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# Stem cell regulation by *Arabidopsis* WOX genes

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**Short summary:** In plants, specialized WUSCHEL homeobox (WOX) genes act as master regulators of stem cell pluripotency. This manuscript reveals important insight into the diversification of WOX protein function. The ability to maintain shoot and floral stem cell activity is confined to the members of the WUS clade, excluding WOX4. These specific functions are determined outside the homeodomain and require a canonical WUS-box for interaction with TPL/TPR corepressors.

**Running title:** *WOX* genes and the stem cells

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**Abstract**

Gene amplification followed by functional diversification is a major force in evolution. A typical example of this is seen in the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) gene family, named after the *Arabidopsis* stem cell regulator *WUSCHEL*. Here we analyze functional divergence in the *WOX* gene family. Members of the WUS clade, except the cambium stem cell regulator *WOX4*, can substitute for *WUS* function in shoot and floral stem cell maintenance to different degrees. Stem cell function of WUS requires a canonical WUS-box, necessary for interaction with TPL/TPR corepressors, whereas the repressive EAR domain is dispensable and the acidic domain seems only to be required for female fertility. In contrast to the WUS clade, members of the ancient WOX13 and the WOX9 clades cannot support stem cell maintenance. Although the homeodomains are interchangeable between WUS and WOX9 clade members, a WUS-compatible homeodomain together with canonical WUS-box is not sufficient for stem cell maintenance. Our results suggest that WOX function in shoot and floral meristems of *Arabidopsis* is restricted to the modern WUS clade, suggesting that stem cell control is a derived function. Yet undiscovered functional domains in addition to the homeodomain and the WUS-box are necessary for this function.

Key words: *Arabidopsis thaliana*, shoot meristem, stem cells, *WOX* genes, *WUSCHEL*

## INTRODUCTION

The *Arabidopsis thaliana WUSCHEL (WUS)* homeobox gene is a key regulator of the stem cell niche in the shoot meristem, from which all above ground organs are derived. *WUS* expression is restricted to a small group of cells, the Organizing Center (OC), underneath the stem cells (Laux et al., 1996; Mayer et al., 1998). Stem cell homeostasis requires a dynamic feedback loop: signaling from the OC involving WUS protein movement (Daum et al., 2014; Yadav et al., 2011) maintains pluripotency of the overlying stem cells and directly activates transcription of the signaling peptide gene *CLV3*, which in turn represses *WUS* transcription via receptor kinase signaling (Clark et al., 1997; Fletcher et al., 1999; Lenhard and Laux, 2003; Schoof et al., 2000). At least a part of the function of WUS in the OC appears to be mediated via direct transcriptional repression of *ARR* genes, resulting in enhanced intracellular cytokinin signal transduction (Leibfried et al., 2005). In determinate floral meristems, WUS directly activates transcription of the *AGAMOUS (AG)* gene, which in turn represses *WUS* (Lenhard et al., 2001; Lohmann et al., 2001), leading to termination of the stem cells at the end of flower development. Furthermore, WUS is also required during development of female and male organs (Deyhle et al., 2007; Groß-Hardt et al., 2002), where it acts through positive regulation of *WINDHOSE* gene expression (Lieber et al., 2011).

*WUS* is the founding member of the plant specific *WUS homeobOX* (*WOX*) gene family that share a specific N-terminal homeodomain variant (Haecker et al., 2004) and that is present in representatives of green algae, bryophytes, lycophytes, ferns, and seed plants (Nardmann and Werr, 2012). The resulting phylogeny identifies three major branches: an ancient clade consisting of *WOX13*-related genes, present in some green algae and throughout all land plant genomes, and two derived WOX9 and WUS clades (Deveaux et al., 2008; Nardmann et al., 2009; van der Graaff et al., 2009). The series of events from the ancient type of *WOX13*-

related genes to new family members is unresolved, but predates seed plants that contain members in all three clades (Nardmann and Werr, 2013).

Members of the WUS clade variably contain three conserved sequence motifs at their carboxy terminal ends: the WUS-box, an EAR domain, and an acidic domain (Figure 1A). The canonical WUS-box with the core sequence TL-LFP(MILV) (Fig. 1B; (Haecker et al., 2004) is restricted to the WUS clade and has been shown to interact with TOPLESS-type corepressors (WUS, WOX1, WOX5) (Kieffer et al., 2006; Pi et al., 2015; Zhang et al., 2014) to mediate gene repression via histone deacetylation (WOX5) (Pi et al., 2015). In addition, some WUS clade members contain an EAR domain (WUS, WOX5, WOX7), which can also function in transcriptional repression (Ohta et al., 2001), or an acidic domain (WUS, WOX4, WOX5, WOX6, WOX7), which is a potential eukaryotic transcriptional activation domain (Ma and Ptashne, 1987). *WUS*, *WOX4*, and *WOX5* are regulators of stem cell maintenance in the shoot and floral meristems, cambium, and root meristem, respectively, and *WOX1* and *WOX3* redundantly contribute to marginal and plate meristem activity in leaves (Nakata et al., 2012). Aside from stem cell regulation, WUS clade members function in diverse developmental processes, including embryo patterning (*WOX1,2,3,5*) (Breuninger et al., 2008) and reproductive development (*WUS*) (Deyhle et al., 2007; Groß-Hardt et al., 2002); *WOX6* (Park et al., 2005).

Gene numbers in the WUS clade vary between species. In *Arabidopsis thaliana* there are *WUS* and *WOX 1-7* with defined discrete phylogenetic subbranches, of which WOX2, 3 and 4 according to their presence in gymnosperms and angiosperms and supported by conserved expression patterns in the apical embryo domain, plate and marginal meristems or the vascular cambium, respectively, are possibly plesiomorphic for seed plants. A gene amplification at the base of the angiosperms gave rise to *WUS* and its closest relative *WOX5*,

which comprise a bifurcated phylogenetic sub-branch that also contains single gymnosperm and leptosporangiate fern representatives (Nardmann and Werr, 2013). *WOX1*, except for its presence in the basal angiosperm *Amborella trichopoda*, was only found in extant eudicots, where it acts redundantly with *WOX3* during leaf development (Nakata et al., 2012). *WOX1* was possibly lost in monocots, where grass genomes manifested a *WOX3* duplication (Nardmann and Werr, 2013). *WOX6* and *7* comprise close relatives of *WOX1* and *WOX5*, respectively, and are restricted to *Arabidopsis*, possibly due to recent whole genome duplications (Blanc and Wolfe, 2004).

The WOX9 clade contains WOX8, 9, 11 and 12 proteins that have a modified WUS-box (Figure 1B) and a clade specific 56-60 amino acid residue long C-terminal extension. WOX8 and WOX9 are expressed during embryogenesis in the zygote and after the zygotic division in the basal descendants. Both genes are required for embryo and shoot development (Breuninger et al., 2008; Skylar et al., 2010).

The ancient WOX13 clade, containing WOX10 (a putative pseudogene), WOX13, and WOX14, only shares the homeodomain and in case of WOX13 also the acidic domain with other WOX clades. *WOX13* and *WOX14* genes have been reported to function in root development and floral transition in *Arabidopsis* (Romera-Branchat et al., 2012). In the moss *Physcomitrella patens*, *WOX13* is required for cellular reprogramming during stem cell initiation (Sakakibara et al., 2014).

To address functional divergence within the WOX family, we compared the ability of *WOX* genes to replace *WUS* in stem cell maintenance, and analyzed the essential sequences. We show that the potential to maintain shoot and floral meristem stem cells is restricted to members of the WUS clade, with the exception of WOX4, which, similar to the WOX9 and

WOX13 clades, cannot substitute for WUS. Our studies suggest that specific WOX functions are determined outside the homeodomain, with the canonical WUS-box being absolutely essential for stem cell maintenance and interaction with TPL/TPR proteins, whereas the EAR domain is largely dispensable and the acidic domain appears to be only required for female fertility. We also find that the ability to function in stem cell maintenance is restricted to the WUS clade by additional, unidentified protein domains.

## RESULTS

### WUS clade members can partially substitute for WUS in shoot and floral meristem stem cell maintenance

First, we analyzed whether members of the WOX family can replace WUS in regulating stem cell maintenance. To this end, we expressed *WOX* coding regions in the OC of shoot and floral meristems of the putative null allele *wus-1* by using the *WUS* promoter and the *pOp*/*LhG4* expression system (Moore et al., 1998). All *WOX* cDNAs were cloned into the *pOp* vector in the same way and the integrity of all constructs was confirmed by sequencing. Phenotypic analysis was performed in the F1 generation, after crossing *pWUS:LhG4 pOp:WOXpOp:GUS wus-1*/+ to *wus-1*/+ and selecting homozygous *wus-1* plants containing each transgene (verified by PCR). For simplicity, we refer to, for example, plants of the genotype *pWUS:LhG4 pOp:WUS-pOp:GUS* as *pWUS::WUS*. At least 10 independent transformants were analyzed for each construct and the results of representative lines with confirmed normal expression patterns of the transgene are presented. RT-PCR and the tandem *pOp:GUS* expression reporter gene were used to verify the expression pattern for each construct (Figure S1). GUS activity levels were similar in all cases, with the exception of *pOp:WOX2*, *6* and *12*, which had slightly higher expression.

10-day-old wild-type seedlings display an active primary shoot meristem, formed during embryogenesis, and have produced several leaves (Figure 1C; Table 1). Indeterminate shoots bolt about 3 weeks after germination and give rise to multiple flowers, each of which contains 4 sepals, 4 petals, 5/6 stamens, and 2 fused carpels.

In contrast to wild-type, *wus-1* seedlings lack a primary shoot meristem and instead display an empty apex (Figure 1C; Table 1). Subsequently leaves are initiated across the terminated apex in a disorganized manner rather than a rosette as in wild-type (Laux et al., 1996). About 5 weeks after germination, adventitious shoots appear at random positions across the enlarged apex. In addition to their abnormal position, these shoot are thinner and terminate prematurely compared to wild-type shoots. The adventitious shoots occasionally give rise to floral meristems, which again terminate prematurely after the formation of a single stamen (Figure 1C; Table 1).

All *wus-1* plants expressing a *pWUS::WOX* transgene were analyzed with respect to: (1) the presence of an active primary shoot meristem in 10-day-old seedlings, denoted as "primary shoot", (2) the delayed formation of a shoot in a central position of the seedling apex, denoted as "delayed central shoot", which typically were analyzed around 29 days after germination. These delayed shoots resemble wild-type primary shoots in that they are initiated in the apex center, form a rosette of leaves, and are thicker compared to adventitious shoots of untransformed *wus-1* plants, (3), the activity of the inflorescence meristem, as judged by the number of flowers/branch, and (4) the activity of floral meristems, as judged by the number of floral organs formed in whorls three and four.

As expected, expression of *pWUS::WUS* suppressed premature termination of shoot and floral meristems in *wus-1* (Figure 1C; Figure 2B and 2C; Table 1). In these plants, we observed slightly enlarged shoot meristems (not shown), and increased activities of inflorescence and floral meristems (Figure 1C; Figure 2B and 2C; Table 1), suggestive of a higher activity of the *WUS* transgene compared to wild-type. These plants were fertile and formed normal seeds, indicating that the transgene also complemented in ovule and stamen development. However,

gynoecia contained extra carpels and were misshapen and shorter than wild-type, consistent with *WUS* over-expression. Seed production was occasionally low depending on growth conditions, which might explain the failure of a *WUS* transgene to complement *wus-1* fertility defects in a previous study (Ikeda et al., 2009).

First, we analyzed members of the WUS clade, *WOX1* through *WOX6* (*WOX7* was omitted because it is highly homologous and functionally equivalent to *WOX5*; data not shown). 10-day-old *pWUS::WOX1,2,3,5,6/+ wus-1* seedlings lacked a primary shoot meristem indistinguishably from untransformed *wus-1*, but subsequently gave rise to a delayed central shoot that showed indeterminate growth (Figure 2C; See also Figure S2; Table 1). This suggests that *WOX1-3* and *WOX5-6* cannot efficiently replace WUS function in shoot meristem formation during embryogenesis, but are sufficient to enable formation of an indeterminate shoot during postembryonic development. Floral meristem activity was partially recovered by the expression of *WOX3, 6* and, to a weaker extent, *WOX1*, *WOX2* and *WOX5* (Figure 2A-C; Table 1). The gynoecia in these plants were abnormal: p*WUS:WOX1*/+ carpels did not fuse, and p*WUS:WOX3*/+ gynoecia were minute in size (Figure 2A and B).

Since previous studies of plants carrying two copies of p*WUS::WOX5* revealed a much stronger rescue of *wus-1* defects (Sarkar et al., 2007) than observed here for the plants with only one transgene copy, we asked whether an increase in transgene dosage could provide a more complete complementation. However, this was only the case for *WOX2* in addition to *WOX5* (Figure S3, compare to Figure S2H) but not for any other *WOX* transgene (not shown), suggesting that the reduced stem cell activity of WOX proteins compared to WUS is likely due to qualitative rather than quantitative differences.

In contrast to all other members of the WUS clade, *pWUS::WOX4* was unable to complement any *wus-1* defect (Figure 2C; See also Figure S2M; Table 1), even when the transgene was homozygous (not shown).

Taken together, *WOX* genes are able to replace *WUS* functions in shoot meristem stem cell regulation in a ranked order: *WUS* (complete rescue) > *WOX6* > *WOX1*,*2*,*3* > *WOX5* > *WOX4* (no complementation).

**The WUS-box but not the EAR motif or the acidic domain is essential in stem cell maintenance**

Stem cell maintenance requires WUS to act as both a repressive and an activating transcription factor (Leibfried et al., 2005; Yadav et al., 2011). In order to investigate which domain(s) of the WUS protein are essential for stem cell maintenance, we analyzed complementation of *wus-1* defects by modified WUS protein variants (Figure S4A). Expression levels from the *pWUS* promoter of all variants were similar to, or higher than, that of the *pWUS::WUS* reference as determined by the levels of the tandem GUS reporter and RT-PCR of the transcripts (Figure S5A and S5B).

When the WUS-box was deleted from the *WUS* cDNA (*pWUS::WUSΔWB*) no aspect of *wus-1* stem cell defects is complemented (Figure 3A and 3C; See also Figure S5C and S5D; Table 2). Furthermore, changing the highly conserved "TL" residues (Figure S4A) within the WUS-box into “AG” (*WUSmWB*), thereby mimicking the WB sequence present in the WOX9 clade, abolishes the ability of *WUS* cDNA to complement *wus-1* (Figure 3C; Table 2), with the exception that the stamen number was slightly increased (Table 2). Together, these results

suggest that the WUS-box is indispensable for WUS function in stem cell maintenance, with a critical requirement for the conserved "TL" residues.

In contrast to the WUS-box, deleting the EAR motif (*pWUS::WUSΔEAR*; Figure S4A), had hardly any effect on the capability to restore stem cell regulation (Figure 3A-C; See also Figure S5C and S5E; Table 2). This indicates that the EAR domain in the WUS protein, despite its repressive activity in protoplast transcription assays (Ikeda et al., 2009), is largely dispensable for WUS function in shoot and floral meristems.

When the acidic region was deleted, the resulting *pWUS::WUSΔAc* transgene (Figure S4A) had the capacity to restore stem cell defects at all stages analyzed (Figure 3A-C; See also Figure S5C and S5F; Table 2), although only weakly (23,8%) in the case of the embryonic (primary) meristem. However, *pWUS::WUSΔAc wus-1* flowers did not produce any seeds, despite forming stamens and gynoecia. Reciprocal crosses between *pWUS::WUSΔAc wus-1* and wild-type indicate that *pWUS::WUSΔAc* contained functional pollen but was female sterile (Table S1). Thus, the acidic domain is not required for WUS function in stem cell regulation, but is required for female gametophyte development, where previous studies had shown its role in megaspore mother cell and integument formation (Groß-Hardt et al., 2002; Lieber et al., 2011).

**The WUS-box and the EAR motif are required for protein-protein interactions with TPL/TPR co-repressors**

Previous studies showed that the C-terminal domain of WUS, which encompasses the WUS-box, EAR motif, and acidic domain, mediates interaction between WUS and the transcriptional co-repressors TOPLESS (TPL) and TOPLESS-RELATED 4 (TPR4)

respectively (Kieffer et al., 2006) and that this interaction is essential in flower development (Causier et al., 2012). To establish the range of interactions between WUS and the five TPL/TPR members (Long et al., 2006), we performed yeast two-hybrid experiments. These assays identified TPR1 and TPR2 as novel interactors of WUS and confirmed the previously reported TPL and TPR4, whereas no interaction was found with TPR3 (Figure 3D).

Deletion of the WUS-box (WUSΔWB), or changing its "TL" residues to "AG" (WUS-mWB), abolished the WUS-TPL interactions (for the constructs used see Figure S4B; Figure 3D). Surprisingly, deletion of the EAR domain (WUSΔEAR) also abolished interaction with TPL in the yeast system (Figure 3D), although this domain had only a minor effect in meristem maintenance (Figure 3A-C, See also Figure S5C and S5E, Table 2). By contrast, deletion of the acidic domain (WUSΔAc) or changing the WUS-box sequence from TL**P**LFPMH to TL**Q**LFPMH (WUSqWB) did not disrupt the interaction with TPL (Figure 3D). The latter might appear surprising because exchanging of a turn-inducing proline is expected to have a significant effect on the three dimensional protein domain structure. On the other hand, this finding is consistent with the weak conservation at this position between WUS paralogs even within the WUS clade. We then asked whether the constructs that do not interact with TPL, namely WUSΔWB, WUS-mWB and WUSΔEAR, might still be able to interact with other members of the TPL/TPR family, but found that this is not the case (Figure 3D).

**Divergent and conserved functions between WUS, WOX9, and WOX13 subclades**

In contrast to the majority of members of the WUS clade, members of the WOX9 (*WOX8* and *WOX9*) and ancient (*WOX13*) clades did not rescue any *wus-1* defect when expressed from the *WUS* promoter (Figure 2C; See also Figure S2N-Q; Table 1), indicating that these subclades are diverged from WUS activity.

*WOX8* and *WOX9* function in axis patterning during early embryogenesis (Breuninger et al., 2008) and *WOX13* has been reported to have a role during flowering, root development, and fertility (Deveaux et al., 2008; Romera-Branchat et al., 2012). To delineate the functional differences between them and WUS, we replaced the *WUS* homeodomain with the one from *WOX8, WOX9,* and *WOX13* (Figure S4C). The resulting *pWUS::WUS-WOX8HD* and, to a weaker extent, *pWUS::WUS-WOX9HD* transgenes were able to partially complement the meristem defects of *wus-1*. These results indicate that the homeodomains of WOX8 (and to a lesser degree of WOX9) are interchangeable with that of WUS, although the activities of the resulting proteins are much reduced (Figure 4; See also Figure S6A; Table 3). By contrast, a *pWUS::WUS-WOX13HD* transgene was unable to substitute for WUS protein (Figure 4; See also Figure S6A; Table 3). Thus, while WUS and WOX9 clade members appear to have maintained equivalent homeodomains, they have diverged from the ancient clade WOX13 homeodomain.

*WOX8* and *WOX9* possess an altered WUS-box compared to the WUS clade (Figure 1B): the essential “TL” residues of the canonical WUS-box are replaced by "AG" residues that, in the context of WUS variants, abolished stem cell regulation (see Fig. 1C) . Because WOX8 and WUS homeodomains are interchangeable, and because the EAR and acidic motifs of WUS are dispensable for stem cell maintenance, we asked whether the canonical WUS-box can provide stem cell function to *WOX8*. As with all other transgenic lines, expression levels of these constructs were confirmed with RT-PCR and GUS assays (Figure S5; Figure S6B and S6C). We found that *pWUS::WOX8-wusWB* (Figure S4C) was unable to restore any aspect of stem cell maintenance in *wus-1* (Figure 4; See also Figure S6A; Table 3). To exclude that the WOX9 clade specific C-terminal domain interfered with stem cell regulation, we additionally deleted this domain (*pWUS::WOX8wsbxΔD*; Figure S4C), but this construct also did not

rescue any *wus-1* stem cell defects (Figure 4; Table 3). Thus, the canonical WUS-box, albeit absolutely required in the context of the WUS protein, is not sufficient to provide stem cell maintenance in a WOX8 backbone.

In a complementary set of experiments, we addressed whether *WUS* is able to replace *WOX8* in embryo development. *wox8*-/-*wox9*+/- plants segregate 25% homozygous embryos that display multiple developmental defects and arrest at the globular stage (Breuninger et al., 2008)(Figure 5C and 5D). As a positive control, transformation of *pWOX9:WOX8* restored wild-type development in *wox8 wox9* double mutants (genotyped by PCR, data not shown). By contrast, we did not find a single viable *pWOX9:WUS* T1 plant (n=46) homozygous for *wox8 wox9*, suggesting that *WUS* cannot substitute for *WOX8* in embryo development. Surprisingly, replacing the WOX8 type WUS-box with the one from the *WUS* gene, giving p*WOX9:WOX8-wusWB*, restored wild-type-like development in *wox8 wox9* plants (14% of T1 plants, n=21; Figure 5E and 5F). This suggests that the WOX8-specific modifications of the canonical WUS-box are not important for WOX8 function in embryo patterning.

## DISCUSSION

WOX proteins share a conserved homeodomain and fulfill a variety of functions in *Arabidopsis* development. To address their functional divergence, we have studied the ability of proteins of all three WOX clades side-by-side to function in stem cell regulation and the requirement for conserved protein motifs in this process.

### Functional WOX protein motifs

Our results show that in stem cell maintenance the homeodomain appears interchangeable within the WUS clade, but also between WUS and WOX8/9. This suggests that, in the shoot meristem relevant WUS targets for stem cell maintenance are not discriminated based on homeodomain DNA-specificity and that yet unknown tissue or developmental stage specific factors provide transcriptional specificity to WOX proteins, reminiscent of animal homeodomain transcription factor complexes (Mann et al., 2009; Prince, 2002). By contrast, the homeodomain of the ancient clade member WOX13 cannot substitute for the WUS homeodomain. This can be explained by the recent observation that the WOX13 homeodomain is unable to provide intercellular protein movement in the context of the WUS proteins (Daum et al., 2014). The preservation of the ancestral *WOX13*-type genes in all plant genomes suggest a role in basic cellular processes such as cell growth, which has been suggested from *WOX13* studies in *Physcomitrella patens* (Sakakibara et al., 2014).

Our protein variant experiments demonstrate that the canonical WUS-box is absolutely essential for both stem cell maintenance and for its interaction with TPL/TPR co-repressors, consistent with previous studies of WUS and WOX5 stem cell functions (Causier et al., 2012; Pi et al., 2015). The EAR domain has been reported to provide an interaction surface with TPL repressors in diverse proteins (Szemenyei et al., 2008) and, in the context of the WUS

protein, to be sufficient for transcriptional repression in protoplast assays (Ikeda et al., 2009). However, we find that the EAR domain is largely dispensable for shoot meristem maintenance, supporting previous observations (Ikeda et al., 2009). The presence of the EAR domain exclusively in WUS, WOX5, and WOX7 but its absence in the rest of the WUS clade might suggest a supporting function specifically in the apical shoot and root meristems, which could play a more important role under specific growth conditions.

What is the minimal requirement of a WOX protein to function in shoot meristem stem cell maintenance? Because the WUS-WOX8HD protein is functional in the shoot meristem, whereas WOX8-wusWB or WOX8wsbxΔD are not, we conclude that the combination of a WUS-compatible homeodomain and a canonical WUS-box is not sufficient for stem cell function. Although we cannot exclude the possibility that sequences in the WOX8 backbone are incompatible with WUS function, an alternative interpretation is that yet unidentified sequences in the WUS protein, and by inference in WOX5, are essential for stem cell maintenance in the apical meristems. This hypothesis is supported by recent finding that an unconserved region between the homeodomain and WUS-box plays an essential role in determining WUS protein mobility (Daum et al., 2014).

Stretches of acidic residues are usually associated with transcriptional activation (Ma and Ptashne, 1987) and WUS has been shown to function as direct transcriptional activator of *CLV3* in stem cells and of the *AGAMOUS* (*AG*) gene to terminate floral meristem activity (Lohmann et al., 2001; Yadav et al., 2011). Furthermore, in transient assays, the acidic domain has been shown to be essential for transcriptional activation (Ikeda et al., 2009). Surprisingly, we find that the deletion of the acidic domain only slightly impairs *WUS* function in the floral meristem, indicating that transcriptional activation of the *AG* gene by

WUS relies on a still unidentified mechanism rather than an "acidic blob" transactivation domain (Ma and Ptashne, 1987). This supports previous findings that in plants, the acidic domain is not necessary for *AG* expression (Ikeda et al., 2009). The acidic domain of WUS is, however, essential for female gametophyte development, where WUS is required for megaspore mother cell and integument formation (Groß-Hardt et al., 2002; Lieber et al., 2011). The use of different transcriptional activating domains between ovules and meristems is consistent with previous findings that *WUS* function involves different effector genes in these processes (Groß-Hardt et al., 2002; Lieber et al., 2011).

**Clade-divergences**

Only members of the WUS clade, with the exception of WOX4, which all contain the canonical WUS-box (TL-LFPM*) are able to function in maintaining shoot meristem stem cells, displaying a range of activity with *WUS* (complete rescue)>*WOX6*>*WOX1*,*2*,*3* >*WOX5*. Compared to the shoot meristem, restoring floral meristem activity with WUS clade members was generally less efficient. Since all available evidence suggests that similar mechanisms operate in shoot and floral stem cell maintenance (Schoof et al., 2000), we conclude that floral meristems are more sensitive to compromised WUS activity compared to the shoot meristem, consistent with phenotypes reported for an allelic series of *wus* mutants (Graf et al., 2010).

*WOX4* is the only member of the WUS clade that is completely unable to replace *WUS* in shoot or floral meristem maintenance, and previous studies showed that unlike *WUS*, *WOX4* could not replace PRS/WOX3 function in leaf and flower development (Ji et al., 2010a; Shimizu et al., 2009). *WOX4* functions in stem cell regulation of the vascular cambium (Hirakawa et al., 2010; Ji et al., 2010a; Ji et al., 2010b). However, whereas *WUS* and *WOX5* repress differentiation in stem cells from neighboring signal centers, *WOX4* is expressed in

the cambium and primarily promotes cell division, suggesting a different mode of action. The larger functional divergence of *WOX4* compared to other WUS clade members is surprising because vasculature coevolved with apical growth (Kenrick and Crane 1997), i.e. involves shoot and root apical meristem activity.

In rice, however, it is *WOX4* rather than the *WUS* ortholog *TAB*, that enables vegetative shoot meristem maintenance, in addition to its function in vascular development (Ohmori et al., 2013; Tanaka et al., 2015), whereas TAB1 and WOX4 both are essential for axillary meristems formation (Tanaka et al., 2015).

In the leptosporangiate fern *Ceratopteris richardii*, the single WUS clade member *CrWUL* is transiently expressed in the developing vasculature, which suggests that the association of members in the WUS clade with the vascular cambium possibly predated seed plants (Nardmann and Werr, 2013). One possible interpretation is that seed plants preserved this ancestral cambium trait in their WOX4 lineage, whereas other gene amplifications in the WUS clade were subject to neofunctionalization that diverged transcriptionally into different stem cell niches but retained similar stem cell regulating protein functions.

Members of the WOX9 and ancient WOX13 clades are nonfunctional in stem cell maintenance, which is not surprising because both lack a canonical WUS-box (and also an EAR domain). It is thus likely that the members of these clades do not act via recruitment of TPL/TPR proteins, which is supported by WOX8/9 interactome studies (unpublished).

Similar to *wus-1*, the *Nicotiana sylvestris* mutant of the WOX1 homolog *LAMINA1* (*LAM1*) that is defective in leaf blade growth can be complemented by the *A. thaliana* WUS clade genes but not by WOX9 and WOX13 (Lin et al., 2013; Tadege et al., 2011). The latter could, however, substitute for *LAM1* when fused to either a SRDX or the WUS-box repressor

domain, supporting the pivotal role of transcriptional repression by WOX proteins for meristem maintenance (Lin et al., 2013).

Our results show that for WOX8 function in embryogenesis, the non-canonical WUS-box is not essential, consistent with the reported function of WOX8 as transcriptional activator rather than a repressor (Lin et al., 2013). This suggests that the canonical WUS-box may have evolved by amino-acid sequence changes neutral for ancestral WOX function and gained the potential to interact with TPL/TPR proteins to repress transcription.

Together, the functional diversification between major clades of the *WOX* gene family with meristem promoting activity apparently confined to members of the WUS clade. Because WUS function in shoot and floral meristems involved its intercellular movement (Yadav et al., 2011; Daum et al., 2014), it is plausible that other WUS clade members might also be mobile and, in turn, that the protein domains determining WUS mobility are within the conserved features of the WUS clade. The molecular basis for the difference in WOX4 activity remains to be elucidated and might relate to the functional peculiarity of the vascular cambium releasing phloem or xylem cells to either side.

Given the presence of *WOX2, WOX3* and *WOX4* orthologs in seed plants (Nardmann and Werr, 2013) or *WUS* and *WOX5* orthologs within angiosperms (Nardmann et al., 2009), the functional differences elaborated here between genes from three main phylogenetic clades support that adaptations in the *WOX* gene family correlate with the evolution of plant stem cell niches and that gene amplifications in the WUS clade relate to their diversification in seed plants. By contrast, the interchangeability of WUS clade members is reminiscent of the animal HOX-paradox, i.e. evolution of transcriptional specificity rather than diversification of

protein function (Prince, 2002) and indicates similar constraints of selection in animal as in plant evolution.

## MATERIAL AND METHODS

### Plant Material, Transformation and Cloning details

The *wus-1* mutant and plant growth conditions have been described previously (Laux et al., 1996). We used the *pOpL* two-component expression system (Moore et al., 1998) for all transgenic experiments. Generation of the *pOp:WUS* (MT69) and *pOp:WUS-pOp:GUS* (MT72) (Schoof et al., 2000) and of *pWUS:LhG4* were previously described (Groß-Hardt et al., 2002). All constructs for the *wus-1* rescue experiment were prepared by introducing the respective *WOX* cDNA or *WUS*/*WOX8* constructs into the *pOpL* system as was previously done with *WUS* cDNA. Constructs were electroporated into *Agrobacterium* strain GV3101 (pMP90) (Koncz and Schell, 1986) and transformed into L*er wus-1* plants containing *pWUS:LhG4* by the floral dip method (Clough and Bent, 1998). The *WOX8*/*WUS* constructs, for the rescue of the *wox8 wox9* mutant, had the promoter of *WOX9*, which was previously described (Breuninger et al., 2008). All mutant alleles and transgenic lines used are listed in Table S2. Details of the cloning are available upon request.

### GUS staining and Microscopy

GUS staining was performed as described (Schoof et al., 2000). After 15 hours of staining material was cleared in 70 % ethanol before photography using a Leica MZ12 binocular or a Leica DC300 camera. Nomarski microscopy analysis of embryos was performed using a chloral hydrate solution (containing glycerin, chloral hydrate, and water in a ratio of 1:8:3) on a Zeiss Imager A1 with DIC optics.

### Genetic analysis, PCR genotyping

To obtain plants with single copies of each transgene, *pWUS:LhG4 pOp:WOXpOp:GUS wus-1*/+ plants were crossed to *wus-1*/+. The F1 plants were genotyped for homozygosity at *wus-1* and for both the driver and the acceptor constructs by PCR and then submitted to phenotypic analysis.

**RT-PCR analysis**

Total RNA was extracted from the above-ground parts of seedlings or from inflorescence tissues using the RNeasy Plant mini kit (Qiagen). Total RNA was reverse transcribed with SUPERSCRIPT III reverse transcriptase (Invitrogen) and oligo-dT primer. Primers used for RT-PCR in this study are listed in Table S3.

**Yeast two hybrid assay**

The WUS bait constructs, containing WT and modified versions of the WUS coding sequence, were constructed in pGBT9 (Clontech). Full-length TPL/TPR prey vectors were constructed by recombining PCR-generated fragments into pGADT7-Rec (Clontech). Baits were transformed into yeast HF7c or AH109 cells and preys were transformed into yeast Y187 cells (Clontech). Following mating between bait and prey yeast strains, diploids were selected on minimal SD medium lacking tryptophan and leucine. Interactions were assayed on SD medium lacking tryptophan, leucine and histidine, with the addition of 5mM 3-aminotriazole.

Author contribution:

Majority of Investigation, A.D.; Investigation contribution, J.N. and W.W. (contributed the homeodomain swap experiment), E.C., B.C. and B.D. (contributed the yeast-two hybrid

experiments); Investigation help, E.G. and J.C.; Majority of Conceptualization, A.D. and T.L.; Writing, A.D., W.W., B.D. and T.L.

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**References:**


Blanc, G., and Wolfe, K.H. (2004). Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. Plant Cell 16:1679-1691.

Breuninger, H., Rikirsch, E., Hermann, M., Ueda, M., and Laux, T. (2008). Differential expression of WOX genes mediates apical-basal axis formation in the Arabidopsis embryo. Dev Cell 14:867-876.

Causier, B., Ashworth, M., Guo, W., and Davies, B. (2012). The TOPLESS interactome: a framework for gene repression in Arabidopsis. Plant Physiol 158:423-438.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. (1997). The CLAVATA1 gene encodes a putative receptor-kinase that controls shoot and floral meristem size in Arabidopsis. Cell 89:575-585.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743.

Daum, G., Medzihradszky, A., Suzaki, T., and Lohmann, J.U. (2014). A mechanistic framework for noncell autonomous stem cell induction in Arabidopsis. Proc Natl Acad Sci U S A 111:14619-14624.

Deveaux, Y., Toffano-Nioche, C., Claisse, G., Thareau, V., Morin, H., Laufs, P., Moreau, H., Kreis, M., and Lecharny, A. (2008). Genes of the most conserved WOX clade in plants affect root and flower development in Arabidopsis. BMC Evol Biol 8:291.

Deyhle, F., Sarkar, A.K., Tucker, E.J., and Laux, T. (2007). WUSCHEL regulates cell differentiation during anther development. Dev Biol 302:154-159.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science 283:1911-1914.

Graf, P., Dolzblasz, A., Wurschum, T., Lenhard, M., Pfreundt, U., and Laux, T. (2010). MGOUN1 encodes an Arabidopsis type IB DNA topoisomerase required in stem cell regulation and to maintain developmentally regulated gene silencing. Plant Cell 22:716-728.

Groß-Hardt, R., Lenhard, M., and Laux, T. (2002). WUSCHEL signaling functions in interregional communication during Arabidopsis ovule development. Genes & Development 16:1129-1138.

Haecker, A., Groß-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131:657-668.

Hirakawa, Y., Kondo, Y., and Fukuda, H. (2010). TDIF peptide signaling regulates vascular stem cell proliferation via the WOX4 homeobox gene in Arabidopsis. Plant Cell 22:2618-2629.

Ikeda, M., Mitsuda, N., and Ohme-Takagi, M. (2009). *Arabidopsis* WUSCHEL Is a Bifunctional Transcription Factor That Acts as a Repressor in Stem Cell Regulation and as an Activator in Floral Patterning. Plant Cell 21:3493-3505.

Ji, J., Shimizu, R., Sinha, N., and Scanlon, M.J. (2010a). Analyses of WOX4 transgenics provide further evidence for the evolution of the WOX gene family during the regulation of diverse stem cell functions. Plant Signal Behav 5:916-920.

Ji, J., Strable, J., Shimizu, R., Koenig, D., Sinha, N., and Scanlon, M.J. (2010b). WOX4 promotes procambial development. Plant Physiol 152:1346-1356.

Kieffer, M., Stern, Y., Cook, H., Clerici, E., Maulbetsch, C., Laux, T., and Davies, B. (2006). Analysis of the Transcription Factor WUSCHEL and Its Functional Homologue in Antirrhinum Reveals a Potential Mechanism for Their Roles in Meristem Maintenance. Plant Cell 18:560-573.

Koncz, C., and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Mol. Gen. Genet. 204:383-396.

Laux, T., Mayer, K.F., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122:87-96.

Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 438:1172-1175.

Lenhard, M., Bohnert, A., Jürgens, G., and Laux, T. (2001). Termination of Stem Cell Maintenance in Arabidopsis Floral Meristems by Interactions between WUSCHEL and AGAMOUS. Cell 105:805-814.

Lenhard, M., and Laux, T. (2003). Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. Development 130:3163-3173.

Lieber, D., Lora, J., Schrempp, S., Lenhard, M., and Laux, T. (2011). Arabidopsis WIH1 and WIH2 genes act in the transition from somatic to reproductive cell fate. Curr Biol 21:1009-1017.

Lin, H., Niu, L., McHale, N.A., Ohme-Takagi, M., Mysore, K.S., and Tadege, M. (2013). Evolutionarily conserved repressive activity of WOX proteins mediates leaf blade outgrowth and floral organ development in plants. Proc Natl Acad Sci U S A 110:366-371.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell 105:793-803.

Long, J.A., Ohno, C., Smith, Z.R., and Meyerowitz, E.M. (2006). TOPLESS regulates apical embryonic fate in Arabidopsis. Science 312:1520-1523.

Ma, J., and Ptashne, M. (1987). A new class of yeast transcriptional activators. Cell 51:113-119.

Mann, R.S., Lelli, K.M., and Joshi, R. (2009). Hox specificity unique roles for cofactors and collaborators. Curr Top Dev Biol 88:63-101.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., and Laux, T. (1998). Role of WUSCHEL in Regulating Stem Cell Fate in the *Arabidopsis* Shoot Meristem. Cell 95:805-815.

Moore, I., Galweiler, L., Grosskopf, D., Schell, J., and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. PNAS 95:376-381.

Nakata, M., Matsumoto, N., Tsugeki, R., Rikirsch, E., Laux, T., and Okada, K. (2012). Roles of the Middle Domain-Specific WUSCHEL-RELATED HOMEOBOX Genes in Early Development of Leaves in Arabidopsis. Plant Cell 24:519-535.

Nardmann, J., Reisewitz, P., and Werr, W. (2009). Discrete Shoot and Root Stem Cell-Promoting WUS/WOX5 Functions Are an Evolutionary Innovation of Angiosperms. Mol Biol Evol 26:1745-1755.

Nardmann, J., and Werr, W. (2012). The invention of WUS-like stem cell-promoting functions in plants predates leptosporangiate ferns. Plant Mol Biol 78:123–134.

Nardmann, J., and Werr, W. (2013). Symplesiomorphies in the WUSCHEL clade suggest that the last common ancestor of seed plants contained at least four independent stem cell niches. New Phytol 199:1081-1092.

Ohmori, Y., Tanaka, W., Kojima, M., Sakakibara, H., and Hirano, H.Y. (2013). WUSCHEL-RELATED HOMEOBOX4 is involved in meristem maintenance and is negatively regulated by the CLE gene FCP1 in rice. Plant Cell 25:229-241.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13:1959-1968.

Park, S.O., Zheng, Z., Oppenheimer, D.G., and Hauser, B.A. (2005). The PRETTY FEW SEEDS2 gene encodes an Arabidopsis homeodomain protein that regulates ovule development. Development 132:841-849.

Pi, L., Aichinger, E., van der Graaff, E., Llavata-Peris, C.I., Weijers, D., Hennig, L., Groot, E., and Laux, T. (2015). Organizer-Derived WOX5 Signal Maintains Root Columella Stem Cells through Chromatin-Mediated Repression of CDF4 Expression. Dev Cell 33:576-588.

Prince, V. (2002). The Hox Paradox: More complex(es) than imagined. Dev Biol 249:1-15.

Romera-Branchat, M., Ripoll, J.J., Yanofsky, M.F., and Pelaz, S. (2012). The WOX13 homeobox gene promotes replum formation in the Arabidopsis thaliana fruit. Plant J 73:37-49.

Sakakibara, K., Reisewitz, P., Aoyama, T., Friedrich, T., Ando, S., Sato, Y., Tamada, Y., Nishiyama, T., Hiwatashi, Y., Kurata, T., et al. (2014). WOX13-like genes are required for reprogramming of leaf and protoplast cells into stem cells in the moss Physcomitrella patens. Development 141:1660-1670.

Sarkar, A., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R., and Laux, T. (2007). Conserved factors regulate signalling in *Arabidopsis thaliana* shoot and root stem cell organizers. Nature 446:811-814.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jurgens, G., and Laux, T. (2000). The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100:635-644.

Shimizu, R., Ji, J., Kelsey, E., Ohtsu, K., Schnable, P.S., and Scanlon, M.J. (2009). Tissue specificity and evolution of meristematic WOX3 function. Plant Physiol 149:841-850.

Skylar, A., Hong, F., Chory, J., Weigel, D., and Wu, X. (2010). STIMPY mediates cytokinin signaling during shoot meristem establishment in *Arabidopsis* seedlings. Development 137:541-549.

Szemenyei, H., Hannon, M., and Long, J.A. (2008). TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science 319:1384-1386.

Tadege, M., Lin, H., Niu, L., and Mysore, K.S. (2011). Control of dicot leaf blade expansion by a WOX gene, STF. Plant Signal Behav 6:1861-1864.

Tanaka, W., Ohmori, Y., Ushijima, T., Matsusaka, H., Matsushita, T., Kumamaru, T., Kawano, S., and Hirano, H.Y. (2015). Axillary Meristem Formation in Rice Requires the WUSCHEL Ortholog TILLERS ABSENT1. Plant Cell 27:1173-1184.

van der Graaff, E., Laux, T., and Rensing, S.A. (2009). The WUS homeobox-containing (WOX) protein family. Genome Biol 10:248.

Yadav, R.K., Perales, M., Gruel, J., Girke, T., Jonsson, H., and Reddy, G.V. (2011). WUSCHEL protein movement mediates stem cell homeostasis in the Arabidopsis shoot apex. Genes Dev 25:2025-2030.

Zhang, F., Wang, Y., Li, G., Tang, Y., Kramer, E.M., and Tadege, M. (2014). STENOFOLIA recruits TOPLESS to repress ASYMMETRIC LEAVES2 at the leaf margin and promote leaf blade outgrowth in *Medicago truncatula*. Plant Cell 26:650-664.

**Figure legends**

**Figure 1. *Arabidopsis WOX* gene structure and *wus-1* phenotypes**

**(A)** Schematic representation of constructs used for *wus-1* complementation tests. Boxes represent WOX protein domains: Ac, acidic domain; C, WOX8 clade C-terminal domain; E, EAR domain; HD, homeodomain; N, WOX8-type N-terminal domain; P, PEST domain; WB, WUS-box; blue strap, „AG" residues .

**(B)** Comparison of WUS-box sequences (modified from Haecker et. al., 2004).

**(C)** The phenotypes of 10-day-old seedlings (top panel), 40-day-old adult plants (middle), and flowers (bottom panel) of the control genotypes used in the study.

Scale bars: (C) 2 cm (middle panel), 3 mm (upper and lower panels)

**Figure 2. Complementation of *wus-1* stem cell defects by different *WOX* genes**

**(A-B)** Comparison of gynoecia phenotypes, showing unfused carpels in a p*WUS::WOX1* flower at an early stage of fruit development (A); and an elevated carpel number in a p*WUS::WUS* flowers and two types of underdeveloped gynoecia in p*WUS::WOX3* flowers at a fully elongated gynoecia stage (B). Wild-type and *wus-1* gynoecia are included for comparison.

**(C)** Complementation of *wus-1* by the indicated constructs. "Central Shoot" combines all plants that form either a primary or a delayed shoot in the center of the epicotyl as given in Table 1.

***, statistically different to *wus-1* with p<0,001. Unmarked; statistically not significant. Probabilities for primary meristem and central shoot determined by Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing. Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

Scale bars: (A-B) 3 mm

**Figure 3. Complementation of *wus-1* stem cell defects and interactions with TPL proteins by WUS variants**

**(A)** 10-day-old seedling phenotypes, showing that embryonic (primary) meristem formation is rescued by the expression of *pWUS::WUSΔEAR* and *pWUS::WUSΔAc* transgenes, but not by *pWUS::WUSΔWB*.

**(B)** Silique phenotypes. *pWUS::WUSΔEAR* transgene causes rescue of gynoecia development similar to *pWUS::WUS*, whereas the siliques of *pWUS::WUSΔAc* were smaller.

**(C)** Complementation of *wus-1* by the indicated constructs. "Central Shoot" combines all plants that form either a primary or a delayed shoot in the center of the epicotyl as given in Table 2.

*, **, ***, statistically different to *wus-1* with $p<0.05$, $p<0.01$, $p<0,001$, respectively. Unmarked; statistically not significant. Probabilities for primary shoot and central shoot determined by Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing. Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

**(D)** Two-hybrid assays showing interaction between TPL/TPR as preys and different WUS variants as baits. As controls, TPL/TPR preys were tested against an LBD37 bait (At5g67420; positive control) and a HDA19 bait (At4g38130; negative control).

Scale bars: (A-B) 3 mm

**Figure 4. Complementation of *wus-1* stem cell defects by different WUS/WOX8 clade chimeras**

Complementation of *wus-1* by indicated constructs. "Central Shoot" combines all plants that form either a primary or a delayed shoot in the center of the epicotyl as given in Table 3.

*, **, ***, statistically different to *wus-1* with $p<0.05$, $p<0.01$, $p<0,001$, respectively. Unmarked; statistically not significant. Probabilities for primary shoot and central shoot determined by Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing. Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

**Figure 5. Complementation of *wox8 wox9* embryo defects by chimeric *WOX8* proteins**

**(A, C, E)** Globular embryo phenotype and **(B, D, F)** heart embryo phenotype of wild-type **(A-B),** *wox8 wox9* **(C-D)** and *wox8 wox9* p*WOX9:WOX8-wusWB* **(E, F)** plants.

Arrows point to the embryos, which were outlined with thin white line

Scale bars: 20µm

**TABLES**

**Table 1. Meristem activity of *wus-1-/-* plants expressing different *WOX* transgenes**

| genotype | primary shoot [a] (%) | delayed central shoot [b] (%) | flowers/branch[c] | | flower organ numbers | | | | n |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | stamens | | carpels | | |
| | | | median | m.d. | median | m.d. | median | m.d. | |
| wild-type | 100$^{***}$ | n.a. | 11,99$^{***}$ | 4,34 | 5$^{***}$ | 0 | 2$^{***}$ | 0 | 18 |
| *wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 0,79$^{†††}$ | 1,17 | 1$^{†††}$ | 0 | 0$^{†††}$ | 0 | 51-154 |
| *pWUS::WUS wus-1* | 89,9$^{***}$ | 10,1$^{***}$ | 15,95$^{***}$ | 9,12 | 7$^{***}$ | 1,5 | 4$^{***}$ | 0 | 54-60 |
| *pWUS::WOX1 wus-1* | 0$^{†††}$ | 50$^{***,†}$ | 4,56$^{***}$ | 2,38 | 3$^{***,†††}$ | 1,5 | 0$^{†††}$ | 0 | 16-20 |
| *pWUS::WOX2 wus-1* | 0$^{†††}$ | 93,3$^{***}$ | 4,50$^{***}$ | 1,93 | 3$^{***,††}$ | 1,5 | 0$^{†††}$ | 0 | 15-16 |
| *pWUS::WOX3 wus-1* | 0$^{†††}$ | 70$^{***}$ | 6,23$^{***}$ | 2,96 | 5$^{***}$ | 1,5 | 0$^{***,†††}$ | 0 | 21-26 |
| *pWUS::WOX4 wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 1,42$^{†††}$ | 0,72 | 1$^{†††}$ | 0 | 0$^{†††}$ | 0 | 37-42 |
| *pWUS::WOX5 wus-1* | 0$^{†††}$ | 40$^{***,††}$ | 3,16$^{***}$ | 1,26 | 3$^{***,†††}$ | 1,5 | 0$^{†††}$ | 0 | 14-17 |
| *pWUS::WOX6 wus-1* | 0$^{†††}$ | 100$^{***}$ | 17,41$^{***}$ | 5,76 | 5$^{***}$ | 1,5 | 2$^{***}$ | 0 | 8-10 |
| *pWUS::WOX8 wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 1,81$^{††}$ | 0,87 | 1$^{†††}$ | 1,5 | 0$^{†††}$ | 0 | 7-8 |
| *pWUS::WOX9 wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 1,40$^{†††}$ | 1,04 | 1$^{†††}$ | 0 | 0$^{†††}$ | 0 | 21-23 |
| *pWUS::WOX12 wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 0,98$^{†††}$ | 1,45 | 1$^{†††}$ | 0 | 0$^{†††}$ | 0 | 7-8 |
| *pWUS::WOX13 wus-1*[d] | 0$^{†††}$ | 0$^{†††}$ | 0,76$^{†††}$ | 1,13 | 1$^{†††}$ | 0 | 0$^{†††}$ | 0 | 31-34 |

[a], 10-day-old seedlings with a primary shoot meristem
[b], shoot initiated in the center of the epicotyl, morphologically indistinguishable from the primary wild-type shoot, but initiated later than 20 days post germination
[c], 90-day-old dry plants were analyzed
[d], flowers on all stems were counted. In all other cases, the 3 first flowers were counted.
n, number of plants.
m.d., mean absolute deviation
Statistically different to *wus-1-/-* with p<0,001 $^{***}$
Statistically different to wild-type with P<0,05 $^{†}$, <0,01 $^{††}$, <0,001 $^{†††}$
Probabilities for a) and b) determined from Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing.
Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

**Table 2. Meristem activity of *wus-1-/-* plants expressing WUS variants**

| genotype | primary shoot [a] (%) | delayed central shoot [b] (%) | flowers/branch[c] | | flower organ numbers | | | | n |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | stamens | | carpels | | |
| | | | median | m.d. | median | m.d. | median | m.d. | |
| wild-type | $100^{***}$ | n.a. | $18{,}89^{***}$ | 3 | $5^{***}$ | 0 | $2^{***}$ | 0 | 10-32 |
| *wus-1-/-*[d] | $0^{\dagger\dagger\dagger}$ | $0^{\dagger\dagger\dagger}$ | $1{,}42^{\dagger\dagger\dagger}$ | 2,1 | $1{,}5^{\dagger\dagger\dagger}$ | 0,7 | $0^{\dagger\dagger\dagger}$ | 0 | 27-39 |
| *pWUS::WUS wus-1* | $87^{***}$ | $13^{***}$ | $34{,}24^{***}$ | 19,2 | $6{,}5^{***}$ | 0,7 | $4^{***,\dagger\dagger\dagger}$ | 0 | 20-46 |
| *pWUS::WUSΔWB wus-1*[d] | $0^{\dagger\dagger\dagger}$ | $0^{\dagger\dagger\dagger}$ | $3{,}23^{\dagger\dagger\dagger}$ | 0,9 | $2^{\dagger\dagger\dagger}$ | 1,5 | $0^{\dagger\dagger\dagger}$ | 0 | 15 |
| *pWUS::WUSmWB wus-1* | $0^{\dagger\dagger\dagger}$ | $7{,}7^{\dagger\dagger\dagger}$ | $4{,}31^{\dagger\dagger\dagger}$ | 2 | $3^{**,\dagger\dagger\dagger}$ | 1,5 | $0^{\dagger\dagger\dagger}$ | 0 | 12-13 |
| *pWUS::WUSΔEAR wus-1* | $68{,}8^{***,\dagger\dagger}$ | $23{,}5^{***}$ | $24{,}30^{***}$ | 9,7 | $6^{***}$ | 1,5 | $4^{***}$ | 1,5 | 13-16 |
| *pWUS::WUSΔAc wus-1* | $23{,}8^{*,\dagger\dagger\dagger}$ | $56{,}2^{***}$ | $9{,}20^{***}$ | 4 | $6^{***}$ | 1,5 | $2^{***}$ | 3 | 15-26 |

[a], 10-day-old seedlings with a primary shoot meristem
[b], shoot initiated in the center of the epicotyl, morphologically indistinguishable from the primary wild-type shoot, but initiated later than 20 days post germination
[c], 90-day-old dry plants were counted
[d], flowers on all stems were counted. In all other cases, the 3 first flowers were counted.
n, number of plants.
m.d., mean absolute deviation
Statistically different to *wus-1-/-* with $p<0{,}05$ $^{*}$, $<0{,}01$ $^{**}$, $<0{,}001$ $^{***}$.
Statistically different to wild-type with $p<0{,}01$ $^{\dagger\dagger}$, $<0{,}001$ $^{\dagger\dagger\dagger}$. Unmarked combinations, statistically not significant.
Probabilities for a) and b) determined from Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing.
Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

**Table 3. Meristem activity of *wus-1-/-* plants expressing chimeric *WUS* and *WOX8* transgenes**

| genotype | primary shoot [a] (%) | delayed central shoot [b] (%) | flowers/branch[c] | | flower organ numbers | | | | n |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | stamens | | carpels | | |
| | | | median | m.d. | median | m.d. | median | m.d. | |
| *wus-1*[d] | 0 | 0 | 0,86 | 1,3 | 1 | 0 | 0 | 0 | 15-27 |
| *pWUS::WUS-WOX8HD wus-1* | 28,6$^{*}$ | 50$^{***}$ | 4,97$^{**}$ | 2,6 | 6$^{***}$ | 1,5 | 0$^{**}$ | 0 | 10-28 |
| *pWUS::WUS-WOX9HD wus-1* | 12,5 | 12,5 | 4,14$^{**}$ | 1,4 | 5$^{***}$ | 1,5 | 0 | 0 | 10-24 |
| *pWUS::WUS-WOX13HD wus-1*[d] | 0 | 0 | 2,08 | 1,2 | 1 | 0 | 0 | 0 | 27 |
| *pWUS::WOX8 wus-1*[d] | 0 | 0 | 1,46 | 0,5 | 1 | 0,7 | 0 | 0 | 10-11 |
| *pWUS::WOX8wusWB wus-1*[d] | 0 | 0 | 1 | 0,7 | 1 | 1,5 | 0 | 0 | 25 |
| *pWUS::WOX8wsbxD wus-1*[d] | 0 | 0 | 2,66 | 2,1 | 1 | 0 | 0 | 0 | 21 |

[a], 10-day-old seedlings with a primary shoot meristem
[b], shoot initiated in the center of the epicotyl, morphologically indistinguishable from the primary wild-type shoot, but initiated later than 20 days post germination
[c], 90-day-old dry plants were counted
[d], flowers on all stems were counted. In all other cases, the 3 first flowers were counted.
n, number of plants.
m.d., mean absolute deviation.
Statistically different to *wus-1-/-* with P<0,05 $^{*}$, <0,01 $^{**}$, <0,001 $^{***}$. Unmarked combinations, statistically not significant.
Probabilities for a) and b) determined from Fisher's Exact test, using the Holm-Bonferroni method to correct for multiple testing.
Probabilities for the other experiments determined using Kruskal-Wallis with Dunn's post-hoc test and corrected for multiple testing using the Holm method.

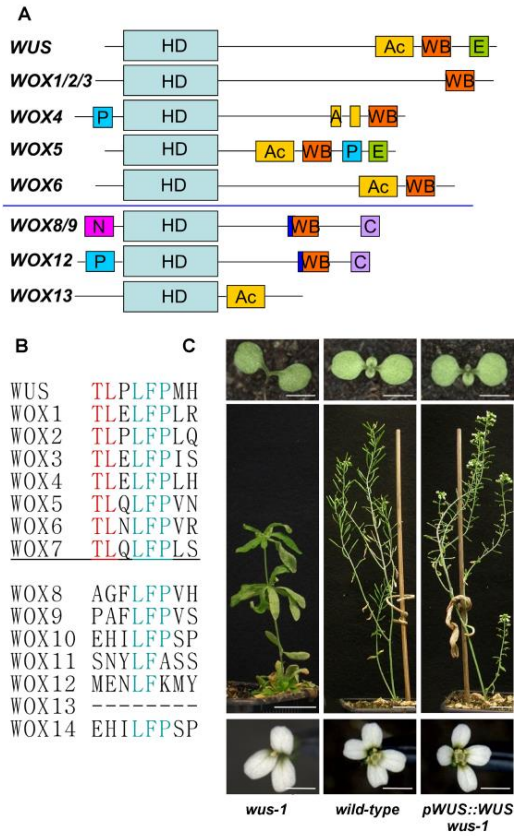
A
WUS
WOX1/2/3
WOX4
WOX5
WOX6
WOX8/9
WOX12
WOX13
HD
Ac
WB
E
P
A
C
N
B
C
WUS TLPLFPMH
WOX1 TLELFPLR
WOX2 TLPLFPLQ
WOX3 TLELFPIS
WOX4 TLELFPLH
WOX5 TLQLFPVN
WOX6 TLNLFPVR
WOX7 TLQLFPLS
WOX8 AGFLFPVH
WOX9 PAFLFPVS
WOX10 EHILFPSP
WOX11 SNYLFASS
WOX12 MENLFKMY
WOX13 --------
WOX14 EHILFPSP
wus-1
wild-type
pWUS::WUS
wus-1

Figure 1.

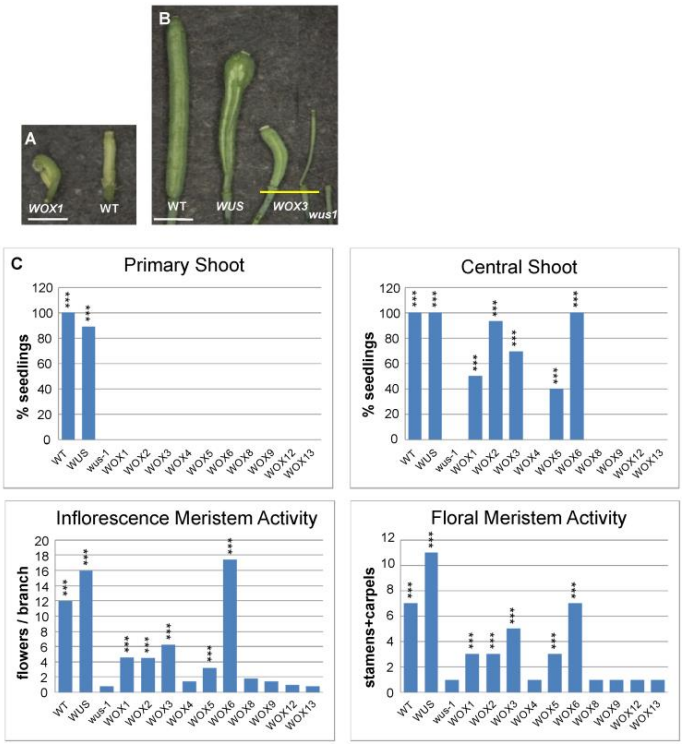
A
WOX1
WT
B
WT
WUS
WOX3
wus1
C
Primary Shoot
% seedlings
Central Shoot
% seedlings
Inflorescence Meristem Activity
flowers / branch
Floral Meristem Activity
stamens+carpels
WT
WUS
wus-1
WOX1
WOX2
WOX3
WOX4
WOX5
WOX6
WOX8
WOX9
WOX12
WOX13

Figure 2.

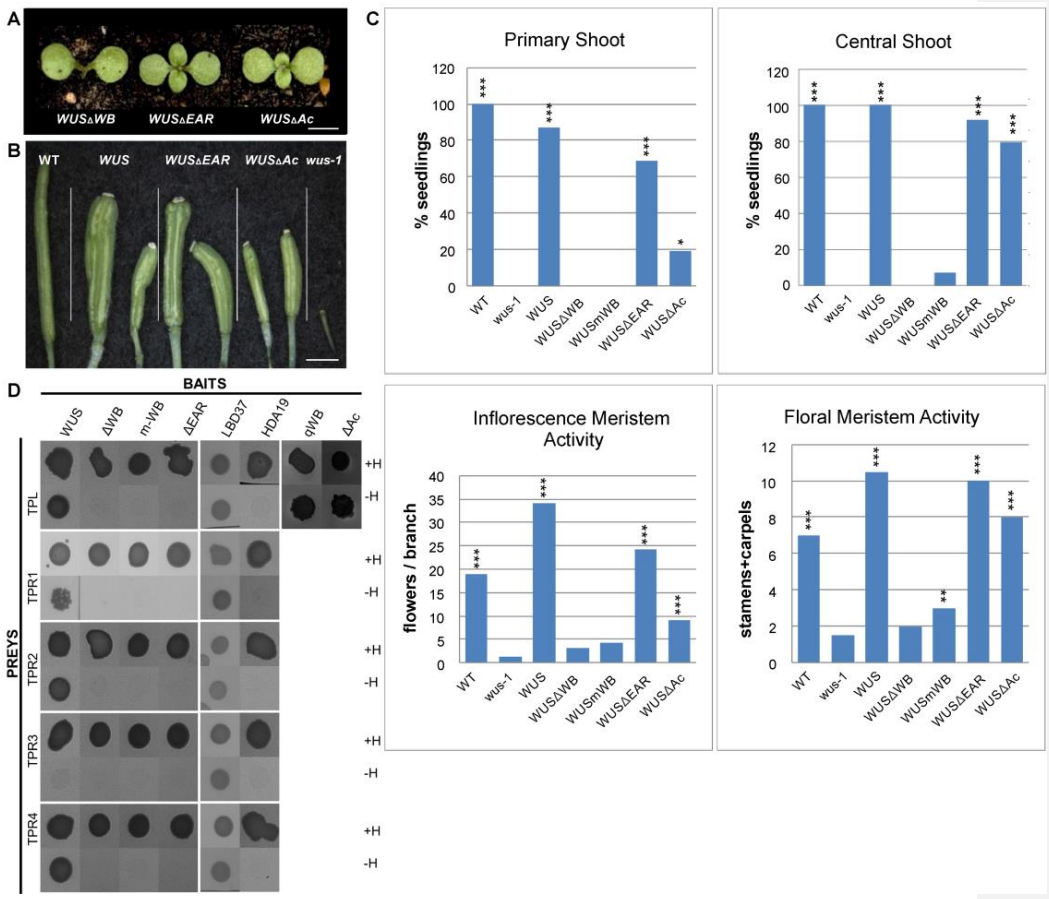
A
WUSΔWB
WUSΔEAR
WUSΔAc
B
WT
WUS
WUSΔEAR
WUSΔAc
wus-1
C
Primary Shoot
% seedlings
Central Shoot
% seedlings
Inflorescence Meristem Activity
flowers / branch
Floral Meristem Activity
stamens+carpels
WT
wus-1
WUS
WUSΔWB
WUSmWB
WUSΔEAR
WUSΔAc
D
BAITS
WUS
ΔWB
m-WB
ΔEAR
LBD37
HDA19
qWB
ΔAc
PREYS
TPL
TPR1
TPR2
TPR3
TPR4
+H
-H

Figure 3.

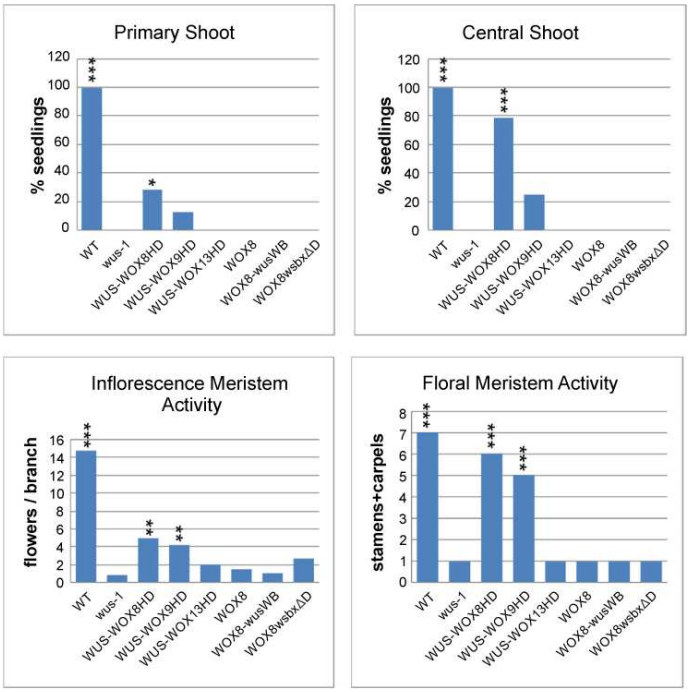
Primary Shoot
% seedlings
120
100
80
60
40
20
0
***
*
WT
wus-1
WUS-WOX8HD
WUS-WOX9HD
WUS-WOX13HD
WOX8
WOX8-wusWB
WOX8wsbxΔD
Central Shoot
% seedlings
***
***
Inflorescence Meristem Activity
flowers / branch
16
14
12
10
8
6
4
2
0
***
**
**
Floral Meristem Activity
stamens+carpels
8
7
6
5
4
3
2
1
0
***
***
***

Figure 4.

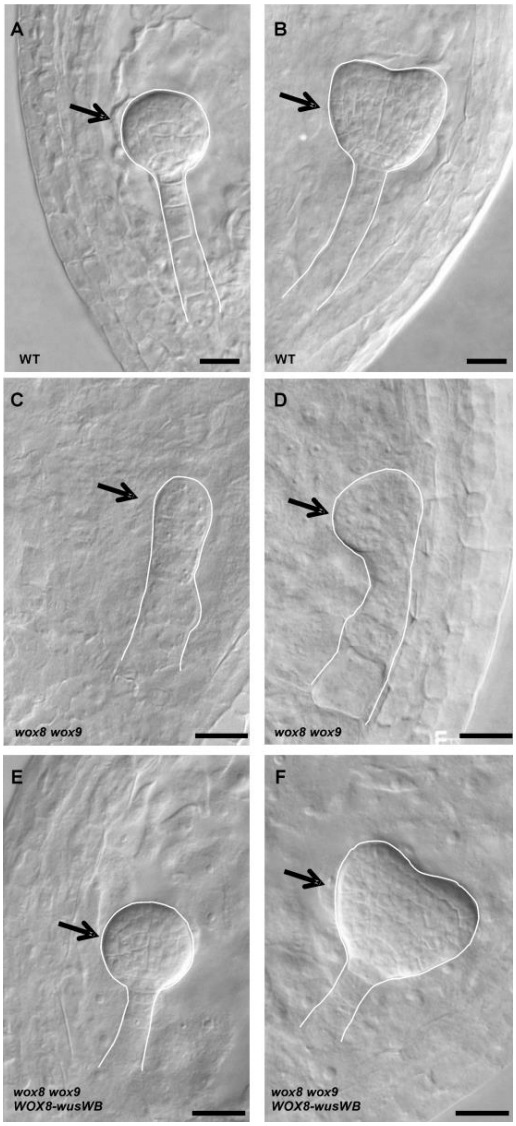
A
WT
B
WT
C
wox8 wox9
D
wox8 wox9
E
wox8 wox9
WOX8-wusWB
F
wox8 wox9
WOX8-wusWB

Figure 5.