

# WOX11 and 12 Are Involved in the First-Step Cell Fate Transition during de Novo Root Organogenesis in *Arabidopsis*<sup>W</sup>

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**De novo organogenesis is a process through which wounded or detached plant tissues or organs regenerate adventitious roots and shoots. Plant hormones play key roles in de novo organogenesis, whereas the mechanism by which hormonal actions result in the first-step cell fate transition in the whole process is unknown. Using leaf explants of *Arabidopsis thaliana*, we show that the homeobox genes *WUSCHEL RELATED HOMEBOX11 (WOX11)* and *WOX12* are involved in de novo root organogenesis. *WOX11* directly responds to a wounding-induced auxin maximum in and surrounding the procambium and acts redundantly with its homolog *WOX12* to upregulate *LATERAL ORGAN BOUNDARIES DOMAIN16 (LBD16)* and *LBD29*, resulting in the first-step cell fate transition from a leaf procambium or its nearby parenchyma cell to a root founder cell. In addition, our results suggest that de novo root organogenesis and callus formation share a similar mechanism at initiation.**

## INTRODUCTION

Unlike animals, many plants have remarkable abilities to regenerate and form an entire plant body from various tissues or organs, or even from a single somatic cell (Birbaum and Sánchez Alvarado, 2008; Sugimoto et al., 2011; Xu and Huang, 2014). Among the different types of plant regeneration, de novo organogenesis, in which adventitious roots and shoots form from wounded or detached plant tissues or organs, is frequently used in basic research and biotechnological breeding as it is a simple and robust in vitro method for plant culture (De Klerk et al., 1999; Duclercq et al., 2011). In recent decades, the regulation of de novo organogenesis in plants has been studied extensively, and phytohormones are considered to be the critical factors affecting this process (Skoog and Miller, 1957; Sangwan et al., 1997; De Klerk et al., 1999; Duclercq et al., 2011; Ikeuchi et al., 2013).

Physiological studies have shown that the phytohormones auxin and cytokinin play major roles in cell fate determination during de novo organogenesis. Auxin is the main hormone inducing de novo root organogenesis, while cytokinin promotes de novo shoot organogenesis (Skoog and Miller, 1957; Duclercq et al., 2011; Correa Lda et al., 2012). Recent research has identified a number of the factors mediating de novo organogenesis, including hormone receptors, proteins involved in hormone signaling and transport, and several transcription factors (Duclercq et al., 2011; Su and Zhang, 2014).

In traditional in vitro tissue cultures, adventitious roots and shoots are usually induced from a pluripotent cell mass known as callus. Previously, it was thought that callus was a group of dedifferentiated cells, but recent research has revealed that it is a group of root meristem tip cells and that the formation of callus and lateral roots is very similar (Sugimoto et al., 2010). The fact that callus formation resembles root formation suggests that this process is actually a type of de novo organogenesis. It was reported that callus is initiated from xylem-pole pericycle cells of root explants and pericycle-like cells of aerial organs, although the exact cell type of pericycle-like cells in aerial organs is not yet known (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010). Genetic approaches by gain-of- or loss-of-function analyses in *Arabidopsis thaliana* led to the identification of genes involved in callus formation. The *aberrant lateral root formation4 (arf4)* mutant, which is defective in forming lateral roots (Celenza et al., 1995), almost completely failed to form callus (Sugimoto et al., 2010). Mutants with loss of function of the Polycomb Repressive Complex 2, which catalyzes genome-wide histone H3 lysine 27 trimethylation (H3K27me3) on chromatin, also showed a block in callus formation from leaf explants, but not from root explants (He et al., 2012). The lateral organ boundaries domain (LBD) transcription factors were recently reported to be important in regulating callus formation (Fan et al., 2012). The *LBD16*, *LBD17*, *LBD18*, and *LBD29* genes were rapidly induced in explants cultured on callus-inducing medium (CIM), and ectopic expression of each of these four genes in *Arabidopsis* was sufficient to trigger callus formation without supplied phytohormones. In addition, suppression of the LBD function resulted in defects in callus formation (Fan et al., 2012). *LBD* genes were shown to be the direct targets of *AUXIN RESPONSE FACTOR7 (ARF7)* and *ARF19* during lateral root formation (Okushima et al., 2007), and the *arf7 arf19* double mutant is defective in producing lateral roots (Okushima et al., 2005).

The results of these previous studies greatly advanced our knowledge of de novo organogenesis. However, a long unanswered

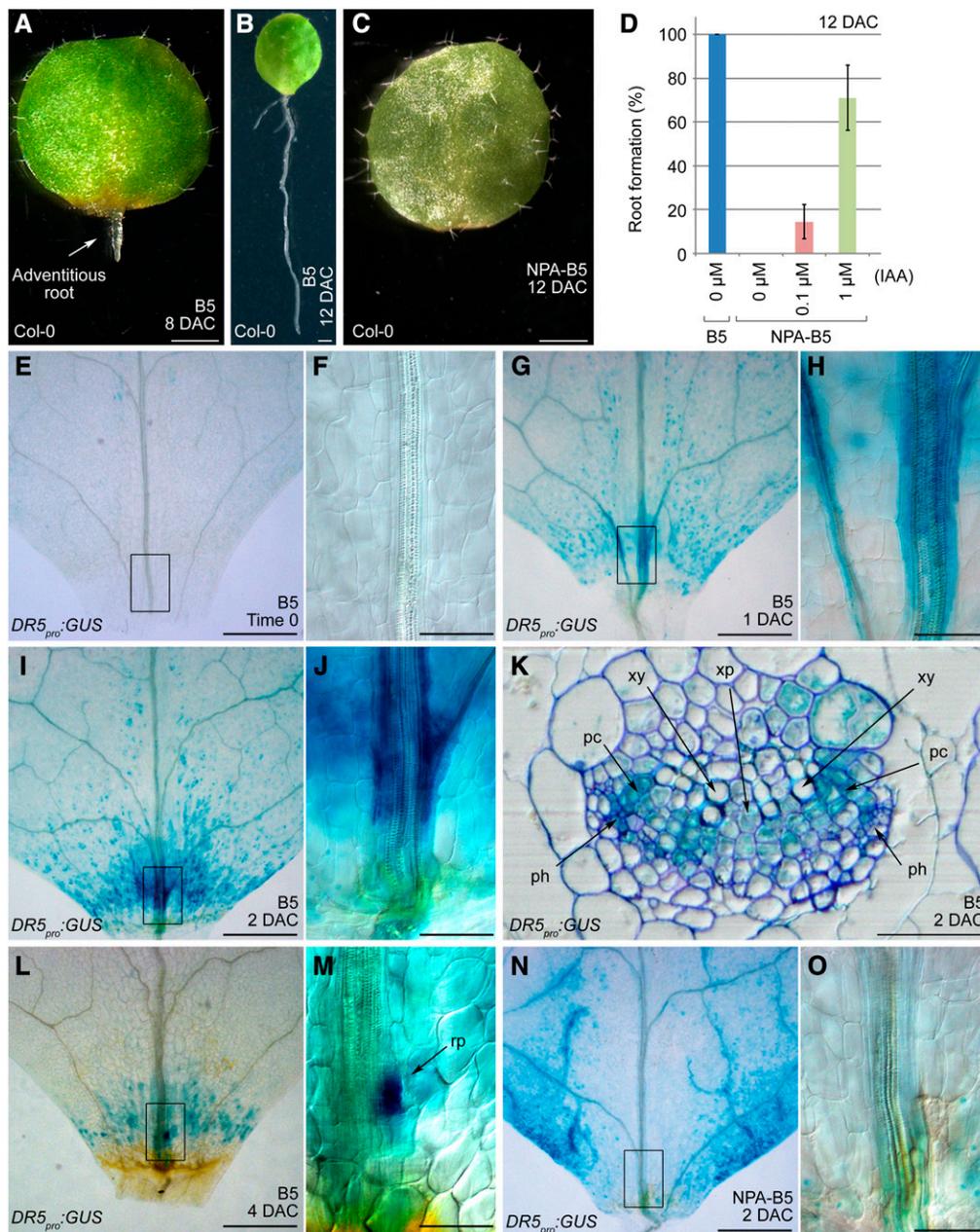
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**Figure 1.** Wound-Induced Auxin Accumulation and Polar Transport Are Essential for Adventitious Root Formation.

(A) and (B) Leaf explants at 8 (A) and 12 DAC (B) on B5 medium.

(C) The 12-DAC leaf explant on NPA-B5. A total of 30 leaf explants were analyzed, and they all failed to form adventitious roots.

(D) Addition of IAA to NPA-B5 could rescue the NPA-caused rooting defect. Bars show  $\text{SD}$  with three biological repeats.  $n = 30$  in each individual repeat.

(E) to (J) GUS staining at time 0 (E) and (F), 1 DAC (G) and (H), and 2 DAC (I) and (J) of  $DR5_{pro}::GUS$  leaf explants cultured on B5 medium.

(K) Transverse section through the GUS-staining region of a 2-DAC  $DR5_{pro}::GUS$  leaf explant grown on B5 medium. Note that GUS staining was mainly concentrated in the procambium and the nearby parenchyma cells. See the similar section without toluidine blue staining in Supplemental Figure 1.

(L) and (M) GUS staining of a 4-DAC  $DR5_{pro}::GUS$  leaf explant on B5 medium. Arrow in (M) indicates an emerging root primordium.

(N) and (O) GUS staining of a 2-DAC  $DR5_{pro}::GUS$  leaf explant cultured on NPA-B5.

(F), (H), (J), (M), and (O) are close-ups of the boxed regions in (E), (G), (I), (L), and (N), respectively. rp, root primordium; xy, xylem; xp, xylem parenchyma cell; pc, procambium; ph, phloem. Bars = 1 mm in (A) to (C), 500  $\mu\text{m}$  in (E), (G), (I), (L), and (N), 100  $\mu\text{m}$  in (F), (H), (J), (M), and (O), and 50  $\mu\text{m}$  in (K).

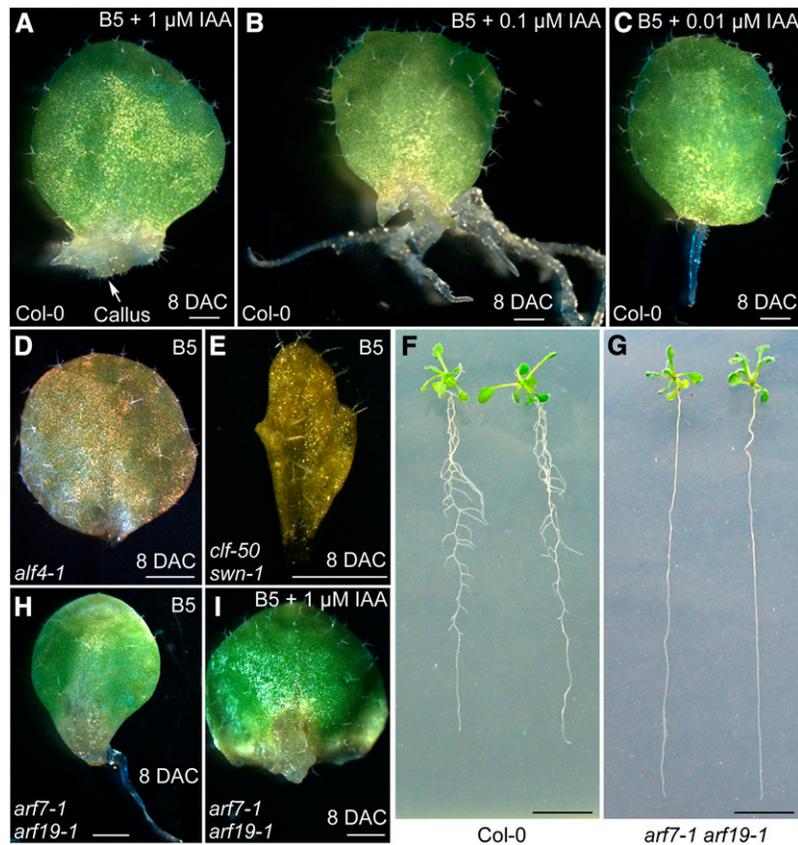
question in this field still remains elusive: What is the mechanism guiding the first-step cell fate transition in de novo organogenesis. Here, we report the elucidation of the regulatory mechanism underlying the first-step cell fate transition, which links the upstream wounding and hormonal signaling to the downstream organ formation, using our simple de novo root organogenesis system that mimics the natural conditions without added hormones. We also show that the mechanism of the first-step cell fate transition for callus initiation is similar to that for de novo root organogenesis.

## RESULTS

### Auxin Accumulation Near Wounds Initiates Adventitious Root Formation from Leaf Explants

In natural conditions, detached organs from some plant species, such as members of the Crassulaceae and Cactaceae, are able

to regenerate a new plant. Different from in vitro tissue culture in which explants regenerate on medium containing phytohormones, plant regeneration in natural conditions relies on the endogenous hormones in the detached tissues or organs. To investigate the molecular mechanisms guiding this process, we attempted to imitate natural conditions by culturing *Arabidopsis* leaf explants on B5 medium (Gamborg et al., 1968) without additional phytohormones. Leaves were cut at the region between the blade and petiole, and this simple method resulted in de novo root organogenesis that regenerated adventitious roots. Usually one adventitious root per leaf explant was visible on the proximal part at 8 d after culture (DAC) (Figure 1A). Roots continued to grow on the medium, and almost every leaf explant had regenerated roots by 12 DAC, with some explants having two or three roots (Figure 1B). On B5 medium containing 1  $\mu\text{M}$  naphthylphthalamic acid (a polar auxin transport inhibitor), hereafter referred to as NPA-B5, rooting from leaf explants was blocked completely (Figure 1C). This phenotype could be rescued by culturing leaf explants on NPA-B5 with exogenous



**Figure 2.** Callus Formation Resembles Adventitious Root Formation.

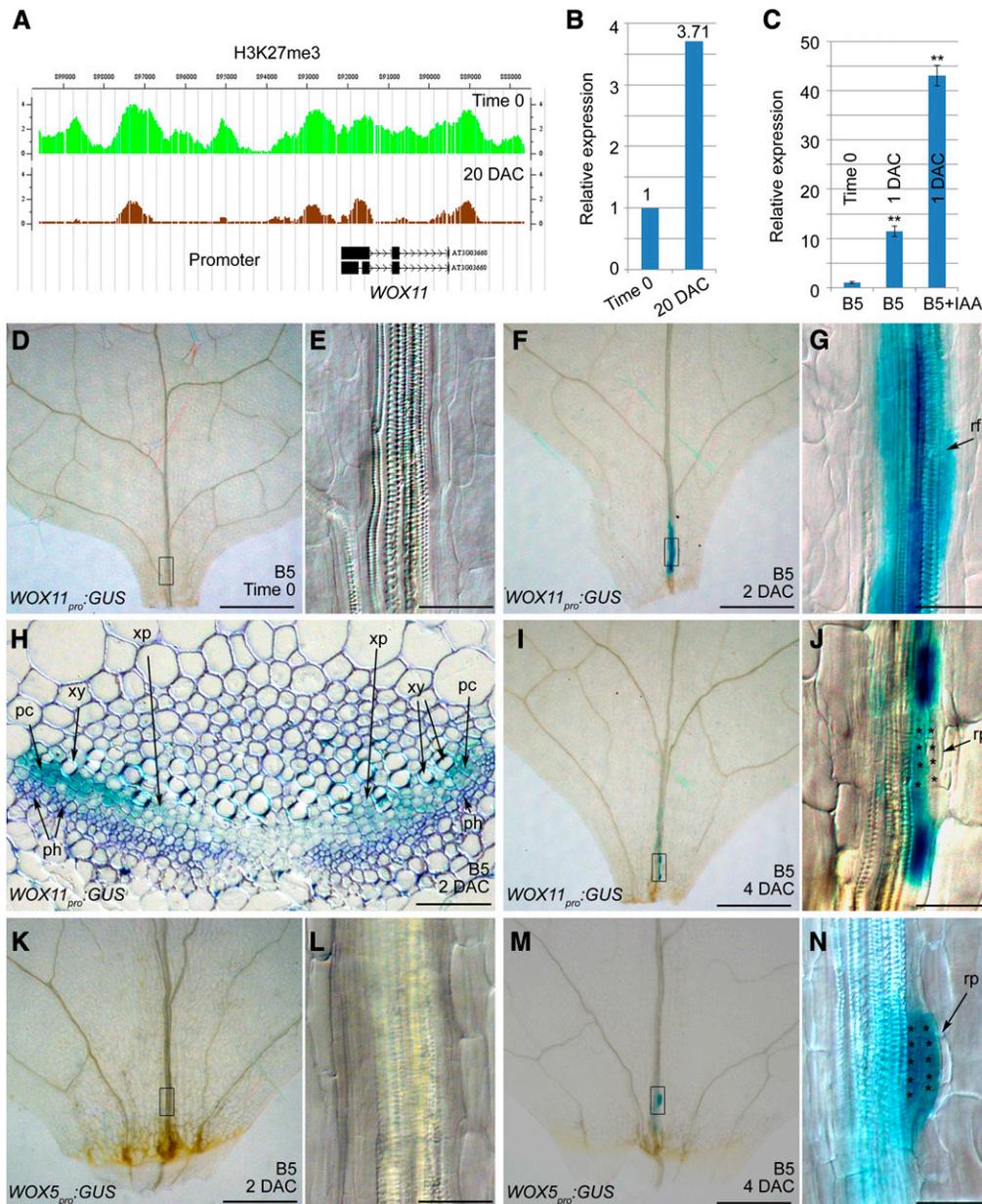
(A) to (C) Phenotypes of the 8-DAC leaf explants on B5 medium containing 1  $\mu\text{M}$  (A), 0.1  $\mu\text{M}$  (B), and 0.01  $\mu\text{M}$  (C) IAA.

(D) and (E) Leaf explants of *alf4-1* (D) and *clf-50 swm-1* (E) mutants, which are defective in adventitious root formation. A total of 30 leaf explants from each mutant were analyzed, and the results were consistent.

(F) and (G) Lateral root formation differs between wild-type Col-0 (F) and *arf7-1 arf19-1* (G). Seedlings were grown on half-strength MS medium for 14 d.

(H) and (I) Leaf explants from the *arf7-1 arf19-1* double mutant on B5 medium (H) or B5 medium containing 1  $\mu\text{M}$  IAA (I). Note that seedlings of the *arf7-1 arf19-1* double mutant produce no lateral roots but can generate adventitious roots normally without IAA or callus with 1  $\mu\text{M}$  IAA. A total of 30 leaf explants were tested, and the results were consistent.

Bars = 1 mm.



**Figure 3.** *WOX11* and *WOX5* Are Differentially Expressed during Adventitious Root Formation.

(A) The level of epigenetic marker H3K27me3 at the *WOX11* locus was dramatically reduced in the 20-DAC leaf explants that produce callus (brown) compared with that in the time-0 leaf explants (green). Note that the high level of H3K27me3 at a locus usually marks repression of the corresponding gene (Schatlowksi et al., 2008).

(B) *WOX11* expression was upregulated in the 20-DAC leaf explants cultured on CIM compared with that in the time-0 explants. The data in (A) and (B) were generated from previous ChIP-chip and microarray analyses, respectively (He et al., 2012).

(C) qRT-PCR analysis of *WOX11* expression in the 1-DAC leaf explants on B5 media without IAA or with 2  $\mu$ M IAA. The value of time-0 leaf explants was arbitrarily fixed at 1.0. Bars show  $\pm$  SE with three technical repeats. \*\* $P < 0.01$  in two-sample  $t$  test compared with time-0 leaf explants.

(D) to (G) GUS staining of time-0 (D) and (E) and 2-DAC (F) and (G) leaf explants from *WOX11<sub>pro</sub>::GUS* plants, cultured on B5 medium.

(H) Transverse section of a 2-DAC *WOX11<sub>pro</sub>::GUS* leaf explant grown on B5 medium. Note that GUS staining appeared mainly in the procambium cells and some xylem parenchyma cells. See the similar section without toluidine blue staining in Supplemental Figure 1.

(I) and (J) GUS staining of a 4-DAC *WOX11<sub>pro</sub>::GUS* leaf explant cultured on B5 medium.

(K) to (N) GUS staining of 2-DAC (K) and (L) and 4-DAC (M) and (N) *WOX5<sub>pro</sub>::GUS* leaf explants cultured on B5 medium.

(E), (G), (J), (L), and (N) are close-ups of the boxed regions in (D), (F), (I), (K), and (M), respectively. rf, root founder cell; rp, root primordium; xy, xylem; xp, xylem parenchyma cell; pc, procambium; ph, phloem. Asterisks indicate the dividing cells that are in the process of forming a root primordium. Bars = 500  $\mu$ m in (D), (F), (I), (K), and (M) and 50  $\mu$ m in (E), (G), (H), (J), (L), and (N).

indole-3-acetic acid (IAA) (Figure 1D). These results suggest that not only auxin but also its transport are required for adventitious root formation from leaf explants.

Next, we used the *DR5<sub>pro</sub>:GUS* ( $\beta$ -glucuronidase) line (Ulmasov et al., 1997) to monitor the level of endogenous free auxin. Leaf explants from *DR5<sub>pro</sub>:GUS* plants showed only very faint GUS staining prior to culturing (time 0) (Figures 1E and 1F). In 1-DAC explants, GUS staining was visible in both mesophyll and vascular cells near wounds (Figures 1G and 1H) and was even stronger in the 2-DAC leaf explants (Figures 1I and 1J). Analysis of transverse sections showed that the strongest GUS staining in the 2-DAC leaf explants was in the procambium cells, although GUS signals were also visible in some parenchyma cells near the procambium (Figure 1K; Supplemental Figure 1). The regenerating root primordium was visible under a microscope in 4-DAC explants, and the GUS staining became further concentrated in the region in which the new root primordium was forming (Figures 1L and 1M). Compared with that in 2-DAC leaf explants on B5 medium, GUS staining was not detected in similar regions of the same-age leaf explants grown on NPA-B5, although some mesophyll cells of explants grown on NPA-B5 displayed much stronger GUS signals than those in the explants grown on B5 medium (Figures 1N and 1O). These results indicate that regeneration of adventitious roots requires an auxin maximum, and free auxin production, polar transport, and auxin signaling may all be involved.

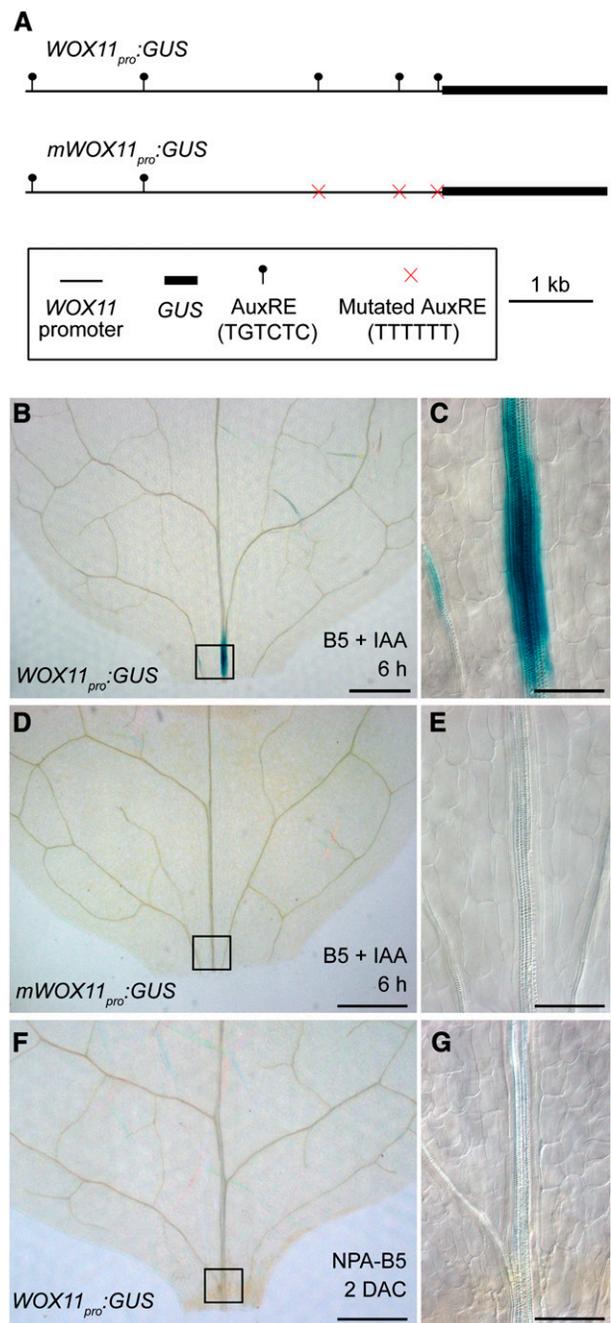
### Callus and Adventitious Roots Share a Similar Genetic Pathway for Initiation

To analyze the effect of auxin on adventitious root formation from leaf explants, we cultured leaf explants on B5 medium containing different concentrations of IAA. Leaf explants cultured on B5 medium with 1  $\mu$ M IAA produced callus (Figure 2A), whereas those cultured on B5 medium with 0.1  $\mu$ M IAA formed several thickened adventitious roots (Figure 2B). Explants cultured on B5 medium with 0.01  $\mu$ M IAA formed only one root (Figure 2C), mimicking those on B5 medium without phytohormones (Figure 1A). These results indicate that formation of callus or adventitious roots from a leaf explant highly depends on the auxin concentration in the medium. This observation is consistent with a previous model in which callus was proposed to resemble the tip of the root meristem (Sugimoto et al., 2010).

To study the genetic pathways involved in the formation of callus, adventitious roots and lateral roots, we analyzed *Arabidopsis* mutants defective in callus and/or lateral root formation. Leaf explants of both *alf4-1* and *clf-50 swn-1* are defective in callus formation (Sugimoto et al., 2010; He et al., 2012), and they also failed to form adventitious roots on B5 medium (Figures 2D and 2E). Whereas the *arf7-1 arf19-1* double mutant produced no lateral roots (Figure 2G) (Okushima et al., 2005) unlike the wild type (Figure 2F), its leaf explants were capable of forming both adventitious roots (Figure 2H) and callus (Figure 2I). These results suggest that adventitious root formation may share similar regulatory mechanisms with callus formation.

### WOX11 Is Directly Induced by Auxin for Stem Cell Fate Transition

Based on the hypothesis that there are similar regulatory mechanisms in the formation of adventitious roots and callus,



**Figure 4.** Auxin Directly Induces *WOX11* Expression.

(A) Diagram of structures of the *WOX11<sub>pro</sub>:GUS* and *mWOX11<sub>pro</sub>:GUS* constructs.

(B) to (E) GUS staining of *WOX11<sub>pro</sub>:GUS* [(B) and (C)] and *mWOX11<sub>pro</sub>:GUS* [(D) and (E)] leaf explants after 6 h cultured on B5 medium containing 1  $\mu$ M IAA. Two independent *WOX11<sub>pro</sub>:GUS* or *mWOX11<sub>pro</sub>:GUS* lines were analyzed and the results were consistent.

(F) and (G) GUS staining was undetectable in 2-DAC leaf plants from *WOX11<sub>pro</sub>:GUS* plants cultured on NPA-B5.

(C), (E), and (G) are close-ups of the boxed regions in (B), (D), and (F), respectively. Bars = 500  $\mu$ m in (B), (D), and (F) and 100  $\mu$ m in (C), (E), and (G).

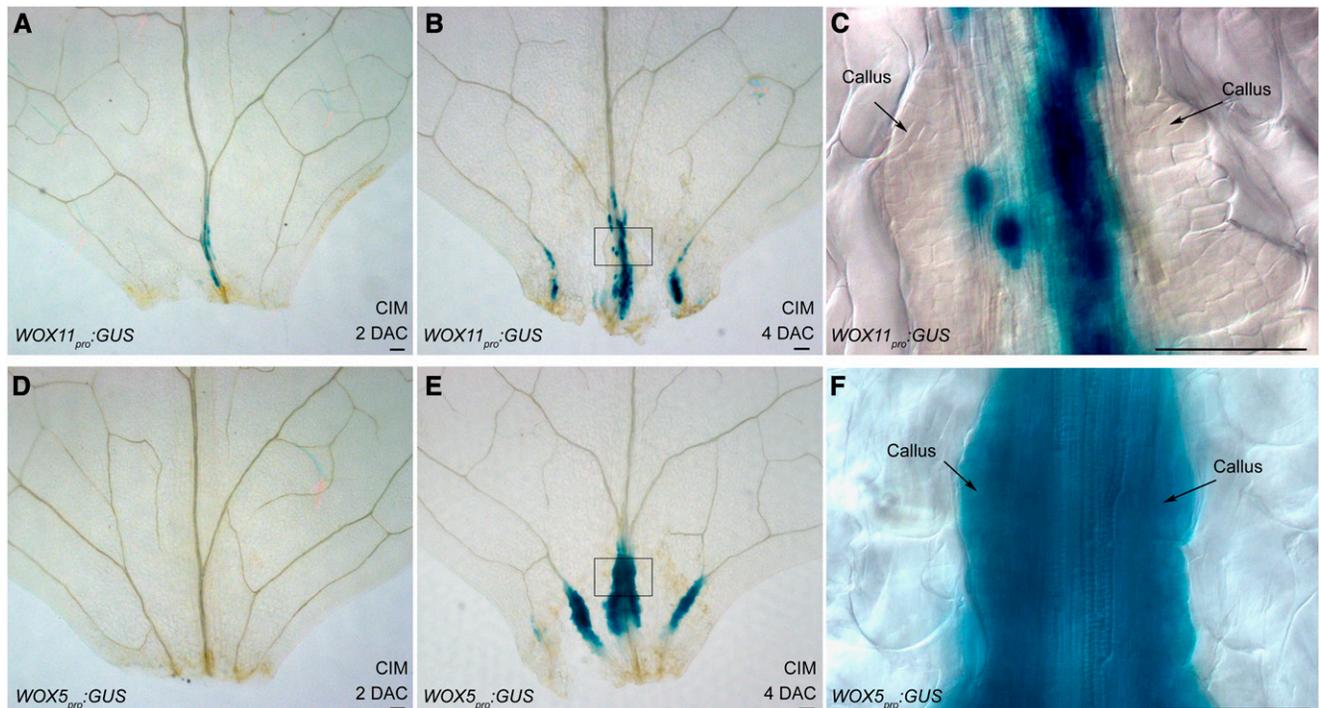
we expected that key genes for initiation of adventitious roots might be identified by analyzing our previously established databases of gene expression and histone methylation during the leaf-to-callus process (He et al., 2012). We selected genes that were differentially expressed between leaf explants and callus, and the identified genes were further evaluated in terms of their IAA responsiveness. *WUSCHEL RELATED HOMEBOX11* (*WOX11*) was one of the genes in the target category as it showed differential expression between leaf explants and callus (Figures 3A and 3B), and its expression was induced by IAA (Figure 3C). Since a previous report showed that the rice (*Oryza sativa*) homolog of *WOX11* is required for the development of the crown root (a kind of adventitious root) (Zhao et al., 2009), we characterized *WOX11* in more detail.

Analysis of GUS staining from leaf explants of the *WOX11<sub>pro</sub>:GUS* marker lines revealed that there was no GUS signal in time-0 leaf explants (Figures 3D and 3E), but a GUS signal was present in vascular tissues near wounds in 2-DAC explants on B5 medium (Figures 3F and 3G), coincident with the region in which auxin was highly accumulated (Figures 1I and 1J). To better understand the *WOX11* expression pattern, we analyzed transverse sections of *WOX11<sub>pro</sub>:GUS* leaf explants. GUS staining was mainly concentrated in the procambium cells and sometimes could also be observed in some xylem parenchyma cells near the procambium (Figure 3H; Supplemental Figure 1). These results were consistent

with the quantitative RT-PCR (qRT-PCR) results (Figure 3C), further indicating that *WOX11* expression is induced by auxin.

In plants, the procambium or cambium is a meristematic tissue that contains adult stem cell populations (Lachaud et al., 1999). Several previous histological observations revealed that both adventitious roots and shoots initiate from the procambium or cambium (Greenwood et al., 2001; Ahkami et al., 2009, 2013; de Almeida et al., 2012; Correa Lda et al., 2012), and callus also initiates from procambium cells (Yu et al., 2010). Thus, it is possible that procambium cells, perhaps together with some parenchyma cells near the procambium in leaf explants, function like the xylem-pole pericycle cells in roots to produce callus, adventitious roots, and adventitious shoots.

We speculated that the expression of *WOX11* marked the transition of stem cell fate from a procambium cell to a root founder cell because these cells initiated divisions to form the root primordium in 4-DAC leaf explants (Figures 3I and 3J). The intensity of GUS staining, which reflected the *WOX11* expression level, was markedly decreased in these small root primordium cells (Figure 3J). To monitor cell fate transition in the newly formed adventitious root primordium, we analyzed leaf explants of the *WOX5<sub>pro</sub>:GUS* transgenic plants because *WOX5* is known to be expressed in cells of the root quiescent center (Gonzali et al., 2005; Sarkar et al., 2007). GUS staining was not detected in root founder cells in

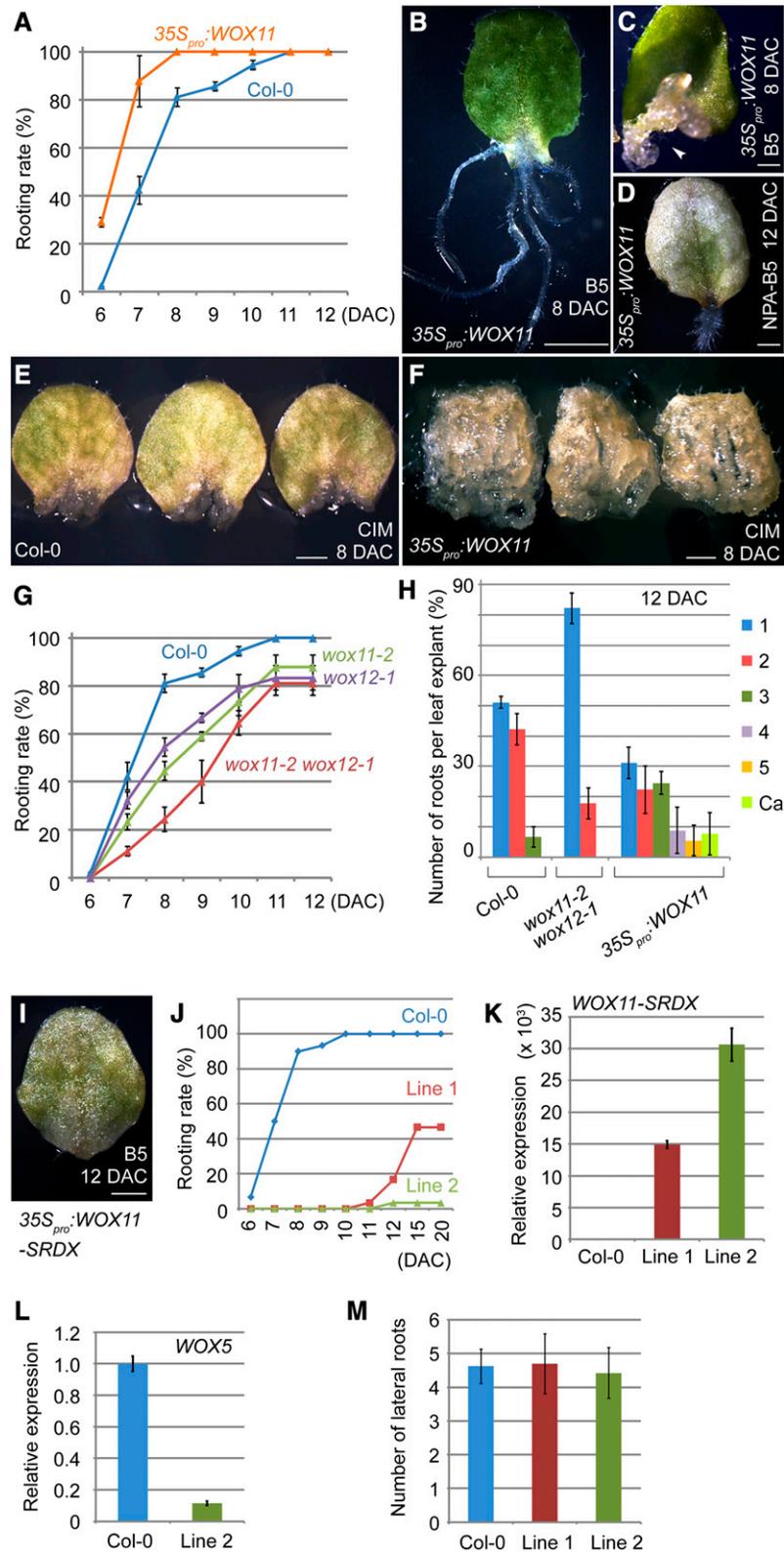


**Figure 5.** *WOX11* and *WOX5* Expression Patterns in Callus Formation.

(A) to (C) GUS staining of the 2-DAC (A) and 4-DAC (B) and (C) leaf explants from *WOX11<sub>pro</sub>:GUS* plants on CIM. GUS staining was found in the vascular tissues near wounds in 2-DAC leaf explants (A) and became stronger at 4 DAC (B) and (C). However, GUS staining was absent in the rapidly proliferating callus cells in the 4-DAC explants (C).

(D) to (F) GUS staining of the 2-DAC (D) and 4-DAC (E) and (F) *WOX5<sub>pro</sub>:GUS* leaf explants cultured on CIM. GUS staining of *WOX5<sub>pro</sub>:GUS* was not detected in the 2-DAC leaf explants (D) but was found in the proliferating callus cells in the 4-DAC explants (E) and (F).

(C) and (F) are close-ups of the boxed regions in (B) and (E), respectively. Bars = 100 μm.



**Figure 6.** WOX11 and WOX12 Are Involved in Adventitious Root and Callus Formation.

2-DAC leaf explants of *WOX5<sub>pro</sub>:GUS* transgenic plants (Figures 3K and 3L), whereas it was clearly visible in root primordium cells of 4-DAC leaf explants (Figures 3M and 3N), indicating the completion of fate transition from root founder cells to root primordium cells. The expression of *WOX5* in the small dividing root primordium cells suggested that these cells may have some quiescent center cell features. Interestingly, the neighboring root founder cells flanking the dividing small root primordium cells continually expressed high levels of *WOX11* (Figure 3J).

To test whether *WOX11* expression was directly induced by auxin, we analyzed the *WOX11* promoter. The promoter harbored at least five auxin response elements (AuxREs) (Figure 4A). Remarkably, *WOX11* expression could be detected in leaf explants of the *WOX11<sub>pro</sub>:GUS* line after ~6 h on B5 medium containing 1  $\mu$ M IAA (Figures 4B and 4C). By contrast, GUS staining of leaf explants from the *mWOX11<sub>pro</sub>:GUS* line, in which three out of the five AuxREs were mutated (Figure 4A), was undetectable after 6 h of IAA induction (Figures 4D and 4E). In addition, no GUS staining was detected in leaf explants of the *WOX11<sub>pro</sub>:GUS* line cultured on NPA-B5 (Figures 4F and 4G). These data indicate that *WOX11* is directly regulated by the auxin signaling pathway.

#### Molecular Evidence for Similar Mechanisms Regulating Adventitious Root and Callus Initiation

*WOX11* and *WOX5* expressions reflect different stages of adventitious root formation from leaf explants. Therefore, if initiation of adventitious roots and of callus share the same regulatory mechanism, the expression patterns of these two genes during callus formation should resemble those during adventitious root formation. Thus, we analyzed the expressions of *WOX11* and *WOX5* during callus formation and compared them with those during adventitious root formation. In 2-DAC

leaf explants of the *WOX11<sub>pro</sub>:GUS* line grown on CIM, there were GUS signals in the vascular bundle of the midvein near wounds (Figure 5A), similar to that observed during the rooting process on B5 medium (Figures 3F and 3G), suggesting that there was cell fate transition to root founder cells. In the 4-DAC explants, small proliferating callus cells with decreased *WOX11* expression appeared (Figures 5B and 5C). *WOX5* was not expressed in 2-DAC leaf explants on CIM (Figure 5D), but it was strongly expressed in the small dividing callus cells in 4-DAC leaf explants (Figures 5E and 5F), suggesting that the cells undergo fate transition from root founder cells to callus cells, which are actually a group of fast-dividing root primordium cells continuously stimulated by the high level of hormones in CIM. Our data showed that the pattern of *WOX11* and *WOX5* expression in callus formation was similar to that during adventitious root formation. The only difference was that both *WOX11* and *WOX5* were expressed more strongly in leaf explants during callus induction on CIM than during adventitious root induction on B5 medium. These results provide further evidence at the cellular and molecular levels that initiation of callus and of adventitious roots share the same genetic pathway.

#### *WOX11* and *WOX12* Act Redundantly in Adventitious Root Formation

To better understand the role of *WOX11* in de novo root organogenesis, we tested the ability of leaf explants from both gain-of-function and loss-of-function *WOX11* plants to regenerate adventitious roots. Compared with that in wild-type Columbia-0 (Col-0), rooting was accelerated in *35S<sub>pro</sub>:WOX11* leaf explants (Figure 6A). In addition, the *35S<sub>pro</sub>:WOX11* leaf explants produced more adventitious roots (Figures 6B and 6H) than did the explants from wild-type Col-0 (Figure 1A). Interestingly, 7.8% of leaf explants

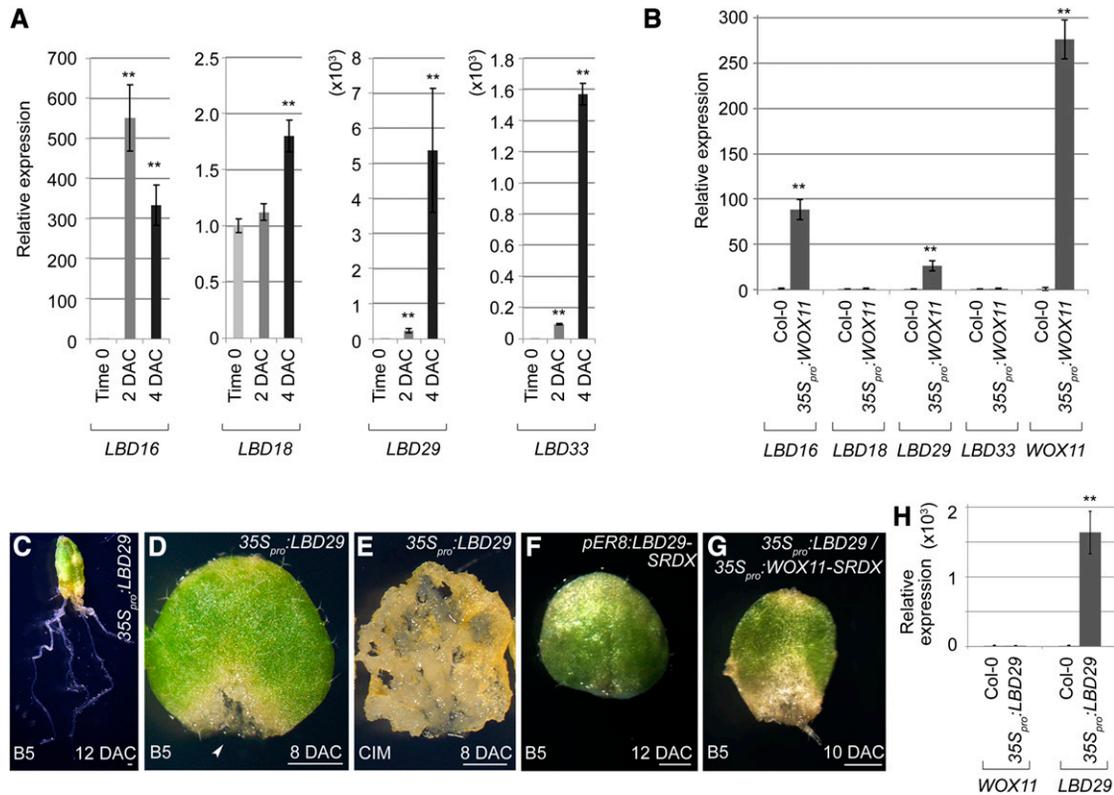
**Figure 6.** (continued).

- (A) Overexpression of *WOX11* accelerated adventitious root formation on B5 medium. Bars show  $\text{SD}$  with three biological repeats.  $n = 30$  in each repeat.
- (B) The 8-DAC leaf explant of *35S<sub>pro</sub>:WOX11*, showing five regenerated adventitious roots on B5 medium.
- (C) The 8-DAC leaf explant of *35S<sub>pro</sub>:WOX11*, showing callus formation (arrowhead) on B5 medium.
- (D) Defective adventitious root formation in 10% of leaf explants (three out of 30 from two independent *35S<sub>pro</sub>:WOX11* lines) on NPA-B5 could be rescued by overexpression of *WOX11*. Shown is a 12-DAC leaf explant.
- (E) and (F) Leaf explants from wild-type (E) and *35S<sub>pro</sub>:WOX11* (F) plants were cultured on CIM. Note that the callus formation in the *35S<sub>pro</sub>:WOX11* leaf explants was markedly accelerated compared with that in the wild-type leaf explants. A total of 30 leaf explants from two independent *35S<sub>pro</sub>:WOX11* lines were tested.
- (G) *WOX11* and *WOX12* functions are involved in de novo root organogenesis. Both *wox11-2* and *wox12-1* displayed delayed adventitious root formation, and the *wox11-2 wox12-1* double mutant showed even slower rooting on B5 medium. Bars show  $\text{SD}$  with three biological repeats.  $n = 30$  in each repeat.
- (H) Quantitative analyses of the adventitious root number per 12-DAC leaf explant grown on B5 medium. Bars show  $\text{SD}$  with three biological repeats.  $n = 30$  in each repeat.
- (I) The 12-DAC leaf explant of the *35S<sub>pro</sub>:WOX11-SRDX* plant Line 2, showing rooting defect on B5 medium.
- (J) Quantitative analyses of the adventitious root formation using leaf explants from two independent *35S<sub>pro</sub>:WOX11-SRDX* transgenic lines, Line 1 and Line 2. A total of 30 leaf explants from each line were analyzed.
- (K) qRT-PCR analyses of *WOX11-SRDX* expression levels in Line 1 and Line 2 of the *35S<sub>pro</sub>:WOX11-SRDX* transgenic plants. Note that the higher *WOX11-SRDX* expression level in Line 2 explants is consistent with its stronger defect in rooting.
- (L) qRT-PCR analysis showed that *WOX5* was only weakly expressed in 4-DAC leaf explants of Line 2, compared with that in wild-type Col-0. In (K) and (L), the value of wild-type leaf explants was arbitrarily fixed at 1.0, and bars show  $\text{SE}$  from three technical repeats.
- (M) Quantitative analyses of the lateral root number per 1 cm in length of the primary root from 12-d-old Line 1 and Line 2 grown on half-strength MS medium. Bars show  $\text{SD}$  from three biological repeats.  $n = 10$  seedlings in each individual repeat.
- Bars = 2 mm in (B) and 1 mm in (C) to (I).

from the  $35S_{pro}::WOX11$  lines produced callus instead of roots on B5 medium (Figures 6C and 6H), similar to the wild-type explants cultured on B5 medium containing 1  $\mu$ M IAA (Figure 2A). Furthermore, a proportion of  $35S_{pro}::WOX11$  leaf explants regenerated adventitious roots on NPA-B5 (Figure 6D), suggesting that overexpression of *WOX11* bypassed the block in auxin signaling. This supports the proposal that *WOX11* is a major response gene of auxin signaling in adventitious root formation. Finally, *WOX11* overexpression caused

rapid callus formation on CIM (Figures 6E and 6F). For wild-type Col-0, the 8-DAC explants only produced small pieces of callus at their proximal parts (Figure 6E), whereas calli formed everywhere on the  $35S_{pro}::WOX11$  explants (Figure 6F). All these results indicate that *WOX11* plays a role in promoting adventitious root and callus formation.

Compared with those of the wild type, leaf explants from the *wox11-2* mutant showed a slight delay in rooting (Figure 6G).



**Figure 7.** *WOX11* Upregulates *LBD16* and *LBD29*.

- (A) qRT-PCR analyses showed that expression levels of the analyzed *LBD* genes were elevated during the culture of leaf explants on B5 medium.
- (B) qRT-PCR analyses of *LBD* and *WOX11* expression using rosette leaves of 12-d-old  $35S_{pro}::WOX11$  seedlings. Two independent  $35S_{pro}::WOX11$  lines were analyzed, and the results were consistent. Values of time-0 leaf explants in (A) and wild-type leaves in (B) were arbitrarily fixed at 1.0. Bars show SE from three technical repeats. \*\* $P < 0.01$  in two-sample *t* test comparing with time-0 leaf explants (A) or wild-type leaves (B).
- (C) The 12-DAC  $35S_{pro}::LBD29$  leaf explant on B5 medium, showing multiple regenerated adventitious roots.
- (D) The 8-DAC  $35S_{pro}::LBD29$  leaf explant cultured on B5 medium, showing callus formation (arrowhead). A total of 40 leaf explants from two independent  $35S_{pro}::LBD29$  lines were analyzed on B5 medium. Among them, eight showed callus growth (D), while the rest regenerated multiple adventitious roots (C).
- (E) The 8-DAC leaf explant of  $35S_{pro}::LBD29$  cultured on CIM, showing robust callus growth. A total of 30 leaf explants from two independent  $35S_{pro}::LBD29$  lines were tested, and all of them showed similar phenotypes.
- (F) The 12-DAC leaf explant of *pER8:LBD29-SRDx*, showing defective adventitious root formation on B5 medium. A total of 70 leaf explants from three independent lines were tested, and 47 of them showed the regeneration defect.
- (G) The 10-DAC leaf explant of  $35S_{pro}::LBD29/35S_{pro}::WOX11-SRDx$ , in which the regeneration defect caused by  $35S_{pro}::WOX11-SRDx$  was partly rescued. The  $35S_{pro}::LBD29/35S_{pro}::WOX11-SRDx$  plants were constructed by crossing a phenotypically tested  $35S_{pro}::LBD29$  line to Line 1 of  $35S_{pro}::WOX11-SRDx$ . The F1 seedlings, after PCR verification, were used for regeneration analysis. Among a total of 16 leaf explants analyzed, 12 formed adventitious roots or callus on B5 medium.
- (H) qRT-PCR analyses of *WOX11* and *LBD29* expression using rosette leaves from 12-d-old  $35S_{pro}::LBD29$  seedlings. Two independent  $35S_{pro}::LBD29$  lines were analyzed, and the results were consistent. Values of wild-type Col-0 leaves were arbitrarily fixed at 1.0, and bars show SE from three technical repeats. \*\* $P < 0.01$  in two-sample *t* test comparing with wild-type leaves.
- Bars = 1 mm in (C) to (G).

WOX12, another member of the WOX family in *Arabidopsis*, shares high protein sequence similarity with WOX11 (Supplemental Figure 2), and a phylogenetic analysis indicated that WOX11 and WOX12 belong to the same clade (Haecker et al., 2004). Like *WOX11*, *WOX12* expression was strongly induced during adventitious root formation, and leaf explants of  $35S_{pro}::WOX12$  also produced more adventitious roots than did the wild-type leaf explants (Supplemental Figure 3). However, like that in *wox11-2*, the rooting process in the *wox12-1* single mutant was mildly affected (Figure 6G). To test the possible functional redundancy of *WOX11* and *WOX12*, we constructed a *wox11-2 wox12-1* double mutant (Supplemental Figure 2). Our data showed that the rooting time of *wox11-2 wox12-1* was more strongly delayed (Figure 6G), and *wox11-2 wox12-1* explants produced fewer roots than did the wild-type Col-0 (Figure 6H). The fact that rooting of the *wox11-2 wox12-1* leaf explants were only partly affected suggests that the *wox12-1* allele may not be strong (Supplemental Figure 2); alternatively, other as yet unknown redundant factor(s) may exist.

Fusion of the repression domain SRDX to a transcription factor can specifically suppress the expression of target genes, resulting in disruption of genetic pathways regulated by the transcription factor, even in the presence of redundant transcription factors (Hiratsu et al., 2003). Therefore, we constructed  $35S_{pro}::WOX11-SRDX$  lines to analyze the role of *WOX11*. The leaf explants from the  $35S_{pro}::WOX11-SRDX$  plants displayed severe rooting defects. In a strong *WOX11-SRDX*-expressing line (Line 2), leaf explants barely formed adventitious roots (Figures 6I to 6K), while in a relatively weak *WOX11-SRDX*-expressing line (Line 1), rooting occurred at a low frequency (Figures 6J and 6K) even with a prolonged culture time. In addition, only very low *WOX5* expression was detected in the Line 2 leaf explants at DAC 4, when they were cultured on B5 medium (Figure 6L). This result is consistent with the fact that rooting in the  $35S_{pro}::WOX11-SRDX$  leaf explants was defective. Interestingly, lateral root initiation on the primary roots was normal in the Line 1 and Line 2 seedlings (Figure 6M). Analyses of the  $35S_{pro}::WOX11-SRDX$  transgenic plants indicate the possibility that *WOX11* may act redundantly with some other *WOX* family members in regulating adventitious root formation.

### WOX11 Promotes Expression of *LBD16* and *LBD29*

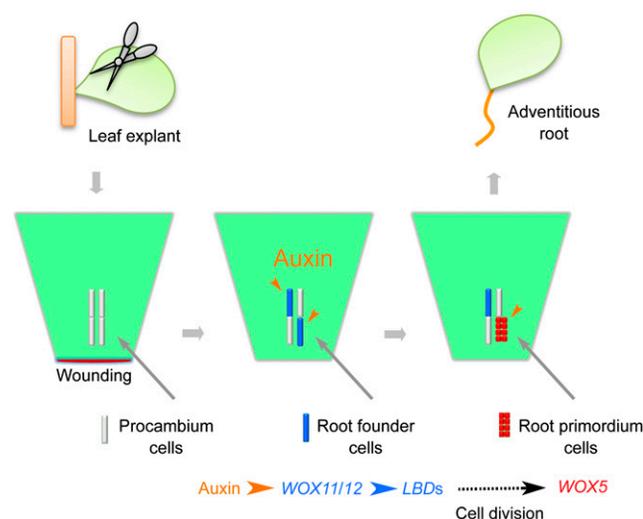
Several *LBD* family genes, such as *LBD16*, *18*, *29*, and *33*, are known to promote callus formation from multiple detached plant tissues (Fan et al., 2012). In an attempt to elucidate the genetic pathway regulating adventitious root formation, we analyzed the expressions of these four genes. During rooting from leaf explants, they were all upregulated, albeit to different levels (Figure 7A). We then determined the expression levels of these *LBD* genes in the leaves of  $35S_{pro}::WOX11$  transgenic plants and found that *LBD16* and *29* were ectopically expressed (Figure 7B).

To confirm the functions of the *LBD* genes in regeneration, we analyzed the phenotypes of the  $35S_{pro}::LBD29$  plants during adventitious root and callus formation. Leaf explants of  $35S_{pro}::LBD29$  lines on B5 medium showed an enhanced ability to regenerate adventitious roots and formed more roots than did the wild type (Figure 7C). A proportion of the leaf explants from

$35S_{pro}::LBD29$  lines was also able to form callus on B5 medium (Figure 7D). In addition,  $35S_{pro}::LBD29$  explants on CIM showed accelerated callus formation (Figure 7E). To analyze the *LBD* functions in callus and adventitious root formation, we constructed *pER8:LBD29-SRDX* transgenic plants, which harbored a chimeric *LBD29-SRDX* fusion under the control of a  $\beta$ -estradiol-inducible promoter. Our results showed that 67% of leaf explants from the *pER8:LBD29-SRDX* transgenic lines failed to form adventitious roots by 12 DAC (Figure 7F). These phenotypes are similar to those observed in the corresponding analyses for *WOX11*. In addition, overexpression of *LBD29* in the  $35S_{pro}::WOX11-SRDX$  background was able to partly rescue the regeneration defect caused by loss of *WOX* functions (Figure 7G). By contrast, upregulation of *WOX11* expression was not detected in leaves of the  $35S_{pro}::LBD29$  transgenic plants (Figure 7H). Taken together, these results indicate that *WOX11* acts upstream of *LBDs*, and *WOX11* regulates adventitious root and callus formation at least partly through the *LBD* pathway.

### DISCUSSION

Our study revealed the cell lineage and molecular mechanisms of de novo root organogenesis. Our data support the following model for the mechanism guiding the first-step cell fate transition (Figure 8). Wounding induces the production of free auxin, which is then highly concentrated in procambium stem cells and their surrounding parenchyma cells via polar auxin transport. The auxin maximum formed in the procambium and parenchyma cells directly induces *WOX11* expression. *WOX11* and *WOX12* then act redundantly to upregulate *LBD16* and *LBD29*. The actions of *WOX11* and *WOX12* together mediate the first-step stem cell fate transition from procambium cells to root founder cells.



**Figure 8.** A Model for de Novo Root Organogenesis.

A cellular and molecular framework of de novo root organogenesis from *Arabidopsis* leaf explants is shown in the model. Note that there are two steps of cell fate transition during adventitious root formation.

It was previously reported that several *LBD* genes participate in lateral root formation (Berckmans et al., 2011; Feng et al., 2012a, 2012b; Lee et al., 2012). During the initiation of root primordia, *LBD18* was found to regulate the cell cycle-controlling gene *E2Fa* and the cell wall-loosening factor gene *EXPANSIN14* (Berckmans et al., 2011; Lee et al., 2012). These findings suggested a possible role for *LBDs* in promoting cell division and in preparation for the second-step cell fate transition from root founder cells to root primordium cells (see the model in Figure 8). In addition, AuxREs are also present in the promoter regions of the *LBD* genes (Okushima et al., 2007), suggesting that expression of these *LBDs* may also depend on the auxin signaling pathway.

Based on the genetic pathways involved, we propose that initiation of adventitious roots shares similar regulatory mechanisms with that of callus, and whether leaf explants produce adventitious roots or callus mainly depends on free auxin levels. Since the processes of lateral root and adventitious root formation after their initiation are rather similar developmentally, it is likely that they share some similar regulation mechanisms, such as those involving *LBD* and *ALF4* genes and others involved in the late root developmental stages. The only possible difference between adventitious root/callus formation and lateral root formation that we found in this study was in the genes involved in the first-step cell fate transition. In particular, adventitious root and callus formation require *WOX11* and *WOX12*, whereas lateral root initiation does not. We showed that lateral roots initiated normally from primary roots of the *35S<sub>pro</sub>:WOX11-SRDX* plants, whereas initiation of adventitious roots was severely defective in leaf explants from the same *35S<sub>pro</sub>:WOX11-SRDX* lines. By contrast, the *arf7 arf19* double mutant showed defective lateral root formation, whereas it had the normal adventitious root and callus formation. *ARF7* and *19* were previously known to directly activate *LBDs* during lateral root formation (Okushima et al., 2007). Because de novo root regeneration from a detached organ is not a usual process in the development of *Arabidopsis* plants, unlike lateral root formation, activation of *LBD* genes during de novo root regeneration may require additional regulatory processes, such as that involving *WOX11/12* action.

The procambium or cambium is thought to be a population of multipotent adult stem cells that are responsible for stem thickening and production of the xylem and phloem in the vascular tissues (Lachaud et al., 1999). Among many different cell types in the leaf explant, procambium cells, probably also including some other parenchyma cells surrounding the procambium, are likely to serve as competent cells that initiate adventitious roots and callus. This is not only supported by several previous studies (Greenwood et al., 2001; Ahkami et al., 2009, 2013; Yu et al., 2010; Correa Lda et al., 2012; de Almeida et al., 2012), but also supported by the specific *WOX11* expression patterns upon auxin induction. It was proposed that callus is initiated from the pericycle-like cells surrounding the vascular tissues when aerial organs are used as explants, but the nature of the pericycle-like cells was unknown (Sugimoto et al., 2010, 2011). Based on our current data, we propose that the procambium cells may serve as one type of pericycle-like cells in aerial organs. *WOX11* is usually expressed only in the protoxylem cells in the apical region of a root under natural growth conditions. However, *WOX11* could be rapidly induced in the xylem-pole

pericycle cells after growing seedlings were moved to medium containing auxin (Supplemental Figure 4). Because the xylem-pole pericycle cells are known to have the potential to initiate callus, the similar *WOX11* expression pattern in xylem-pole pericycle and procambium cells after auxin induction suggests that these cells have similar features in callus formation.

The *WOX11* expression pattern depends on auxin distribution, and auxin induction relies on wounding of explants at the beginning of de novo root organogenesis. Therefore, how wounding induces auxin accumulation and how auxin is specifically transported to certain procambium cells are the next important questions to address.

## METHODS

### Plant Materials and Tissue Culture

The mutants *wox11-2* (SALK\_004777), *wox12-1* (SALK\_087882) (Alonso et al., 2003), *alf4-1* (Celenza et al., 1995), and *arf7-1 arf19-1* (Okushima et al., 2005) are in the Col-0 background, and *clf-50 swm-1* is in the Wassilewskija background (Chanvittana et al., 2004). For construction of the *WOX11<sub>pro</sub>:GUS* transgenic plants, a 4.8-kb fragment upstream to the *WOX11* coding region was subcloned into the pBI101 vector, which was then used for plant transformation. The *mWOX11<sub>pro</sub>:GUS* construct was made by PCR-based modifications of *WOX11<sub>pro</sub>:GUS*. The *DR5<sub>pro</sub>:GUS* line in Landsberg *erecta* and the *WOX5<sub>pro</sub>:GUS* line in Col-0 were described previously (Ulmasov et al., 1997; He et al., 2012). *35S<sub>pro</sub>:WOX11* and *35S<sub>pro</sub>:LBD29* were constructed by insertion of cDNA fragments encoding the full length of *WOX11* and *LBD29*, respectively, into the pMON530 vector. *35S<sub>pro</sub>:WOX11-SRDX* was constructed by first fusing a sequence encoding the SRDX peptide (Hiratsu et al., 2003) with the *WOX11* cDNA. The fusion was then inserted into pMON530. *pER8:LBD29-SRDX* was constructed by insertion of the *LBD29* cDNA-SRDX fusion into the pER8 vector (Zuo et al., 2000). All transgenic lines were obtained by the *Agrobacterium tumefaciens*-mediated transformation to wild-type Col-0. Primers and restriction sites for cloning are listed in Supplemental Table 1.

For generation of adventitious roots or callus, *Arabidopsis thaliana* seeds were grown on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) at 22°C, with 16 h of light. The first two rosette leaves were cut from 12-d-old seedlings and cultured on B5 medium (Gamborg B5 basal medium with 0.5 g/L MES, 3% Suc, and 0.8% agar, pH 5.7), CIM (B5 medium with 0.2 μmol/L kinetin and 2.2 μmol/L 2,4-D), or B5 medium with additional chemicals at 22°C in the dark to induce adventitious roots or callus for phenotype analysis, GUS staining, and qRT-PCR. Leaves from 25-d-old seedlings were used for sectioning analysis.

### GUS Staining, Thin Sectioning, and Microscopy

GUS staining was performed by incubation of leaf explants from the *DR5<sub>pro</sub>:GUS*, *WOX11<sub>pro</sub>:GUS*, *mWOX11<sub>pro</sub>:GUS*, and *WOX5<sub>pro</sub>:GUS* lines at 37°C in GUS assay solution (He et al., 2012) for 2, 12, 12, and 2 h, respectively. For differential interference contrast microscopy observation, the stained tissues were incubated in the chloral hydrate solution (200 g chloral hydrate, 20 g glycerol, and 50 mL water) (Tsuge et al., 1996) at 65°C for ~12 h for tissue transparency. Thin sectioning was performed as previously described (Xu et al., 2003).

### RT-PCR and qRT-PCR

RT-PCR and qRT-PCR were performed according to the methods described previously (Xu et al., 2003; He et al., 2012) using gene-specific

primers. The qRT-PCR results are shown as the relative expression levels, which were normalized against those produced by the primers for *ACTIN*. Primers for RT-PCR and qRT-PCR are listed in Supplemental Table 1.

#### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *WOX11* (At3g03660) and *WOX12* (At5g17810).

#### Supplemental Data

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

**Supplemental Figure 1.** *DR5* and *WOX11* Expression in Leaf Explants during Adventitious Root Formation.

**Supplemental Figure 2.** Identification of the *wox11* and *wox12* Mutants.

**Supplemental Figure 3.** *WOX12* Is Involved in Adventitious Root Formation.

**Supplemental Figure 4.** Auxin Induces *WOX11* Expression in Root Pericycle Cells.

**Supplemental Table 1.** List of Primers Used in This Study.

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#### AUTHOR CONTRIBUTIONS

J. Liu, L.S., Y.X., H.H., and L.X. designed the research. J. Liu, L.S., Y.X., and J. Li performed research. All authors analyzed data. H.H. and L.X. wrote the article.

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