

Two-Step Functional Innovation of the Stem-Cell Factors WUS/WOX5 during Plant Evolution

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Abstract

WUS and WOX5, which are expressed, respectively, in the organizing center (OC) and the quiescent center (QC), are essential for shoot/root apical stem-cell maintenance in flowering plants. However, little is known about how these stem-cell factors evolved their functions in flowering plants. Here, we show that the WUS/WOX5 proteins acquired two distinct capabilities by a two-step functional innovation process in the course of plant evolution. The first-step is the apical stem-cell maintenance activity of WUS/WOX5, which originated in the common ancestor of ferns and seed plants, as evidenced by the interspecies complementation experiments, showing that ectopic expression of fern *Ceratopteris richardii* WUS-like (*CrWUL*) surrounding OC/QC, or exclusive OC-/QC-expressed gymnosperms/angiosperms WUS/WOX5 in *Arabidopsis wus-1* and *wox5-1* mutants, could rescue their phenotypes. The second-step is the intercellular mobility that emerged in the common ancestor of seed plants after divergence from the ferns. Evidence for this includes confocal imaging of GFP fusion proteins, showing that WUS/WOX5 from seed plants, rather than from the fern *CrWUL*, can migrate into cells adjacent to the OC/QC. Evolutionary analysis showed that the WUS-like gene was duplicated into two copies prior to the divergence of gymnosperms/angiosperms. Then the two gene copies (WUS and WOX5) have undergone similar levels of purifying selection, which is consistent with their conserved functions in angiosperm shoot/root stem-cell maintenance and floral organ formation. Our results highlight the critical roles and the essential prerequisites that the two-step functional innovation of these genes performs and represents in the origin of flowering plants.

Key words: experimental evolution, plant molecular evolution, developmental biology, plant stem cells.

Introduction

The development of flowering plants depends on the activity of stem cells situated in specific niches (Dinneny and Benfey 2008; Heidstra and Sabatini 2014; Bennett et al. 2014). In higher plants, stem-cell niches most prominently occur in the shoot apical meristem (SAM) and the root apical meristem (RAM) (Scheres 2001; Weigel and Jürgens 2002; Busch et al. 2010). SAM activity controls the development of the aerial parts of the plant body, whereas RAM activity controls the development of the belowground parts. These plant stem-cell niches are maintained by two small groups of the so-called organizing cells, which occur at a site known as the organizing center (OC) in the SAM and a site known as the quiescent center (QC) in the RAM. The OC and QC provide microenvironments that function in determining the fate of surrounding stem cells (Heyman et al. 2013; Gaillochet et al. 2015).

WUSCHEL (WUS) and WUSCHEL-related homeobox (WOX) genes encode a large family (WOX gene family) of homeodomain (HD)-containing transcription factors that are known to regulate the formation and development of

plant tissues; these genes are found in plant lineages, but not in other organisms such as bacteria or animals. (van der Graaff et al. 2009). Phylogenetic analyses have divided the WOX gene family into three major lineages: the WUS lineage, which contains WUS/WOX5 genes that are mainly present in seed plants (i.e., gymnosperms and angiosperms), the WOX9 lineage (mainly present in vascular plants), and the WOX13 lineage (present in all major plant lineages, including green algae and non-vascular mosses). With the exception of the HD that is common to all WOX family members, other unique motifs are shared only within one of the three lineages of WOX proteins (Deveaux et al. 2008; Nardmann et al. 2009; van der Graaff et al. 2009; Nardmann and Werr 2012; Lian et al. 2014).

In flowering plants, within the SAM, WUS is exclusively expressed in the OC; it controls shoot stem cell development. WOX5 is specifically expressed in the QC within the RAM, where it regulates root stem cell homeostasis (Scheres 2005; Forzani et al. 2014; Zhou et al. 2015). Additionally, WUS is known to be essential for the formation of functional floral organs (Sarkar et al. 2007). The flowering plants are the most diverse group of land plants; with about 350,000 species, they

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comprise about 90% of the plant kingdom. The ancestors of flowering plants emerged in the Triassic Period sometime between 202 and 245 million years ago (Ma). They diversified extensively during the Low Cretaceous, replacing the previously-dominant conifers (Bond and Scott 2010). The floral organs are the defining characteristics of the flowering plants, which increase the successful ratio of fertilization and facilitate the flowering plants rapid propagation after their divergence from the nonflowering plants. However, little is known concerning how flowering plants emerged with flower organs during plant evolution.

The members of the WUS/WOX5 family (WUS lineage) contain the WUS motif and the ERF-associated amphiphilic repression (EAR) motif (Nardmann et al. 2009; van der Graaff et al. 2009; Nardmann and Werr 2012), in addition to the invariably conserved, characteristic HD. The WUS motif is involved in transcriptional repression via cooperation with the EAR motif. Recent work has established that the WUS motif can recruit TPL/TPR corepressors to regulate the genes that control cell differentiation (Ikeda et al. 2009; Lin et al. 2013; Zhang et al. 2014; Pi et al. 2015). The stem-cell factor WUS establishes the shoot apical stem-cell niche through a CLAVATA3 (CLV3)—WUS feedback loop (Mayer et al. 1998; Brand et al. 2000; Schoof et al. 2000; Yadav et al. 2011; Perilli et al. 2012). The cell-to-cell movement of the WUS proteins is essential for this feedback loop (Yadav et al. 2011). Likewise, in the RAM, WOX5 establishes the root stem-cell niche through a feedback circuit involving auxin-related response factors (Sabatini et al. 1999; Blilou et al. 2005; Ding and Friml 2010; Yang et al. 2015). REPRESSOR OF WUSCHEL1 (ROW1) maintains both RAM and SAM development by confining the expression of WUS to the OC, and by confining WOX5 expression to the QC (Han et al. 2008; Han and Zhu 2009; Zhang et al. 2015; Kong et al. 2015). A recent study showed that HAIRY MERISTEM controls the development of the shoot and root stem-cell niches by interacting with, respectively, WUS and WOX5 (Zhou et al. 2015).

A previous study showed that the occurrence of WUS and WOX5 as separate genes was an evolutionary innovation of angiosperms, as only a single copy of WUS/WOX5 was identified in gymnosperms (Nardmann et al. 2009). However, both the separate WUS and WOX5 genes were recently identified in the gymnosperm *Picea abies* (Hedman et al. 2013). Interestingly, WOX5 and WUS have been shown to be functionally interchangeable in *Arabidopsis* shoot and root stem cell maintenance (Sarkar et al. 2007).

Despite the importance of WUS/WOX5 in plant apical stem-cell homeostasis and flower morphogenesis, little is known about how these conserved stem-cell factors evolved these important functions in flowering plants. Here, we expressed various ancestral WUS/WOX5 from extant plant species in the *Arabidopsis* WUS or WOX5 knockout mutants with the goal of studying the function of these stem-cell factors and elucidating their underlying evolutionary processes during the course of plant evolution. Our results reveal that a two-step functional innovation of WUS/WOX5 endowed these stem-cell factors with two distinct capabilities: apical stem-cell maintenance activity and intercellular mobility.

These innovations enable WUS/WOX5 to noncell-autonomously regulate shoot and root stem-cell niches of the flowering plant and to control floral organ formation. This evolutionary innovation also seems to have been a critical prerequisite step that facilitated the emergence of functional floral organs in the origin of flowering plants.

Results

Survey of the WUS and WOX5 Genes in Plant Kingdom

To obtain a broad view of the evolution of the WOX gene family, we surveyed the sequences of WOX family members in the plant kingdom. We used the full-length protein sequence of *Arabidopsis thaliana* WUS (AtWUS) as a query (Nardmann et al. 2009) to search against the available genome sequences from the bacterium *Bacillus anthracis* and 13 species from the plant kingdom that represented unicellular green algae, mosses, lycophytes, ferns, gymnosperms, and angiosperms (fig. 1A). We found that the number of WOX family genes increased substantially with the emergence of the vascular plant lineages (i.e., lycophytes, ferns, gymnosperms, and angiosperms) (WOX number > 10), in contrast to the nonvascular plant lineages (i.e., mosses and green algae) (WOX number ≤ 2) (fig. 1B). Note that only five WOX genes were identified in the fern *Ceratopteris richardii*, which likely reflects the incomplete status of the genome sequence of this species.

The deduced amino acid sequences of the WOX genes were aligned, and a phylogenetic tree was constructed (fig. 1C). The tree is similar to that of a previous report (Nardmann et al. 2009; Nardmann and Werr 2012) and can be divided into three major lineages according to the features of the conserved domain: the WOX13, the WOX9, and the WUS lineages (fig. 1C). The WOX13 lineage proteins are present in all major plant lineages, including the green algae and mosses. In addition to the conserved HD, the WOX13 proteins contain the WOX13 OG domain (supplementary fig. S1, Supplementary Material online). The WOX9 lineage is present in vascular plants; these proteins possess motifs that proteins of the other WOX lineages lack, such as the LQxG WOX8 motif (supplementary fig. S1, Supplementary Material online). The WUS lineage is mainly present in the seed plants. WUS lineage proteins are characterized by their WUS motifs (supplementary fig. S1, Supplementary Material online). Only one WOX protein, belonging to the WOX13 lineage, was identified in the green alga *Ostreococcus tauri* (Deveaux et al. 2008). However, the fern *C. richardii* (Cr) was found to have one or two WOX genes from each of the three lineages (fig. 1C). Notably, one WOX family member in *C. richardii* was grouped in the WUS lineage, because, in addition to its HD, this *C. richardii* WUS-like (CrWUL) protein also contains a WUS motif common to the WUS lineage and an EAR motif specific to the WUS/WOX5 proteins (Nardmann and Werr 2012) (supplementary fig. S1, Supplementary Material online). Rather than a single copy of WUS-like gene was found in the fern *C. richardii*, both the WUS and WOX5 genes are present in seed plants, including the gymnosperm *P. abies* and other angiosperm species (fig. 1C) (Hedman et al. 2013). This is consistent with the timing of the first whole-genome

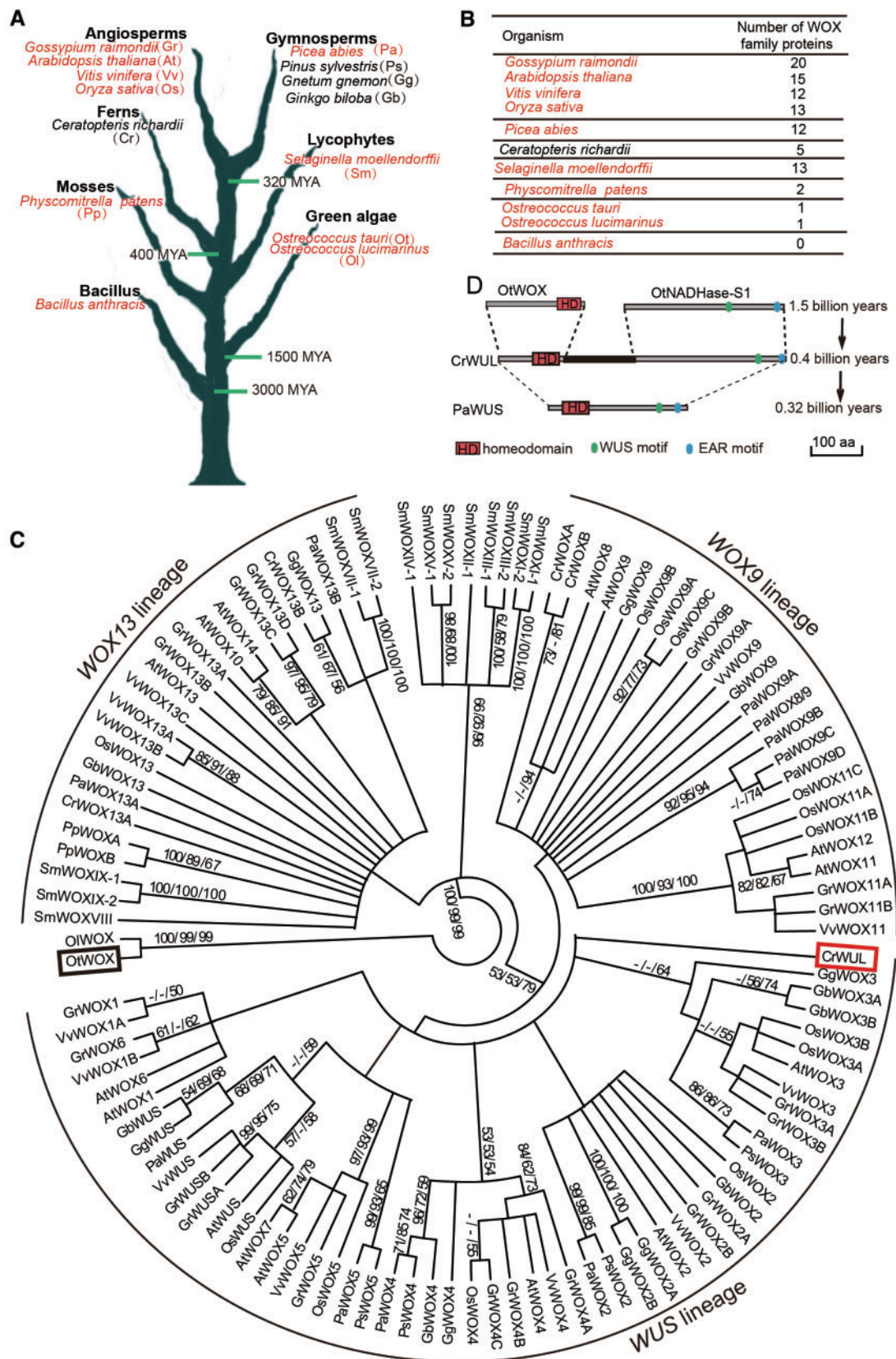


Fig. 1. Evolution and origin of the WUS/WOX5 genes. (A) Summary of the investigated plant species, from green algae to angiosperms, according to evolutionary history. Red indicates species whose genomes have been fully sequenced. (B) Number of WOX proteins in various organisms. The distribution of the WOX members showed that the number of the WOX genes is substantially increased in the vascular plants (i.e., lycophytes, ferns, gymnosperms, and angiosperms) as compared with the nonvascular plants (i.e., mosses and green algae). (C) Phylogenetic analysis of the WOX family in the plant kingdom. The tree was divided into three lineages: the WUS lineage, the WOX9 lineage, and the WOX13 lineage. The black box highlights the single WOX identified in the green alga *O. tauri*. The red box highlights the single copy of a WUS-like gene, belonging to the WUS

duplication event that was postulated to have occurred at the base of the seed plant lineage approximately 320 Ma (Jiao et al. 2011).

Although no WUS/WOX5 member was found in the green alga *Ostreococcus tauri*, we identified an nicotinamide adenine dinucleotide reduced dehydrogenase subunit 1 protein (OtNADHase-S1) that contains both WUS and EAR motifs, but no HD, that exists in *Ostreococcus tauri* (OtWOX) (fig. 1D and supplementary fig. S2, Supplementary Material online). Therefore, the fern *C. richardii* WUS-like (CrWUL) predecessor may have originated through a gene-fusion event that generated a protein containing all three conserved domains of WUS/WOX5. Moreover, the third, functionally essential HD is apparently missing in all plant species that originated prior to the fern lineage in which we theorize an important gene-fusion event occurred (supplementary fig. S3, Supplementary Material online). Notably, the fern CrWUL has a much longer protein sequence (591aa) than the WUS/WOX5 protein sequences of seed plants such as the gymnosperm *P. abies* WUS (PaWUS, 285aa) and PaWOX5 (207aa) (supplementary fig. S4, Supplementary Material online), among others (supplementary fig. S1, Supplementary Material online).

The Apical Stem-Cell Maintenance Functions of WUS and WOX5 in Shoots

All WOX genes in seed plants are known to have diverged from a single common ancestral WOX gene predecessor. With the goal of exploring which of the *Arabidopsis thaliana* WOX genes that evolved from the single green-algae-like ancestor function in maintaining shoot apical stem-cell niches (similar to WUS genes function), we analyzed all of the members of the WOX family with genetic complementation experiments using the *Arabidopsis wus-1* mutant, which carries a loss-of-function mutation in WUS. In accordance with previous reports, we found that, in contrast to wild-type *Arabidopsis*, *wus-1* mutant plants have a defective shoot meristem that terminates prematurely in an aberrant flat structure (fig. 2A and B, mid-upper panels) and fails to develop into a normal inflorescence (fig. 2A and B, upper panels) (Laux et al. 1996; Sarkar et al. 2007). Furthermore, the green fluorescent protein (GFP) of enhancer trap line J2341, which showed specific expression in the SAM of shoots (Kim et al. 2005) and in the distal meristem of roots in wild-type *Arabidopsis* (Ding and Friml 2010), was absent from *wus-1* mutant background (fig. 2A and B, mid-lower panels), confirming the aborted development of the shoot meristem in the *wus-1* mutant. Additionally, in contrast to the wild-type, *wus-1* mutants showed severe defects in floral organ development, with only a single stamen and no gynoecium (fig. 2A

and B, lower panels). Genetic complementation experiments showed that the expression of AtWOX5 and AtWUS driven by the native WUS promoter rescued both the premature termination of the shoot meristem and the floral organ developmental defects in *Arabidopsis wus-1* (supplementary fig. S5a and b, Supplementary Material online). Other WOX members of WUS lineage, including AtWOX1, AtWOX2, or AtWOX3, when driven by *Arabidopsis* WUS promoter, could also partially rescue the aborted SAM with an indeterminate inflorescence meristem (supplementary fig. S5c–e, Supplementary Material online). Notably, the expression of the WUS lineage AtWOX4 failed to rescue the *Arabidopsis wus-1* phenotype (supplementary fig. S5f, Supplementary Material online). AtWOX9 (WOX9 lineage) and AtWOX13 (WOX13 lineage) were also unable to rescue *wus-1* shoot meristem defects (supplementary fig. S5g and h, Supplementary Material online). These results indicate that the ability to maintain the *Arabidopsis* shoot stem-cell niche is not a general property of all the AtWOX family members that ultimately diverged from the green-algae-like ancestor.

We next explored the functional origin of WUS and WOX5 proteins that maintain the *Arabidopsis* shoot apical stem-cell niche and floral organ development during plant evolution. Interspecies genetic complementation experiments revealed that expression of OtWOX (green alga), which lacks the WUS and EAR motifs, in *Arabidopsis wus-1* was not able to rescue the phenotype with defective shoot meristem in aberrant flat structure and floral organ containing a single stamen and no gynoecium (fig. 2C). Strikingly, despite containing both WUS and EAR motifs, expression of the fern CrWUL protein in the *Arabidopsis wus-1* mutant, using the WUS promoter, still failed to complement its defects in SAM maintenance or flower organ formation (fig. 2D). However, the expression of gymnosperm proteins, including PaWUS/PaWOX5, WUS from *G. biloba* (GbWUS), and WOX5 from *P. sylvestris* (PsWOX5), in *Arabidopsis wus-1* mutant rescued *Arabidopsis* defects in both shoot apical stem-cell maintenance and flower organ formation (fig. 2E–H). Expression of GrWUS and GrWOX5 from the angiosperm *Gossypium raimondii* rescued the *Arabidopsis wus-1* phenotypes, suggesting that GrWUS/GrWOX5 and AtWUS/AtWOX5 are functionally equivalent in determining shoot apical stem-cell fate and in flower morphogenesis (fig. 2I and J). These results imply that the functional WUS/WOX5 predecessor, maintaining the *Arabidopsis* SAM and floral organ development, might have originated in the common ancestor of the gymnosperms/angiosperms after its divergence from the fern lineage. These successful interspecies functional complementation experiments in which WUS/WOX5 proteins from various seed plants expressed in *Arabidopsis wus-1* shoots reveals

FIG. 1. Continued

lineage, in the fern *Ceratopteris richardii* (CrWUL). A phylogenetic analysis was performed using the NJ/MP/ML method with full-length protein sequences. Bootstrap values are shown on the branch points. Ot, *Ostreococcus lucimarinus*; Ot, *Ostreococcus tauri*; Pp, *Physcomitrella patens*; Sm, *Selaginella moellendorffii*; Cr, *Ceratopteris richardii*; Gb, *Ginkgo biloba*; Gg, *Gnetum gnemon*; Ps, *Pinus sylvestris*; Pa, *Picea abies*; Os, *Oryza sativa*; Vv, *Vitis vinifera*; At, *Arabidopsis thaliana*; Gr, *Gossypium raimondii*. (D) Alignment of CrWUL with OtWOX and OtNADHase-S1 indicates that the three conserved domains common to WUS/WOX5 might have originated in fern via gene fusion. The PaWUS protein from the gymnosperm *P. abies* is substantially smaller than the fern CrWUL. Scale bars: 100 amino acids (aa).

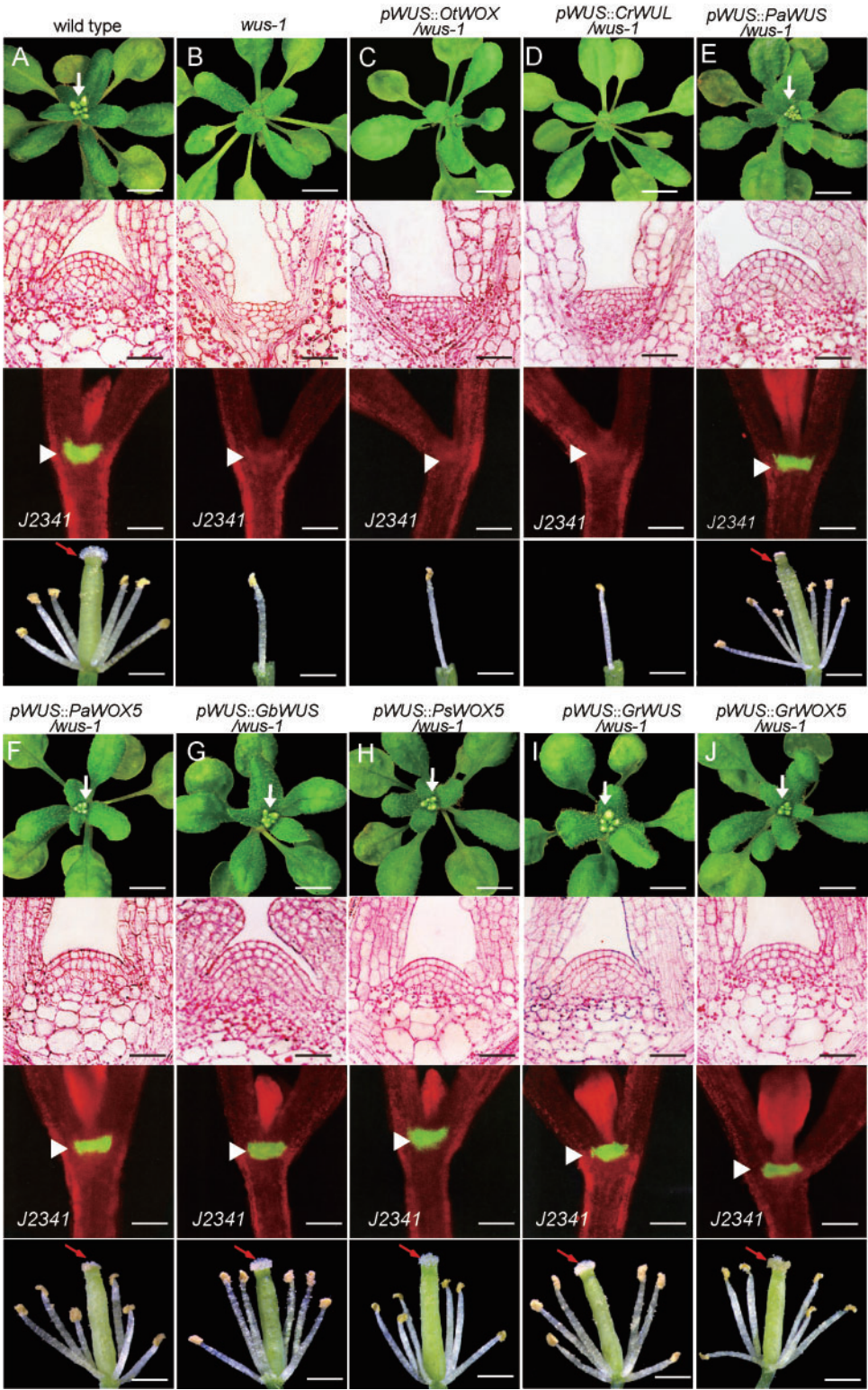


Fig. 2. Evolutionary analysis of WUS/WOX5 function in the maintenance of the *Arabidopsis* shoot apical stem-cell niche and in floral organ development. (A, B) In contrast to the wild-type shoot (A), the *wus-1* mutant (B) had defective shoot meristem development (upper panel) that terminated prematurely in an aberrant flat structure (mid-upper panel). The GFP of enhancer trap line J2341, which showed specific expression in the SAM of wild-type *Arabidopsis* shoots, was absent from the *wus-1* mutant (mid-lower panel). Flowers with four sepals and four petals removed (lower panel). Wild-type flowers contain six stamens and central gynoecium (A), while *wus-1* flowers had only one central stamen present and lacked central gynoecium (B). (C–J) Interspecies complementation experiments with WUS/WOX5 orthologues from the green alga lineage to the angiosperm lineage expressed in *Arabidopsis wus-1* mutants showed that *OtWOX* (green alga) and *CrWUL* (fern) are unable to maintain the shoot stem cell-niche and regulate floral organ morphogenesis (C, D), while the WUS/WOX5 orthologues from seed plants can rescue the defective *wus-1* shoot meristem niche and floral organ phenotypes. White arrows indicate the shoot apex. Red arrows indicate the gynoecium. Scale bars: 1 cm (upper panel), 20 μ m (mid-upper panel), 100 μ m (mid-lower panel), and 1 mm (lower panel).

that the shoot apical stem-cell maintenance function of these proteins has been evolutionarily conserved since the separation of angiosperms and gymnosperms.

The Apical Stem-Cell Maintenance Functions of WUS and WOX5 in Roots

We performed a similar functional analysis of WOX5 in the maintenance of root stem-cell homeostasis using *wox5-1*, a loss-of-function mutant for *AtWOX5*. In contrast to wild-type *Arabidopsis*, *wox5-1* mutants have a defective root distal meristem that seems to have undergone premature differentiation and is characterized by the accumulation of starch granules (fig. 3A and B, upper panel). Besides being absent from *wus-1* mutants, the GFP of enhance-trap line J2341 showed specific expression in wild-type root distal stem cells (DSCs) and was also absent from *wox5-1* mutant roots (fig. 3A and B, lower panel), confirming the aborted development of DSCs in *wox5-1* roots. *AtWOX5* and *AtWUS*, as driven by WOX5 promoter, were able to complement the DSC defects in the *wox5-1* mutant (supplementary fig. S6a and b, Supplementary Material online). No other WOX members from any of the three lineages had the ability to maintain the *Arabidopsis* root stem-cell niche (supplementary fig. S6c–h, Supplementary Material online).

Similar to the results found in the shoot meristem, *OtWOX* (green alga), driven by *Arabidopsis* WOX5 promoter, failed to rescue the *Arabidopsis wox5-1* mutant (fig. 3C). The fern *CrWUL*, which contains the conserved HD and the WUS/EAR motifs as in the seed plant WUS/WOX5 proteins (supplementary fig. S3, Supplementary Material online), was not able to complement the *Arabidopsis wox5-1* defects when also driven by native WOX5 promoter (fig. 3D). Both WUS and WOX5 from seed plants, including those from both gymnosperms and angiosperms, were able to replace the function of *AtWOX5* in maintaining the *Arabidopsis* root distal meristem stem cell niche (fig. 3E–J), implying that functional WUS/WOX5 molecular in flowering plant root stem-cell niche maintenance might originate in the recent common ancestor of gymnosperm/angiosperm after the divergence from fern lineage, and the function has been highly conserved during evolution course after the gymnosperms/angiosperms split.

Intercellular Mobility and Biological Activity of WUS in Shoots

Even though both the seed plant WUS and the fern *CrWUL* contain the three conserved domains of WUS/WOX5 subclade, we want to know why the seed plant WUS proteins, but not the fern *CrWUL*, were able to maintain the shoot apical stem-cell niche and control flower formation in *Arabidopsis*. Given that the fern *CrWUL* is much longer than the seed plant WUS proteins (supplementary fig. S1, Supplementary Material online), we began by investigating how this change of the molecular mass might have influenced WUS/WOX5 function. Using a *Arabidopsis* transcriptional reporter *pWUS::ER-GFP* construct, we found that WUS is specifically expressed in the OC driven by WUS promoter, which is localized to the L3 and deeper layers of the SAM

(Yadav et al. 2013); it is not expressed in the L1 or L2 layers (fig. 4A). To observe the distribution and mobility of the WUS proteins in the *Arabidopsis* shoot meristem, we further generated various chimeric WUS-GFP proteins using three WUS protein-coding sequences from fern and seed plants. These fusion WUS-GFP proteins were driven from the native *Arabidopsis* WUS promoter. In both *pWUS::AtWUS-GFP* and *pWUS::PaWUS-GFP* transgenic lines, we observed bright fluorescence signals in OC cells, indicating WUS expression. We also observed relatively weak fluorescence signals in adjacent cells of the L1 and L2 layers, indicating the mobility of these fusion proteins (fig. 4B). By contrast, *pWUS::CrWUL-GFP* signals were strictly restricted to the OC, with no detectable signals in the L1 and L2 layers (fig. 4B). Genetic complementation experiments also showed that the mobile seed plant *AtWUS-GFP* and *PaWUS-GFP* fusion proteins, but not the immobile fern *CrWUL-GFP* fusion protein, were able to rescue the *Arabidopsis wus-1* mutant defects in SAM and floral organ development (fig. 4C). Combined with the sequence analysis (fig. 1D), it seems plausible that inability of the fern *CrWUL* to maintain the SAM and flower development in *Arabidopsis* may result from a reduction in its mobility owing to its relatively larger molecular mass. These results suggest that the intercellular mobility of WUS protein may have originated in the common ancestor of gymnosperms and angiosperms after their divergence from the fern lineage.

Then we generated a truncated version of *CrWUL* (Mini-*CrWUL*) by deleting sequences that are not found in gymnosperm *PaWUS* (supplementary fig. S4, Supplementary Material online). When we expressed the Mini-*CrWUL-GFP* using the WUS promoter, the protein was observed to migrate into the L1 and L2 layers from the OC (fig. 4D). Moreover, the mobile Mini-*CrWUL-GFP* driven by *Arabidopsis* WUS promoter was able to rescue the *Arabidopsis wus-1* SAM and floral organ defects (fig. 4E). These results indicate that the WUS predecessor somehow became shorter during the evolution of seed plants after they split from the fern lineages, and thereby gained the intercellular mobility that now appears to be crucial for shoot meristem and floral organ development in flowering plant species.

Although the lack of migration appeared to interfere with the function of the fern *CrWUL* to complement the *Arabidopsis wus-1* mutant, it remained unclear as to whether *CrWUL* itself possesses the biological activity to maintain shoot apical stem cells and control flower development. The *CrWUL-GFP* proteins were strictly retained in the OC and were unable to migrate into the L1 and L2 layers to function in stem-cell maintenance activity when driven by the WUS promoter (fig. 4B). It was therefore difficult for us to assess their own biological activity. To address this issue, we took advantage of the fact that *CLV3* is known to be specifically expressed in the L1 and L2 layers (fig. 4F) (Ogawa et al. 2008). Thus, to mimic the WUS proteins moving into these layers and to test its shoot apical stem-cell maintenance activity, we simultaneously and ectopically expressed fern *CrWUL-GFP* in the OC and the L1 and L2 layers by using both the *CLV3* and WUS promoters. In *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP* plants, GFP signals were detected in both

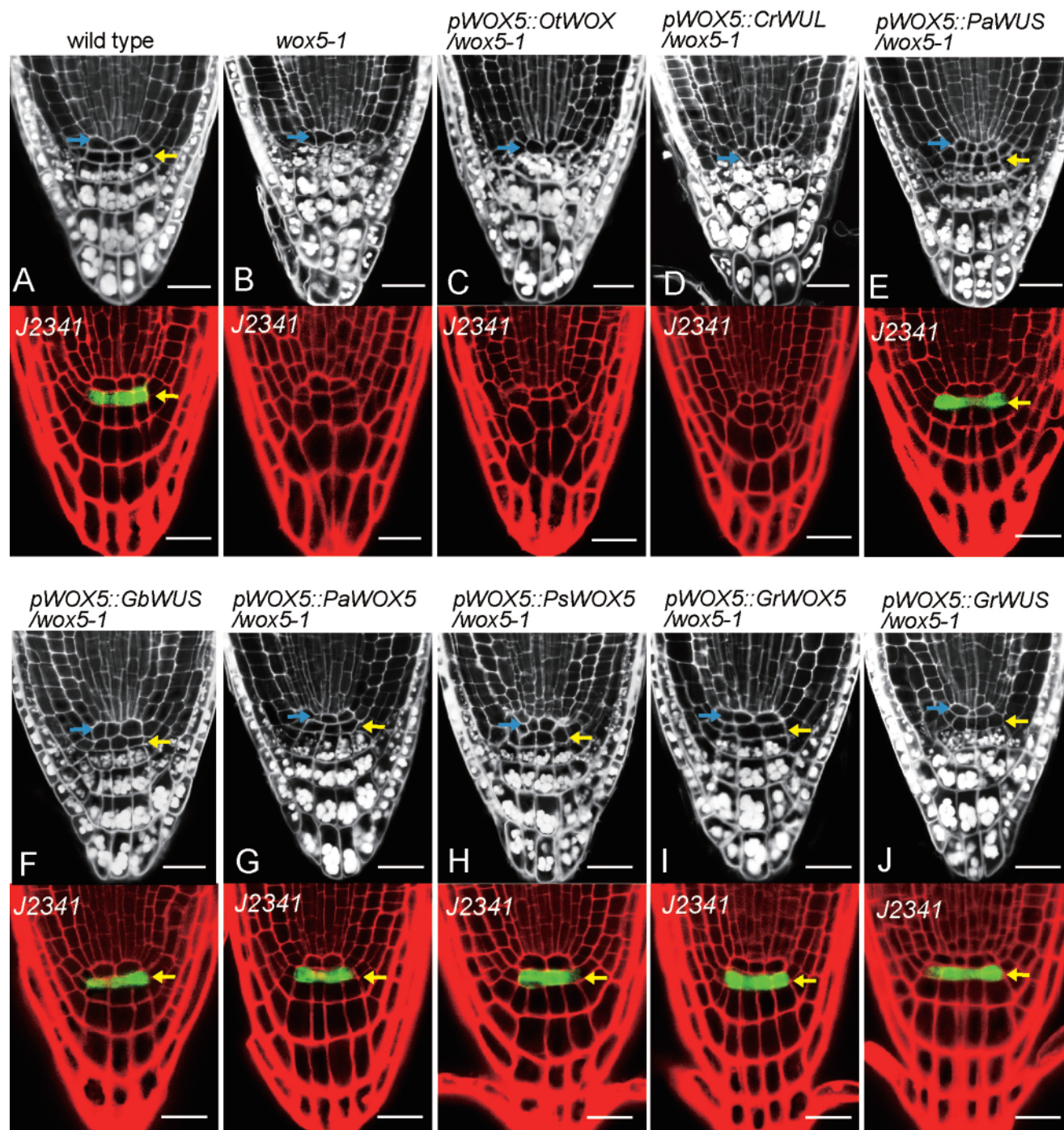


Fig. 3. Evolutionary analysis of WOX5/WUS function in the maintenance of the *Arabidopsis* root stem-cell niche. (A) Wild-type root showing a normal root stem-cell niche. In the upper panel, undifferentiated DSCs (yellow arrowheads) below the QC (blue arrowheads) are characterized by the absence of starch, whereas white starch granules stained by the mPS-PI method are visible in differentiated columella cells below the DSCs. (B) The *wox5-1* mutant failed to maintain root stem cells, lacked DSCs, and showed premature differentiation, as visualized by the accumulation of starch granules in the cell tier below the QC (upper panel). In contrast to the single tier of GFP signals in the wild-type root DSCs, the DSC marker J2341 in *wox5-1* roots showed no GFP expression (lower panel). (C–J) Interspecies complementation with WUS/WOX5 orthologues from the green alga lineage to the angiosperm lineage expressed in *Arabidopsis wox5-1* mutants showed that OtWOX (green alga) and CrWUL (fern) are unable to maintain the *Arabidopsis* root stem cell niche, whereas WUS/WOX5 orthologues from seed plants are able to rescue the defective *wox5-1* root meristem. Scale bars, 20 μm.

the OC and L1 and L2 layers (fig. 4G). In contrast to the *pWUS::CrWUL-GFP* construct (fig. 4C), the *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP* constructs were able to complement *Arabidopsis wus-1* defects (fig. 4H), suggesting that fern

CrWUL has acquired the shoot apical stem-cell maintenance activity, and, further, that this activity is independent of inter-cellular mobility. Combined with the previous interspecies complementation experiments, our results suggest that the

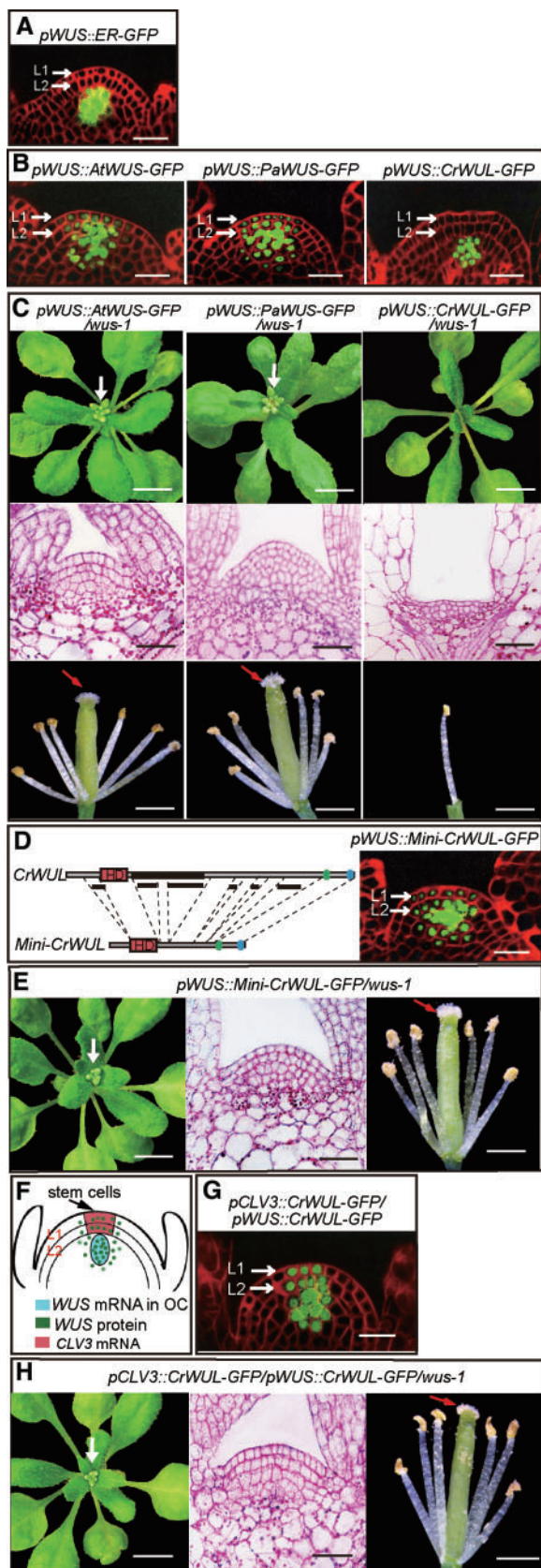


FIG. 4. The evolutionary innovations of functional WUS with apical stem-cell maintenance activity and intercellular mobility in *Arabidopsis* shoots. (A) Lateral view of the SAM with the confocal microscopy showing the specific expression of the transcriptional reporter *pWUS::ER-GFP* in the OC, but not the L1 or L2 cell layers,

shoot apical stem-cell maintenance activity was present in the common ancestor of ferns/seed plants prior to the acquisition of its intercellular mobility.

Intercellular Mobility and Biological Activity of WOX5 in Roots

We used the same approach to examine how the WOX5 protein, a homologue of WUS, evolved its function in regulating the root stem-cell niche. The transcriptional reporter *pWOX5::ER-GFP* indicated that WOX5 was expressed exclusively in the QC (Chen et al. 2011; Zhang et al. 2015). We generated chimeric WOX5-GFP proteins using coding sequences from three different plant lineages representing the ferns, gymnosperms, and angiosperms. These fusion constructs used the *Arabidopsis* native WOX5 promoter. We found that seed plant WOX5 proteins—specifically, the gymnosperm PaWOX5 and angiosperm AtWOX5—were able to move into cells surrounding the QC, including the DSC, whereas the fern CrWUL was restricted to the QC and produced no detectable signal in the DSC layer (fig. 5A). These results suggest that the intercellular mobility of WOX5 was an evolutionary innovation of seed plants that has been conserved strongly following the gymnosperm/angiosperm split.

Interspecies complementation experiments showed that, when using the WOX5 promoter, the expression of the seed plant angiosperm AtWOX5-GFP and gymnosperm PaWOX5-GFP, but not the immobile fern CrWUL-GFP, in *Arabidopsis* *wox5-1* mutants, resulted in the rescue of root meristem defects (fig. 5B), a situation that may partially result from the differences in the molecular masses of WOX5 variants. To test this hypothesis, we expressed the truncated Mini-CrWUL-GFP using the WOX5 promoter. The GFP signals were detected in both the QC and DSC (fig. 5C), similar to the distribution of the gymnosperm PaWOX5-GFP and the angiosperm AtWOX5-GFP (fig. 5A). Finally, the *pWOX5::Mini-CrWUL-GFP* construct was able to rescue the *wox5-1* defect in the DSC (fig. 5D). These results suggest that the intercellular

FIG. 4. Continued

in wild-type *Arabidopsis* shoots. (B) When driven by *Arabidopsis* WUS promoter, the seed plant WUS-GFP proteins (left and middle panels), but not the fern CrWUL-GFP protein (right panel), migrated into the L1 and L2 layers from the OC in wild-type *Arabidopsis* shoots. (C) In contrast to the *pWUS::AtWUS-GFP* and *pWUS::PaWUS-GFP* constructs, the *pWUS::CrWUL-GFP* construct failed to rescue the aborted SAM and floral organ defect in *wus-1* plants. (D) In *pWUS::Mini-CrWUL-GFP* plants, the GFP signals were detected both in the OC and in L1 and L2 layers, in *Arabidopsis* shoots. (E) The *pWUS::Mini-CrWUL-GFP* construct was able to rescue the *wus-1* mutant shoot meristem and flower defects. (F) Schematic representation of *Arabidopsis* shoot stem-cell niche maintenance. Modified from Laux (Cell 113, 281–283, 2003). (G) In *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP* plants, GFP signals were simultaneously detected in the OC and the L1 and L2 layers. (H) The *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP/wus-1* construct was able to rescue the defective shoot meristem and floral organ defects in *wus-1* mutant plants. Scale bars: 1 cm (C, E, and H, upper panels), 1 mm (C, E, and H, lower panels), 20 μ m (other panels).

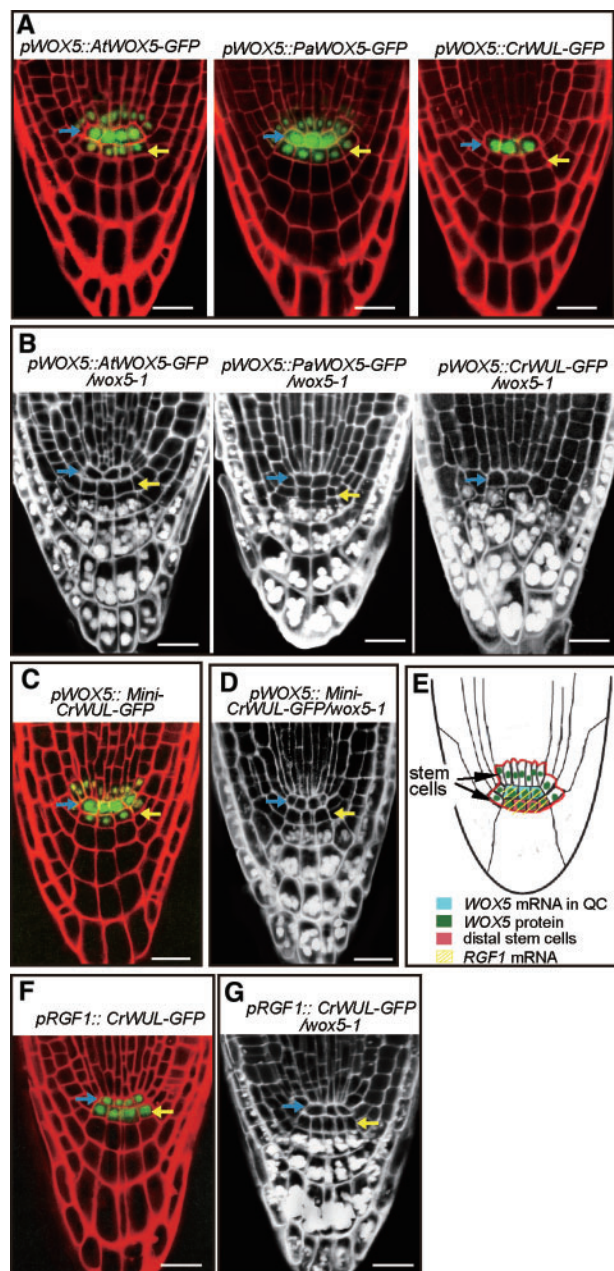


Fig. 5. The evolutionary innovations of functional WOX5 with apical stem-cell maintenance activity and intercellular mobility in *Arabidopsis* roots. (A) Lateral view of the root apical meristem (RAM) showing the seed plant WOX5-GFP proteins (left and middle panels) driven by the native WOX5 promoter, were detected in DSCs. However, the fern CrWUL-GFP protein (right panel) as driven by the native WOX5 promoter, was restricted to the QC. Blue arrow, QC position; Yellow arrow, DSC layer. (B) In contrast to the *pWOX5::AtWOX5-GFP* and *pWOX5::PaWOX5-GFP* constructs, the *pWOX5::CrWUL-GFP* construct was unable to rescue the *wox5-1* aborted DSC. (C) In *pWOX5::Mini-CrWUL-GFP* plants, the GFP signals were detected in the whole root stem-cell niche, including the DSCs. (D) The *pWOX5::Mini-CrWUL-GFP* construct was able to rescue *wox5-1* root defects. (E) Schematic representation of *Arabidopsis* root stem-cell niche maintenance. The red line outlines the root stem-cell niche. Modified from Sarkar et al. (2007). (F) In *pRGF1::CrWUL-GFP* plants, the GFP signals were simultaneously detected in the QC and DSCs. (G) The *pRGF1::CrWUL-GFP* construct was able to rescue the *wox5-1* defects. Scale bars, 20 μ m.

mobility of WOX5 may have been acquired via a decrease in the molecular mass of an ancestral WUS/WOX5 precursor during the evolution of seed plants after they diverged from the fern lineage.

Despite of the immobility of the fern CrWUL protein in root, we want to know whether CrWUL itself possesses root apical stem-cell maintenance regulatory activity. We investigated this by mimicking the movement of the seed plant (gymnosperms and angiosperms) WOX5 proteins in *Arabidopsis*. In order to mimic the movement, we directly expressed fern CrWUL-GFP in the QC and DSC using the *Arabidopsis* RGF1 promoter, which is known to induce expression in the QC and DSC (Matsuzaki et al. 2010) (fig. 5E). In *pRGF1::CrWUL-GFP* plants, GFP signals were observed in both the QC and the DSC (fig. 5F). Furthermore, the *pRGF1::CrWUL-GFP* construct was able to rescue the aborted DSC in the *wox5-1* mutant (fig. 5G). Together, these results indicate that fern CrWUL itself possesses the root apical stem-cell maintenance activity. Notably, only the fern CrWUL and seed plant WUS/WOX5 proteins contain the carboxyl-terminal EAR motif, which is lacked in other WOX proteins (supplementary fig. S1, Supplementary Material online), suggesting that this motif might be responsible for the conserved role of WOX in controlling root stem-cell maintenance activity. Finally, we conclude that this root apical stem-cell maintenance activity may have originated in the common ancestor of ferns and seed plants prior to the capacity for intercellular mobility, and suggesting that capacity has been highly conserved during the course of plant evolution after the fern/seed plant split.

Evolution of WUS/WOX5 Driven by Positive Selection

To examine the driving force of WUS/WOX5 evolution, we analyzed the WUS and WOX5 sequences from the green algae to seed plants. Specifically, we used WUS and WOX5 genes from seed plants, WUS-like (i.e. WUL) genes from the ferns, and the WOX genes from the lower plants (moss and green algae) (fig. 6A). We estimated the ratio of nonsynonymous to synonymous substitution rate (termed ω), which is a measure of the driving force behind molecular evolution, with $\omega < 1$ values indicating purifying selection, $\omega = 1$ neutral evolution, and $\omega > 1$ positive selection (Nei and Kumar 2000). Two evolutionary analyses were conducted using codeml in the PAML package (Yang 2007). First, we constrained the branch (branch A shown in red in fig. 6A) ancestral to all of the WUS/WOX5 genes as the foreground and conducted branch-site models. The branch-site null model A (null hypothesis) fixed the ω for the branch A at 1 (i.e., neutral evolution), whereas the branch-site model A (alternative hypothesis) estimated the same ω allowing it to exceed 1 (i.e., positive selection). A likelihood ratio test between the two models was significant ($P = 0.007$; fig. 6B), suggesting that positive selection probably had an effect to increase the evolutionary rate of the WUS/WOX5 ancestor after the separation of ferns and seed plants. We also observed that a single copy of WUS-like gene (WUL) was duplicated into two copies (WUS and WOX5) in angiosperms and gymnosperms (fig. 6A). Thus, positive selection may have played an important role in the functional

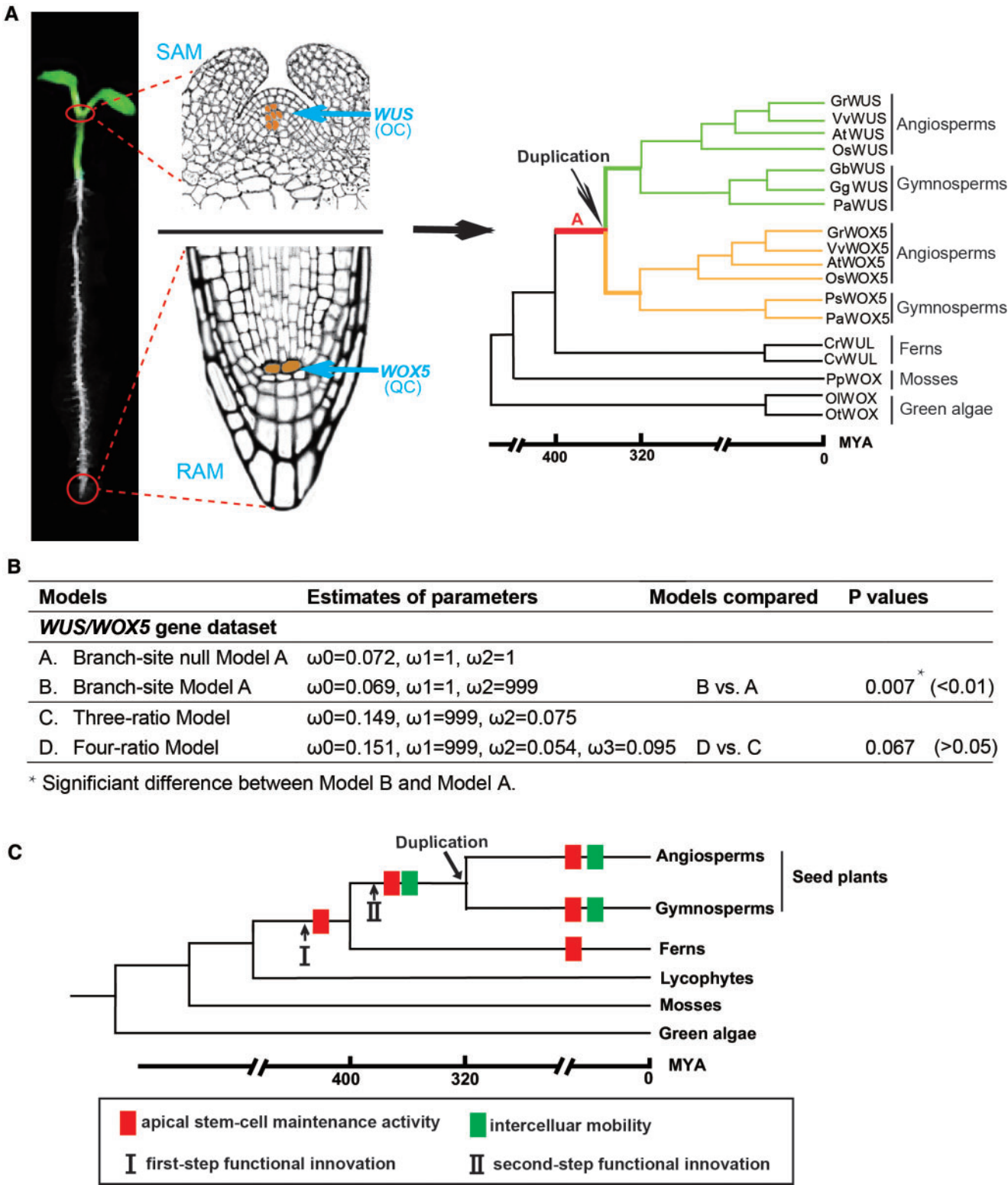


FIG. 6. Selection test and schematic drawing of capabilities acquired for a two-step functional evolution of WUS/WOX5 subfamily. (A) Phylogeny of WUS and WOX5 genes during plant evolution. Left, a one-week-old *Arabidopsis* seedling with its shoot and root apical tissues highlighted by two red circles. Middle, the enlarged structures of the *Arabidopsis* shoot and root apical meristems with the WUS expression domain in OC (upper) and WOX5 expression domain in QC (lower). Right, Construction of phylogenetic tree with WUS (upper) and WOX5 (lower) lineages using the NJ, MP, and ML methods. (B) Selection tests suggest that the positive selection had an effect to increase the evolutionary rate of the WUS/WOX5 ancestor after the split of ferns and seed plants, whereas similar levels of purifying selection on the separate WUS and WOX5 genes were observed in seed plants, indicating the functional conservation of these stem-cell factors in the evolution course after duplication. (C) An evolutionary scheme representing the two-step functional innovation of WUS/WOX5 proteins during plant evolution. WUS/WOX5 proteins gained the apical stem-cell maintenance activity in the recent common ancestor of ferns and seed plants; the intercellular mobility was subsequently acquired in the common ancestor of seed plants, after divergence from the fern lineage.

innovations of the *WUS*/*WOX5* genes. Second, we tested the possibility of differential selection on the *WUS* and *WOX5* genes by comparing a three-ratio model with a four-ratio model (fig. 6B). The three-ratio model assumes three ω values: branch A in red has ω_1 , branches in green have ω_2 , branches in yellow also have ω_2 , and other branches have ω_0 , whereas the four-ratio model assumes one additional ω_3 for the branches in yellow (all *WOX5* genes) and ω_2 for the branches in green (all *WUS* genes) (fig. 6A). Both ω_2 (0.054) and ω_3 (0.095) estimated by the four-ratio model are lower than 1 (fig. 6B), suggestive of purifying selection in both *WUS* and *WOX5* genes. A likelihood ratio test between the two models was not significant ($P = 0.065$; fig. 6B), indicating similar levels of purifying selection on the *WUS* and *WOX5* genes. Thus, during the evolution of seed plants, purifying selection of *WUS*/*WOX5* genes may have resulted in the functional conservation of these stem-cell factors in flowering plant shoot/root apical stem-cell maintenance and in flower organ formation. This supposition is concordant with the results of our functional assays of successful inter-species complementation with the seed plant *WUS*/*WOX5* expressed in the *Arabidopsis wus-1* and *wox5-1* mutants (fig. 2E–J and 3E–J).

Discussion

Our results showed that the two-step functional innovation of the stem-cell factors *WUS*/*WOX5* plays a pivotal role in flowering plant shoot/root stem cell homeostasis and floral organ development during plant evolution. As depicted in the model (fig. 6C), the first-step functional innovation is the apical stem-cell maintenance activity of *WUS*/*WOX5* originated in the recent common ancestor of ferns and seed plants, which is evidenced by the successful inter-species complementation experiments, with the fern *CrWUL* ectopically expressed surrounding the OC/QC or exclusively OC-/QC-expressed the seed plants *WUS*/*WOX5* in *Arabidopsis WUS* or *WOX5* knockout mutants. The confocal microscope imaging of GFP fusion proteins revealed that the intercellular mobility of *WUS*/*WOX5*, as the second functional innovation, was furtherly acquired in the recent common ancestor of gymnosperms/angiosperms after they diverged from the fern lineage. These two capabilities of *WUS*/*WOX5*, originated from two-step evolutionary innovation, have been evolutionarily conserved after the divergence of the gymnosperms/angiosperms. Our findings therefore provide evolutionary insight into the origin of these noncell-autonomous stem-cell factors.

Instead of the single copy of *WUS*-like gene identified in fern, both the *WUS* gene and its homologous *WOX5* gene is present in seed plants—gymnosperms and angiosperms—suggesting a duplication event occurred after the divergence of ferns/seed plants, but prior to the separation of the angiosperms/gymnosperms (fig. 6C). Moreover, the duplication event may have occurred since the gain of the intercellular mobility function and then both of the discrete *WUS* and *WOX5* proteins have inherited both of the functional capacities developed in the two-step functional innovation history of these genes, which results in the interchangeable function

of *WUS* and *WOX5* in *Arabidopsis*. After the duplication, both the *WUS* and *WOX5* genes underwent similar levels of purifying selection, which was consistent with the conserved functions of gymnosperms/angiosperms *WUS*/*WOX5* in *Arabidopsis* shoot/root stem-cell maintenance and flower organ formation, as shown by interspecies complementation experiments (figs. 2E–J and 3E–J).

Through plant evolution, one of the most important events was the emergence of the flowering plants. Because the flowering plants contain floral organs, which are conducive to efficient fertilization, they were capable of rapid reproduction, allowing their populations to spread more rapidly than their predecessors. Our results show that the functional *WUS*, which was acquired by a two-step evolutionary innovation process in plant evolution, is essential for flower organ formation; therefore, suggesting that the two-step functional innovation of *WUS*/*WOX5* should be considered to have played an important role in facilitating the emergence of the flowering plants.

Although neither OC/QC structures nor *WOX5*/*WUS* genes are present in the moss *P. patens*, the *WOX13*-like genes are known to be involved in the reprogramming of leaf and protoplast cells into stem cells (Barlow 1994; Harrison et al. 2009; Sakakibara et al. 2014). However, the *WOX13* lineage seems to have no function in flowering plant apical stem-cell maintenance. In ferns, there is a single large pyramidal cell in the root meristem that is referred to as the apical cell. The apical cell is a QC-like cell that alternates cleavage events along its four faces; it is the ultimate source of all cells in the meristem. However, in contrast to the QC cells in seed plants, the fern apical cell has mitotic activity. The fern *C. richardii WUS*-like gene is transcribed in the whole root tip instead of in this apical cell (Nardmann and Werr 2012), suggesting the cell-autonomous action of the *CrWUL* in ferns. Interestingly, it has been reported that in *Arabidopsis*, when *AtWUS* was expressed under the control of the *CLV3* promoter, transgenic plants showed significantly enlarged shoot meristems with massive increases in their stem cell populations, due to an unbalanced positive-negative feedback loop (Brand et al. 2002; Yadav et al. 2010). The meristem of *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP* plants are comparable to wild-type meristem (fig. 4G). These findings indicate that, beyond an inability in intercellular movement, *CrWUL* may still behave differently from seed plant *AtWUS* in controlling *Arabidopsis* shoot stem cell activities. In gymnosperm and angiosperm species, the OC/QC is histologically apparent (Barlow 1994), but studies have shown that the gymnosperm *G. gnemon WUS* was transcribed in the OC and its surrounding cells, including in the L1 layer (Nardmann et al. 2009), whereas the angiosperm *Arabidopsis WUS* and *WOX5* are exclusively expressed in the OC and QC and then move to the adjacent cells to control the fate of their surrounding stem cells. This information suggests that the noncell-autonomous action of these conserved stem-cell factors might emerge after the divergence of the gymnosperms/angiosperms. It also implies that the coevolution between the OC/QC structural formation and functional *WUS*/*WOX5* acquired by the two-step evolutionary innovation plays a

critical role in the origin of the noncell-autonomous regulation mechanism that the flowering plants used to maintain the apical stem-cell and flower organ development. In addition to WUS/WOX5, how the noncell-autonomous activities of other essential plant transcriptional regulators such as SHORTROOT (SHR), KNOTTED1/SHOOT MERISTEMLESS (KN1/STM), and LEAFY (LFY) through plant evolution remain unresolved questions (Sessions et al. 2000; Gallagher et al. 2004; Daum et al., 2014).

Although plants and animals separated very early and evolved independently, they show similar mechanisms in stem cell control. In animals, morphogens are expressed in specific niches and then migrate into adjacent cells to set up concentration gradients that drive the stem cells to differentiate into different cell types, thereby forming all of the tissues and organs of the body. But it remains to be shown how these noncell-autonomous molecules in animals originated during the evolution of animal kingdom.

Materials and Methods

Search for WOX Family Members

The accession numbers or IDs of WOX genes from the following plant species can be found in Nardmann et al. (2009): *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Physcomitrella patens*, *Ginkgo biloba*, *Gnetum gnemon*, *Pinus sylvestris*, *Oryza sativa*, and *Arabidopsis thaliana*. The accession numbers or IDs of the WOX proteins in *C. richardii* can be found in Nardmann and Werr (2012). WOX genes in other plant species were identified from the following databases: *Selaginella moellendorffii* and *Vitis vinifera* from the National Center for Biotechnology Information (NCBI), *Gossypium raimondii* from <http://www.cottongen.org/> (Wang et al. 2012), and *P. abies* from both NCBI and <http://congenie.org/>. The accession numbers or IDs of identified WOX genes are given in supplementary table S1, Supplementary Material online. Multiple sequence alignments were performed using ClustalX (Thompson et al. 1997).

Evolutionary Analysis

WUS/WOX5 genes were translated into protein sequences and subsequently aligned with ClustalX (Thompson et al. 1997). Neighbor joining (NJ) and maximum-parsimony (MP) phylogenetic analyses were conducted with MEGA v5.05 (Tamura et al. 2011). Maximum likelihood (ML) phylogenetic analysis was undertaken with PhyML v3.0 (Guindon and Gascuel 2003). NJ analysis was performed using the protein Poisson distances and the pairwise deletion of gap sites. MP analysis used the default parameters. The best-fitting substitution model for the ML analysis was selected with the jModelTest2 program (Darriba et al. 2012). For each of three phylogenetic analyses, 1,000 bootstrap replicates were used to evaluate the reliability of the phylogenetic trees. The estimates of the ratios of nonsynonymous and synonymous nucleotide substitution rates were calculated with the codeml program implemented in PAML (Yang 2007). Likelihood ratio tests were used to compare nested models.

Plant Materials and Growth Conditions

The *Arabidopsis* mutant and transgenic lines used were as follows: *wox5-1* (SALK_038262), *wus-1* (NASC ID: N15), J2341 (NASC ID: N9118), and *pWUS::ER-GFP* (NASC ID: N23897). Transgenic plants with *pWUS::AtWUS-GFP* in a wild-type background were kindly provided by Dr. J. U. Lohmann (University of Heidelberg, Germany) (Daum et al. 2014). Seeds were surface-sterilized with 0.1% HgCl₂, germinated on Murashige and Skoog (MS) medium for 2 weeks, transferred to soil, and grown in an Intellus control system (Percival) with a 16/8-h light/dark cycle at 22 °C and 70% humidity.

Vector Construction, Plant Transformation, and Rescue Analysis

To generate plasmids for genetic complementation analysis, WUS-coding sequences from different plant species were cloned into pQG110 containing either a 4.8-kb WOX5 promoter or a 5.6-kb WUS promoter. To detect the movement of WUS/WOX5 proteins, GFP was fused in-frame to the C terminus of various WUS open reading frames and then driven by the WUS or WOX5 promoter. The primers used in the generation of these constructs are detailed in supplementary table S2, Supplementary Material online. Transgenic *Arabidopsis* plants were generated by the floral dip method (Han et al. 2008) and selected on solid, half-strength MS medium plates containing 50 mg/ml of the appropriate antibiotics.

For rescue analysis, we extracted the RNA from the *pWUS::WOX* or *pWOX5::WOX* rescue/non-rescue transgenic lines, and the cDNA was reverse-transcribed from 5 µg of the total RNA as previously reported (Han et al. 2008). Then we used the forward primer from 5'-untranslated region sequence of WUS mRNA or WOX5 mRNA and the reverse primer from the different WOX CDS for quantitative real-time polymerase chain reaction (QRT-PCR) analysis with the housekeeping gene *UBQ5* used as the internal standard. We took care to ensure that the mRNA expression levels of *pWUS::WOX* or *pWOX5::WOX* constructs in the nonrescued transgenic lines used for phenotypic analysis were highly expressed and were comparable to the expression level in the rescued transgenic lines to exclude the possibility that the nonrescued phenotype resulted from the insufficient expression of the WOX genes in the *wus-1* or *wox5-1* mutants. The primers used for analysis are listed in supplementary table S4, Supplementary Material online.

Starch Staining

The starch granules and cell walls in *Arabidopsis* root tips (7 days old) were stained via the modified pseudo-Schiff propidium iodide staining (mPS-PI) method and imaged with a confocal microscope, as previously described (Truernit et al. 2008). In brief, whole seedlings were fixed in 50% methanol/10% acetic acid at 4 °C for up to 24 h. Tissues were then rinsed briefly with ddH₂O and incubated in 1% periodic acid at room temperature for 40 min. The tissue was then rinsed twice with ddH₂O and incubated in Schiff reagent with propidium iodide (100 mM sodium metabisulphite, 0.15 N HCl, and 100 mg/ml propidium iodide) for 2 h until the

plants were visibly stained. More than three samples were transferred onto microscope slides and covered with chloral hydrate solution (4 g chloral hydrate, 1-ml glycerol, and 2-ml water). The slides were incubated overnight at room temperature, after which excess chloral hydrate was removed. The seedlings were mounted in Hoyer's solution (30-g gum arabic, 200-g chloral hydrate, 20-g glycerol, and 50-ml water). The slides were left undisturbed for at least 3 d prior to observation (excitation 488 nm, emission 520–720 nm).

Histological Analysis

To prepare semithin sections, shoot tips were stained in 1% (w/v) periodic acid solution containing Schiff's reagent; these were fixed overnight in 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in phosphate-buffered saline, pH 7.2, at 4 °C. The specimens were then dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) and embedded in Spurr's resin (Spi-Chem). The tissue was mounted in ddH₂O and sectioned at a thickness of 2 µm on a Leica RM 2265 microtome. The sections were observed under bright-field optics using a Leica DMRE microscope.

Confocal Microscopy

For confocal microscopy, *Arabidopsis* shoot tips were stained with 50-µg/ml FM4-64FX (Invitrogen, F34653) (Reddy et al. 2004). *Arabidopsis* root tips were stained with 10-µg/ml propidium iodide for 5 min (Sarkar et al. 2007), washed briefly in ddH₂O, and then visualized at 720–760 nm for FM4-64FX, at 600–640 nm for propidium iodide, and at 500–560 nm for GFP on an LSM 710 NLO confocal microscope with DuoScan functionality.

To confirm the enhancer trap reporter J2341 (a GAL4-GFP enhancer trap reporter) is present in both rescued and nonrescued transgenic lines, we extracted the genomic DNA, utilized the PCR-based genotyping and sequencing of the GFP and GAL4 to confirm that the J2341 reporter was introduced in both the rescued and nonrescued transgenic lines before they were used for confocal microscope imaging. To confirm the *pCLV3::CrWUL-GFP/pWUS::CrWUL-GFP* lines used for analysis with the strong expression of CrWUL-GFP fusion proteins in both central zone and OC, we utilized the confocal microscopy for selection of these transgenic lines with strong GFP fluorescence under 488 nm of excitation wavelength, and at least three independent transformants were used for phenotypic analysis.

Supplementary Material

Supplementary figures S1–S6 and tables S1–S4 are available at *Molecular Biology and Evolution* online.

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