WUSCHEL-RELATED HOMEOBOX4 Is Involved in Meristem Maintenance and Is Negatively Regulated by the CLE Gene FCP1 in Rice[W]

Yoshihiro Ohmori,[a,1] Wakana Tanaka,[a] Mikiko Kojima,[b] Hitoshi Sakakibara,[b] and Hiro-Yuki Hirano[a,2]

[a] Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-8654, Japan
[b] RIKEN Plant Science Center, Tsurumi, Yokohama 230-0045, Japan

The shoot apical meristem is the ultimate source of the cells that constitute the entire aboveground portion of the plant body. In *Arabidopsis thaliana*, meristem maintenance is regulated by the negative feedback loop of *WUSCHEL-CLAVATA* (*WUS-CLV*). Although *CLV*-like genes, such as *FLORAL ORGAN NUMBER1* (*FON1*) and *FON2*, have been shown to be involved in maintenance of the reproductive meristems in rice (*Oryza sativa*), current understanding of meristem maintenance remains insufficient. In this article, we demonstrate that the *FON2-LIKE CLE PROTEIN1* (*FCP1*) and *FCP2* genes encoding proteins with similar CLE domains are involved in negative regulation of meristem maintenance in the vegetative phase. In addition, we found that *WUSCHEL-RELATED HOMEOBOX4* (*WOX4*) promotes the undifferentiated state of the meristem in rice and that *WOX4* function is associated with cytokinin action. Consistent with similarities in the shoot apical meristem phenotypes caused by overexpression of *FCP1* and downregulation of *WOX4*, expression of *WOX4* was negatively regulated by *FCP1* (*FCP2*). Thus, *FCP1/2* and *WOX4* are likely to be involved in maintenance of the vegetative meristem in rice.

INTRODUCTION

The aboveground aerial parts of plants are derived from the shoot apical meristem (SAM), and their development ultimately depends on SAM function. In the SAM, the stem cells divide and produce descendant cells both to self-maintain and to provide cells for the differentiation of lateral organs. The balance between self-maintenance and differentiation of the cells is essential for stem cell homeostasis in the SAM.

The genetic mechanism of this homeostasis is well understood in *Arabidopsis thaliana* (reviewed in Barton, 2010; Ha et al., 2010; Aichinger et al., 2012). Stem cell identity is positively regulated by WUSCHEL (WUS) protein, the expression of which is negatively regulated by the CLAVATA (CLV) signaling pathway, consisting of a peptide signal molecule processed from CLV3 protein and its receptors, such as CLV1 (Mayer et al., 1998; Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000; Reddy and Meyerowitz, 2005; Yadav et al., 2010). In turn, WUS promotes expression of *CLV3*, which is localized in the stem cell region. This negative feedback loop of WUS-CLV3 in the stem cell niche is the framework of SAM maintenance in *Arabidopsis*.

WUS encodes a type of homeobox protein, classified into the WOX protein family (Mayer et al., 1998). *CLV3* encodes a small protein that has a conserved CLE domain (Fletcher et al., 1999). *CLV3* is expressed in the apical domain of the central region of the meristem, whereas *WUS* is expressed in the region underneath the *CLV3*-expressing domain, called the organizing center (OC). After processing and chemical modifications, the peptide corresponding to the CLE domain acts as a signal molecule through three kinds of receptor complexes consisting of CLV1, CORYNE-CLV2, and RECEPTOR-LIKE PROTEIN KINASE2 (Clark et al., 1997; Kondo et al., 2006; Müller et al., 2008; Ogawa et al., 2008; Ohyama et al., 2009; Kinoshita et al., 2010). The signal is transmitted to the *WUS* gene via phosphorylation/dephosphorylation and unknown mechanisms (Williams et al., 1997; Song et al., 2006; Betsuyaku et al., 2011). The WUS protein moves from the OC to the stem cell region and directly binds the *CLV3* promoter to activate expression of *CLV3* (Yadav et al., 2011).

A phytohormone, cytokinin, is a key factor in stem cell homeostasis. *WUS* expression is positively regulated by cytokinin; in turn, *WUS* promotes cytokinin action by repressing the genes encoding negative regulators of cytokinin signaling, such as *ARABIDOPSIS* RESPONSE REGULATOR7 (ARR7) and ARR15 (Leibfried et al., 2005; Gordon et al., 2009). An active form of cytokinin produced by epidermal cells of the SAM acts as a positional cue to promote *WUS* expression together with ARABIDOPSIS HISTIDINE KINASE4, a cytokinin receptor, the expression domain of which is superimposed on the OC (Chickarmane et al., 2012). Thus, robust stem cell homeostasis can be achieved by a negative/positive feedback loop between CLV3/cytokinin and WUS.

In contrast with *Arabidopsis*, our understanding of stem cell maintenance is insufficient in other plants. Nevertheless, the genetic mechanism underlying the negative regulation of stem cell proliferation is likely to be conserved in both rice (*Oryza*

sativa) and maize (*Zea mays*). Mutations in the genes *FLORAL ORGAN NUMBER* (*FON*) in rice and *fasciated ear2* (*fea2*) and *thick tassel dwarf1* (*td1*) in maize cause an enlargement of the size of either the flower or inflorescence meristem, resulting in an increase in floral organ numbers or thickening of inflorescences (Taguchi-Shiobara et al., 2001; Suzaki et al., 2004; Bommert et al., 2005). Rice *FON1* and maize *td1* encode an Leucine-rich repeat–like receptor kinase that is closely related to *Arabidopsis* CLV1, and *fea2* encodes a CLV2-like Leucine-rich repeat protein. Rice *FON2/FON4* encodes a small secreted protein with a CLE domain related to that of CLV3 and is expressed in the apical region of the meristem in a CLV3-like pattern (Chu et al., 2006; Suzaki et al., 2006). Because no effect is observed when FON2 is overexpressed in the *fon1* mutant, the FON2 signal may be mediated through the FON1 receptor (Suzaki et al., 2006). In addition, it has been shown that *FON2 SPARE1* (*FOS1*), encoding another CLE protein, redundantly regulates stem cell maintenance with *FON2* in *indica* rice and wild species in the *Oryza* genus (*japonica* rice has a mutation in *FOS1*) (Suzaki et al., 2009). Thus, the CLV-like signaling pathway is likely to be involved in meristem maintenance in grasses. In addition to this pathway, cytokinin plays an important role in rice because loss of function of *LONELY GUY*, which encodes an enzyme responsible for conversion of the cytokinin precursor into active forms, results in failed maintenance of the floral meristem (Kurakawa et al., 2007).

In contrast with the reproductive phase, however, mutations in these genes do not bring about obvious defects in the vegetative phase in either rice or maize, suggesting that other genes are required for maintenance of the vegetative meristems. Putative candidates for these genes are *FON2-like CLE PROTEIN1* (*FCP1*) and *FCP2* in rice. Both *FCP1* and *FCP2* encode proteins with a CLE domain, very similar to that of FON2 (Suzaki et al., 2008). Regenerating shoots overexpressing *FCP1* fail to maintain the SAM. This effect is also observed in the *fon1* mutant background, suggesting that FCP1 may act through a receptor different from FON1. These findings suggest that at least two independent pathways are involved in the negative regulation of meristem maintenance in rice, depending on the type of the meristem (Suzaki et al., 2008, 2009). This situation contrasts with that in *Arabidopsis*, where a single CLV pathway regulates all types of meristem. In addition, the gene that promotes stem cell maintenance has not yet been identified in plants other than *Arabidopsis*.

Class 1 Knotted-like homeobox (KNOX) genes, such as *Arabidopsis SHOOT MERISTEMLESS* (*STM*), rice *ORYZA SATIVA HOMEOBOX1* (*OSH1*), and maize *Knotted1* (*Kn1*), promote the undifferentiated state of the meristem (Long et al., 1996; Sato et al., 1996; Vollbrecht et al., 2000; Tsuda et al., 2011). Loss-of-function mutants of *STM* and *Kn1* fail to form or maintain the meristem during embryogenesis (Long et al., 1996; Vollbrecht et al., 2000). In the rice *osh1* mutant, the SAM is formed but cannot be maintained after germination. In the double mutant of *osh1* and *d6*, which encodes the related homeobox protein OSH15, the SAM is not established, suggesting that both genes are responsible for formation or maintenance of the SAM in the embryo (Tsuda et al., 2011). *KNOX* genes promote cytokinin accumulation by upregulating the expression of genes for its

biosynthesis in both *Arabidopsis* and rice (Jasinski et al., 2005; Yanai et al., 2005; Sakamoto et al., 2006).

WUSCHEL-RELATED HOMEOBOX (*WOX*) genes are involved in various aspects of development in *Arabidopsis*, such as maintenance of the root apical meristem, embryogenesis, and vascular development (Haecker et al., 2004; Wu et al., 2005; Sarkar et al., 2007; Breuninger et al., 2008; Hirakawa et al., 2010; Ji et al., 2010; Suer et al., 2011). Among these genes, *WOX4* promotes proliferation of stem cells in the procambium/cambium in *Arabidopsis*, and *WOX4* itself is upregulated in response to TDIF (for tracheary element differentiation inhibitory factor) mediated by TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) (Hirakawa et al., 2008, 2010; Etchells and Turner, 2010; Ji et al., 2010; Suer et al., 2011). TDIF is a CLE peptide processed from proteins encoded by the CLE41 and CLE44 genes (Ito et al., 2006). In rice, *WOX* genes are involved in maintenance of the root apical meristem and crown root development (Kamiya et al., 2003; Zhao et al., 2009). Although the expression patterns of other *WOX* genes have been reported, current understanding of the function of *WOX* genes is insufficient in rice (Nardmann and Werr, 2006; Dai et al., 2007).

In this work, we first confirmed that *FCP1* is a negative regulator of meristem maintenance using inducible RNA silencing and overexpression experiments. Second, we revealed that rice *WOX4* acts positively on the indeterminacy of the meristem and is associated with cytokinin function. Third, the expression of *WOX4* was found to be negatively regulated by *FCP1*. Both induced overexpression of *FCP1* and induced RNA silencing of *WOX4* resulted in a reduction in the expression of *OSH1* and *FON2*, suggesting that *WOX4* is responsible for meristem maintenance by promoting the undifferentiated indeterminate state and by preventing premature differentiation.

RESULTS

FCP1 Overexpression Inhibits Meristem Maintenance

To examine the effect of constitutive expression of *FCP1*, we first introduced a construct that induces constitutive expression of *FCP1* (*pACT1-FCP1*) into rice calli derived from the scutellum and then regenerated shoots. The resulting shoots showed abnormal morphology and lacked a normal SAM structure (Figures 1C and 1D). Although this observation suggested that *FCP1* overexpression inhibits maintenance of the SAM, the detailed function of *FCP1* could not be determined from the analysis of regenerated shoots.

To elucidate further the function of *FCP1* and *WOX4* (see below), we developed an inducible expression system in rice using a modified pINDEX vector that induces the gene of interest in trans by application of dexamethasone (DEX) (see Supplemental Figure 1 online). In the DEX induction experiment, we first selected transformants (T0 plants) in which each transgene was effectively induced by application of DEX and then examined effect of DEX induction by comparing T1 siblings derived from the same T0 plant throughout in this study (see Methods).

T1 seedlings with *pACT1-GVG>FCP1* were germinated and grown for 7 d in the presence or absence of DEX. The shoot phenotype and the SAM morphology in seedlings without DEX
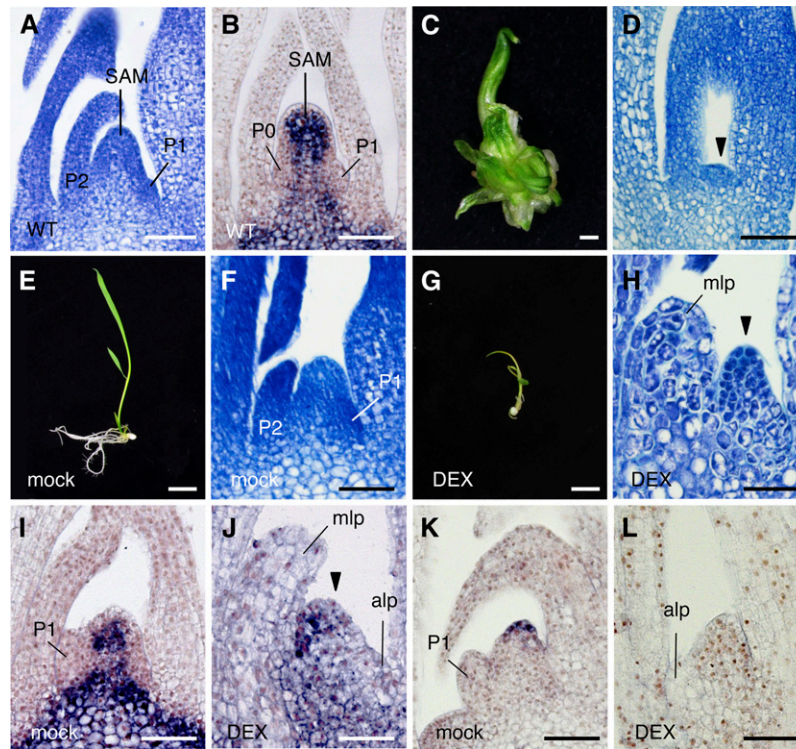
Figure 1. Effects of Constitutive and Inducible Overexpression of *FCP1*.

(A) and **(B)** Shoot apex **(A)** and in situ hybridization showing spatial expression pattern of *OSH1* **(B)** in a wild-type seedling grown in soil 30 d after germination.
(C) and **(D)** Generating shoot **(C)** with *pACT1-FCP1* and its shoot apex **(D)**. Arrowhead indicates a terminated meristem **(D)**.
(E) and **(F)** Transgenic shoot with *pACT1-GVG>FCP1* (without DEX).
(G) and **(H)** Transgenic shoot with *pACT1-GVG>FCP1* (10 µM DEX). Arrowhead indicates an abnormal meristem.
(I) and **(J)** Spatial expression pattern of *OSH1* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1*. Mock **(I)**; 50 µM DEX **(J)**. Arrowhead indicates an abnormal meristem.
(K) and **(L)** Spatial expression pattern of *FON2* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1*. Mock **(K)**; 50 µM DEX **(L)**. Seedlings were grown for 7 d from germination in the presence or absence of DEX (**[E]** to **[L]**).
alp, abnormal leaf primordia; mlp, malformed leaf primordia; P1-P5, plastochrons 1 to 5; WT, the wild type. Bars = 50 µm in **(A)**, **(B)**, **(D)**, **(F)**, and **(H)** to **(L)**, 1 mm in **(C)**, and 1 cm in **(E)** and **(G)**.

treatment were indistinguishable from those in seedlings under normal growth conditions (Figures 1A, 1B, 1E, and 1F). By contrast, abnormal shoots were produced in DEX-treated seedlings: The leaves were small and pale green, and crown roots were not formed (Figure 1G). Observation of a longitudinal section of the shoot apex revealed that the SAM was morphologically abnormal and exhibited a reduced number of cells. The leaf primordia were enlarged and had large vacuolated cells (Figure 1H).

Next, we examined two marker genes of the meristem, *OSH1* and *FON2*. *OSH1* was expressed in the meristem except for the L1 layer and leaf initiation sites in both seedlings grown in soil and those without DEX treatment (Figures 1B and 1I). Expression of *OSH1* was downregulated, and its spatial expression pattern was disturbed in the SAM in seedlings grown in the presence of DEX (Figure 1J). *FON2* is expressed in a small number of cells in the apical region of the meristem, probably corresponding to the stem cell region (Suzaki et al., 2006). This

expression pattern of *FON2* was confirmed in mock-treated seedlings (Figure 1K). By contrast, *FON2* expression disappeared completely from the SAM in DEX-treated seedlings (Figure 1L). These results suggest that stem cell maintenance is compromised by induced overexpression of *FCP1*.

RNA Silencing of *FCP1* and *FCP2*

We tried to examine the effect of loss of function of *FCP1*. Despite extensive screening, however, we failed to obtain any knockout lines of *FCP1*. We therefore inhibited expression of the endogenous *FCP1* gene by inducible RNA silencing (see Supplemental Figure 2 online; *pACT1-GVG>FCP1:RNAi*) using a pAID3 vector, which was made in this study (see Supplemental Figure 1 online). Although slight abnormalities, such as twisted leaves, were observed in the seedlings with induced silencing of *FCP1*, the SAM was largely normal in morphology and size, and leaf primordia were initiated normally (Figures 2A and 2B). We

hypothesized that this finding was due to the existence of another CLE gene, *FCP2*, the CLE domain of which is highly similar to that of FCP1 with only one amino acid difference (Suzaki et al., 2006).

We therefore made a construct to silence *FCP1* and *FCP2* simultaneously using pAID3 (*pACT1-GVG>FCP1-FCP2:RNAi*) (see Supplemental Figure 2 online) and examined transgenic plants (T1) with *pACT1-GVG>FCP1-FCP2:RNAi* (Figures 2C to 2P). A longitudinal section revealed that leaf initiation was inhibited: P1 and P2 leaf primordia were not detected in the DEX-treated seedlings (Figure 2F). The P3 and P4 leaves that were observed would have been formed before a significant effect of cosilencing of *FCP1* and *FCP2*. Similar morphological abnormalities in the SAM and leaf primordia were observed in other

DEX-treated seedlings (see Supplemental Figures 3A to 3G online). In rice, three true leaves are formed in embryogenesis (Itoh et al., 2005). Therefore, the phenotype of small yellow seedlings suggests that growth of these leaves is inhibited by cosilencing of *FCP1* and *FCP2* (Figure 2E).

OSH1 was expressed more strongly and widely in DEX-treated seedlings with *pACT1-GVG>FCP1-FCP2:RNAi* than in mock-treated seedlings (Figures 2G and 2I). In mock-treated seedlings, *OSH1* expression was downregulated at the future leaf initiation site (P0; Figures 2G, 2H, and 2N), as in normal seedlings (Figure 1B). This downregulation of *OSH1* was invariably detected in mock-treated seedling (see Supplemental Figures 4A to 4D online). By contrast, *OSH1* was ectopically expressed at this P0 site in DEX-treated *pACT1-GVG>FCP1-FCP2:RNAi*
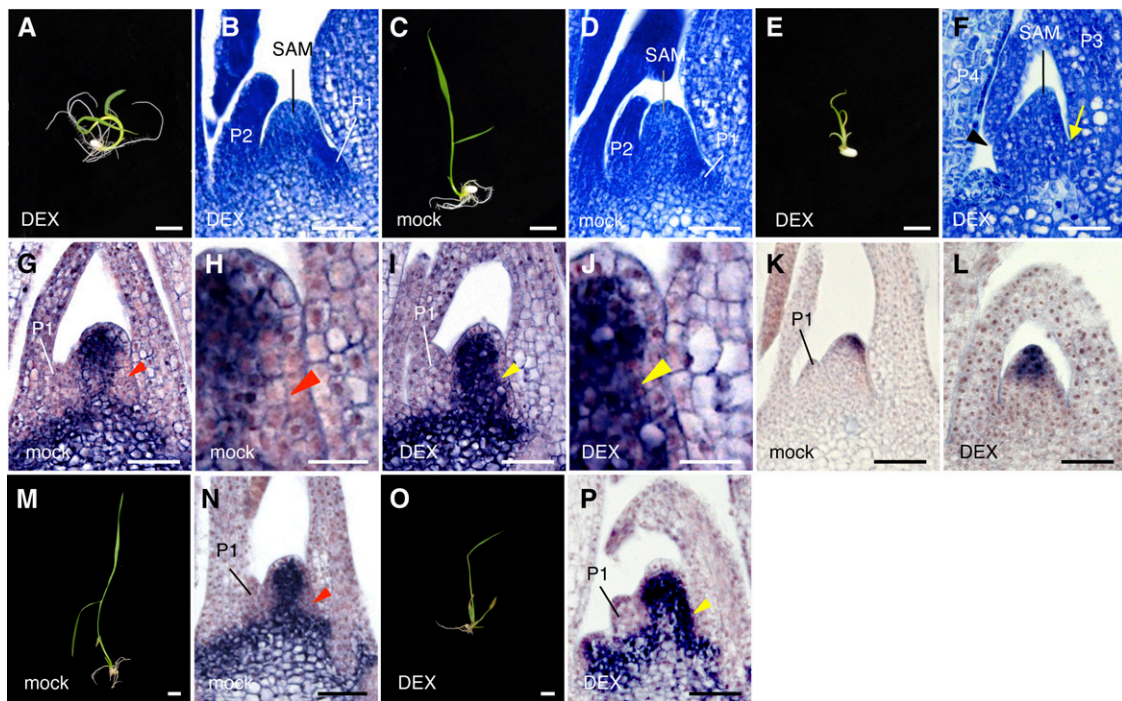

Figure 2. Effects of Inducible Silencing of *FCP1* and Cosilencing of *FCP1-FCP2*.

(A) and **(B)** Transgenic shoot with *pACT1-GVG>FCP1:RNAi* (25 µM DEX).

(C) and **(D)** Transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (mock).

(E) and **(F)** Transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (25 µM DEX). Arrow and arrowhead indicate the positions for the formation of P1 and P2 primordia, respectively. Note that no leaf primordium is observed in these positions.

(G) and **(H)** Spatial expression pattern of *OSH1* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (mock). **(H)** is a magnified view of part of **(G)**. *OSH1* is downregulated in the P0 site (red arrowheads).

(I) and **(J)** Spatial expression pattern of *OSH1* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (50 µM DEX). **(J)** is a magnified view of part of **(I)**. Note that *OSH1* is expressed in the P0 site (yellow arrowheads).

(K) Spatial expression pattern of *FON2* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (mock).

(L) Spatial expression pattern of *FON2* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (50 µM DEX).

(M) and **(N)** Shoot **(M)** and spatial expression pattern of *OSH1* **(N)** in a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (mock). *OSH1* is downregulated in the P0 site (red arrowhead in **[N]**).

(O) and **(P)** Shoot **(O)** and spatial expression pattern of *OSH1* **(P)** in a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi* (25 µM DEX). Yellow arrowhead in **(P)** indicates the P0 site, where *OSH1* is ectopically expressed.

Seedlings were grown for 7 d from germination in the presence or absence of DEX (**[A]** to **[L]**) or for 5 d from germination in MS medium and treated without or with DEX for 5 d (**[M]** to **[P]**). Bars = 1 cm in **(A)**, **(C)**, **(E)**, **(M)**, and **(O)**, 50 µm in **(B)**, **(D)**, **(F)**, **(G)**, **(I)**, **(K)**, **(L)**, **(N)**, and **(P)**, and 20 µm in **(H)** and **(J)**.

seedlings (Figures 2I and 2J). We often observed this ectopic *OSH1* expression in other DEX-treated seedlings with this construct (see Supplemental Figures 4E to 4G online). These observations suggest that the SAM fails to recruit leaf founder cells and that the undifferentiated state was maintained throughout the meristem due to cosilencing of *FCP1* and *FCP2*. In addition, expression of *FON2* was elevated and the expression domain was expanded in the DEX-treated seedlings (Figure 4L), compared with the mock-treated ones (Figure 4K), suggesting that the stem cell population was likely to be increased.

We then checked the effect of DEX treatment on the SAM in a mild condition. Seedlings were grown for 5 d in the absence of DEX, transferred to a new solution containing 25 μM DEX, and then grown for a further 5 d. The apparent shoot phenotype was largely normal, although the leaves were a bit smaller (cf. Figure 2O with Figure 2M). However, in these seedlings, *OSH1* was also strongly expressed at the P0 site (Figure 2P), confirming the above result.

Taking into account the results of induced *FCP1* overexpression, *FCP1* seems to be a negative regulator of meristem maintenance.

Identification of *WOX4* and Its Spatial Expression Patterns

Because *FCP1* negatively regulates stem cell maintenance, it is possible that genes responsible for the positive regulation of meristem maintenance would be downregulated in the regenerating shoots with *pACT1-FCP1* (Figure 1C). Here, we focused on the rice *WOX* genes, especially those encoding proteins containing a WUS box (Haecker et al., 2004). We examined expression levels of *WOX* genes in *pACT1-FCP1* regenerating shoots by quantitative real-time PCR (qRT-PCR) analysis. We found that *WOX4*, an ortholog of *Arabidopsis WOX4* (Nardmann and Werr, 2006), was significantly downregulated in the regenerating shoots with *pACT1-FCP1*, compared with those with empty vector (Figure 3). An increase in *WOX5* expression was observed in *pACT1-FCP1* shoots. This upregulation probably results from the ectopic expression of *WOX5* in the adventitious root primordia, which were included in the regenerating shoot samples, because the *WOX5* (*QHB*) expression domain was highly expanded by the application of FCP1 CLE peptide in our previous study (Suzaki et al., 2008). The change in the levels of *WUS* expression was not statistically significant (Figure 3).

Thus, we focused on *WOX4* for further work. In rice and *Arabidopsis*, WOX4 proteins are well conserved with respect to not only homeoboxes, but also 5′WOX1/4 and WUS boxes (see Supplemental Figure 5 online) (Haecker et al., 2004; Vandenbussche et al., 2009). We analyzed the spatial expression patterns of *WOX4* by in situ hybridization. In the shoot apex, *WOX4* was expressed in the meristem and the leaf primordia in the vegetative shoot apex (Figures 4A and 4B). No signal was detected when sense probe was used (Figure 4C). Expression of *WOX4* was also detected in the axillary meristems (Figures 4D and 4E). Although *WOX4* was expressed in the whole region of the P1 and P2 leaf primordia, its expression was restricted to narrow regions in subsequent leaf development (Figures 4F and 4H). Rice develops two types of vasculature, large and small vascular
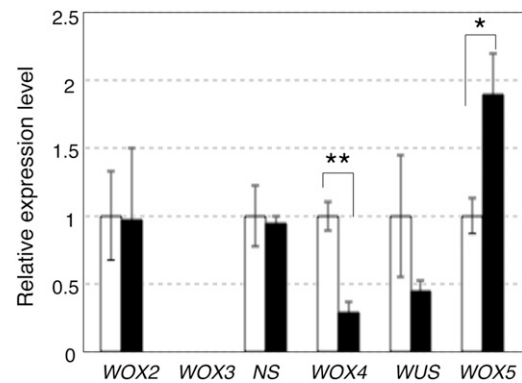

Figure 3. Real-Time PCR Analysis of Rice *WOX* Genes.

RNA was isolated from regenerating shoots with empty vector (white) or those with *pACT1-FCP1* (black) (see Figure 1C), and the relative expression of each *WOX* gene was measured by real-time PCR (*n* = 4; four RNA samples independently isolated). Student's *t* test; **P < 0.01 and *P < 0.05. The difference in *WUS* expression is not statistically significant.

bundles, in the leaf (Nishimura et al., 2002). In the P3 primordia, *WOX4* expression was detected in the putative procambium of the large vascular bundle (Figure 4H). *WOX4* was subsequently expressed in developing large vascular bundles (Figures 4J and 4K), and the expression became restricted to the cells between the xylem and the phloem in a later stage (Figure 4M). *WOX4* expression was also detected in putative procambium of the small vascular bundle in P4 and P5 (Figures 4F and 4K). These *WOX4* signals were not detected when sense probe was used (Figures 4G, 4I, and 4L). These observations suggest that *WOX4* is also involved in vascular development in rice, as in other plants such as *Arabidopsis*.

In the reproductive phase, *WOX4* was expressed predominantly in the inflorescence and branch meristems (Figure 4N). During flower development, strong expression was detected in the floral meristem, whereas weaker expression was observed in the primordia of the lateral floral organs such as the lemma and sterile lemma (Figure 4O).

Downregulation of *WOX4* Fails to Maintain the SAM

To elucidate the function of *WOX4*, we first downregulated *WOX4* expression constitutively. Although shoot-like structures were regenerated from calli with *pACT1-WOX4:RNAi*, the morphology of these structures was abnormal (Figure 5A). A cross section of the shoot apex revealed that the SAM was reduced in size or flattened, suggesting premature termination of the meristem (Figures 5B and 5C). These shoot phenotypes and the SAM morphology in *pACT1-WOX4:RNAi* plants was similar to those in *pACT1-FCP1* plants (Figures 1C and 1D), suggesting that *FCP1* and *WOX4* play opposite functions in maintaining the SAM of regenerated shoots.

We then examined the effect of silencing *WOX4* using an inducible RNA interference (RNAi) system (see Supplemental Figure 2 online; *pACT1-GVG>WOX4:RNAi*). qRT-PCR analysis indicated that *WOX4* was clearly downregulated by the application
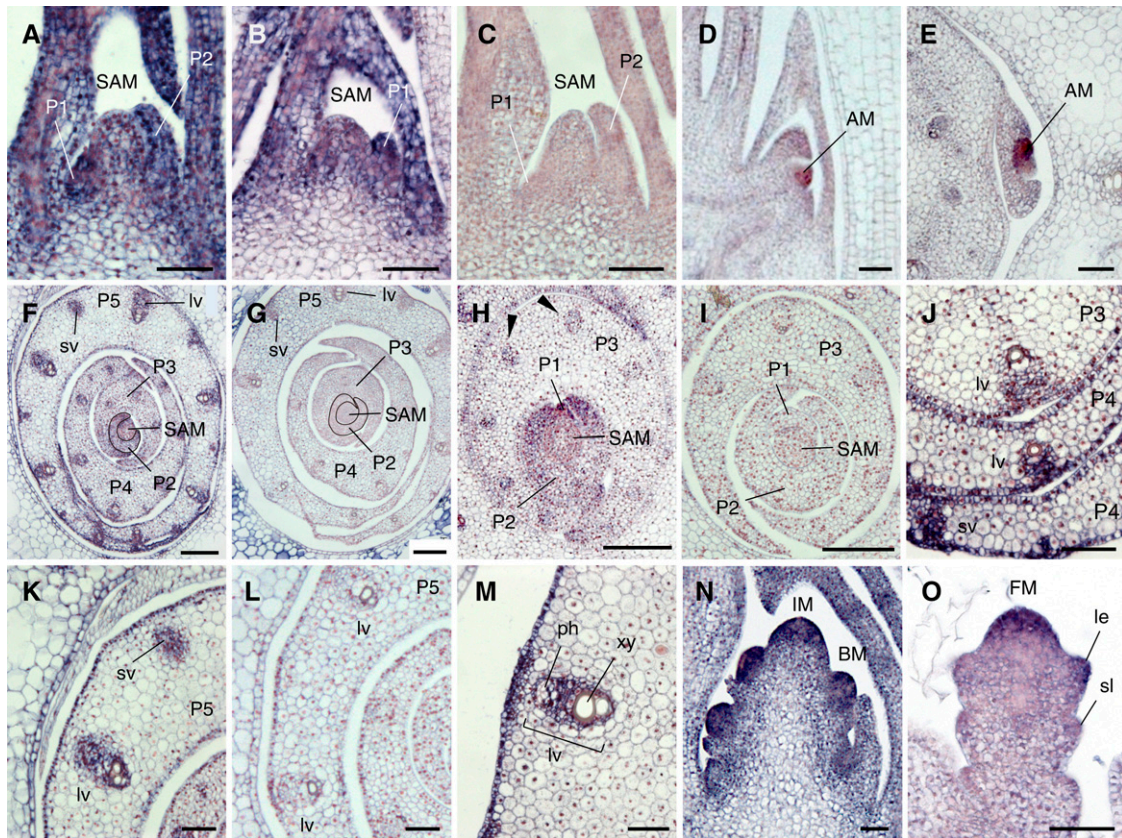
Figure 4. Temporal and Spatial Expression Pattern of *WOX4* in the Shoot Apex and Developing Leaf.

(A) and **(C)** Longitudinal section of the shoot apex of a seedling grown in soil for 14 d.

(B) Longitudinal section of the shoot apex of a control seedling grown in MS medium without DEX 7 d after germination.

(D) and **(E)** Longitudinal **(D)** and transverse **(E)** section of an axillary bud.

(F) to **(I)** Transverse section of the shoot apex with leaf primordia and developing young leaves. Arrowheads in **(H)** indicate putative procambium. P2 primordium is outlined in **(F)** and **(G)**.

(J) to **(M)** Close-up view of vascular bundles in developing young leaves.

(N) Longitudinal section of a young inflorescence.

(O) Longitudinal section of a developing flower.

(A), **(B)**, **(D)** to **(F)**, **(H)**, **(J)**, **(K)**, and **(M)** to **(O)** Antisense probe of *WOX4*.

(C), **(G)**, **(I)**, and **(L)** Sense probe of *WOX4*.

AM, axillary meristem; BM, branch meristem; FM, floral meristem; IM, Inflorescence meristem; lv, large vascular bundle; le, lemma; ph, phloem; P1 to P5, plastochrons 1 to 5; sl, sterile lemma; sv, small vascular bundle; xy, xylem. Bars = 50 µm in **(A)** to **(E)** and **(J)** to **(O)** and 100 µm in **(F)** to **(I)**.

of DEX in *pACT1-GVG>WOX4:RNAi* lines (see Supplemental Figure 6A online). When *pACT1-GVG>WOX4:RNAi* transgenic plants were grown in the presence of DEX for 7 d from germination, the shoots were very small (Figure 5G), compared with mock-treated plants (Figure 5D). In addition, the SAM became smaller and morphologically abnormal, and the leaf primordium did not initiate properly (Figures 5H and 5I; compare with mock-treated shoot apex in Figures 5E and 5F). In addition, gaps were observed among the cells (Figure 5H). These gaps were observed in other *pACT1-GVG>WOX4:RNAi* transgenic seedlings treated with DEX (see Supplemental Figure 7 online) but not observed in the mock-treated seedlings (Figure 5E). The formation of gaps may reflect uncoordinated growth of cells and tissues in the plants with a reduced level of *WOX4*.

In situ experiments showed that *OSH1* expression was downregulated in the SAM of these DEX-treated shoots (Figures 5J to 5L). This *OSH1* downregulation was also observed similarly in the shoot apex of seedlings that were mildly treated with DEX (Figures 5M to 5O). Expression of *FON2* was also downregulated by DEX treatment (Figures 5P to 5R). These results suggest that *WOX4* is required for maintenance of the undifferentiated cell state and stem cell identity in rice.

In addition to the abnormalities in the SAM, cells in the leaf primordia were enlarged and vacuolated, implying that expression of *WOX4* in the leaf primordia is necessary for normal development of the leaf (Figures 5K, 5L, 5Q, and 5R). These results resembled those of the experiments using *pACT1-GVG>FCP1* plants.
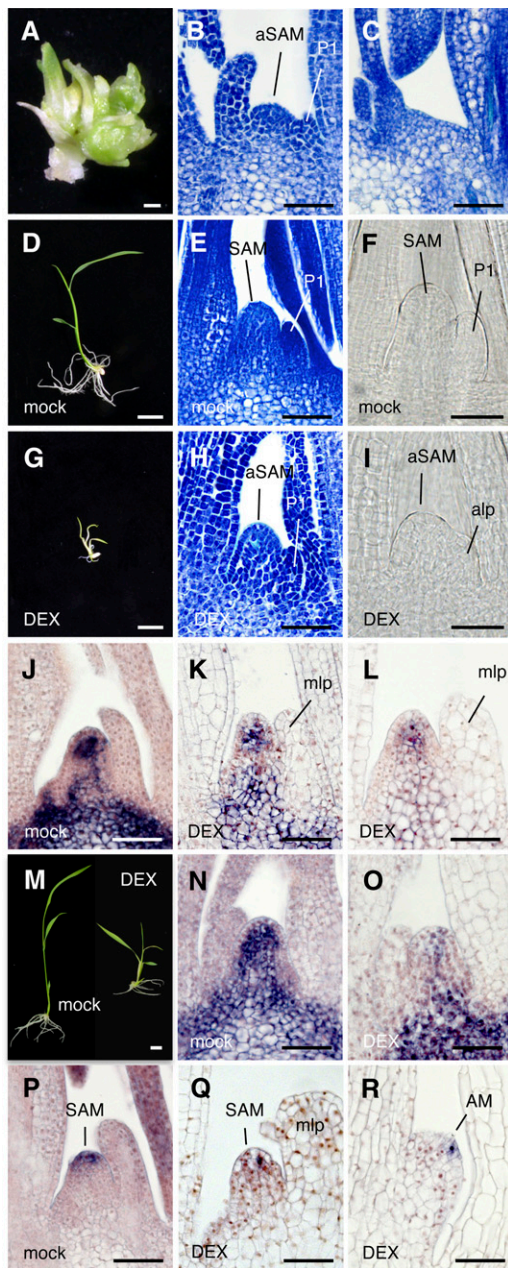
Figure 5. Effects of Constitutive and Inducible RNA Silencing of *WOX4*.

(A) to **(C)** Transgenic shoot with *pACT1-WOX4:RNAi*.

(D) to **(F)** Transgenic shoot with *pACT1-GVG>WOX4:RNAi* (mock).

(G) to **(I)** Transgenic shoot with *pACT1-GVG>WOX4:RNAi* (25 μM DEX).

(J) to **(L)** Spatial expression pattern of *OSH1* in the shoot apex of a transgenic shoot with *pACT1-GVG>WOX4:RNAi*. Mock **(J)**; 25 μM DEX (**[K]** and **[L]**).

(M) Shoot phenotypes of a transgenic shoot with *pACT1-GVG>WOX4: RNAi*.

(N) and **(O)** Spatial expression pattern of *OSH1* in the shoot apex of a transgenic shoot with *pACT1-GVG>WOX4:RNAi*. Mock **(N)**; 25 μM DEX **(O)**.

(P) and **(Q)** Spatial expression pattern of *FON2* in the shoot apex of a transgenic shoot with *pACT1-GVG>WOX4:RNAi*. Mock **(P)**; 25 μM DEX **(Q)**.

Taking these results altogether, it is likely that *WOX4* is a positive regulator of meristem maintenance and probably antagonizes *FCP1* function.

FCP1 Negatively Regulates *WOX4* Expression

To examine the genetic relationship between *WOX4* and *FCP1*, we examined the expression pattern of *WOX4* after induced overexpression or silencing of *FCP1*. In the presence of DEX, expression of *WOX4* was downregulated in both the meristem and the leaf primordia of transgenic seedlings carrying *pACT1-GVG>FCP1* (Figures 6A and 6B). By contrast, *WOX4* expression was upregulated in transgenic seedlings with *pACT1-GVG>FCP1-FCP2:RNAi* (Figures 6C and 6D). Strong downregulation of *WOX4* was observed in abnormal shoot-like structures formed in calli constitutively expressing *FCP1* (Figures 6E and 6F). These results suggest that expression of *WOX4* is negatively regulated by *FCP1*.

To confirm negative regulation of *WOX4* by *FCP1*, we performed qRT-PCR analysis. The results indicated that induction of *FCP1* by the application of DEX caused downregulation of *WOX4* expression (see Supplemental Figure 6B online), confirming the results of in situ analysis. By contrast, *WOX4* was not changed by DEX application in the *pACT1-GVG>FCP1-FCP2: RNAi* seedlings (see Supplemental Figure 6C online). This inconsistency seems to result from a difference in temporal expression pattern of *WOX4* and *FCP1* in leaf development: *FCP1* expression is restricted to only the P1 and P2 primordia (Suzaki et al., 2008), whereas *WOX4* expression was maintained in the later stages of leaf development (Figure 4). The young leaves, in which *WOX4* expression should not be regulated by *FCP1*, occupied a large portion of the shoot apices used for the qRT-PCR experiments.

Constitutive Expression of *WOX4* Mimics Cytokinin Action in Calli

Next, we examined the effects of constitutive expression of *WOX4*. Calli with *pACT1-WOX4* proliferated both in the callus induction medium and in the selection medium, as did ordinary calli, such as nontransformed calli or calli transformed with genes that do not inhibit shoot regeneration. After transferring the calli into regeneration medium, the ordinary calli produced green spots, from which shoots were regenerated. By contrast, although the calli with *pACT1-WOX4* produced green spots,

(R) Spatial expression pattern of *FON2* in the axillary bud of a transgenic shoot with *pACT1-GVG>WOX4:RNAi* (25 μM DEX).

The seedlings were grown for 7 d from germination in the presence or absence of DEX (**[D]** to **[L]** and **[P]** to **[R]**) or for 5 d from germination in MS medium and treated without or with DEX for 5 d (**[M]** to **[O]**). alp, abnormal leaf primordia; AM, axillary meristem; aSAM, abnormal SAM; mlp, malformed leaf primordia. Bars = 1 mm in **(A)**, 1 cm in **(D)**, **(G)**, and **(M)**, 50 μm in **(B)**, **(C)**, **(E)**, **(F)**, **(H)** to **(L)**, and **(N)** to **(R)**.
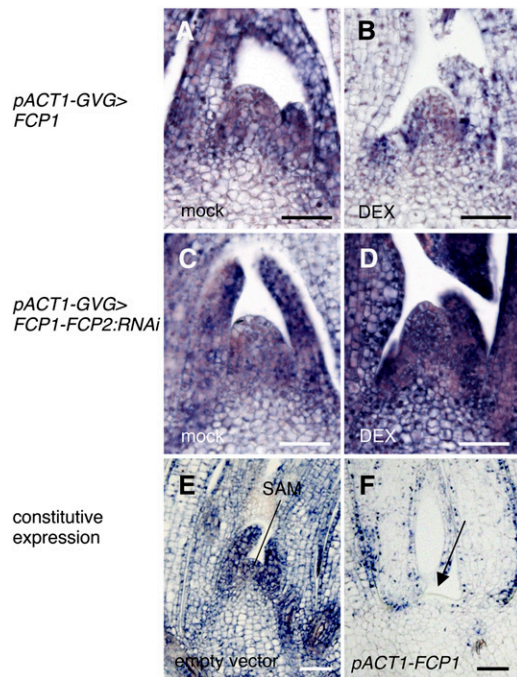
Figure 6. Analysis of the Effect of the *FCP1* and *FCP2* Genes on the Expression of *WOX4*.

(A) and **(B)** Expression of *WOX4* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1*. Mock **(A)**; 10 µM DEX **(B)**.
(C) and **(D)** Expression of *WOX4* in the shoot apex of a transgenic shoot with *pACT1-GVG>FCP1-FCP2:RNAi*. Mock **(C)**; 10 µM DEX **(D)**.
(E) and **(F)** Expression of *WOX4* in the shoot apex of regenerating shoot with empty vector **(E)** or *pACT1-FCP1* **(F)**. Arrow indicates a terminated meristem **(F)**.
Bars = 50 µm.

they either remained green without regeneration or regenerated morphologically abnormal shoot-like structures (Figure 7A).

The phenotypes of *WOX4*-expressing calli were similar to those of calli grown on culture medium containing a high concentration of cytokinin (Figure 7B). Under an appropriate concentration of cytokinin (8 µM), shoots were regenerated. At higher concentrations of cytokinin, by contrast, normal shoot formation was inhibited or only green calli proliferated. Thus, this finding raised the possibility that green calli or abnormal shoot-like structures overexpressing *WOX4* contained a higher content of cytokinin.

We therefore determined the content of cytokinin in the calli. Although the content varied depending on the callus sample (probably due to the state of individual calli), overall, cytokinins, including active forms such as isopentenyl adenine (iP) and *cis*-zeatin (cZ), accumulated at higher levels in *WOX4*-expressing calli than in calli with control vector (Figure 7C; see Supplemental Table 1 online). Whereas iP and *trans*-zeatin (tZ)-type cytokinins are the major forms in *Arabidopsis*, cZ is also an active cytokinin in rice and maize (Veach et al., 2003; Kudo et al., 2012). The level of tZ was undetectable in calli with *pACT1-WOX4* and in those with empty vector (see Supplemental Table 1 online). Consistent

with the higher cytokinin levels, shoots were regenerated from *WOX4*-expressing calli that were placed on hormone-free media, whereas the ordinary calli did not show any shoot regeneration (Figure 7D).

We tested the effect of FCP1 peptide on calli expressing *WOX4*. Calli with abnormal shoot-like structures that had been grown on the regeneration agar medium for 1 month were transferred into regeneration liquid medium and grown in the presence or absence of FCP1 peptide (20 µM) for 7 d with shaking (Figure 7E). As a result, normal shoots with expanded leaves were grown from the abnormal shoot-like structures in the presence of FCP1 peptide, but such shoots were not observed in the absence of FCP1 peptide. Thus, it seems likely that FCP1 peptide suppresses the effect of *WOX4* overexpression in the abnormal shoot-like structures.

DISCUSSION

FCP1/FCP2 Is Likely to Be a Negative Regulator of Meristem Maintenance in Rice

We have shown that, similar to *CLV3* in *Arabidopsis*, *FON2* negatively regulates stem cell identity in the floral meristem of rice (Chu et al., 2006; Suzaki et al., 2006). Although the floral meristem is strongly affected in null *fon2* mutants or *FON2*-overexpressing transgenic plants, no defects are observed in the meristem of their vegetative phases, implying that there are other factors that regulate maintenance of the vegetative SAM. In a previous study (Suzaki et al., 2008), we suggested that *FCP1* is a candidate to be such a factor, based on the observation that regenerated shoots overexpressing *FCP1* fail to maintain the SAM. In this article, using an inducible gene expression system, we demonstrated that FCP1 is a negative regulator in maintenance of the vegetative SAM. First, induction of *FCP1* in seedlings germinated from seeds inhibited maintenance of the SAM that had formed normally during embryogenesis. Second, cosilencing of *FCP1* and *FCP2* resulted in promotion of meristem activity. The shoot phenotypes and the SAM morphologies in both *FCP1*-overexpresssing and *FCP1/2*-silenced plants are consistent with changes in the expression of meristem markers, such as *OSH1* and *FON2*.

OSH1 promotes indeterminacy of the meristem and maintains the undifferentiated state of the cells (Sato et al., 1996; Tsuda et al., 2011). A loss-of-function mutant of *OSH1* fails to maintain the SAM after germination (Tsuda et al., 2011). A reduction or enhancement in the expression levels of *OSH1* was observed in the vegetative SAM of *FCP1*-overexpressing or *FCP1/2*-silenced plants, respectively. It is therefore likely that *OSH1* may be involved in the action of FCP1/FCP2 in the meristem to maintain the undifferentiated state of cells. In addition, failed downregulation of *OSH1* in the leaf founder cells (P0 site) was observed in *FCP1/2*-silenced plants. This suggests that *FCP1/2* probably acts not only on the central region of the meristem, but also on the peripheral region where leaves initiate. Thus, *FCP1/2* may be required to function in the downregulation of *OSH1* in the P0 site to establish founder cells for leaf differentiation in wild-type rice.
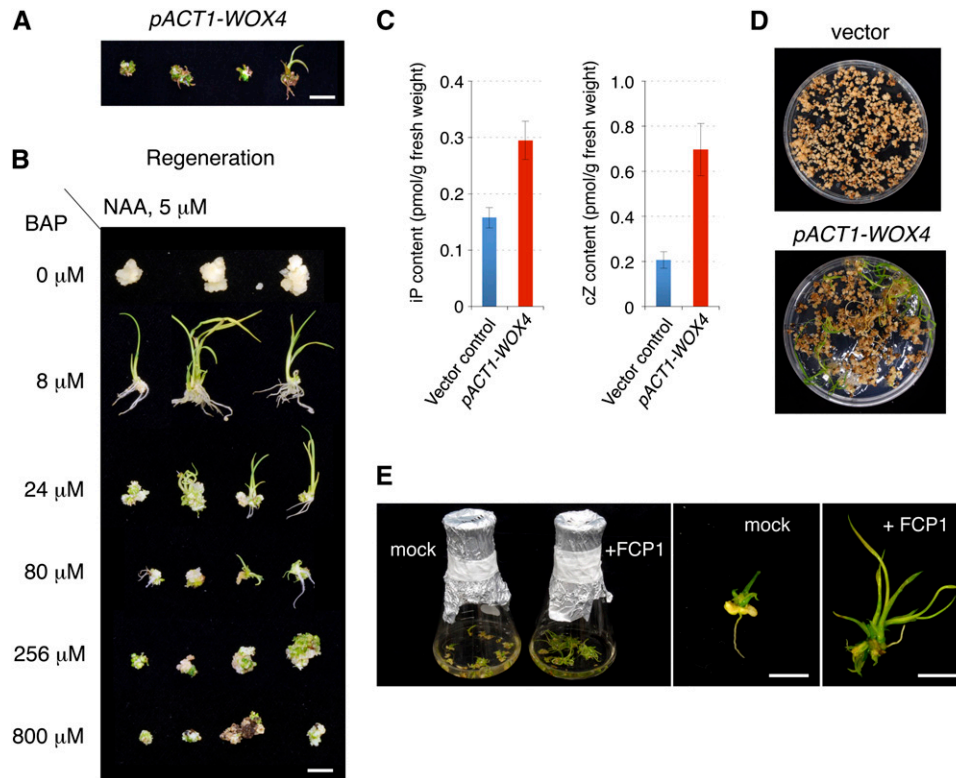
Figure 7. *WOX4* Overexpression and Cytokinin Action.

(A) Phenotypes of calli constitutively overexpressing *WOX4*.

(B) Phenotypes of calli and regenerating shoots grown on different concentrations of BAP. NAA, naphthalene acetic acid.

(C) Content of iP and cZ in calli with empty vector (blue) or with *pACT1-WOX4* (red). tZ was undetectable in either type of calli. Error bars indicate SE; *n* = 6.

(D) Shoot formation from calli overexpressing *WOX4* in a hormone-free medium.

(E) Effect of FCP1 peptide on calli overexpressing *WOX4*. Calli with abnormal shoot-like structures (as in **[A]**) were grown in regeneration liquid medium in the absence or presence of FCP1 peptide (20 µM) for 7 d with shaking (left). Shoot regeneration (shown on the right) was observed in the presence of FCP1 peptide, whereas no change was observed in the absence of FCP1.

Bars = 5 mm in **(A)**, **(B)**, and **(E)**.

FON2 is expressed in the tip of the meristem, and its expression domains are expanded in the floral meristem of the *fon1* mutant (Suzaki et al., 2006). This *FON2* behavior, similar to that of *CLV3* in *Arabidopsis*, suggests that the region expressing *FON2* corresponds to stem cells in rice. Expression of *FON2* was strongly reduced by the induced overexpression of *FCP1*, whereas the expression domain of *FON2* was expanded by the induced cosilencing of *FCP1* and *FCP2*. Therefore, it is likely that *FCP1* (and probably *FCP2*) negatively regulates stem cell identity.

Both plants overexpressing *FCP1* and those with *FCP1/2* silenced by DEX induction showed similar phenotypes of small shoots with small pale green leaves; however, these phenotypic similarities seem to result from different consequences of the loss or gain of *FCP1/2* function. In *FCP1*-overexpressing plants, leaf initiation would be inhibited because of a reduction in meristematic activity, as shown by reduced *OSH1* expression. In *FCP1/2*-silenced plants, by contrast, the SAM may fail to recruit leaf founder cells because of the ectopic expression of *OSH1*, which would invade the P0 site. As a result, *FCP1/2* may promote leaf initiation by repressing *OSH1* expression properly at the flank of the meristem.

WOX4 Seems to Be a Positive Regulator of Maintenance of the SAM

The induced RNA silencing of *WOX4* led to small aberrant shoots with yellow leaves, a reduction in SAM size, and a decrease in the expression level of both *OSH1* and *FON2*, suggesting that the function of SAM was compromised. Therefore, *WOX4* is likely to be involved in the positive regulation of meristem maintenance in rice. The upregulation and downregulation of *WOX4* in *FCP1*-silenced and *FCP1/2*-overexpressing seedlings, respectively, demonstrates that *WOX4* is likely to be negatively regulated by *FCP1*. This idea is supported by the observation that exogenous application of FCP1 peptide suppressed the effect of *WOX4* overexpression in calli. The induced overexpression of *FCP1* and RNA silencing of *WOX4* resulted in the downregulation of *OSH1* and *FON2*, suggesting that *FCP1* and *WOX4* have opposite roles in SAM maintenance. Taking these observations

together, it is probable that one of the roles of *FCP1* in meristem maintenance is regulation of *WOX4*, which promotes meristem activity (see Supplemental Figure 8 online).

The function of *WOX4* may be associated with cytokinin action. Constitutive expression of *WOX4* resulted in increased levels of active cytokinins, such as isopentenyl adenine and *cis*-zeatin. It has been shown that *OSH1* promotes the expression of genes involved in cytokinin biosynthesis (Sakamoto et al., 2006). Therefore, an increase in cytokinin may result from the upregulation of *OSH1* expression in calli that overexpress *WOX4* (see Supplemental Figure 8 online). Consistent with increased levels of cytokinin, calli overexpressing *WOX4* showed phenotypes similar to those of calli grown in a higher concentration of cytokinin and were able to regenerate shoots without the addition of cytokinin to the culture medium.

The combined action of *FCP1* and *WOX4* in rice is similar to that of *CLV3* and *WUS* that regulates stem cell maintenance in *Arabidopsis*. Although the FCP1-WOX4 pathway is likely to be involved in meristem maintenance as discussed above, there seem to be a few differences between FCP1-WOX4 and CLV-WUS. First, no enlargement of the SAM was observed in *FCP1/2*-silenced plants. This contrasts with the enlargement of the SAM caused by overproliferation of stem cells in a loss-of-function mutant of *CLV3* in *Arabidopsis* (Clark et al., 1995; Reddy and Meyerowitz, 2005). Downregulation of *FCP1/2* may not be directly linked to overproliferation of stem cells, although upregulation of *FON2* expression was observed in *FCP1/2*-silenced plants. Second, leaf initiation was inhibited in *FCP1/2*-silenced plants, whereas no such inhibition is observed in the *Arabidopsis clv3* mutant where, instead, an increased number of floral organs is formed from the floral meristem. As discussed above, inhibition of leaf initiation in *FCP1/2*-silenced plants may result from the ectopic expression of *OSH1* in the P0 site. Third, expression of *WOX4* was seen throughout the SAM, and this expression overlaps with that of *FCP1* (Suzaki et al., 2008). This contrasts with the expression patterns of *CLV3* and *WUS*, which are involved in communication between the different domains of the meristem. Thus, the FCP1-WOX4 pathway must act on the meristem in rice in a manner that differs from that of the CLV3-WUS pathway in *Arabidopsis*.

In rice, loss of function of *FON2* causes an enlargement of the floral meristem and an increase in the number of floral organs. Therefore, *FON2* seems to act on the floral meristem in rice, as *CLV3* does in *Arabidopsis*. It would be of great interest to identify the gene that is involved in positive regulation of the floral meristem, similar to *Arabidopsis WUS*, and to elucidate the regulatory genetic pathway that maintains stem cells.

Roles of *WOX4* in Leaf Development

WOX4 is likely to be involved in leaf development. *WOX4* was strongly expressed in the P1 and P2 primordia. Downregulation of *WOX4* by induced RNA silencing of *WOX4* or by over-expression of *FCP1* caused malformed leaf primordia with enlarged and vacuolated cells. Therefore, *WOX4* is likely to be required to maintain the leaf primordia during early developmental stages, when cells divide and proliferate properly, probably by inhibiting premature differentiation of the cells.

In the P3 primordia, expression of *WOX4* was restricted to the putative procambium and subsequently to the region between the differentiating xylem and phloem. In *Arabidopsis*, *WOX4* promotes stem cell proliferation in the procambium and cambium (Hirakawa et al., 2010). The temporal and spatial expression patterns of rice *WOX4* are highly similar to those of *Arabidopsis WOX4*, suggesting that these orthologous genes have similar roles in the regulation of vascular development. Although, like other monocots, rice has no distinct layer corresponding to the cambium, the expression pattern of rice *WOX4* implies that the cells between the xylem and the phloem might have characteristics similar to those of the procambium or cambium in eudicots. Further studies would be required to clarify whether *WOX4* is involved in vascular development in rice.

The similar expression patterns of *WOX4* in developing leaves of both rice and *Arabidopsis* imply that the ancestral function of *WOX4* might be associated with the regulation of vascular development. Unlike in rice, *WOX4* is not expressed in the SAM in *Arabidopsis*, and no abnormalities in the SAM have been reported in the null *wox4* mutant (Hirakawa et al., 2010). It is therefore possible that *WOX4* might have been recruited to regulate meristem maintenance during the evolution of rice. Alternatively, meristem maintenance might be an ancestral function of *WOX4* that has been lost in the lineage of *Arabidopsis*. It would be of great interest to know how the *WOX4* function associated with meristem maintenance is conserved among Angiosperms.

METHODS

Plasmid Construction

pINDEX4 was a kind gift from P. Ouwerkerk (Leiden University) (Ouwerkerk et al., 2001). The rice (*Oryza sativa*) *ACTIN1* promoter (Zhang et al., 1991), which induces strong expression in rice, was inserted in the *Apa*I site of pINDEX4, and the resulting plasmid was named pAID1 (see Supplemental Figure 1 online). To make constructs easily, the Gateway rfC cassette was inserted in the *Spe*I site of pAID1 (named pAID2; see Supplemental Figure 1 online). The cassette for RNAi silencing, which was derived from the pANDA vector (Miki and Shimamoto, 2004), was inserted in the same site of pAID1 (pAID3; see Supplemental Figure 1 online). Full or a partial cDNA of *WOX4* was amplified using the primers listed in Supplemental Table 2 online and inserted in the Gateway entry vector pENTR-D/TOPO (Invitrogen). Concerning *FCP1* and *FCP2*, plasmids constructed in a previous study were used for further construction (Suzaki et al., 2006). *FCP1* cDNA was inserted in pAID2 (*pACT1-GVG>FCP1*; see Supplemental Figure 2 online) by the LR reaction. For inducible RNA silencing, a partial *FCP1* cDNA, a ligated fragment of partial *FCP1* and *FCP2* cDNA, and a partial cDNA fragment specific to *WOX4* (341 bp; from the initiation codon, 20 to 361) were inserted in pAID3 (*pACT1-GVG>FCP1:RNAi*, *pACT1-GVG>FCP1-FCP2:RNAi*, and *pACT1-GVG>WOX4:RNAi*; see Supplemental Figure 2 online) by the LR reaction. A partial *WOX4* cDNA was similarly inserted in pANDA-EG1 (*pACT1-WOX4:RNAi*; see Supplemental Figure 2 online) (Suzaki et al., 2008).

Transformation and Tissue Culture

The rice cultivar Taichung65 (T65) was used as a host for the transformation. The constructs were introduced into *Agrobacterium tumefaciens* strain EHA101 or EHA103 and transformed into scutellum-derived calli of T65, according to the method described by Hiei et al. and/or Ozawa (Hiei

et al., 1994; Ozawa, 2012). To make the transgenic plants, shoots were regenerated from hygromycin-resistant calli in regeneration medium containing 8 μM 6-benzylaminopurine (BAP) and 5 μM naphthalene acetic acid.

To examine the effect of cytokinin on shoot regeneration, different concentrations of BAP were used instead of 8 μM BAP in regeneration medium (Hiei et al., 1994). FCP1 peptides (REVPTGDPIHH) were synthesized and purified to at least 95% using HPLC by TaKaRa and added to the medium as described previously (Suzaki et al., 2006). *WOX4*-overexpressing calli with abnormal shoot-like structures (grown in regeneration agar medium for 30 days) were transferred into liquid regeneration medium (20 mL) either containing or not containing 20 μM FCP1 peptide in a 100-mL conical flask. The flask was incubated at 28°C under 16 h light/8 h dark cycles with shaking (100 rotations/minute).

Selection of Transgenic Plants Used for DEX Induction

For inducible overexpression or RNAi silencing, transformants that showed efficient DEX induction were selected. Leaf sections ($\sim$1-cm length, four to five sections for each sample) cut off from regenerated shoots (T0 generation) were immersed in sterile water in the presence (50 μM) or absence of DEX overnight. RNA was isolated, and the levels of RNA transcript from the cDNA or the DNA fragment (for RNA silencing) under the control of 4UAS in each construct were analyzed by RT-PCR. For each construct, five T0 lines showing high induction of transgene expression by DEX application were selected from $\sim$25 to 30 independent transformants.

The effect of DEX induction was examined using T1 seedlings germinated from seeds, which were harvested from the same T0 plants. The concentration of DEX and length of treatment are indicated in each figure legend.

In Situ Hybridization

OSH1 and *FON2* probes were prepared using the plasmid described in the original studies (Sato et al., 1996; Suzaki et al., 2006). To generate probes for the *WOX4* transcript, partial cDNA fragments (341 bp; from the initiation codon, 20 to 361) were amplified with the primers listed in Supplemental Table 2 online and cloned into pENTR-D/TOPO (Invitrogen). This fragment contained the *WOX4*-specific region ($\sim$250 bp) and a part of the homeodomain region in which similarity is not high among rice *WOX* members. After linearization of the plasmids with *Not*I, RNA was transcribed by T7 RNA polymerase and labeled with digoxigenin (Roche). Preparation of sections and hybridization experiments were performed by the methods described by Suzaki et al. (2004).

qRT-PCR Analysis

To determine the expression levels of *WOX* genes in regenerating shoots, whole shoots including adventitious root primordia, which carried *pACT1-FCP1* and empty vector, were used. For DEX induction experiments, the shoot apices (<3 mm diameter $\times$ 7- to 8-mm height), which include leaf primordia and a few young leaves, were excised from the DEX- or mock-treated seedlings and used for RNA isolation. Total RNA was isolated using Treasure (Bioline), and the RNA extract was treated with DNase I (Invitrogen) to remove genomic DNA. The first strand of cDNA was synthesized from 1 μg of total RNA using a PrimScript RT reagent kit (TaKaRa). The primers used for RT-PCR are listed in Supplemental Table 2 online. qRT-PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems) with Power SYBR Green RT-PCR Master Mix (Applied Biosystems). Four RNA samples, which were independently isolated from three to five regenerated shoots, were used for qRT-PCR analysis, and the PCR reaction was performed twice for each sample. The *ACTIN1* gene was used as a control.

Cytokinin Quantification in the Calli Overexpressing *WOX4*

Calli carrying *pACT1-WOX4* and empty vector were grow on selection medium containing auxin (2 mg/L 2,4-D) for 30 d (Hiei et al., 1994) and then on hormone-free Murashige and Skoog (MS) medium containing 3% Suc for 30 d. A few calli (corresponding to 80 to 100 mg) were harvested and used for cytokinin quantification. The quantification was repeated six times for each construct. Extraction and determination of cytokinins from calli were performed as described previously using ultraperformance liquid chromatography–tandem mass spectrometry (AQITY UPLC system/ Xevo-TQS; Waters) with an ODS column (Aquity UPLC BEH C18, 1.7 μm, 2.1 $\times$ 100 mm; Waters) (Kojima et al., 2009).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: AB354584 (*FCP1*), AB354585 (*FCP2*), AJ556181 (Os *WOX4*), NM_103605 (At *WOX4*), AB218894 (Os *WUS*), AM234749 (Os *WOX2*), AM490244 (Os *WOX3*), AM490243 (Os *NS*), AK102203 (*QHB*), AB245090 (*FON2*), and AK107637 (*OSH1*).

Supplemental Data

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

Supplemental Figure 1. Vectors Used for Inducible Overexpression or Downregulation.

Supplemental Figure 2. Plasmid Constructs Used in This Study.

Supplemental Figure 3. Morphologies of the Shoot Apex in Seedlings Carrying *pACT1-GVG>FCP1-FCP2:RNAi* after DEX Treatment.

Supplemental Figure 4. *OSH1* Expression Patterns in the Shoot Apex of Seedlings Carrying *pACT1-GVG>FCP1-FCP2:RNAi*.

Supplemental Figure 5. Comparison of Rice WOX4 and *Arabidopsis* WOX4.

Supplemental Figure 6. qRT-PCR Analysis of the *WOX4* and *WUS* Genes of Rice.

Supplemental Figure 7. The Shoot Apices of *pACT1-GVG>WOX4*: *RNAi* Transgenic Plants after DEX Treatment.

Supplemental Figure 8. Model of Genetic Interactions Involved in Maintenance of the Vegetative Meristem in Rice.

Supplemental Table 1. Cytokinin Content in Calli with *pACT1-WOX4* or Empty Vector.

Supplemental Table 2. Primers Used in This Study.

AUTHOR CONTRIBUTIONS

Y.O. and H.-Y.H. designed the research. Y.O., W.T., M.K., H.S., and H.-Y.H. performed the research. Y.O. and H.-Y.H. wrote the article.

REFERENCES

Aichinger, E., Kornet, N., Friedrich, T., and Laux, T. (2012). Plant stem cell niches. Annu. Rev. Plant Biol. **63:** 615–636.

Barton, M.K. (2010). Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. Dev. Biol. **341:** 95–113.

Betsuyaku, S., Takahashi, F., Kinoshita, A., Miwa, H., Shinozaki, K., Fukuda, H., and Sawa, S. (2011). Mitogen-activated protein kinase regulated by the CLAVATA receptors contributes to shoot apical meristem homeostasis. Plant Cell Physiol. **52:** 14–29.

Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S., and Werr, W. (2005). *thick tassel dwarf1* encodes a putative maize ortholog of the *Arabidopsis CLAVATA1* leucine-rich repeat receptor-like kinase. Development **132:** 1235–1245.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. Science **289:** 617–619.

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.

Chickarmane, V.S., Gordon, S.P., Tarr, P.T., Heisler, M.G., and Meyerowitz, E.M. (2012). Cytokinin signaling as a positional cue for patterning the apical-basal axis of the growing *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci. USA **109:** 4002–4007.

Chu, H., Qian, Q., Liang, W., Yin, C., Tan, H., Yao, X., Yuan, Z., Yang, J., Huang, H., Luo, D., Ma, H., and Zhang, D. (2006). The *floral organ number4* gene encoding a putative ortholog of *Arabidopsis* CLAVATA3 regulates apical meristem size in rice. Plant Physiol. **142:** 1039–1052.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. Development **121:** 2057–2067.

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.

Dai, M., Hu, Y., Zhao, Y., Liu, H., and Zhou, D.-X. (2007). A *WUSCHEL-LIKE HOMEOBOX* gene represses a *YABBY* gene expression required for rice leaf development. Plant Physiol. **144:** 380–390.

Etchells, J.P., and Turner, S.R. (2010). The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. Development **137:** 767–774.

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.

Gordon, S.P., Chickarmane, V.S., Ohno, C., and Meyerowitz, E.M. (2009). Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proc. Natl. Acad. Sci. USA **106:** 16529–16534.

Ha, C.M., Jun, J.H., and Fletcher, J.C. (2010). Shoot apical meristem form and function. Curr. Top. Dev. Biol. **91:** 103–140.

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.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. **6:** 271–282.

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.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., and Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. Proc. Natl. Acad. Sci. USA **105:** 15208–15213.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., and Fukuda, H. (2006). Dodeca-CLE peptides as suppressors of plant stem cell differentiation. Science **313:** 842–845.

Itoh, J.-I., Nonomura, K.-I., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H., and Nagato, Y. (2005). Rice plant development: From zygote to spikelet. Plant Cell Physiol. **46:** 23–47.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005). KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr. Biol. **15:** 1560–1565.

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

Kamiya, N., Nagasaki, H., Morikami, A., Sato, Y., and Matsuoka, M. (2003). Isolation and characterization of a rice *WUSCHEL*-type homeobox gene that is specifically expressed in the central cells of a quiescent center in the root apical meristem. Plant J. **35:** 429–441.

Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H., and Sawa, S. (2010). RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in *Arabidopsis*. Development **137:** 3911–3920.

Kojima, M., Kamada-Nobusada, T., Komatsu, H., Takei, K., Kuroha, T., Mizutani, M., Ashikari, M., Ueguchi-Tanaka, M., Matsuoka, M., Suzuki, K., and Sakakibara, H. (2009). Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography-tandem mass spectrometry: An application for hormone profiling in *Oryza sativa*. Plant Cell Physiol. **50:** 1201–1214.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H., and Sakagami, Y. (2006). A plant peptide encoded by *CLV3* identified by in situ MALDI-TOF MS analysis. Science **313:** 845–848.

Kudo, T., Makita, N., Kojima, M., Tokunaga, H., and Sakakibara, H. (2012). Cytokinin activity of cis-zeatin and phenotypic alterations induced by overexpression of putative cis-Zeatin-O-glucosyltransferase in rice. Plant Physiol. **160:** 319–331.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445:** 652–655.

Leibfried, A., To, J.P.C., 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.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. Nature **379:** 66–69.

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.

Miki, D., and Shimamoto, K. (2004). Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiol. **45:** 490–495.

Müller, R., Bleckmann, A., and Simon, R. (2008). The receptor kinase CORYNE of *Arabidopsis* transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. Plant Cell **20**: 934–946.

Nardmann, J., and Werr, W. (2006). The shoot stem cell niche in angiosperms: Expression patterns of WUS orthologues in rice and maize imply major modifications in the course of mono- and dicot evolution. Mol. Biol. Evol. **23**: 2492–2504.

Nishimura, A., Ito, M., Kamiya, N., Sato, Y., and Matsuoka, M. (2002). *OsPNH1* regulates leaf development and maintenance of the shoot apical meristem in rice. Plant J. **30**: 189–201.

Ogawa, M., Shinohara, H., Sakagami, Y., and Matsubayashi, Y. (2008). *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. Science **319**: 294.

Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M., and Matsubayashi, Y. (2009). A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. Nat. Chem. Biol. **5**: 578–580.

Ouwerkerk, P.B., de Kam, R.J., Hoge, J.H., and Meijer, A.H. (2001). Glucocorticoid-inducible gene expression in rice. Planta **213**: 370–378.

Ozawa, K. (2012). A high-efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). Methods Mol. Biol. **847**: 51–57.

Reddy, G.V., and Meyerowitz, E.M. (2005). Stem-cell homeostasis and growth dynamics can be uncoupled in the *Arabidopsis* shoot apex. Science **310**: 663–667.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., and Matsuoka, M. (2006). Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. Plant Physiol. **142**: 54–62.

Sarkar, A.K., 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.

Sato, Y., Hong, S.K., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y., and Matsuoka, M. (1996). A rice homeobox gene, *OSH1*, is expressed before organ differentiation in a specific region during early embryogenesis. Proc. Natl. Acad. Sci. USA **93**: 8117–8122.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jürgens, 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.

Song, S.-K., Lee, M.M., and Clark, S.E. (2006). POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for *Arabidopsis* shoot and floral stem cells. Development **133**: 4691–4698.

Suer, S., Agusti, J., Sanchez, P., Schwarz, M., and Greb, T. (2011). *WOX4* imparts auxin responsiveness to cambium cells in *Arabidopsis*. Plant Cell **23**: 3247–3259.

Suzaki, T., Ohneda, M., Toriba, T., Yoshida, A., and Hirano, H.-Y. (2009). *FON2 SPARE1* redundantly regulates floral meristem maintenance with *FLORAL ORGAN NUMBER2* in rice. PLoS Genet. **5**: e1000693.

Suzaki, T., Sato, M., Ashikari, M., Miyoshi, M., Nagato, Y., and Hirano, H.-Y. (2004). The gene *FLORAL ORGAN NUMBER1* regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to *Arabidopsis* CLAVATA1. Development **131**: 5649–5657.

Suzaki, T., Toriba, T., Fujimoto, M., Tsutsumi, N., Kitano, H., and Hirano, H.-Y. (2006). Conservation and diversification of meristem maintenance mechanism in *Oryza sativa*: Function of the *FLORAL ORGAN NUMBER2* gene. Plant Cell Physiol. **47**: 1591–1602.

Suzaki, T., Yoshida, A., and Hirano, H.-Y. (2008). Functional diversification of CLAVATA3-related CLE proteins in meristem maintenance in rice. Plant Cell **20**: 2049–2058.

Taguchi-Shiobara, F., Yuan, Z., Hake, S., and Jackson, D. (2001). The *fasciated ear2* gene encodes a leucine-rich repeat receptor-like protein that regulates shoot meristem proliferation in maize. Genes Dev. **15**: 2755–2766.

Tsuda, K., Ito, Y., Sato, Y., and Kurata, N. (2011). Positive auto-regulation of a *KNOX* gene is essential for shoot apical meristem maintenance in rice. Plant Cell **23**: 4368–4381.

Vandenbussche, M., Horstman, A., Zethof, J., Koes, R., Rijpkema, A.S., and Gerats, T. (2009). Differential recruitment of WOX transcription factors for lateral development and organ fusion in petunia and *Arabidopsis*. Plant Cell **21**: 2269–2283.

Veach, Y.K., Martin, R.C., Mok, D.W.S., Malbeck, J., Vankova, R., and Mok, M.C. (2003). *O*-glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. Plant Physiol. **131**: 1374–1380.

Vollbrecht, E., Reiser, L., and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, knotted1. Development **127**: 3161–3172.

Williams, R.W., Wilson, J.M., and Meyerowitz, E.M. (1997). A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. Proc. Natl. Acad. Sci. USA **94**: 10467–10472.

Wu, X., Dabi, T., and Weigel, D. (2005). Requirement of homeobox gene *STIMPY/WOX9* for *Arabidopsis* meristem growth and maintenance. Curr. Biol. **15**: 436–440.

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

Yadav, R.K., Tavakkoli, M., and Reddy, G.V. (2010). WUSCHEL mediates stem cell homeostasis by regulating stem cell number and patterns of cell division and differentiation of stem cell progenitors. Development **137**: 3581–3589.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Curr. Biol. **15**: 1566–1571.

Zhang, W., McElroy, D., and Wu, R. (1991). Analysis of rice *Act*1 5′ region activity in transgenic rice plants. Plant Cell **3**: 1155–1165.

Zhao, Y., Hu, Y., Dai, M., Huang, L., and Zhou, D.-X. (2009). The WUSCHEL-related homeobox gene *WOX11* is required to activate shoot-borne crown root development in rice. Plant Cell **21**: 736–748.