and Okada, 2001; Nardmann et al., 2004). The function of the apical-most portion of the lateral stripe of PRS1 accumulation across the dome of the floral meristem (Fig. 5G) described herein is unknown. Considering that sepals are initiated from founder cells located at the meristem peripheral zone and not from apical domains, in the absence of plant cell migration it seems highly unlikely that this apical stripe of PRS1 marks the location of sepal precursor cells. We speculate that this lateral stripe of PRS1 accumulation does indeed initialize founder cells throughout its extent from the meristem periphery to the apical dome; however, only those initials located in the PZ are in the correct position to receive the developmental signal(s) that imparts sepal identity.

When driven by the *ML1* and *SCR1* promoters, *PRS1* transcripts are not limited to the lateral domains of the floral meristem but are expressed throughout the periphery and crown of the stage 3 floral meristem. However, no adverse phenotypic effects were observed from this ectopic PRS1 accumulation. Aside from previously described prs1 mutant phenotypes, no additional developmental abnormalities were observed in C2 and C3 transgenic plants. However, these data do not rule out the possibility that full PRS1 function requires that its accumulation is limited to a lateral stripe in the stage 3 meristem. In this interpretation, the ectopic expression of PRS1 outside the lateral foci into all adaxial and abaxial domains of the floral meristem may impede PRS1 function in C2 (p*M1*-PRS1 ~ GFP) and C3 (p*SCR1*-PRS1 ~ GFP) transgenic plants.

Evolution of *WOX* Gene Function

When expressed in the PRS1 functional domain, the shoot meristem stem cell organizer WUS1 completely rescues all prs1 mutant phenotypes. These genetic complementation data suggest that WUS1 and PRS1 can interact with identical or functionally homologous cofactors and regulate the transcription of similar target genes in the PZ. Previous microarray analyses of the maize *PRS1* ortholog *NS1* (Zhang et al., 2007) suggested some homology with downstream components of WUS1 function, also identified in microarray analyses (Leibfried et al., 2005). Indeed, these analyses implicated a role for cytokinin two-component response pathways during both NS1 and WUS1 function and revealed the shared regulation of an orthologous, jasmonate-induced *lectin* gene of unknown function. Taken together, these data suggest significant overlap in WUS1 and PRS1 function and that the subfunctionalization of these *WOX* family members has primarily occurred via the evolutionary divergence of their respective promoter specificities and resulting expression domains rather than by changes in protein functional motifs. Another interpretation is that lateral organ initials and meristematic stem cells are not interchangeable or identical but display common attributes that are dependent upon shared WOX functions. A probable link between stem cells expressing *WUS1* and founder cells expressing *PRS1* is that both cell populations are comprised of rapidly dividing initial cells that are responsive to cytokinin signaling.

In their elegant analysis of WOX5 function, Sarkar et al., (2007) demonstrated that WUS1 can compensate for loss of WOX5 function in the Arabidopsis root meristem, and WOX5 rescues the shoot meristem termination phenotype of wus1 mutants. These results suggested that stem cells in the root and shoot meristems are essentially interchangeable. Similar analyses revealed that WOX9/STIP function, which is required for embryogenesis beyond early stages, can be completely complemented by expression of the *STPL/WOX8* homolog under the *STIP* promoter (Wu et al., 2007). Perhaps more unexpectedly, our data further suggest that both meristematic stem cells and lateral organ initials are inherently similar in nature and are regulated by related developmental mechanisms. These data are surprising in that unlike the undifferentiated stem cells of shoot meristems, founder cells occupying the PZ are generally believed to have already begun the process of differentiation to form lateral organs (for review, see Fleming, 2006; Barkoulas et al., 2007).

However, previous models suggested that NS/PRS1 function initializes founder cells without specifying any particular organ fate such that their eventual differentiation into lateral leaf domains, stipules, or sepals is dependent upon positional signals that are received later in development (Scanlon et al., 1996; Nardmann et al., 2004). The rescue of WOX5 root stem cell phenotypes (Sarkar et al., 2007) and PRS1 lateral organ phenotypes by the shoot meristem stem-cell organizing WUS1 protein further suggests that the stem cell populations of root meristems, shoot meristems, and lateral organ initials are essentially equivalent at their conception. Thereafter, stem cell functions in roots, shoots, and organ founder cells are wholly determined by stage-specific and domain-specific positional signals. In this way, the activities of multiple growth regulators and transcription factors such as *KNOX*, together with high levels of the phytohormone cytokinin, induce stem cells initialized by WUS1 function to assume shoot meristematic fate in the CZ. In contrast, cells initialized by PRS1 in the lateral domain of the PZ are signaled by transcription factors, including *ASSYMETRIC LEAVES1*, and phytohormones such as auxin and gibberellin to form lateral organ founder cells. This model stipulates that the conserved WUS/WOX5/PRS1 gene function is during organization of plant initial cells, a process that is ultimately blind to the eventual developmental fate of these cells.

MATERIALS AND METHODS

Plant Growth Conditions and Sepal Counts

Arabidopsis (*Arabidopsis thaliana*) plants were sown in solid (0.7% agar) or liquid medium containing 1/2 Murashige and Skoog basal medium (Sigma-Aldrich) plus 1% Suc, pH 5.7, and grown at 22° C under long-day conditions. Transformants were selected on 1/2 Murashige and Skoog medium containing 50 mg/L kanamycin and then transplanted into water-saturated RediEarth medium (Sun Gro Horticulture). T1 and T2 Arabidopsis transgenic seedlings were used for further experiments. Sepal counts and size evaluations were conducted on stage 12 flowers (i.e. just prior to bud opening; Smyth et al., 1991) from at least 600 plants of each genotype analyzed, as described previously (Nardmann et al., 2004).

Plasmid Constructs and Transformations

Expression constructs containing the *ML1* and *SCR1* promoters and either the GFP or GUS reporter construct in the pBI101 vector were generously obtained from D. Jackson (Cold Spring Harbor Laboratory) and P. Benfey (Duke University) and modified for use in these experiments. All constructs were generated using the pBI101 vector and introduced into *Agrobacterium tumefaciens* strain LBA4404 or GV3101 by electroporation. Arabidopsis ecotype Landsberg *erecta* and null mutant prs1 plants in the Lansdberg *erecta* background (described in Matsumoto and Okada, 2001) were transformed by floral dip (Clough and Bent, 1998). Genomic DNA was isolated from

Arabidopsis whole seedlings (Columbia ecotype) or from young leaves with Extract-*N*-Amp Plant PCR kits (Sigma-Aldrich). The *PRS1* promoter sequence was PCR-amplified from diluted DNA and ligated in frame with PRS1, *GUS*, and/or *GFP* reporter genes in the pBI101 vector. All primers utilized in are summarized in Supplemental Table S1.

Total RNA isolation and cDNA synthesis were performed on Arabidopsis whole seedlings (Columbia ecotype) as described (Nardmann et al., 2004); full-length coding regions of *PRS1*, *WUS1*, and *WOX4* were amplified by reverse transcriptase PCR and ligated into the pBI101 vector.

Microscopy and Imaging

Floral meristem inflorescences were dissected and mounted in water for viewing in a TCS SP2 confocal laser-scanning microscope (Leica Microsystems). GFP signals were observed using excitation at 488 nm and collection at 504 to 526 nm. Background autofluorescence was collected in the range of 613 to 648 nm. Detection of GUS expression was performed with 5-bromo-4-chloro-3-indolyl-d-glucuronidase as described previously (Sieburth and Meyerowitz, 1997). The stained tissues were fixed in 50% ethanol, 10% formaldehyde, and 5% acetic acid embedded in Paraplast Plus (McCormick Scientific) and sectioned at 8-μm thickness as described (Sylvester and Ruzin, 1994). Deparaffinized sections were analyzed and photographed under light microscopy using a Z1-Apotome (Carl Zeiss). Saffranin-fast green stained transverse sections of Arabidopsis vegetative shoots were obtained using the method of Johansen, according to the protocol described in Sylvester and Ruzin (1994).

Antibody Production and Immunohistolocalization Analyses

Full-length NS1 protein was produced in *Escherichia coli* using a GST Gene Fusion system (GE Healthcare). *NS1* cDNA was fused upstream of a GST and cloned into the pGEX5-1 vector. For immunization, NS1∼GST fusion protein accumulation was induced in *E. coli* and purified from whole protein extracts on glutathione Sepharose columns using the RediPack GST Purification Module kit. Affinity-purified NS1 polyclonal antibodies were generated against the NS1 protein by Sigma-Genosys. Immunohistolocalization analyses were performed on transverse sections of 14-d-old maize (*Zea mays*) vegetative seedling shoot apices using a 1:350 dilution of affinity-purified rabbit anti-NS1 antiserum as primary antibody, as described (Scanlon et al., 1996).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NC003070 and NC003071.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Summary of oligonucleotide primers used in this project.

ACKNOWLEDGMENTS

We thank Jae-Yean Kim and David Jackson (Cold Spring Harbor Laboratory) and Philip Benfey (Duke University) for generously sharing layer-specific promoters and expression plasmids. Thanks to Eddy Yeh (Iowa State University) for technical assistance, and to the Brad Barbazuk (University of Florida) and Pat Schnable (Iowa State University) laboratories for sharing data prior to publication. We are grateful to Carol Bayles (Life Sciences Core Laboratories Center, Cornell University) and Helene Javot (Plant Cell Imaging Center, Boyce Thompson Institute) for advice and technical expertise in confocal imaging microscopy and imaging.

Received October 3, 2008; accepted December 3, 2008; published December 10, 2008.

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