The Shoot Stem Cell Niche in Angiosperms: Expression Patterns of WUS Orthologues in Rice and Maize Imply Major Modifications in the Course of Mono- and Dicot Evolution

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Introduction

Angiosperms, flowering plants, comprise the largest group of plants today with more than 235,000 species in over 300 families, with fossil records suggesting that many of the modern families and genera may already have existed 75 MYA. Angiosperms are considered as a monophyletic group, a true clade, comprising 2 classes—the monocotyledons and the dicotyledons—which diverged over 150 MYA (Wikstrom and Kenrick 2001). The monophyletic origin relative to the tremendous number of species suggests that ancestral angiosperm genomes were prone to rapid diversification in the course of evolution. The evolution of maize (Zea mays L.) is a special example because it was forced by human selection through domestication over the last 6,000–10,000 years (reviewed in Doebley 2004). Maize still hybridizes with its ancestral wild teosinte relatives Z. mays ssp parviglumis or ssp. mexicana, and pioneering genetic analyses led to the identification of few major quantitative trait loci (QTL) that account for major changes in the plant architecture of maize compared with the wild species such as the maize female inflorescence, the ear being unique in the plant kingdom. Some QTLs have also been resolved into individual genes often encoding transcription factors such as teosinte branched1 (tb1; Doebley et al. 1997), teosinte glume architecture1 (tga1; Wang et al. 2005), and zf2 (Z. mays FLORICAULA/LEAFY2), a candidate gene for a QTL controlling ear rank differences between maize and teosinte (Bomblies et al. 2003).

The gross morphological changes based on single loci raise the question how common are regulatory networks elaborated in the dicot model species Arabidopsis in monocots such as maize. Comparative approaches in the MADS box gene family have provided some evidence that basic principles of the ABC model of flower development such as B-function genes are conserved in angiosperms (Zahn et al. 2005) and applicable to maize (Whipple et al. 2004) despite the different architecture of the grass flower.

In the shoot apex, a mechanism regulating the balance between indeterminate (meristem maintenance) and determinate growth (leaf development) also appears to be ancient. KNOX (knotted1-like homeobox) genes are expressed in a meristem-specific manner and are downregulated in primordial cells (Vollbrecht et al. 1993) by the activity of MYB orthologues (ARP: ASYMMETRIC LEAVES1, ROUGH SHEATH2, PHANTASTICA; Waites et al. 1998; Timmermans et al. 1999; Tsiantis et al. 1999; Byrne et al. 2000), recently reviewed in Piazza et al. (2005). This KNOX–ARP pathway is not only conserved between mono- and dicots but was also independently recruited into microphyll development in lycophytes (Harrison et al. 2005). In contrast, there are major differences in other aspects of leaf development between mono- and dicots. Two paralogous loci in maize NARROW SHEATH1/NARROW SHEATH2 (NS1/2) encode homologues to the Arabidopsis PRESSED FLOWER (PRS) gene (Nardmann et al. 2004). NS1/2 and PRS have maintained their marginal expression domain in lateral organ primordia. The prs mutant phenotype, however, is mostly restricted to lateral sepal of the flower in Arabidopsis, whereas the maize homologues contribute to the specification of a lateral leaf domain at the flank of the shoot apical meristem (SAM) during the vegetative phase (Scanlon 2000). Some remarkable differences exist between meristems of dicots and monocots. In dicot species including Arabidopsis, the SAM appears to be 3 layered, with a tunica comprising 2 clonal layers (L1 + L2) and the corpus commonly designated as the L3 layer (Szymkowiak and Sussex 1996; Evans and Barton 1997). In contrast, monocots such as maize have only 1 histologically apparent single tunica layer (L1) and the inner corpus (Abbe et al. 1951; Steffensen 1968). Radially, in the Arabidopsis SAM, the central stem cell zone can be distinguished from the peripheral zone, where cells begin to differentiate. Vegetative leaves in Arabidopsis originate from few founder cells specified in a spiral phyllotaxy in the peripheral zone (Irish and Sussex 1992), whereas in contrast, the maize leaf may be traced back to approximately 200 leaf founder cells (Poethig 1984), which are recruited from...
the whole circumference of the shoot apex and give rise to an alternate phyllotaxy.

The maintenance of pluripotent stem cells in the SAM of Arabidopsis depends on the WUSCHEL/CLAVATA feedback loop. The WUSCHEL (WUS) homeobox gene provides a key function for the maintenance of stem cell homeostasis in the SAM (Mayer et al. 1998). WUS acts cell autonomously and gene expression is restricted to a few cells in the L3 layer considered to be the stem cell—organizing center (OC). Whereas WUS promotes stem cell fate, it is antagonized by CLAVATA (CLV) signaling (Brand et al. 2000). Acting in a single pathway, 3 CLV genes (CLV1–3) encode a heterodimeric transmembrane receptor kinase (CLV1/2; Clark et al. 1997; Jeong et al. 1999) and its corresponding polypeptide ligand (CLV3; Fletcher et al. 1999). WUS activity is restricted via CLV signaling via a yet unknown signal transduction pathway (Brand et al. 2000).

The recent cloning of THICK TASSEL DWARF1 (TD1; Bommer et al. 2005) and FLORAL ORGAN NUMBER (FON1; Suzuki et al. 2004), CLV1 orthologues from maize and rice, respectively, and of FASCIATED EAR2 (FAE2), a CLV2 orthologue from maize (Taguchi-Shiobara et al. 2001), has suggested that CLV signaling is conserved in monocots. The expression patterns of these genes as well as the phenotypes of corresponding loss-of-function mutants, however, imply major modifications in the control of meristem size in grasses. In Arabidopsis, CLV1 transcription is confined to the L2 and L3 layers of the SAM throughout the plant life cycle (Clark et al. 1997). In contrast, TD1 transcripts are not detected in the vegetative SAM but found in early leaf primordia (Bommer et al. 2005). During the reproductive phase, however, TD1 is transcribed throughout the inflorescence meristem (IM), the descending spikelet-pair meristems (SPMs), spikelet meristems (SMs), and floral meristem (FMs). During later stages of floral development, TD1 transcripts accumulate in differentiated organs such as glumes, lemma, and palea, reminiscent of the expression pattern in leaf primordia during vegetative phase (Bommer et al. 2005). Similarly, the rice orthologue FON1 is transcribed throughout the FM but also in differentiated lateral floral organs (Suzuki et al. 2004).

As CLV1 signaling is thought to restrict WUS activity, the differences in the transcription patterns of CLV1 orthologues between maize and rice or Arabidopsis raised the question whether the expression pattern of WUS orthologues in grasses was also conserved. WUS is the founding member of the so-called WOX (WUS homeobox) gene family encoded by 15 genes in the Arabidopsis genome (Haecker et al. 2004). Here, we describe the identification of WUS orthologues in maize and rice by a detailed phylogenetic comparison of the WUS/WOX gene family between the dicots Arabidopsis thaliana and Populus trichocarpa and 2 monocot species Oryza sativa and Z. mays. The allotetraploid maize genome contains 2 WUS paralogs (ZmWUS1 and ZmWUS2), whereas a single WUS orthologue is present in the smaller rice genome (OsWUS). Consistent with TD1/FON1 expression data, the expression patterns of WUS orthologues in both grass species imply that major changes during angiosperm evolution have occurred and raise doubts about the uniqueness of the WUS/CLV antagonism in the maintenance of the shoot stem cell niche in grasses.

**Materials and Methods**

Cloning of ZmWUS1 and ZmWUS2

To amplify the homeobox of the maize WUS orthologues, polymerase chain reaction (PCR) was performed on genomic DNA of Rscm2 maize using the primer pair ZmHD1 (5′-TGGACCCCAACGACARAT-3′) and ZmHD2 (5′-GCGTTRGGTTGATGAAARTARA-3′). Isolation of total RNA followed the protocol of Chomczynski and Sacchi (1987), and rapid amplification of the 3′-cDNA ends was then performed on RNA of Rscm2 female inflorescences with primers corresponding to the homeobox using the First-Choice RLM-RACE Kit (Ambion, Austin, TX) according to the manufacturer’s protocol; 3′-RACE was performed with primers ZmWUSa (5′-CTCTACTACGGCTGGCCATCC-3′) and ZmWUSb (5′-GACAGAGTCGGAGGTAAGAC-3′). Analysis of the 3′-RACE products revealed 3′-ends of 2 different genes we named ZmWUS1 and ZmWUS2. For 5′-RACE, primers specific for ZmWUS1 were used: ZmWUS1a (5′-CAATCGGAATCTGTGTCGAGCATCAC-3′) and ZmWUS1b (5′-GAACACCTGGAGGCGGCAAGG-3′). 5′-RACE for ZmWUS2 was performed with the primers ZmWUS2a (5′-CCATTATCGTCGCCGGAGGACGAGACAGTCT-3′).

All PCR products were cloned into pCRII TOPO (Invitrogen, Carlsbad, CA) and sequenced. ZmWUS2 was mapped to chromosome 10, between markers bnl1450 and asg19b, using the DNA Kit of 94 IBM Lines (http://www.maizegenetics.org/dna_kits.htm).

**Computational and Database Analysis**

Analysis of DNA and protein sequences was performed using the Wisconsin GCG software package version 7.0 (University of Wisconsin Genetics Computer Group). Homology searches were performed using TBLASTN at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), DNA Data Bank Japan (http://www.ddbj.nig.ac.jp), or the Plant Genome Database (http://plantgdb.org) with default parameters. Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw) and BOXSHADE (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The PHYLIP (http://evolution.genetics.washington.edu/phylip.html) program was used for phylogenetic and molecular evolutionary analyses based on the maximum likelihood method. Sequences selected for the phylogenetic tree in figure 1A are as follows (accession numbers in parentheses): ZmNS1 (AJ536578); ZmNS2 (AJ472083), and Os-WOX5 (Q8WOF1); AtWOX1–AtWOX14 as published recently (Haecker et al. 2004). Accession numbers for the remaining homeodomains (HDs) are listed in table 1. Accession numbers of the genes encoding the proteins for the sequence alignment are ZmWUS1 (AM234744), ZmWUS2 (AM234745), OsWUS (AB218894), ROA (AY162209), TER (AF481951), PrWUS (AM234747), and AtWUS (At1g17950).

**In situ Hybridization**

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 the Leica RM 2145 rotary microtome, and 7-μm sections were used for hybridization. Probes for in situ hybridization were cloned either in sense or antisense orientation to the T7 promoter and then used as a template for synthesis of digoxigenin-labeled RNA probes by T7 RNA polymerase as described (Bradley et al. 1993). In situ probes for the grass WUS genes were chosen to contain sequences downstream of the homeobox and amplified via PCR: ZmWUS1 pos. 294–951 of accession AM234744, ZmWUS2 pos. 267–978 of accession AM234745, OsWUS pos. 435–870 of accession AB218894. In situ probes for TDI and FON1 were used as published (Suzuki et al. 2004; Bommert et al. 2005). The probe for KNI corresponded to the mRNA sequence of accession AY312169 (Vollbrecht et al. 1993).

Light Microscopy and Image Processing

Images were taken using an Axioskop microscope equipped with an Axioacam camera (Zeiss, Oberkochen, Germany). Pictures were processed using Adobe Photoshop version 7.0.

Results

WUS Orthologues in Rice and Maize

Whereas members of the WUS/WOX gene family from rice could be identified from the genome sequence (Goff et al. 2002), the maize family members had to be isolated using a combinatorial approach: firstly, reverse transcriptase (RT)–PCR was performed on cDNAs prepared from immature embryos, roots, shoots, and inflorescences with degenerate primers designed to amplify the conserved HD-encoding sequences of WUS/WOX relatives (Haecker et al. 2004). In situ probes for TDI and FON1 were used as published (Suzuki et al. 2004; Bommert et al. 2005).

Margins and Is Transiently Expressed in the SAM

OsWUS, the Single Rice WUS Orthologue Marks Leaf Margins and Is Transiently Expressed in the SAM

As phylogenetic analyses revealed a single WUS homologue in rice, OsWUS, RNA in situ hybridizations were performed in young rice seedlings, which uncovered remarkable differences between the expression patterns of WUS relatives in comparison to the relatives encoded by the Arabidopsis thaliana (At), Oryza sativa (Os), and Petunia hybrida (Pt) genomes. (B) Protein sequence comparison of Arabidopsis WUS (AtWUS) with its orthologue in Populus (PwWUS), Antirrhinum (ROA), Petunia (TER), the 2 maize WUS paralogues, ZmWUS1 or ZmWUS2, and the single rice WUS orthologue (OsWUS). The extra tyrosine residue specific for the WUS HD is indicated by an arrow. The positions of the HD, the WUS boxes, and the EAR-like domain are indicated by gray lines.
OsWUS and those established for the WUS gene in Arabidopsis (Mayer et al. 1998). OsWUS transcripts are detected in young leaf primordia with a pronounced preference for lateral leaf margins. Depicted in figure 2A + B are leaf primordia P1, P2, and P3, and highest OsWUS transcript levels are evident at the very tip of P2 and P1 lateral margins. In the center of the shoot apex, where WUS is exclusively expressed in the Arabidopsis SAM, OsWUS transcripts were undetectable in the majority of consecutive sections (total 15) through the rice SAM. However, in a small number of consecutive sections (5 out of 15), OsWUS expression was also detected in the center of the vegetative SAM in 1–2 sections at the height of the P0 phytomer (fig. 2A). This may be best explained by a transient expression of OsWUS in the shoot apex in contrast to the stable OC-type expression pattern of WUS in Arabidopsis. During later stages of phytomer development, OsWUS is expressed at the abaxial face of emerging axillary meristems (fig. 2C).

These differences in expression patterns and the identification of 2 WUS orthologues in maize, ZmWUS1 and ZmWUS2, raised questions concerning the expression domains of the maize paralogues and whether there are grass-specific alterations in the WUS expression domains compared with Arabidopsis. The expression patterns of the maize ZmWUS1 and ZmWUS2 differ considerably and will be described separately below.

### The ZmWUS1 Pattern in the Seeding SAM Is Dynamic

Transcriptional activity of ZmWUS1 was not detected earlier than the coleoptilar stage embryo. ZmWUS1 was transcribed in a few cells underlying the emerging coleoptile (fig. 3A + B) in a domain initially including the L1 layer but later extending to subtending cell layers (fig. 3A–C). Expression then ceased after the initiation of the second embryonic leaf, even though the maize embryo develops additional 4 leaves prior to seed dormancy. ZmWUS1 transcriptional activity was again detected in the seeding SAM after germination, although this expression was weak in the center of the apex with transcript maxima detectable at lateral positions of the apex (fig. 3D). Multiple series of longitudinal and transverse sections showed that transcript maxima dynamically differed between opposing flanks of the apex (data not shown). Moreover, consecutive (7 μm thick) transverse sections showed that the height of this ZmWUS1 domain in the center of the apex changes relative to the apical tip in the course of development, as does the relative height of P0 and P1 (fig. 3E). The vegetative maize phytomer comprises a leaf attached to a node, an internode, and an axillary bud. Although located close to the midrib position of the subtending phytomer, the axillary meristem clonally relates to the innode above. The lateral ZmWUS1 maxima, therefore, coincide either with the midrib positions of the emerging leaf primordia, P1 and P0, or with the positions of the axillary meristems at the opposite face of the apex.

To elucidate the position of the lateral ZmWUS1 maxima, we performed side-by-side in situ hybridization experiments with a Knotted1 (KN1) probe (Vollbrecht et al. 1993). The ZmWUS1 transcription domains at the height of the P0 and P1 phytomers in the shoot apex are shown in figure 3E, which also depicts adjacent sections hybridized with the KN1 probe. The P1 sections in figure 3E show a downregulation of KN1 expression at the left flank of the SAM corresponding with the midrib of the emerging P1 leaf. ZmWUS1 activity is absent in these primordial cells but overlaps with the KN1 expression in a central domain of the apex. However, in a deeper section, ZmWUS1 expression extends toward the flank of the SAM opposite to the P1 midrib. Therefore, ZmWUS1 transcripts are absent in founder cells of the P1 leaf sheath/blade but are transcribed in cells of the prospective node/internode in the center of the shoot apex and in the subtending axillary meristem, which is located opposite to the P1 midrib. Consequently, the expression maxima at the flanks of the apex (see fig. 3D) relate to the formation of axillary meristems and not to the specification of leaf founder cells at the prospective midrib position, and subsequently, the ZmWUS1 expression domain is confined to the abaxial face of the axillary bud (fig. 3G).

In summary, the ZmWUS1 transcription domain in the center of the shoot apex initiates as a disc-shaped expression domain at the height of the new P0 phytomers, which is reminiscent of the transient expression of the single OsWUS orthologue in the center of the rice apex. The expression of ZmWUS1 in these cells either persists or is reactivated during subsequent phytomer development, shifting to deeper layers of the SAM. The transcription domain extends laterally to the position of axillary meristems in P1 (fig. 3F). Transcription ceases thereafter, except for activity in axillary meristems (fig. 3G). Similar to the transient OsWUS expression in the rice SAM, the dynamic ZmWUS1 expression pattern in the maize shoot apex does not reflect the stable OC-type expression domain of WUS in Arabidopsis. However, ZmWUS1 expression appears to be correlated with the initiation of leaf phytomers and associated with the initiation of the SAM in the embryo or the establishment of axillary meristems at the flank of the vegetative apex.

#### ZmWUS2 Is Activated in the P1 Leaf Primordium

The in situ hybridization results obtained with the ZmWUS2 probe were in striking contrast to the dynamic

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ZmWUS1 expression pattern in the maize shoot apex. Although RT–PCR experiments indicated a low ZmWUS2 transcript level in the embryo, this was not substantiated on a cellular level. The median longitudinal section depicted in figure 4A shows 2 points of ZmWUS2 transcriptional activity at the flank of the apex positioned at the height of the P1 primordium. Both longitudinal and transverse sections indicate that expression extends into detached leaf primordia, where ZmWUS2 transcription exerts a preference for lateral leaf domains with a maximum of expression at the marginal tip (fig. 4B), whereas transcription is undetectable in the SAM. In consequence, ZmWUS2 is transcribed in an expression pattern similar to that of the single OsWUS orthologue in young leaf primordia, which also exhibits a maximum in lateral leaf margins. As monitored by cyclinB gene expression (data not shown), the ZmWUS2 expression pattern in fully detached P2 or P3 leaf primordia showed a significant correlation with ongoing cell divisions in lateral leaf domains or growth zones residing along the proximal–distal axis (Sylvester et al. 1990). Notably, ZmWUS2 transcripts as well as those of ZmWUS1 and OsWUS were detected at the position of axillary meristems and were confined to the abaxial face of the axillary bud (fig. 4C).

WUS Expression Patterns in Comparison to the Expression Domains of CLV1 Orthologues in Maize and Rice

The number of stem cells in the Arabidopsis SAM is controlled through CLV signaling. Because TD1 and FON1 have been identified in maize and rice, respectively, (Suzaki et al. 2004; Bommert et al. 2005) to be orthologues of CLV1 in Arabidopsis, we therefore compared the TD1 and FON1 expression patterns during the vegetative phase with those of the ZmWUS1/ZmWUS2 paralogues or the single rice orthologue OsWUS.

In maize, longitudinal and transverse sections through the seedling plume show TD1 transcripts in a ring-shaped expression domain in outer cell layers of the SAM and in lateral domains of the P1 leaf (fig. 4D + E). In older leaves, transcripts were confined to the lateral leaf margins as observed for ZmWUS2 (compare fig. 4B with 4F). In longitudinal sections, the highest ring of the TD1 expression domain corresponds to the positions of the P0 phytomer (fig. 4D). From side-by-side comparisons with KN1, it is evident that TD1 transcripts are detected in a mutually exclusive expression pattern compared with that of KN1 in P0 and P1 (fig. 4G). Therefore, the ZmWUS1 expression domain, which is located within the KN1 domain in P0 or P1 (compare fig. 3E), hardly overlaps with transcriptional activity of TD1. However, the TD1 expression pattern significantly overlaps with that of ZmWUS2 in P1, both in the outer cell layers of the apex and in prospective lateral leaf domains (compare fig. 4F with 4A). Especially striking is the coexpression of TD1 and ZmWUS2 in lateral leaf margins during subsequent leaf development.

A similar transcription pattern is observed for FON1 in the rice seedling apex: FON1 transcripts are absent from the apical tip and in the central rib zone of the SAM but, similar to TD1, are found in outer cell layers of the apex at the height of the new phytomer (fig. 4–H–J). This expression pattern contradicts that initially published for the rice seedling SAM (Suzaki et al. 2004). This discrepancy may reside in median versus lateral sections (see complete series in Supplementary Material online), but the FON1 expression data are highly consistent and fully agree with those of TD1, the maize orthologue. Both are consistent with transcriptional activity of grass CLV1 orthologues in peripheral layers of the shoot apex, in prospective primordial cells but not in central domains of the SAM. According to their transcription patterns, neither FON1 nor TD1 are therefore prone to control pulses of OsWUS activity or the dynamic ZmWUS1 expression in the center of the vegetative apex in rice or maize, respectively.

The rice FON1 expression data also substantiate a possible antagonism with OsWUS in leaf margins because similar to TD1/ZmWUS2, the rice CLV1 orthologue FON1 is expressed at the margins of leaf primordia (fig. 4J). In both grass species, WUS and CLV1 orthologues are therefore preferentially transcribed in lateral domains of young leaf primordia, which at this early stage of development still have to expand laterally to enclose the apex. Later in development in both grass species, the transcription patterns of CLV1 and WUS orthologues have not diverged but consistently focus to leaf margins, which may be best explained by corecruitment.

The Expression Patterns of CLV1 and WUS Orthologues in Grasses Merge during Reproductive Development

The architecture of the maize inflorescences is complex, and SPM and SM emerge before lastly FMs are
FIG. 3.—ZmWUS1 transcription patterns. (A) Schematic drawing of maize embryo stages. From left to right: early transition stage, coleoptilar stage, and leaf stage 1. ZmWUS1 transcription begins during the coleoptilar stage (B) and shifts from the outer tunica layers to the inner corpus during leaf stage 1 (C). (D) Longitudinal sections through 1 seedling apex. The positions of the individual sections front (I), median (II), and back (III) are indicated in the schematic drawing depicted in (E). Note the relative height of the ZmWUS1 expression domain in P0 (I, front) and P1 (III, back). (E) Side-by-side sections hybridized with the KN1 or ZmWUS1 probes at the height of the P0 or P1 phytomers. Relative positions are indicated by the corresponding numbers in the schematic drawing to the left. Note KN1 downregulation on the midrib side (left in section 7) in contrast to its overlap with ZmWUS1 activity at the right flank (section 8) in the prospective axillary meristem of P1. (F) Cycling ZmWUS1 activity in the vegetative apex. From left to right, transcription starts in P0 in a small domain central within the SAM, shifting to deeper layers. Expression then spreads laterally toward the prospective axillary meristem in P1 before activity is regained in the next phytomer (now P0). (G) ZmWUS1 transcription at the abaxial face of an axillary bud. axm: axillary meristem; mr: midrib.
initiated (fig. 5A); secondary axes in the male tassel are due to the activity of early branch meristems (BM). Both maize WUS paralogues show meristem-specific expression during the reproductive phase (fig. 5B–G); however, significant differences exist between ZmWUS1 and ZmWUS2. Although active in axillary meristem anlagen (see fig. 3G), no ZmWUS1 expression was detected in the mature IM of the male tassel or the female ear (fig. 5D). ZmWUS1 transcripts were found in the descending SPM, SM, and FM (fig. 5B + C). In SPMs, SMs, and early FMs, ZmWUS1 transcription is confined to a small OC-type central domain (fig. 5B) such as is observed in the early embryo. In the late FM, the ZmWUS1 expression domain extends toward the L1 layer similar to the shift of WUS expression from the L3 layer in the IM to the L2 layer in the FM in Arabidopsis (Mayer et al. 1998). The expression pattern, therefore, reflects histological differences between 1 or 2 tunica layers in maize and Arabidopsis meristems, respectively. Later in female flower development, ZmWUS1 expression was detected in the outer cell layers of the gynoecium in the upper floret and still simultaneously in the center of the lower floret (fig. 5C), which subsequently aborts in the female ear inflorescence.

In contrast, ZmWUS2 transcripts are found in the IM (fig. 5G) often with a slight preference for the L1 layer and with mRNA being evenly distributed through tunica and
A corpus of descending SPMs, SMs, or FMs (fig. 5). This pattern of \textit{ZmWUS2} expression closely resembles that of \textit{TD1} (Bommert et al. 2005), reflecting some L1 layer preference in the IM and the transcriptional activity throughout SPM, SM, or FM. With the exception of the IM, where transcripts are absent, \textit{ZmWUS1} is therefore always expressed in a sub-population of cells also expressing \textit{ZmWUS2}.

Similar results were obtained for \textit{OsWUS} and \textit{FON1} expression in rice despite the different architecture of the panicle. The rice inflorescence is branched similarly to the tassel of maize. Differing from the maize tassel, however, the main-axis meristem (IM) does not produce any spikelets but aborts after having initiated 10 or more primary branch meristems. These lateral branches produce numerous so-called spikelets that develop into single bisexual flowers (FM) or secondary branch meristems (Itoh et al. 2005). \textit{OsWUS} is expressed in rice BMs (fig. 5H) and, as for \textit{ZmWUS2} in the maize IM, exhibits some preference for the outer L1 layer. The L1 preference is lost in the FM where \textit{OsWUS} expression was detected uniformly through the meristem (fig. 5I), redolent of the \textit{ZmWUS2} expression pattern in SPMs, SMs, or FMs. On the transcriptional level, the \textit{OsWUS} expression pattern always overlaps with \textit{FON1} transcription, being active in all layers of the IMs, BMs, and FMs in the rice inflorescence (Suzaki et al. 2004). Consistently and in contrast to during the vegetative phase, the expression of \textit{WUS} ortho/paralogues in rice or maize, therefore, is meristem specific during the reproductive phase and coincides with the expression domains of grass \textit{CLV1} orthologues.

### Discussion

The Expression Patterns of Rice and Maize \textit{WUS} Orthologues Do Not Support a Stable OC-Type Expression Domain in the Grass SAM

Based on the phylogeny of the \textit{WOX} gene family, orthologues to the \textit{Arabidopsis WUS} gene were identified in maize and rice. None of the isolated grass \textit{WUS} orthologues displays a transcription pattern in the vegetative SAM which resembles the stable OC-type expression domain of \textit{WUS} in \textit{Arabidopsis}. In contrast, their expression patterns relate to the specification of new phytomers, which has 2 aspects in the grass culm. The first aspect is the recruitment of leaf founder cells from the whole circumference of the SAM before the leaf primordium detaches from the shoot apex. The second aspect is that growth of the culm depends on the coordinated elongation of the detached leaf sheath and the internode enclosed (Abbe et al. 1951; Steffensen 1968). Essentially, the single rice \textit{OsWUS} orthologue shares an expression pattern with its 2 maize paralogues. However, the different expression patterns of the 2 paralogues in maize identified distinct functions in the apex. \textit{ZmWUS1} is expressed in cells of the prospective nodal/internodal tissue in the center of the SAM and shifts to deeper layers with elaboration of the new phytomer, whereas \textit{ZmWUS2} is transcribed in cells recruited for leaf primordia and has a maximum in basal lateral leaf margins. \textit{ZmWUS2} therefore shares expression in leaf primordia and the preference in lateral leaf margins with \textit{OsWUS}, whereas
ZmWUS1 may relate to the pulsing OsWUS transcription in the SAM center. The expression patterns of the maize WUS paralogues therefore support the hypothesis that gene duplications are preserved by subfunctionalization (Lynch and Force 2000). After duplication, mutations have resulted in a reduction in their joint expression levels and patterns of activity compared with that of the single ancestral WUS gene, which formerly may have been expressed similarly to OsWUS, the single WUS orthologue in rice.

The different expression patterns of grass WUS orthologues compared with that of WUS in Arabidopsis acquire significance due to the correlated changes in the expression patterns of the grass CLV1 orthologues, TD1 and FON1 in maize and rice, respectively (Suzaki et al. 2004; Bommer et al. 2005). Both orthologues are transcribed at the flank of the SAM in cells that have been recruited into leaf primordia but not in the center of the SAM where ZmWUS1 and OsWUS are transiently expressed. TD1 transcription initiates at P₀ and then overlaps with ZmWUS2 expression in the P₁ phytomer and lateral leaf domains after the detachment of the primordium. The activation of ZmWUS2 in P₁ within the TD1 expression domain suggests that pluripotency is acquired de novo in cells already determined for the primordial fate. The OsWUS and FON1 expression patterns in rice leaf primordia conform to this assumption as well as to the assumption that the grass CLV1 orthologues TD1 and FON1 act to antagonize ZmWUS2 or OsWUS activity in prospective primordial cells. Later in leaf development, ZmWUS2 and TD1 in maize and OsWUS and FON1 in rice are cotranscribed in leaf margins. The marginal patterns of WUS and CLV1 orthologues in maize and rice are reminiscent of the narrow sheath1 and narrow sheath2 (NS1/NS2) gene expression patterns in maize, encoding paralogues of the PR5 gene in Arabidopsis (Nardmann et al. 2004). Maize ns1/ns2 double mutants are affected in the development of the basal lateral domains of leaves, and the similarity in transcription pattern suggest that ZmWUS2 and OsWUS may contribute to leaf development and be antagonized by TD1 and FON1, respectively.

The recent identification of ROSULATA (ROA), which encodes the WUS orthologue in A. majus, also confirms that in dicots, ROA is not only involved in SAM maintenance but also acts outside the SAM (Kieffer et al. 2006). In the roa mutant, the petioles of leaves are frequently ventralized or appear radially symmetrical and the leaf blades are less expanded than in wild type. The roa mutants also have short internodes, which are comparable to deficiencies in the elongation of internodes in wus-1 mutant inflorescences in Arabidopsis. WUS functions in dicots therefore contribute to the elongation of the shoot axis, which could relate to ZmWUS1 transcriptional activity within the SAM during development of the maize leaf phytomer.

Grass WUS Orthologues Display a Meristematic Expression Pattern during Reproductive Growth

In contrast to the vegetative phase, ZmWUS1, ZmWUS2, and OsWUS are expressed in meristems during the reproductive phase. The expression patterns of ZmWUS2 and TD1 considerably overlap in all meristem types of the male or female inflorescence and exert a preference for the L1 layer in the IM, whereas transcriptional activity in the SPM, SM, and FM is constitutive throughout the meristems. In contrast, ZmWUS1 transcripts are absent in the ear or tassel IM but mark a small OC-type domain in SPM, SM, and early FM before the expression spreads to the larger ZmWUS2/TD1 coexpression domain during later stages of floral development. Concerning the absence of ZmWUS1 activity in the IM, it should be borne in mind that both maize paralogues are coexpressed during early stages of axillary meristem development as is observed during the initiation of SPMs, SMs, and FMs. Although indeterminate, the IMs of ear or tassel are elaborated meristems, and ZmWUS2 activity may be sufficient for their maintenance. Similar to that in maize, FON1 and OsWUS are coexpressed in BMs and FMs of the rice panicle. WUS and CLV1 orthologues of maize and rice are therefore coexpressed in all reproductive meristems, where fascination and supernumerary floral organs in td1 or foni loss-of-function mutants provide evidence for meristem enlargement (Suzaki et al. 2004; Bommer et al. 2005).

Conforming to the stem cell concept in Arabidopsis, excessive WUS activity could explain the meristem enlargement in clv mutants (Schoof et al. 2000; Waites and Simon 2000). However, differences in the expression patterns of maize WUS paralogues could not be detected in the td1 mutant background (data not shown), whereas WUS expression shifts from the L3 to the L2 layer in Arabidopsis (Schoof et al. 2000). This may relate to differences in the shoot meristem architecture between maize and Arabidopsis. Whereas the OC-type, WUS expression domain in the Arabidopsis SAM subtends a 2-layered tunica, maize meristems possess only a single tunica layer (Abbe et al. 1951; Steffensen 1968). High levels of TD1 or ZmWUS2 transcript are detected in this single tunica layer of the mature IM, and both genes are cotranscribed throughout all layers of the descending SPMs, SMs, or FMs. ZmWUS2 transcription, therefore, is not restricted to a subdomain of the TD1 expression domain in reproductive meristems but overlaps with TD1 expression and shares its transcription specificity with IM L1 layer or leaf margins. A preference for the tunica layer and the leaf margin suggests that an extracellular ligand must be preferentially perceived from subtending cell layers, in contrast to Arabidopsis SAM, where CLV3 is presented top-down from the L1/L2 tunica to the L3 layer.

The expression patterns of TD1 and FON1 during the reproductive phase and the mutant phenotypes suggest that they may control activity of grass WUS orthologues in reproductive meristems. By analogy, this assumption also implies that TD1 and FON1 may restrict ZmWUS2 and OsWUS activity in cells recruited for leaf primordia, where the expression domains overlap during the vegetative phase. Whereas the Arabidopsis leaf can be traced back to a few founder cells within the apex (Irish and Sussex 1992), cells for the grass leaf are recruited from the whole circumference of the SAM (Poethig 1984). The strikingly different expression patterns of grass WUS/CLV1 orthologues relative to those in Arabidopsis during the vegetative phase therefore may relate to fundamentally different developmental programs and the peculiar architecture of the grass culm. However, the maize or rice vegetative expression patterns signify
an evolutionary freedom to recruit the WUS/CLV1 feedback circuit for developmental pathways at the periphery of the SAM without affecting stem cell homeostasis.

The Evolution of the Stem Cell Niche and WUS/CLV1 Coexpression

The present data set based on 2 grass species raises concerns about the general applicability of a concept based on 2 major signaling pathways to control stem cell homeostasis in the shoot as is suggested by the WUS/CLV antagonism in Arabidopsis. The similarity between the various rice and maize expression patterns implies that the differences existed prior to the separation of the 2 grass lineages Oryzoideae and Panicoideae and may be specific for grasses. It would be intriguing to establish the WUS/CLV expression patterns in the third grass radiation, the Pooidae. However, information pertaining to an even more ancestral situation would be obtained from analysis of presumptive basal angiosperms such as Amborella, considered to be a sister to both the dicot and the monocot lineage of angiosperms (Qui et al. 1999; Soltis et al. 1999). A likely scenario is that stem cell homeostasis in an ancestral angiosperm precursor involved multiple pathways acting in parallel to specify stem cell fate and to maintain the stem cell niche. Recent data from Arabidopsis support this view, where STIMPY (STIP or WOX9) and SPLAYED (SYD) act through WUS and presumably contribute to the stem-cell-promoting pathway (Kwon et al. 2005; Wu et al. 2005). Interestingly, our phylogenetic analysis revealed 3 WOX9 orthologues to exist in maize and 2 in rice, which may contribute similar or additional functions. Alternatively, the class III AtHD-Zip protein CORONA (CNA) acts in parallel to CLV signaling and thus independently limits stem cell number (Green et al. 2005). These data indicate that redundantly acting pathways are functional in Arabidopsis and the maintenance of stem cell homeostasis in the SAM by WUS/CLV1 signaling may represent only one outcome of evolutionary selection. In an ancestral angiosperm species, the expression of WUS and CLV1 orthologues may consequently have been restricted to developing new phtomers without affecting stem cell homeostasis in the shoot apex. Cotranscription of ZmWUS2/TD1 and OsWUS/FON1 throughout the plant life cycle and the accompanying changes from leaf-associated to meristem-specific patterns suggest that the entire feedback loop may be an ancient phenomenon.

The absence of TD1 or FON1 transcripts in the center of the vegetative apex raises the question how ZmWUS1 or OsWUS activity is controlled in the SAM. Phylogenetically, the TD1 and FON1 proteins group within 1 branch together with CLV1 (Bommert et al. 2005; DeYoung et al. 2006). However, this unique CLV1 branch is rooted together with a second branch B (Bommert et al. 2005), which contains additional and closely related transmembrane receptor kinase proteins encoded by the genomes of maize, rice, and Arabidopsis. Three closely CLV1-related genes BAM1–3 (barely any meristem1–3) have recently been described in Arabidopsis to be involved in multiple developmental processes including leaf development (DeYoung et al. 2006). Still ongoing research on CLV1 relatives in maize and rice indicates that the subbranch B contains additional grass-specific kinase gene family members (Durantini D and Werr W, unpublished data), which root independently from their closest dicot relatives. Depending on their expression domain, such grass-specific transmembrane receptor kinase family members could be candidates for the control of the dynamic ZmWUS1 or the transient OsWUS expression in the center of the grass SAM. However, the identification of such grass-specific receptor kinase candidates also raises the question how stem cell homeostasis in the maize or rice SAM is positively controlled during the vegetative phase, when a stem cell OC is not provided by expression of WUS orthologues in grasses. Possibly, one of the other WOX gene family members has been recruited for this purpose, or other signaling pathways, which have not yet been identified, may be involved.

In summary, the transcription patterns of WUS and CLV1 orthologues in maize and rice imply that the feedback mechanism controlling stem cell homeostasis in the Arabidopsis SAM has been subject to significant adaptations in the course of plant speciation. The recruitment of ZmWUS2/OsWUS and TD1/FON1 transcription to leaf phytomer development during the vegetative phase in maize as well as rice, without affecting SAM function, implies that other pathways to maintain stem cell homeostasis exist in grass shoot meristems. The expression patterns imply 2 distinct functions in the grass phytomer: one in the center of the apex and the other at the SAM periphery, where cells are recruited into primordia. The data from grasses raise some concern about the generalization of a shoot stem cell niche based on only 2 major antagonistic functions. However, the correlation of transcription patterns strongly supports that the CLV1/WUS antagonism is an ancient angiosperm feature and may still be used to control the activity of WUS orthologues in rice and maize such as it does in Arabidopsis.

Supplementary Material

Supplementary material is available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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Literature Cited


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