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Arabidopsis WUSCHEL Is a Bifunctional Transcription Factor That Acts as a Repressor in Stem Cell Regulation and as an Activator in Floral Patterning [W]

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Most transcription factors act either as activators or repressors, and no such factors with dual function have been unequivocally identified and characterized in plants. We demonstrate here that the *Arabidopsis thaliana* protein WUSCHEL (WUS), which regulates the maintenance of stem cell populations in shoot meristems, is a bifunctional transcription factor that acts mainly as a repressor but becomes an activator when involved in the regulation of the *AGAMOUS* (*AG*) gene. We show that the WUS box, which is conserved among *WOX* genes, is the domain that is essential for all the activities of WUS, namely, for regulation of stem cell identity and size of floral meristem. All the known activities of WUS were eliminated by mutation of the WUS box, including the ability of WUS to induce the expression of *AG*. The mutation of the WUS box was complemented by fusion of an exogenous repression domain, with resultant induction of somatic embryogenesis in roots and expansion of floral meristems as observed upon ectopic expression of *WUS*. By contrast, fusion of an exogenous activation domain did not result in expanded floral meristems but induced flowers similar to those induced by the ectopic expression of *AG*. Our results demonstrate that WUS acts mainly as a repressor and that its function changes from that of a repressor to that of an activator in the case of regulation of the expression of *AG*.

INTRODUCTION

Plants maintain populations of pluripotent stem cells in their meristems throughout their lives, and leaves and flowers are formed continuously from the meristems. The WUSCHEL (WUS) protein plays a central role in the maintenance of stem cell populations in shoot and floral meristems (Laux et al., 1996; Mayer et al., 1998; Veit, 2004). WUS positively regulates the size of each shoot meristem by maintaining the appropriate number of pluripotent stem cells in each shoot meristem. It acts in cooperation with CLAVATA (CLV) proteins by feedback regulation between WUS and CLV3. WUS positively regulates the expression of *CLV3*, and CLVs negatively regulate the sizes of meristems by suppressing the expression of *WUS* (Schoof et al., 2000; Reddy, 2008). Thus, *wus-1* mutant *Arabidopsis thaliana* plants lose shoot meristems after several leaves have formed because the population of stem cells has not been maintained in meristems and the numbers of floral organs are decreased (Laux et al., 1996). By contrast, the ectopic expression of *WUS* increases the size of shoot meristems and induces ectopic accumulation of stem cells, with resultant formation of adventitious shoots and somatic embryos in root tissues (Zuo et al., 2002; Gallois et al., 2004). In addition, WUS negatively regulates the expression of *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, whose products negatively regulate cytokinin signaling (Leibfried et al., 2005). Since cytokinin signaling influences meristem function, phyllotaxis, and the formation of flowers in *Arabidopsis* (Giulini et al., 2004; Leibfried et al., 2005), it seems likely that WUS regulates meristem function via the activation of cytokinin signaling.

In floral meristems, WUS affects not only the size of floral meristems but also the differentiation-related fate of floral stem cells, acting in concert with the AGAMOUS (AG) protein (Lenhard et al., 2001; Lohmann et al., 2001). WUS activates the expression of *AG*, while AG represses the expression of *WUS* to reduce the size of stem cell populations in floral meristems, but induces the formation of stamens and carpels at the center of flowers (Lenhard et al., 2001; Lohmann et al., 2001).

The *AG* gene and the *ARR* genes have been reported to be direct targets of WUS. WUS is known to regulate the expression of *AG* by binding to its second intron and to regulate *ARR7* by binding to its 5′-upstream region (Lohmann et al., 2001; Leibfried et al., 2005). Moreover, WUS acts as a repressor of the transcription of *ARR* genes but as an activator of the transcription of *AG* (Lohmann et al., 2001; Leibfried et al., 2005). These data suggest that WUS acts as a bifunctional transcription factor. However, neither activation or repressive activity of WUS protein nor the functional domain involved in the bifunctional activity has been investigated.

The C-terminal region of WUS is necessary for its biological function and includes an acidic region, a WUS box, and an EAR-like motif (Kieffer et al., 2006). The EAR motif is a repression domain that is conserved in plants (Ohta et al., 2001), and its

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presence supported the proposed role of WUS as a repressor. However, the functions of WUS at the molecular level and those of the three domains in the C-terminal region remain to be characterized.

In this study, we examined the functions of the three domains in the C-terminal region of WUS by loss-of-function and domain complementation analyses. We show here that WUS is a bifunctional transcription factor and that the WUS box is a repression domain that is essential for all the activities of WUS, including the activation activity to induce the expression of *AG*. WUS mainly acts as a transcriptional repressor but becomes an activator when involved in the regulation of the expression of *AG.* Moreover, the WUS box plays a crucial role in the dual functions of WUS.

RESULTS

Functional Analysis and Identification of Functional Domains of WUSCHEL

Transient expression assays, with an effector composed of the coding region of *WUS* fused with that of the yeast GAL4 DNA binding domain (*Pro35S:GAL4DB-WUS*) and a luciferase reporter gene with a GAL4 binding site (*Pro35S-GAL4:LUC*; Hiratsu et al., 2004) revealed that WUS had strong repressive activity and suppressed the activation of the reporter gene, which was under the control of the cauliflower mosaic virus (CaMV) 35S promoter, by >90% in leaves of *Arabidopsis* (Figures 1A and 1C). The C-terminal region of WUS, which is required for WUS activity (Kieffer et al., 2006), contains an acidic region, a WUS box (TLPLFPMH) and an EAR-like (SLELRLN) motif (Figure 1B). Since the EAR motif is a repression domain that is conserved in plant transcription factors (Ohta et al., 2001), we mutated the EAR-like motif and replaced the two Leu residues in SLELRLN by Ala residues (to yield SLEARAN in a mutant protein designated WUSm2; Figures 1A and 1B). We repeated the above-described transient expression assays with the mutant protein. Contrary to our expectations, our results showed that mutation of the EAR-like motif did not interfere with the repressive activity of WUS (Figure 1C), indicating that WUS might contain an additional repression domain. Therefore, we mutated the WUS box (TLPLFPMH), which is conserved in WUS orthologs and in WUSCHEL-RELATED HOMEOBOX (WOX) proteins (Haecker et al., 2004; Kieffer et al., 2006), replacing the two Leu residues in the WUS box by Ala residues (to yield TAPAFPMH in a mutant protein designated WUSm1; Figures 1A and 1B). The results of transient expression assays showed that *WUS* with a single pair of mutations, namely, either WUSm1 or WUSm2, had repressive activity similar to that of WUS, but a *WUS* gene with both of the m1 and the m2 pairs of mutations, which encoded WUSm1m2, lacked repressive activity when fused to a GAL4 DNA binding domain (Figure 1C). Thus, WUS appeared to have two repression domains, the WUS box and the EAR-like motif, in our transient expression system.

The *WUSm1m2* construct, which encoded a derivative of WUS that lacked repressive activity, had, by contrast, weak activation activity. This activation activity disappeared when the acidic

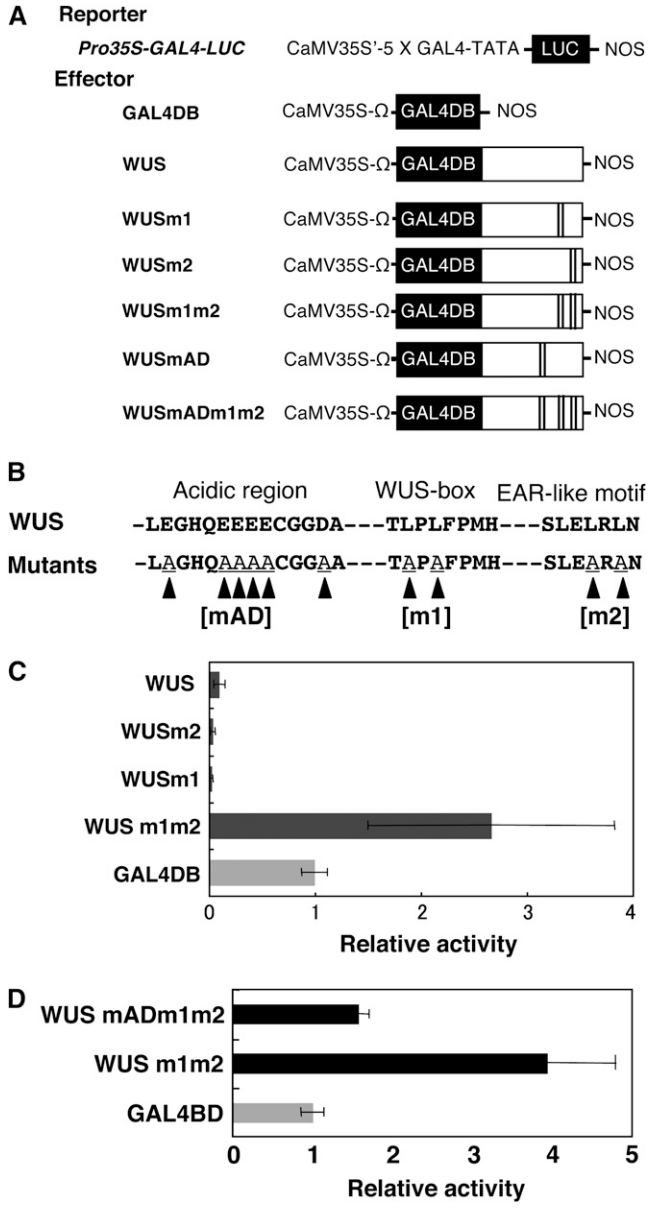

Figure 1. The Repressive Activity of WUS and Its Repression Domains.

(A) Schematic representation of the constructs used in transient expression analysis. Effector constructs encoded the gene for WUS or a mutant derivative (white box) fused to the GAL4 DNA binding domain (GAL4DB; shown as a black box), as indicated. Lines within the white boxes indicate sites of amino acid replacement. Each effector construct was driven by the CaMV 35S promoter and Ω translation enhancer sequence.
(B) Amino acid sequence of the C-terminal region of WUS and the positions of the acidic region, the WUS box, and the C-terminal EAR-like motif. Mutations introduced into the acidic region (mAD), WUS box (m1), and EAR-like motif (m2) are indicated by arrowheads.
(C) and **(D)** Relative luciferase activities after cobombardment of *Arabidopsis* leaves with GAL4DB-fused WUS effectors shown in **(A)** and the *Pro35S-GAL4:LUC* reporter gene. The relative activity due to *Pro35S:GAL4DB* (GAL4DB) was set as 1. **(C)** and **(D)** are independent experiments. Error bars indicate SD (n = 3).

region LEGHQEEEECGGDA of *WUSm1m2* was mutated to yield a gene, designated *WUSmADm1m2*, that encoded WUS with mutations all three domains (Figures 1A, 1B, and 1D). Thus, observation indicated that the acidic region of WUS was the only activation domain in the WUS protein.

Roles of the Various Domains in the Biological Activity of WUS

We analyzed the functional involvement of the acidic activation domain and the two repression domains, the EAR-like and WUS box domains, in the activity of WUS in transgenic plants. The ectopic expression of *WUS* (*Pro35S:WUS*) induced stem cell identity, with formation of somatic embryos and adventitious shoots on root tissues of seedlings, as reported previously (Figure 2A; Zuo et al., 2002; Gallois et al., 2004). The expression of *Pro35S:WUSm2* and that of *Pro35S:WUSmAD*, separately, induced the formation of somatic embryos in the root regions of seedlings, with occasional formation of adventitious floral meristem-like and floral organ-like structures on floral stems and pedicels, similar to those in *Pro35S:WUS* plants (Figures 2A, 2C, 2E, 2M, 2O, and 2Q). We analyzed 54, 34, and 26 independent lines of *Pro35S:WUS*, *Pro35S:WUSm2*, and *Pro35S:WUSmAD* plants, respectively. All the seedlings of these transgenic lines formed adventitious organs in roots. Thus, mutation of either the EAR-like motif or the acidic domain did not destroy the ability of WUS to induce stem cell identity. Unlike mature *Pro35S:WUS* and *Pro35S:WUSmAD* plants, which had twisted and narrow leaves, mature *Pro35S:WUSm2* plants had rounded juvenile-like leaves (Figures 2G, 2I, and 2K).

By contrast, the seedlings, the adult plants and flowers of the *Pro35S:WUSm1m2* and *Pro35S:WUSm1* lines were basically normal (Figures 2N, 2P, and 2R). We analyzed 80 and 36 independent lines of *Pro35S:WUSm1m2* and *Pro35S:WUSm1* plants, respectively. These indicated that the mutations in the WUS box had eliminated the ability of ectopically expressed WUS to induce somatic embryos. Several lines of these plants had defects in the shoot apical meristems (SAMs) of seedlings, and adult plants exhibited a late-flowering phenotype (Figures 2B, 2D, 2F, 2H, 2J, and 2L). Because these features are similar to those of *wus-1* mutants (Figures 4F, 4L, and 4R), it seems possible that ectopic expression of mutated WUS might compete, in terms of activity, with endogenous WUS. These features might have been due to a dominant-negative effect of the mutations in the WUS box, indicating that the WUS box is a functional domain of WUS required for induction and maintenance of shoot stem cell identity.

Next, we investigated the contribution of putative repression domains to the regulation of downstream genes. Consistent with the morphological phenotype of the seedlings of *Pro35S:WUS* and *Pro35S:WUSm2* plants, the expression of the *ABI3*, *FUS3*, and *LEC1* genes, which are expressed during zygotic and somatic embryogenesis, was considerably enhanced. By contrast, no similar enhanced expression was observed in the seedlings of *Pro35S:WUSm1* and *Pro35S:WUSm1m2* plants (Figure 2S). Similar to an earlier report, the expression of *CLV3* in *Pro35S:WUS* plants was >100-fold higher than in wild-type plants (Figure 2T; Schoof et al., 2000). Enhanced expression of *CLV3* was also evident in *Pro35S:WUSm2* plants, although the level of expression was lower than that in *Pro35S:WUS* plants. By contrast, no activation of expression of *CLV3* was observed in either *Pro35S:WUSm1* or *Pro35S:WUSm1m2* plants (Figure 2T). These results indicate that the WUS box is essential for the regulation of the expression of the *ABI3*, *FUS3*, *LEC1*, and *CLV3* genes but the EAR-like motif is not.

We examined the role of the WUS box in the regulation of the expression of *ARR* genes, which are direct targets of WUS (Leibfried et al., 2005). The levels of transcripts of *ARR6* and *ARR7* were lower in the seedlings of *Pro35S:WUS* plants than in the wild type, probably as a result of suppression by the ectopic expression of *WUS* (Figure 2U). The levels of expression of *ARR* genes were also depressed in the seedlings of *Pro35S:WUSm2* plants. By contrast, levels of expression were higher than or similar to those in the wild type in seedlings of both *Pro35S:WUSm1* and *Pro35S:WUSm1m2* plants (Figure 2U). Since *ARR* genes are direct targets of WUS (Leibfried et al., 2005), our results indicate that the repressive activity of the WUS box is necessary for the direct repression of *ARR* genes. By contrast, the EAR-like motif might not function as a repression domain in regulation of the expression of *ARR* genes in transgenic *Arabidopsis* plants, even though WUSm1, which has an EAR-like motif, did exhibit repressive activity in a transient expression assay when fused with the GAL4 DNA binding domain (Figure 1C).

Role of the WUS-Box in Activation of the *AG* Gene

Lohmann et al. (2001) demonstrated that WUS activates the expression of *AG* by direct interaction with the second intron of the gene. We investigated whether the acidic region and/or the WUS box, demonstrated to be a functional repression domain of WUS as described above, might be involved in the activation of the expression of *AG*. We introduced a reporter gene for β-glucuronidase (GUS) driven by 779 bp of the 3′-end region of the second intron of *AG* (*ProAG:GUS*) into *Arabidopsis* with the construct that encoded WUS or a mutant derivative of WUS, driven by 2248 bp of the 5′-upstream region of the site of initiation of transcription of the *LEAFY* (*LFY*) gene (constructs were designated *ProLFY:WUS*, *ProLFY:WUSm1*, *ProLFY:WUSm2*, *ProLFY:WUSm1m2*, and *ProLFY:WUSmAD,* respectively). The expression of *ProAG:GUS* did not affect the morphology of floral tissues, and we detected GUS activity only at the center of young floral meristems (Figures 3A and 3B). By contrast, the flowers of *ProAG:GUS* and *ProLFY:WUS* plants had broadly expanded GUS activity in their floral meristems, which spread still further at later stages (Figure 3C). Moreover, the expression of *ProLFY:WUS* induced abnormal flowers with increased numbers of floral organs, in which the numbers of stamens and carpels were elevated but those of petals and sepals were reduced or zero, similar to those described previously by Lohmann et al. (2001) (Figure 3D). We analyzed 18 independent transgenic lines of *ProLFY:WUS* plants and observed such abnormal flowers in all the lines. Such abnormal flowers are considered to be due to the ectopic expression of *AG* in response to the ectopic expression of the *WUS* gene (*ProLFY:WUS*) that is driven by the *LFY* promoter (Lohmann et al., 2001).

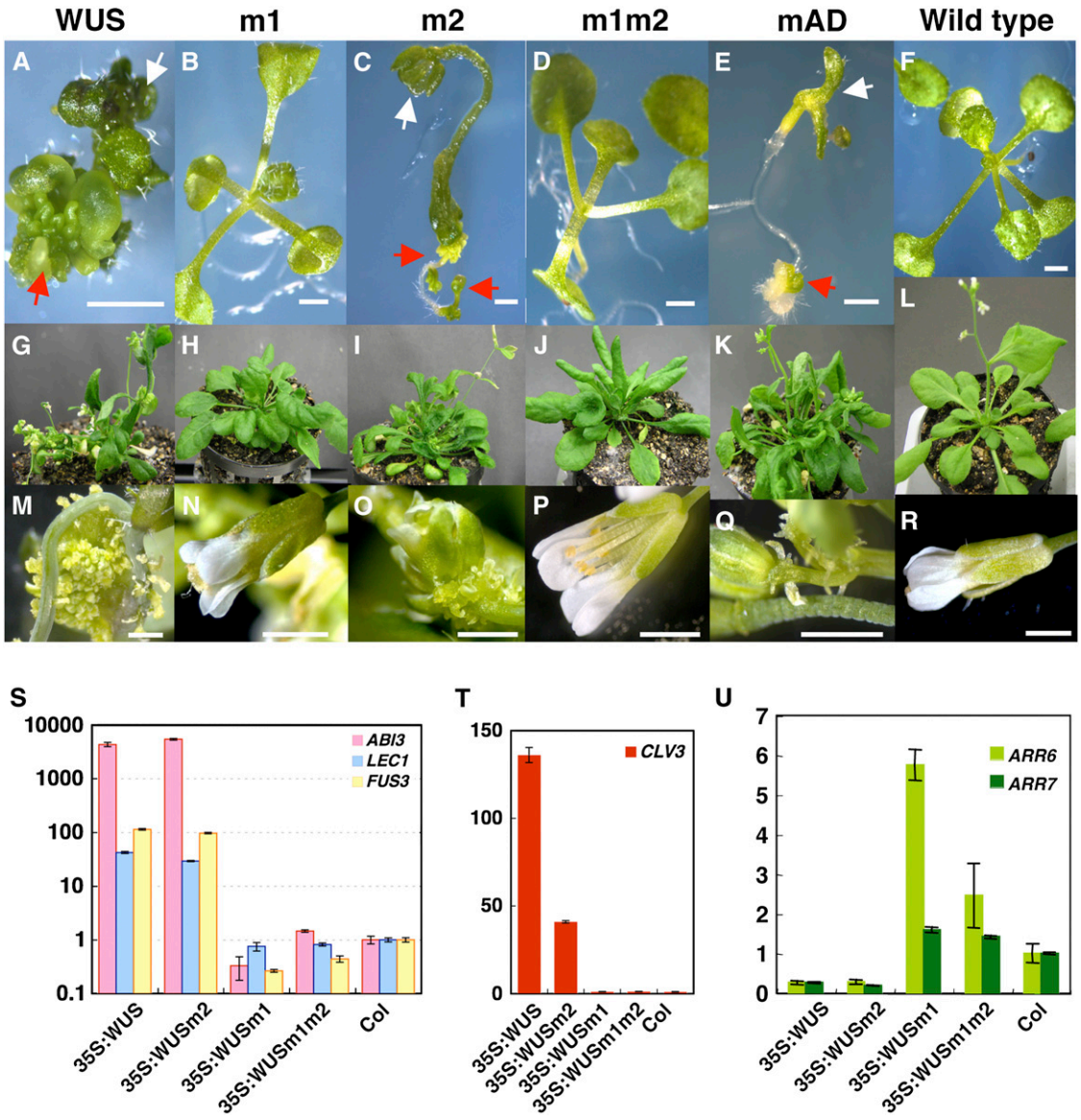

Figure 2. Effects of the Ectopic Expression of Mutant WUS Constructs.

(A) to **(R)** Seedlings (**[A]** to **[F]**), rosette plants (**[G]** to **[L]**), and flowers (**[M]** to **[R]**) of *Pro35S:WUS* (**[A]**, **[G]**, and **[M]**), *Pro35S:WUSm1* (**[B]**, **[H]**, and **[N]**), *Pro35S:WUSm2* (**[C]**, **[I]**, and **[O]**), *Pro35S:WUSm1m2* (**[D]**, **[J]**, and **[P]**), *Pro35S:WUSmAD* (**[E]**, **[K]**, and **[Q]**), and wild-type (**[F]**, **[L]**, and **[R]**) plants. White and red arrows indicate original SAMs and somatic embryos induced on root organs, respectively. Bars = 1 mm.

(S) to **(U)** Regulation of the expression of downstream genes in plants that expressed WUS and mutant derivatives of WUS, as indicated.

(S) Levels of expression of the *ABI3* (red bars), *LEC1* (blue bars), and *FUS3* (yellow bars) genes, as determined by RT-PCR analysis, in seedlings of wild-type (Col), *Pro35S:WUS*, *Pro35S:WUSm2*, *Pro35S:WUSm1*, and *Pro35S:WUSm1m2* plants.

(T) Levels of expression (red bars) of the *CLV3* gene, as determined by RT-PCR, in seedlings of *Pro35S:WUS* plants, mutant derivative lines, and the wild type (Col).

(U) Repression of the expression of *ARR* genes in transgenic plants that expressed *WUS* and mutant derivatives. Levels of expression of the *ARR6* (light green bars) and *ARR7* (dark green bars), as determined by RT-PCR analysis, in seedlings of *Pro35S:WUS* plants, mutant derivative lines, and the wild type (Col). The level of expression of the gene for ubiquitin was used for normalization of the results. The level in wild-type (Col) seedlings was set as 1. Error bars indicate SD (n = 3).

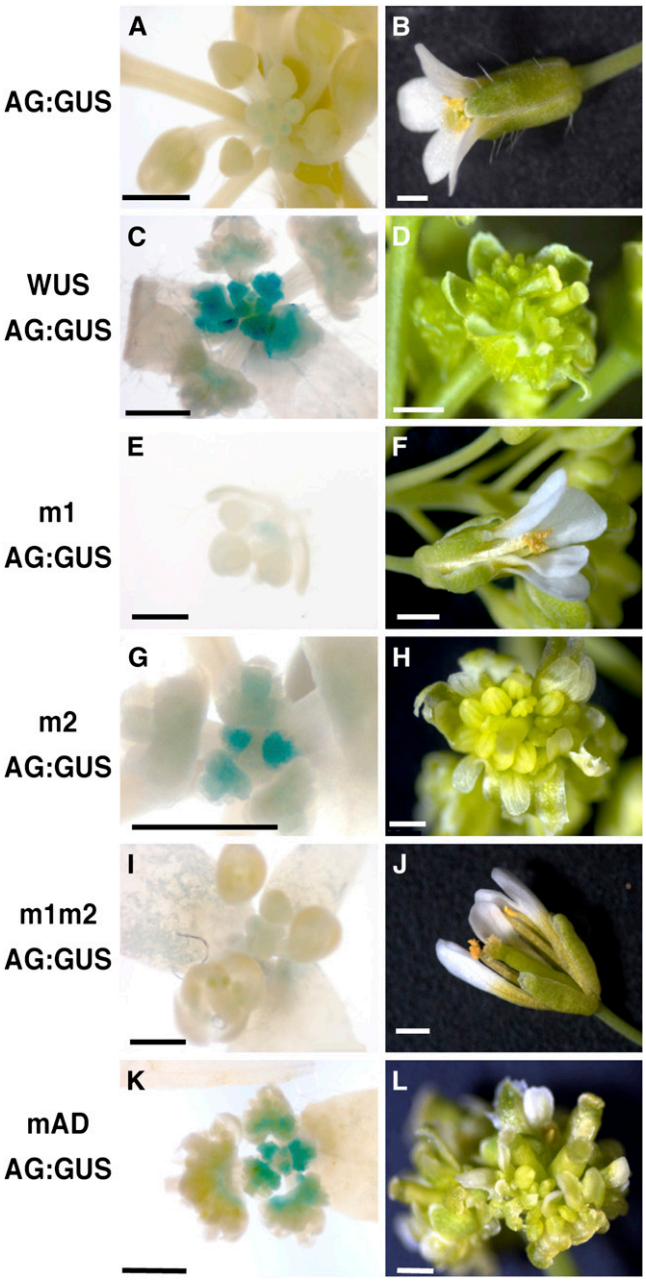

Figure 3. Regulation of the Activity of the Promoter of the *AG* Gene by WUS.

Flowers and results of staining for GUS activity in plants after transformation with *ProAG:GUS* (**[A]** and **[B]**), with *ProLFY:WUS ProAG:GUS* (**[C]** and **[D]**), with *ProLFY:WUSm1 ProAG:GUS* (**[E]** and **[F]**), with *ProLFY:WUSm2 ProAG:GUS* (**[G]** and **[H]**), with *ProLFY:WUSm1m2 ProAG:GUS* (**[I]** and **[J]**), and with *ProLFY:WUSmAD ProAG:GUS* (**[K]** and **[L]**). Bars = 0.5 mm.

The activity of the ectopically expressed *AG* might expand whorl one and whorl two, with resultant formation of numerous stamens and carpels on those whorls (Figures 3C and 3D).

The floral phenotype and the patterns of GUS expression in *ProAG:GUS ProLFY:WUSmAD*, and *ProAG:GUS ProLFY:WUSm2* plants were basically similar to those in *ProAG:GUS ProLFY:WUS* plants (Figures 3G, 3H, 3K, and 3L). Twenty of 24 independent lines of *ProAG:GUS ProLFY:WUSmAD* and 10 of 14 independent lines of *ProAG:GUS ProLFY:WUSm2* plants had flowers similar to those observed in *ProLFY:WUS* plants, respectively. Moreover, as in plants that ectopically expressed *AG*, carpeloid sepals and stameloid petals were observed in some flowers of *ProLFY:WUSmAD* and of *Pro35S:WUSmAD* plants (see Supplemental Figures 1A and 1B online; Mizukami and Ma, 1992). These results indicate that either the acidic region or the EAR-like motif is unnecessary for regulation of the expression of *AG* and, furthermore, that expression of *AG* can be induced ectopically by the ectopic expression of WUS in the absence of its acidic region.

By contrast to the above observations, 31 of 41 independent lines of *ProAG:GUS ProLFY:WUSm1* plants and 30 of 46 independent lines of *ProAG:GUS ProLFY:WUSm1m2* plants had normal flowers and patterns of GUS expression were basically similar to those in a wild-type background (Figures 3E, 3F, 3I, and 3J). Among the transgenic lines, six and four lines of *ProLFY:WUSm1* plants and 14 and two lines of *ProLFY:WUSm1m2* plants occasionally developed flowers that lacked carpels or had double flowers, respectively (Figure 3F; see Supplemental Figure 1C online). In the transgenic lines that produced double flowers, no activity of the *ProAG:GUS* reporter gene was detected (see Supplemental Figure 1D online). These phenotypic features might have resulted from suppression of the *AG* gene by the dominant-negative effect of *WUSm1* or *WUSm1m2*. The results indicated that the WUS box is necessary for the activation of the expression of the *AG* gene.

Complementation Analysis

For further examination of functional domains of WUS, we performed complementation analysis of *wus-1* mutants. We transformed the heterozygous *wus-1* line with a fragment of *WUS* cDNA derived from Columbia-0 (Col-0), fused with 3003 bp of the 5′-upstream region of the site of initiation of transcription of *WUS* (*ProWUS:WUS*). All seedlings of the eight transgenic lines that expressed *ProWUS:WUS* in the *wus-1* homozygous background resembled wild-type (Landsberg *erecta*) seedlings, with normal shoot apical meristems and yielding rosette plants. Thus, *ProWUS:WUS* complemented the defective SAM phenotype of the *wus-1* mutant (Figures 4A and 4F). Although the extent of complementation varied among the eight *ProWUS:WUS wus-1* lines, the defective *wus-1* phenotypes of rosettes and flowers were rescued by the introduction of the *ProWUS:WUS* construct in each transgenic line (Figures 4G, 4L, 4M, and 4R). However, all *ProWUS:WUS wus-1* plants were male-sterile (Figure 4M). Deyhle et al. (2007) reported the involvement of WUS in the development of anthers. The promoter region in our *ProWUS:WUS* construct might have been insufficient, or the noncoding region of the *WUS* gene might be required for complete

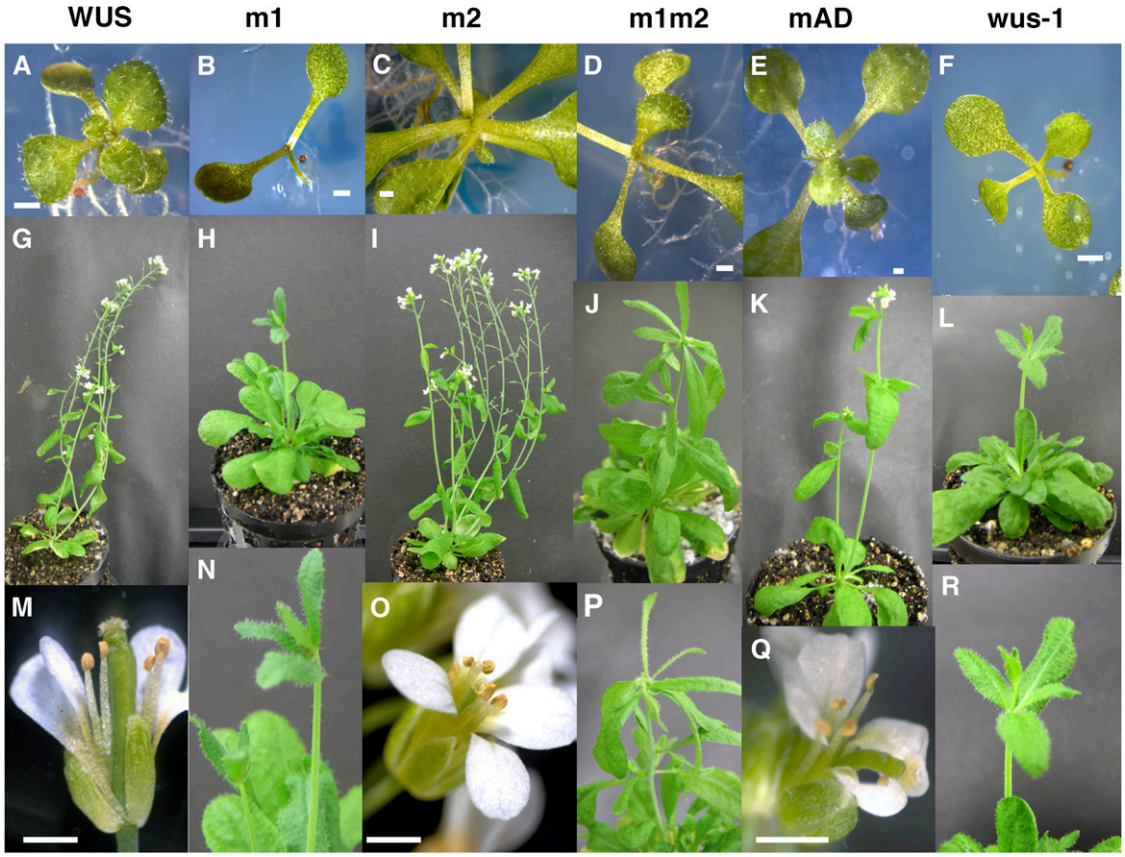

Figure 4. Complementation of the *wus-1* Mutation.

Seedlings (**[A]** to **[F]**), rosette plants (**[G]** to **[L]**), and flowers (**[M]** to **[R]**) of the *wus-1* mutant after transformation with *ProWUS:WUS* (**[A]**, **[G]**, and **[M]**), *ProWUS:WUSm1* (**[B]**, **[H]**, and **[N]**), *ProWUS:WUSm2* (**[C]**, **[I]**, and **[O]**), *ProWUS:WUSm1m2* (**[D]**, **[J]**, and **[P]**), or *ProWUS:WUSmAD* (**[E]**, **[K]**, and **[Q]**) constructs. Nontransformed *wus-1* plants are shown in (**[F]**, **[L]**, and **[R]**). Bars = 1 mm.

complementation (Baurle and Laux, 2005). We isolated five *ProWUS:WUSm2 wus-1* and four *ProWUS:WUSmAD wus-1* lines. As in the case of *ProWUS:WUS*, both *ProWUS:WUSm2* and *ProWUS:WUSmAD* complemented the *wus-1* mutation and all the resultant seedlings formed SAMs (Figures 4C, 4E, and 4F), even though the extent of complementation by the *ProWUS:WUSm2* construct appeared to be weaker than that by *ProWUS:WUS*. The defective floral phenotype of *wus-1* plants was also rescued by introduction of the *ProWUS:WUSm2* or the *ProWUS:WUSmAD* construct, but male sterility was not reversed (Figures 4I, 4K, 4L, 4O, 4Q, and 4R).

Neither the *ProWUS:WUSm1* nor the *ProWUS:WUSm1m2* construct complemented the *wus-1* mutation. We isolated five *ProWUS:WUSm1 wus-1* lines and four *ProWUS:WUSm1m2 wus-1* lines, and all of the resultant plants had a *wus-1*-like phenotype, with defective SAMs, delayed flowering and failure to form floral organs (Figures 4B, 4D, 4F, 4H, 4J, 4L, 4N, 4P, and 4R). The results of our complementation analysis indicate that the WUS box is essential for the maintenance by WUS of populations of pluripotent stem cells in SAMs and for the formation of floral organs and that the EAR-like motif might support the activity of the WUS box.

Repressive Activity of the WUS Box in Transgenic Plants

To examine whether the WUS box acts as a repression domain and is able, when fused to transcriptional activators, to convert them to dominant repressors, as is the case for the SRDX repression domain (Hiratsu et al., 2003), we fused a peptide of 14 amino acids that included a WUS box (WUSB) to the C terminus of the coding region of the *SHOOT MERISTEMLESS* (*STM*) and the *CUP-SHAPED COTYLEDON2* (*CUC2*) genes, respectively (to generate constructs *Pro35S:STMWUSB* and *Pro35S:CUC2WUSB*; Figure 5A). Among 170 independent lines of *Pro35S:STMWUSB* plants analyzed, almost all the seedlings lacked SAMs and had slender cotyledons similar to those of *Pro35S:STMSRDX* plants, in which the SRDX repression domain was fused to the *STM* gene (Figure 5A), but the seedlings were completely different from those of *Pro35S:STM* plants (Figure 5B; see Supplemental Figure 2 online). Among 30 independent lines of *Pro35S:STM* plants analyzed, all the lines had meristems and grew into rosette plants. These results indicate that the phenotype of *Pro35S:STMWUSB* is unlikely to be due to cosuppression, but rather is the effect of the repressive activity of WUSB.

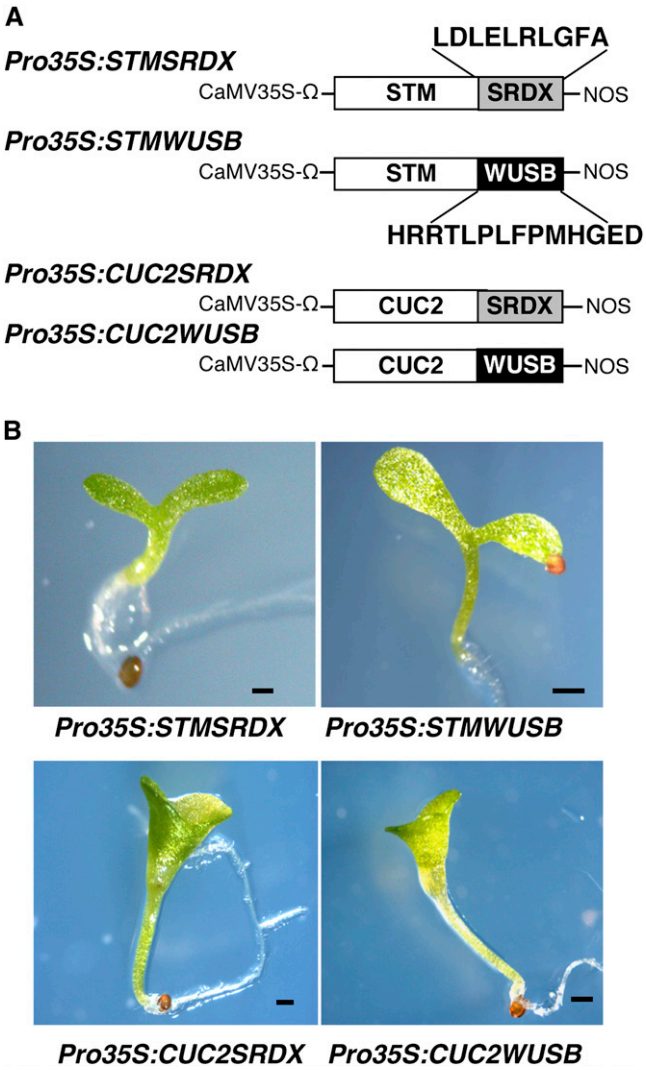

Figure 5. Repressive Activity of the WUS Box.

(A) Schematic representation of the chimeric repressor constructs used for transformations. The genes encode STM and CUC2 (shown as a white box) fused to be the SRDX repression domain (gray box) or the WUSB (black box), respectively. The SRDX and WUSB amino acid sequences are shown. Each construct was driven by the CaMV 35S promoter and Ω translation enhancer sequence.

(B) Phenotypes of seedlings of *Pro35S:STMSRDX*, *Pro35S:STMWUSB*, *Pro35S:CUC2SRDX*, and *Pro35S:CUC2WUSB* plants. Bars = 0.5 mm.

Among 48 independent lines of *Pro35S:CUC2WUSB* plants, 31 seedlings of them had partially or completely fused cotyledons that resembled *Pro35S:CUC2SRDX* cotyledons (Figure 5B). The frequency of fusion in *Pro35S:CUC2WUSB* cotyledons (65%) was similar to that of *Pro35S:CUC2SRDX* cotyledons (63%). Since the *Pro35S:CUC2WUSB* and *Pro35S:STMWUSB* plants resembled *cuc1 cuc2* double mutant and *stm-1* mutant plants, respectively, our results indicate that the WUS box acts as a repression domain, similar to the SRDX repression domain, and can convert transcriptional activators into dominant repressors in transgenic plants.

Functional Complementation of the WUS Box by Fusion of an Exogenous Repression Domain

In an attempt to determine whether loss of function of the WUS box could be rescued by an exogenous repressive activity, we fused the SRDX repression domain to the N terminus and to the C terminus, respectively, of WUSm1 (generating the constructs *Pro35S:SRDXWUSm1* and *Pro35S:WUSm1SRDX*; Figure 6A) and introduced these constructs into *Arabidopsis*. Both *Pro35S:WUSm1SRDX* and *Pro35S:SRDXWUSm1* seedlings resembled *Pro35S:WUS* seedlings and formed adventitious shoots and somatic embryos on the root regions (Figures 2A and 6B to 6D). We observed seedlings resembling those of *Pro35S:WUS* plants in 17 of 112 lines of *Pro35S:WUSm1SRDX* plants and in all 80 lines of *Pro35S:SRDXWUSm1* plants. These observations indicated that loss of the activity of the WUS box in the WUSm1 protein could be complemented by the exogenous repressive activity of SRDX and that the repressive activity of WUS does in fact regulate stem cell identity. Although none of the *Pro35S:SRDXWUSm1* seedlings developed into rosette plants because of the severely abnormal phenotype, >90% of *Pro35S:WUSm1SRDX* lines yielded plants that developed into rosette plants with twisted narrow leaves and occasional adventitious floral meristem-like and floral organ-like structures on floral stems and pedicels, as also observed in the *Pro35S:WUS* line (Figures 2G, 2M, 6E, and 6F). Flowers of *Pro35S:WUSm1SRDX* plants had adventitious floral meristems outside their sepals and increased numbers of floral organs in each whorl of each flower (Figure 6G). The activity of *WUSm1SRDX* appeared to be weaker than that of *SRDXWUSm1* and of the original *WUS* gene since ~50% of *Pro35S:WUSm1SRDX* plants yielded rosettes and mature flowers, even though adventitious shoots formed on their roots. By contrast, none of the *Pro35S:SRDXWUSm1* seedlings developed root tissues and rosettes.

Fusion of the VP16 activation domain (Triezenberg et al., 1988) to WUSm1 (*Pro35S:WUSm1VP16*; Figure 6A) did not complement the activity of the WUS box, although 20 of 50 independent lines had defective shoot meristems, with late flowering and loss of floral organs, as observed in *Pro35S:WUSm1* and *wus-1* mutant plants (Figures 2B, 4F, 4L, 4R, and 6H to 6J). These defects might have been due to a dominant-negative effect of *Pro35S:WUSm1VP16*. The results confirmed that WUS acts as a repressor and not as an activator in the induction of stem cell identity and in maintenance of populations of pluripotent stem cells in shoot and floral meristems.

Molecular Function of the WUS Box in the Formation of Flowers

To examine the molecular function of the WUS box in the regulation of the expression of *AG*, we expressed *WUSm1SRDX* and *WUSm1VP16* under the control of the *LFY* promoter (*ProLFY:WUSm1SRDX* and *ProLFY:WUSm1VP16*) in *ProAG:GUS* transgenic plants and in the wild type (Figure 7A). In 53 of 55 independent transgenic lines, the number of floral organs in *ProLFY:WUSm1SRDX* flowers was elevated just as it was in *ProLFY:WUS* flowers (Figures 7B and 7D), indicating the that size of floral meristems was also regulated by the repressive activity

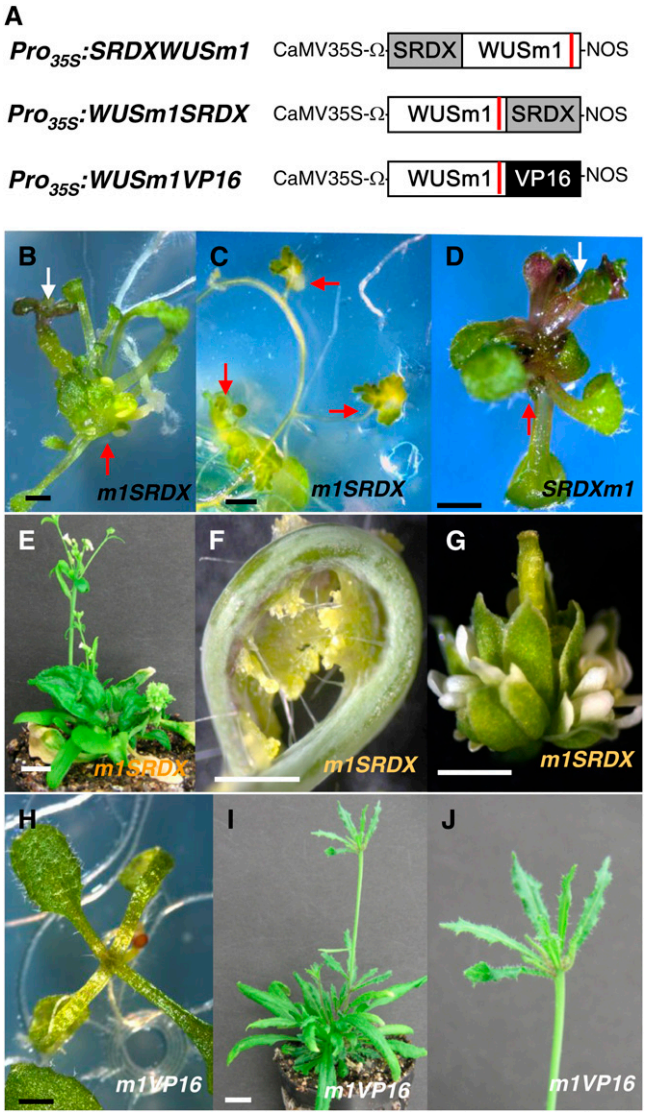

Figure 6. Functional Complementation of the WUS Box.

(A) Schematic representation of the constructs for expression of WUSm1 fused with SRDX and VP16, driven by the CaMV 35S promoter, that were used in functional complementation analysis. Red lines within the white box that indicates WUSm1 correspond to sites of amino acid replacements (Leu to Ala), as also indicated in Figure 1B.
(B) to **(J)** Seedlings (**[B]** to **[D]** and **[H]**), rosette plants (**[E]** and **[I]**), floral stem **(F)**, and flowers (**[G]** and **[J]**) of wild-type plants after transformation with *Pro35S:WUSm1SRDX* (**[B]**, **[C]**, and **[E]** to **[G]**), *Pro35S:SRDXWUSm1* **(D)**, or *Pro35S:WUSm1VP16* (**[H]** to **[J]**). White and red arrows indicate original SAMs and adventitious shoot organs, respectively. Bars = 1 mm in **(B)** to **(D)** and **(F)** to **(H)** and 1 cm in **(E)** and **(I)**.

of WUS. However, unlike the flowers of *ProLFY:WUS* plants, in which only stamens and carpels were formed, most likely because of the ectopic expression of *AG* (Figure 7D; Lohmann et al., 2001), the flowers of *ProLFY:WUSm1SRDX* plants formed sepals and petals, in addition to stamens and carpels (Figure 7B). By contrast to flowers of *ProAG:GUS ProLFY:WUS* plants in which the GUS expression was expanded broadly at later stages (Figure 3C), the GUS expression in the flower of *ProAG:GUS ProLFY:WUSm1SRDX* plants was limited in young floral meristems (Figure 7C). These results indicate that *ProLFY:WUSm1SRDX* does not, apparently, induce the ectopic expression of the *AG* gene in floral organs. It seems unlikely, moreover, that the exogenous repressive activity of SRDX induces activation of the expression of *AG* or complements the activity of the WUS box in the regulation of *AG*.

The *ProLFY:WUSm1VP16* construct did not increase the number of floral organs, which were similar to those of flowers of *ProLFY:WUSm1* plants. However, flowers of 10 of 60 independent lines of *ProLFY:WUSm1VP16* plants formed carpeloid sepals with stigmatic papillae at their tips and staminoid petals (Figures 7E). In addition, two lines had a double flower phenotype, which were similar to those of *apetala1* mutant plants (see Supplemental Figures 1E and 1F online). The formation of such carpel-like organs and staminoid petals is induced by the ectopic

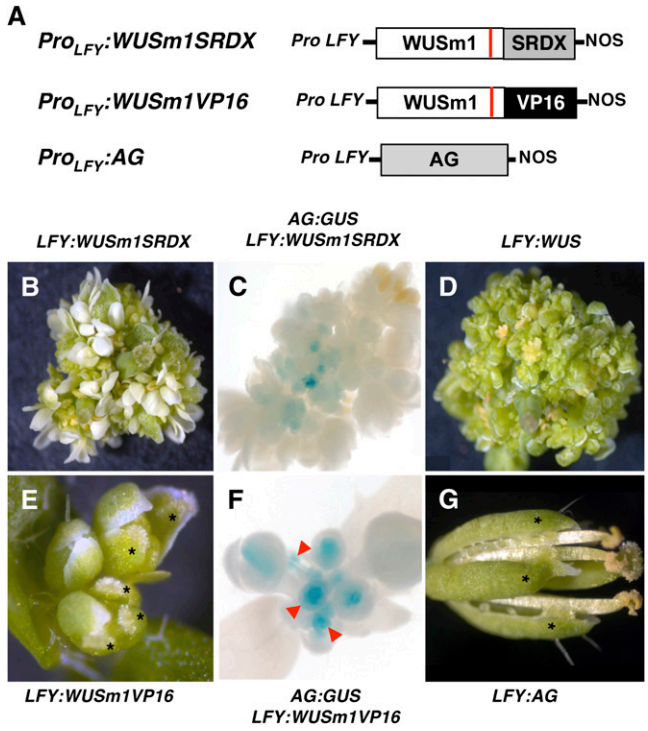

Figure 7. Effects of Functional Complementation of a Mutated WUS Box in Flowers.

(A) Schematic representation of the constructs used for functional complementation analysis in flowers. Red lines within the white box that corresponds to WUSm1 indicate site of amino acid replacement (Leu to Ala) as also indicated in Figure 1B.
(B) to **(E)** Flowers and results of staining for GUS activity in plants after transformation with *ProLFY:WUSm1SRDX* **(B)**, with *ProAG:GUS* and *ProLFY:WUSm1SRDX* **(C)**, with *ProLFY:WUS* **(D)**, with *ProLFY:WUSm1VP16* **(E)**, with *ProAG:GUS*, *ProLFY:WUSm1VP16*, and *ProAG:GUS* **(F)**, and with *ProLFY:AG* **(G)**. Asterisks indicate carpeloid sepals. Red arrowheads indicate GUS staining of the ectopic expression of *ProAG:GUS*. Bars = 0.5 mm.

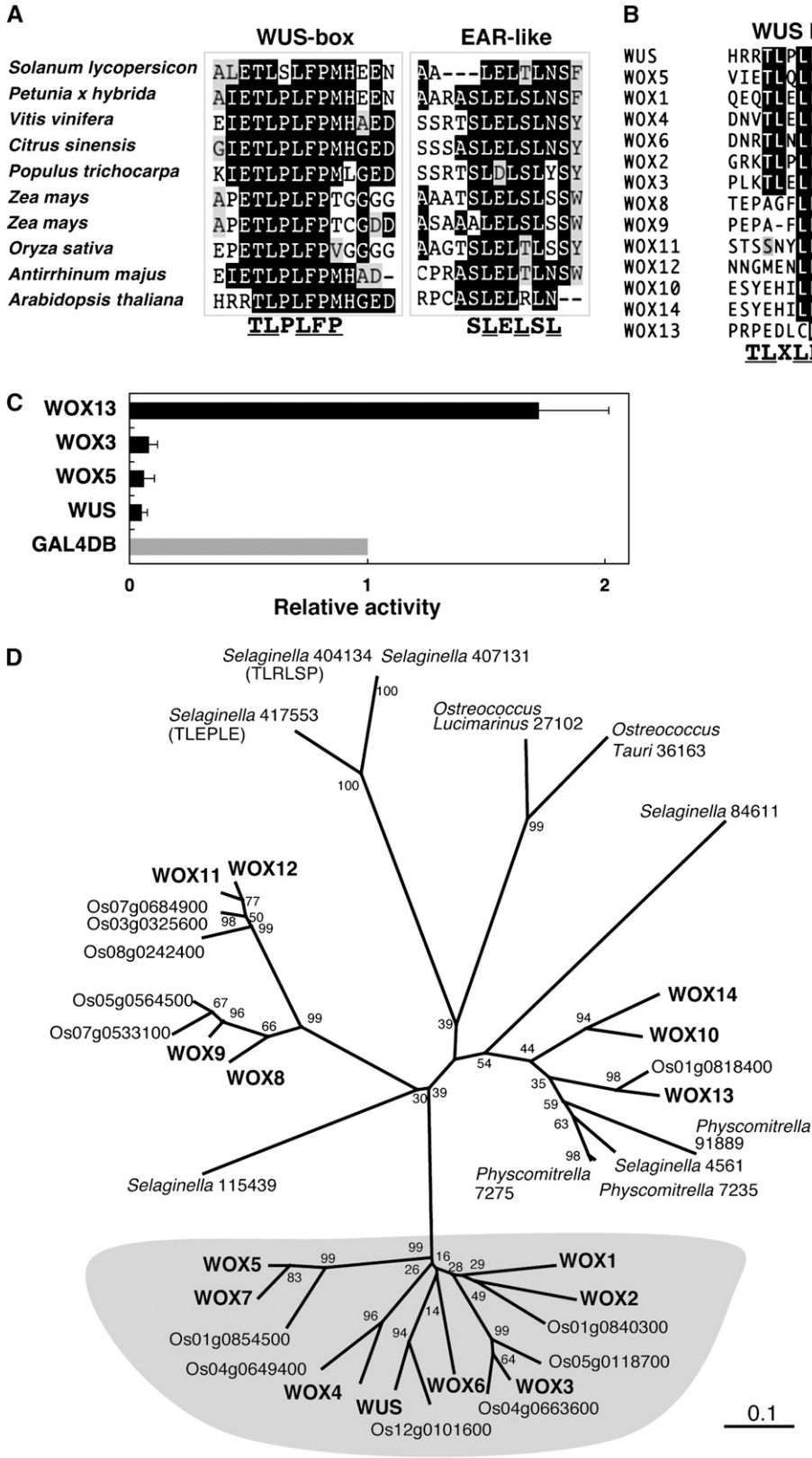

Figure 8. Conservation of the WUS Box in WUS Orthologs and WOX Proteins.

expression of *AG* (Mizukami and Ma, 1992), and we confirmed that the ectopic expression of *AG*, driven by promoter of *LFY* gene (*ProLFY:AG*), induced carpeloid sepals with stigmatic papillae and staminoid petals in transgenic flowers of 15 of 46 independent lines of *ProLFY:AG* plants but did not increase the number of floral organs (Figures 7G; see Supplemental Figure 1G online). In addition, we observed the promoter activity of *AG* in petals and sepals of young floral buds of *ProAG:GUS ProLFY:WUSm1VP16* plants (Figure 7F). These observations indicate that *ProLFY:WUSm1VP16* induces the expression of *AG* in whorls one and two. Our results demonstrate that WUS functions as a transcriptional activator in the regulation of the expression of *AG* in floral organs and, moreover, that the WUS box may be responsible for the activational activity of WUS since the WUS box was necessary for the activation of the expression of *AG* (Figures 3C and 3E).

Conservation of the WUS Box in WOX Proteins

The WUS box is conserved in orthologs of WUS in a variety of plant species and in the WOX proteins of *Arabidopsis* (Figures 8A and 8B; Haecker et al., 2004). The conserved sequence of the WUS box in the orthologs of WUS is TLP/SLFP and resembles the L2R repression domain (TLXLFR) that was recently identified in AtMYBL2 (Matsui et al., 2008; Figure 8A). Seven of 14 *Arabidopsis* WOX proteins contain the TLXLFP motif in their respective WUS boxes, and the presence of this motif suggests that these seven proteins have repressive activity (Figure 8B; Haecker et al., 2004). Phylogenetic analysis indicates that the WOX family can be divided into three groups, with only one group containing the TLXLFP motif in the WUS box (Figure 8D). In a GAL4-fusion transient expression assay, WOX5 and WOX3, which include the TLXLFP motif, had repressive activity, while WOX13, which lacks this motif, did not (Figure 8C). Thus, it appears that the WOX proteins that contain the TLXLFP motif have repressive activity and might act as transcriptional repressors in plants.

DISCUSSION

The WUS Box Is a Functional Domain of WUS

We have demonstrated here that the WUS box is critical for each known function of WUS: namely, complementation of *wus-1* mutants, maintenance and induction of stem cell identity, regulation of the size of shoot and floral meristems, and regulation of the expression of downstream genes, including *ARRs* and *AG*, which are direct targets of WUS. By contrast, the EAR-like motif and the acidic region are not essential for the biological functions of WUS, although the EAR-like motif did have repressive activity in the transient expression assay when WUS was fused with the GAL4 DNA binding domain (Figure 1). The EAR-like motif might, however, support the repressive activity of WUS to some extent because mutation of the EAR-like motif (WUSm2) weakened the activities of WUS, such as its ability to induce the expression of the *CLV3* gene and the formation of shoots and embryos on seedlings, which are associated with the ectopic expression of *WUS*, and complement the *wus-1* mutation. One possible explanation for the inactivity of the EAR-like motif in the WUS protein is related to the position of the motif. We found that, when the SRDX motif was fused to the C-terminal end of WUSm1 (WUSm1SRDX), the resultant derivative had lower activity than when the motif was fused to the N terminus (SRDXWUSm1; Figure 6). This difference suggests that the activity of a repression domain might be weakened when it is located at the C-terminal end of WUS.

WUS Acts Mainly as a Repressor

WUS is one of the factors that induce and maintain stem cell identity in shoot meristems of plants (Laux et al., 1996; Zuo et al., 2002; Gallois et al., 2004). We have shown here that the repressive activity of WUS, derived from the WUS box, is necessary for these activities of WUS (Figures 1 to 4) and that exogenous repressive activity complemented all the activities associated with the WUS box, with the exception of the activation of *AG*, in transgenic plants (Figures 6 and 7). Our results demonstrate clearly that WUS is an active repressor and that the WUS box acts as a repression domain that is necessary for the induction and maintenance of shoot stem cell identity in shoots and floral meristems.

The repressive activity of WUS in transcription is essential for the induction of cell pluripotency and for the positive regulation of the size of meristems. WUS might regulate these phenomena by repressing the expression of genes that positively regulate the differentiation of cells or that negatively regulate the pluripotency of stem cells. Although the direct target of WUS that controls cell pluripotency remains to be identified, our molecular characterization of WUS provides new insight into the regulation of pluripotency of plant cells. Transcription factors that contain a

Figure 8. (continued).

(A) Alignments of WUS boxes and EAR-like motifs from *WUS* orthologs. Conserved sequences are shown below the alignments.

(B) Alignment of the WUS boxes of WOX proteins from *Arabidopsis*. The conserved sequence is shown below the alignment.

(C) Relative luciferase activities after cobombardment of *Arabidopsis* leaves with GAL4DB-fused WOX effectors and the *Pro35S-GAL4:LUC* reporter gene. The relative activity due to *Pro35S:GAL4DB* (GAL4DB; gray bar) was set as 1. Error bars indicate SD ($n = 3$).

(D) Phylogenetic tree of *WOX* genes from *Arabidopsis* (boldface), *Oryza sativa* (Os), *Physcomitrella patens*, *Selaginella moellendorffii*, *Ostreococcus tauri*, and *Ostreococcus lucimarinus.* The numbers next to the braches indicate percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). WOX proteins with WUS box are shown in gray region. Bar with 0.1 represents evolutionary distance of 0.1 amino acid substitutions per site. Accession numbers and detail of coding sequences (CDs) used in the phylogeny are shown in Supplemental Table 2 online. The alignment used to generate this tree is available as Supplemental Data Set 1 online.

WUS box are found only in plants. Thus, the mechanisms for the induction and maintenance of populations of pluripotent stem cells by WUS might be specific to plants.

WUS Acts as a Bifunctional Transcription Factor in the Formation of Flowers

WUS acts as a repressor of transcription, but it also acts as a direct activator of the expression of the *AG* gene (Lohmann et al., 2001). Our experiments with the *ProAG:GUS* reporter and various constructs in which expression of *WUS* or its derivatives was driven by the promoter of the *LFY* gene revealed that the WUS box is necessary for the activation of *AG* in floral organs (Figure 3). The SRDX repression domain was able to complement loss of WUS box activity in the WUSm1 mutant protein, and in *ProLFY:WUSm1SRDX* plants, we observed enlarged floral meristems similar to those observed upon the ectopic expression of WUS. However, the SRDX motif did not appear to restore the ability to activate expression of *AG* because the flowers of *ProLFY:WUSm1SRDX* plants formed abnormally large numbers of petals and sepals (Figures 7B and 7D). By contrast, fusion of the VP16 activation domain to WUSm1 resulted in formation of carpeloid sepals and staminoid petals, as observed in flowers upon ectopic expression of *AG*. However, *ProLFY:WUSm1VP16* did not increase the size of floral meristems (Figures 7E and 7G; see Supplemental Figures 1E and 1F online). Our domain complementation analyses showed that WUS is a bifunctional transcription factor, which acts as a repressor in the regulation of the size of floral meristems and as an activator only in the regulation of the expression of *AG*. The WUS box is involved in both activities, although we have observed only the repressive activity but not the activation activity of WUS box. The change in function of WUS might occur at a very limited site and stage during the formation of flowers.

In animals, there are transcriptional factors that act as both transcriptional activators and repressors, depending on the target gene (Adkins et al., 2006). In plants, the transcriptional activator of tomato (*Solanum lycopersicum*), known as Pti4, represses the expression of PR-10a by forming a complex with the SEBP repressor (González-Lamothe et al., 2008). *Arabidopsis* WRKY53 is a transcription factor that activates or represses the expression of genes, depending on the nature of the promoter sequence of its target genes (Miao et al., 2004). However, neither the repressive activity of WRKY53 nor the evidence for functional conversion within a single molecule has been revealed. Unidentified factors that interact with the WUS box might convert it from a repressor to an activator when WUS binds to the second intron of the *AG* gene.

Evolution of Mechanisms for Maintenance of Stem Cell Populations in Plants

The repressive activity of the WUS box, which is associated with the conserved TLXLFP sequence, is necessary for the maintenance of stem cell populations in shoot meristems (Figures 4B and 8). To date, genes for WUS orthologs and WOX proteins that contain a WUS box with the TLXLFP core sequence have been found only in the genomes of seed plants (spermatophytes) and not in those of *Physcomitrella* (bryophytes; moss), *Selaginella* (pteridophytes; fern), and *Ostreococcus* (green alga) (Figure 8). Apparently, the mechanism for maintenance of shoot stem cell identity by WUS is specific to seed plants. Seed plants maintain multiple stem cells in their SAMs, while the SAMs of ferns, such as *Selaginella*, are very simple, with only one apical cell (stem cell) per SAM. Mosses, such as *Physcomitrella*, develop a SAM on individual gametophytic organs, and there is one gametophore shoot apical cell in each SAM of leafy shoots of mosses. Only seed plants have large populations of pluripotent stem cells in their meristems. The development and evolution of a mechanism for maintenance of populations of pluripotent stem cells might have involved acquisition of the WUS box. WOX orthologs in *Selaginella* have incomplete WUS box–like sequences (TLRLSP and TLEPLE; Figure 8), and it is possible that these sequences might have been the source of the WUS box.

METHODS

Construction of Plasmids

The protein-coding regions of the *WUS*, *STM*, and *CUC2* genes were amplified from an *Arabidopsis thaliana* cDNA library with appropriate primers (see Supplemental Table 1 online). The 5′ upstream region of 3003 bp, which extended from the site of initiation of translation of the *WUS* gene, was used for preparation of the *ProWUS:WUS* gene mutation series constructs. The 5′ upstream region of 2248 bp, which extended from the site of initiation of translation of the *LFY* gene, was used for preparation of the *ProLFY:WUS* mutation series and *ProLFY:GUS* gene constructs. The second intron of *AG* (~800 bp) was used for *ProAG:GUS* gene construct. The mutations in WUS (m1, m2, m1m2, and mAD) were introduced by use of appropriate mutagenic primers (see Supplemental Table 1 online). *35S:WUS-SRDX*, *STM-SRDX*, and *CUC2-SRDX*, were constructed using p35SSRDXG (Mitsuda et al., 2006). Synthetic sense and antisense DNAs of WRBX were annealed and introduced into p35SG vector and used for *STM*-WUSB and *CUC2*-WUSB constructs. The region corresponding to the transgene was transferred to the pBCKH plant expression vector (Mitsuda et al., 2006) using the Gateway system (Invitrogen). Effecter plasmids include the GAL4DB-coding region fused to the coding sequence for a variety of derivatives of WUS and WOX genes, in frame, under control of the CaMV 35S promoter (−800 to +8; CaMV35S). The reporter gene *Pro35S-GAL4:LUC* for transient assay contained a CaMV 35S promoter; five copies (5X) of the GAL4-response element; a minimal TATA region, starting at position −46, of the CaMV 35S promoter; the firefly gene for luciferase; and a nopaline synthase terminator (Fujimoto et al., 2000).

Growth and Transformation of Plants

Arabidopsis Col-0 was used in all experiments except in the case of the *wus-1* mutation (Laux et al., 1996), which was on the Landsberg *erecta* background. Plants were grown in soil at 22°C with 16 h of light daily. Transformations of *Arabidopsis* Col-0 and *wus-1* were performed by the floral dip method (Clough and Bent, 1998).

GUS Staining

Histochemical GUS assays were performed according to a method previously reported (Jefferson, 1987). Transgenic *Arabidopsis* tissues were dipped in a solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide), 50 mM NaH_2PO_4, and 0.1% Tween 20. The samples were incubated 6 h to overnight at 37°C.

Transient Expression Assays

The coding sequences of *WUS* and its mutant derivatives were fused separately to that for GAL4DB (Ohta et al., 2000), and transient expression assays were performed with *Arabidopsis* leaves after particle bombardment, as described previously (Hiratsu et al., 2004). We used 0.8 μg of reporter plasmid and 0.6 μg of effector plasmid for each bombardment. For normalization of the activity of the reporter gene, we used 0.8 μg of a reference plasmid, pPTRL (Fujimoto et al., 2000). After bombardment, samples were incubated for 10 h in darkness, and then luciferase activity was quantified.

Isolation of RNA and Analysis of RNA Expression

Total RNA was isolated with a Plant Mini kit (Qiagen) from 14-d-old seedlings of more than six independent plants in every case. Three micrograms of total RNA, which were treated with DNase I, were subjected to first-strand cDNA synthesis. Quantitative RT-PCR was performed by the SYBR green method using the ABI 7300 real-time PCR system (Applied Biosystems) as described previously (Mitsuda et al., 2005) with appropriate primers (see Supplemental Table 1 online). Relative amounts of transcripts were calculated by an absolute quantification method using the *UBQ1* gene as an internal control. More than three replicates were included in each experiment.

Phylogenetic Analysis of the WOX Family

All putative CDS sequences of WOX family were collected from transcript data sets of rice (*Oryza sativa*), *Physcomitrella patens* ssp *patens* ecotype Gransden, *Selaginella moellendorffii*, *Ostreococcus lucimarinus*, and *Ostreococcus tauri* by TBLASTN search. The CDS sequences that best hit to the *Arabidopsis* WOX family by TBLASTN search against the *Arabidopsis* CDS data set were defined as WOX family in this study. Each CDS data set (TAIR8_cds_20080412 for *Arabidopsis*, RAP2 representative and predicted sequences for rice, transcripts.Phypa1_1.FilteredModels3 for *Physcomitrella*, Selmo1_GeneModels_FilteredModels2_nt for *Selaginella*, O.lucimarinus.FM.na.fasta for *O.lucimarinus*, and O.tauri.FM.na.fasta for *O.tauri*) was downloaded from RAP-DB (http://rapdb.dna.affrc.go.jp/) or Joint Genome Initiative (http://www.jgi.doe.gov/). The accession numbers of sequences used in this analysis are listed in Supplemental Table 2 online. The amino acid sequences translated from collected CDSs were aligned using MAFFT (Katoh et al., 2002) by default settings and manually edited in WUS box and EAR motif followed by local alignment using ClustalW (Chenna et al., 2003). The alignment is available as Supplemental Data Set 1 online. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), and the reliability of each cluster was measured by bootstrap test (1000 replicates) (Felsenstein, 1985) with MEGA4 by default settings (Tamura et al., 2007).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *WUS* (At2g17950), *ABI3* (At3g24650), *LEC1* (At1g21970), *FUS3* (At3g26790), *CLV3* (At2g27250), *ARR6* (At5g62920), *ARR7* (At1g19050), *AG* (At4g18960), *STM* (At1g62360), *CUC2* (At5g53950), *WOX1* (At3g18010), *WOX2* (At5g59340), *WOX3* (At2g28610), *WOX4* (At1g46480), *WOX5* (At3g11260), *WOX6* (At2g01500), *WOX7* (At5g05770), *WOX8* (At5g45980), *WOX9* (At2g33880), *WOX10* (At1g20710), *WOX11* (At3g03660), *WOX12* (At5g17810), *WOX13* (At4g35550), and *WOX14* (At1g20700). The accession numbers of CDSs used in the phylogeny are shown in Supplemental Table 2 online.

Supplemental Data

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

Supplemental Figure 1. Effect of Mutant WUS Constructs on Flowers.

Supplemental Figure 2. Seedlings of *Pro35S:STM* Transgenic Plants.

Supplemental Table 1. Oligonucleotides Used in This Study.

Supplemental Table 2. Accession Numbers and Detail of CDSs Used in the Phylogeny.

Supplemental Data Set 1. Text File of the Alignment Used to Generate the Phylogenetic Tree Shown in Figure 8D.

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