# Transcription Factors WOX11/12 Directly Activate *WOX5/7* to Promote Root Primordia Initiation and Organogenesis[1]

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De novo organogenesis, which gives rise to adventitious roots and shoots, is a type of plant regeneration for survival after wounding. In Arabidopsis (*Arabidopsis thaliana*), two main cell fate transition steps are required to establish the root primordium during de novo root organogenesis from leaf explants. The first step from regeneration-competent cells to root founder cells involves activation of *WUSCHEL-RELATED HOMEOBOX11* (*WOX11*) and *WOX12* (*WOX11/12*) expression by auxin. However, the molecular mechanism controlling the second step of fate transition from root founder cells to root primordium is poorly understood. In this study, we show that the expression levels of *WOX11/12* decrease while those of *WOX5* and *7* (*WOX5/7*) increase during the transition from root founder cells to the root primordium. *WOX11/12* function genetically upstream of *WOX5/7*, and the WOX11/12 proteins directly bind to the promoters of *WOX5/7* to activate their transcription. Mutations in *WOX5/7* result in defective primordium formation. Overall, our data indicate that the expression switch from *WOX11/12* to *WOX5/7* is critical for initiation of the root primordium during de novo root organogenesis.

Plants have powerful regenerative abilities that allow them to reproduce vegetatively by forming a whole plant from somatic cells (Sugimoto et al., 2011; Xu and Huang, 2014; Ikeuchi et al., 2016). De novo organogenesis, a process in which adventitious roots and shoots regenerate from wounded or detached organs, is a type of vegetative reproduction that is widely exploited in modern agricultural applications (Sussex, 2008; Duclercq et al., 2011; Xu and Huang, 2014). De novo root organogenesis commonly occurs in nature, because wounded or detached plant organs can rapidly regenerate adventitious roots to ensure their survival (Bellini et al., 2014; Xu and Huang, 2014; Steffens and Rasmussen, 2016).

Previously, we established a simple method of de novo root organogenesis by culturing leaf explants of Arabidopsis (*Arabidopsis thaliana*) on B5 medium without exogenous hormones (Chen et al., 2014; Liu et al., 2014). In this system, endogenous hormones are sufficient to induce adventitious root formation from leaf explants. Using this method, we revealed that there are at least two steps of cell fate transition required for the formation of the newly regenerated root primordia. In the first step, regeneration-competent cells (i.e. procambium and vascular parenchyma cells) become root founder cells, which are marked by *WUSCHEL-RELATED HOMEOBOX 11* and *12* (hereafter abbreviated as *WOX11/12*). In the second step, root primordium cells, which are marked by *WOX5*, initiate from root founder cells, and this step involves cell division.

The molecular mechanism of the first-step cell fate transition (i.e. from competent cells to root founder cells) involves the activation of *WOX11/12* expression by auxin (Liu et al., 2014), a major hormone that triggers adventitious rooting (Greenwood et al., 2001; De Klerk, 2002; Ahkami et al., 2009; Correa et al., 2012; Liu et al., 2014). Endogenous free auxin is quickly produced in mesophyll cells upon wounding, and then polar transported into competent cells near the wound (Liu et al., 2014). In competent cells, auxin directly up-regulates transcription of *WOX11/12* for priming root founder cells (Liu et al., 2014). Therefore, *WOX11/12* seem to be pivotal genes that turn on the molecular pathway for adventitious rooting. Blocking of *WOX11/12* resulted in rooting defects, and overexpression of *WOX11/12* greatly enhanced the rooting process (Liu et al., 2014).

However, the molecular mechanism of the second-step cell fate transition (i.e. from root founder cells to root primordium) is barely understood. A key question is what molecular mechanism underlies the ability of *WOX11/12* to convey the rooting process from root

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founder cells to root primordium cells. *WOX5* is a molecular marker in the root primordium; however, whether it functions in controlling root primordium formation and how its transcription is activated in the root primordium are unclear. In this study, we show that *WOX11/12* directly activates the transcription of *WOX5* and its most closely related gene, *WOX7* (hereafter, *WOX5/7*; Supplemental Fig. S1A) and that this is essential for the initiation of a normal root primordium. *WOX11/12* and *WOX5/7* belong to two different clades of the WOX family. *WOX11/12* are intermediate-clade *WOX* genes, while *WOX5/7* are in the WUS clade (Supplemental Fig. S1A; Haecker et al., 2004; Sarkar et al., 2007; Mukherjee et al., 2009; Nardmann et al., 2009; van der Graaff et al., 2009; Zhao et al., 2009; Nardmann and Werr, 2013; Lian et al., 2014; Pi et al., 2015; Ge et al., 2016; Zeng et al., 2016). Therefore, we propose that the switch between the two clades of *WOX* genes represents the fate transition from root founder cells to root primordium cells in de novo root organogenesis.

## RESULTS

### *WOX11/12*-to-*WOX5/7* Expression Switch during Root Primordium Initiation

We cultured the Arabidopsis leaf explants on B5 medium without added hormones, and adventitious roots regenerated from the wounded site on the leaf explants (Fig. 1A; Chen et al., 2014; Liu et al., 2014). To analyze the mechanism by which the root primordium initiates from root founder cells, we first analyzed the expression patterns of *WOX* genes during adventitious rooting.

Using $WOX11_{pro}$*:GUS* (Liu et al., 2014) and $WOX12_{pro}$*:GUS* reporter lines, in which the promoters of *WOX11* and *12* were fused to the gene encoding GUS, we found that the expression of *WOX11/12* was activated at the very beginning of adventitious rooting from the leaf explant, when competent cells became root founder cells at 1 d after culture (DAC; Fig. 1, B and C; Supplemental Fig. S2, A and B; Liu et al., 2014). Their expression could be observed during the initial cell divisions at 3 DAC (Fig. 1D; Supplemental Fig. S2C), but decreased when the dome-shaped root primordium formed at 4 DAC (Fig. 1E; Supplemental Fig. S2D; Liu et al., 2014). No *WOX11/12* expression was detected in the newly formed root apical meristem (RAM) at 5 DAC (Fig. 1F; Supplemental Fig. S2E).

We then analyzed the expression patterns of *WOX5/7* in adventitious rooting using $WOX5_{pro}$*:GUS* (Liu et al., 2014) and $WOX7_{pro}$*:GUS* reporter lines. No *WOX5/7* expression was detected during root founder cell formation at 1 DAC (Fig. 1, G and H; Supplemental Fig. S2, F and G). However, *WOX5/7* expression increased during the initial cell divisions at 3 DAC (Fig. 1I; Supplemental Fig. S2H). This expression pattern of *WOX5/7* overlapped with that of *WOX11/12*. The expression of *WOX5/7* continued in the dome-shaped root primordium at 4 DAC (Fig. 1J; Supplemental Fig. S2I), when *WOX11/12* expression decreased.

During the formation of the RAM, *WOX5* expression was gradually confined to the center region (Supplemental Fig. S2J). Expression of *WOX7* was also gradually restricted to the inner region of the newly formed RAM (Fig. 1K). After formation of the RAM, the root tip emerged from the leaf explant. As it emerged, the expression of *WOX5* and *WOX7* continued to be gradually restricted to the stem cell niche (Supplemental Fig. S3), which comprises the quiescent center (QC) and initial cells (Scheres, 2007).

Together, these results show that *WOX11/12* function during the very first event in root founder cell establishment and that *WOX5/7* start to function when root founder cells divide to form root primordium cells.

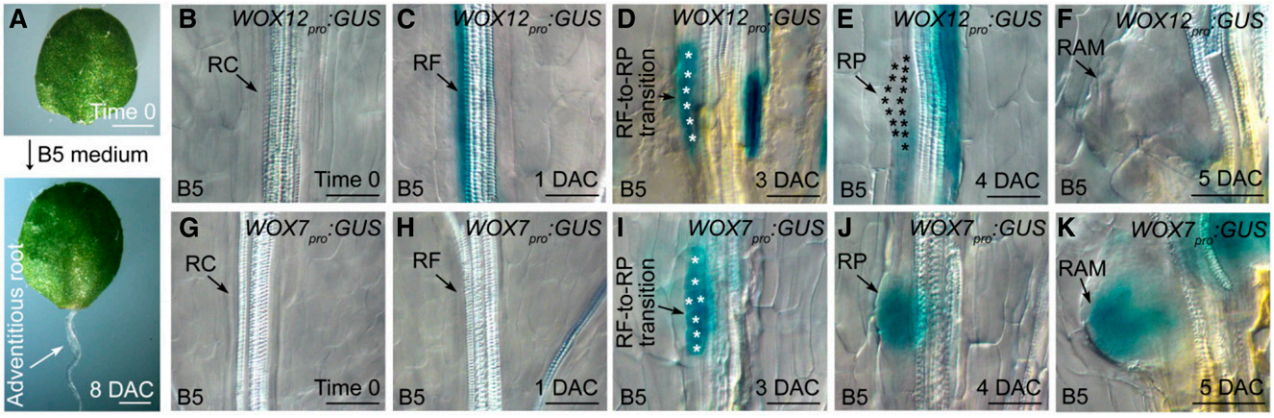


**Figure 1.** Expression patterns of *WOX12* and *WOX7* during adventitious rooting. A, System of de novo root organogenesis used in this study (Chen et al., 2014). B to F, GUS staining of $WOX12_{pro}$*:GUS* in leaf explants at time 0 (B), 1 DAC (C), 3 DAC (D), 4 DAC (E), and 5 DAC (F). G to K, GUS staining of $WOX7_{pro}$*:GUS* in leaf explants at time 0 (G), 1 DAC (H), 3 DAC (I), 4 DAC (J), and 5 DAC (K). White asterisks in D and I indicate transient step in which root founder cells are undergoing cell division to form root primordium. Black asterisks in E indicate dome-shaped root primordia. RC, regeneration-competent cell; RF, root founder cell; RP, root primordium. Bars = 1 mm (A) and 50 $\mu$m (B–K).

### *WOX5/7* Are Involved in Root Primordium Formation

To explore the developmental role of *WOX5/7* in de novo root organogenesis, we first analyzed the phenotypes of adventitious roots formed from leaf explants of the *wox5-1* and *wox7-1* single mutants and the double mutant. The rooting rate was reduced in the *wox5-1* single mutant and further reduced in the *wox5-1 wox7-1* double mutant (Fig. 2A), suggesting that mutations in *WOX5/7* result in slow development of adventitious roots. In addition, the number of adventitious roots that regenerated from leaf explants was reduced in the *wox5-1* single mutant, and this number was further decreased in the *wox5-1 wox7-1* double mutant (Fig. 2B). In *pER8:WOX5* transgenic lines, β-estradiol-induced overexpression of *WOX5* increased the number of roots produced per leaf explant (Fig. 2, C and D). These results indicate that *WOX5* and *WOX7* are involved in de novo root organogenesis.

To further analyze the role of *WOX5/7* at the cellular level, we carefully compared the root primordia in the *wox5-1 wox7-1* double mutant with those in the wild type. In the dome-shaped root primordium with two cell layers from wild-type leaf explants, cells were regularly arranged with coordinated cell division between the two layers (Fig. 3, A and B). However, all root primordia in the *wox5-1 wox7-1* double mutant showed a disorganized and irregularly arranged cell pattern, although cell division had occurred. Some mildly defective root primordia of *wox5-1 wox7-1* showed uncoordinated cell division between the two layers (Fig. 3, C and D). In some severely defective root primordia of *wox5-1 wox7-1*, both anticlinal and periclinal cell divisions were disorganized and the shape of the root primordium was lost (Fig. 3, E and F).

Although most of the root primordia in *wox5-1 wox7-1* were able to form RAMs and finally root tips, the newly formed RAMs in *wox5-1 wox7-1* showed defective stem cell niche formation (Fig. 3, G–J). In the newly formed RAMs in wild-type leaf explants, cells in the outermost layer (Fig. 3, G and H, black asterisks) were destined to form the root cap, and those in the center of the second layer (Fig. 3, G and H, white asterisks) were destined to form the stem cell niche, including QC during the next stage of development (Malamy and Benfey, 1997; Goh et al., 2016). In the newly formed RAMs in *wox5-1 wox7-1* leaf explants, we observed defects with irregular cell division in both the outermost layer and the region where the stem cell niche was going to form (Fig. 3, I and J). The defective RAM patterning resulted in the loss of the QC in the stem cell niche in adventitious root tips regenerated from *wox5-1 wox7-1* leaf explants (Supplemental Fig. S4).

The results of these phenotype analyses indicate that *WOX5/7* are required for proper organization of adventitious root primordium and subsequent stem cell niche establishment in the newly formed RAM.

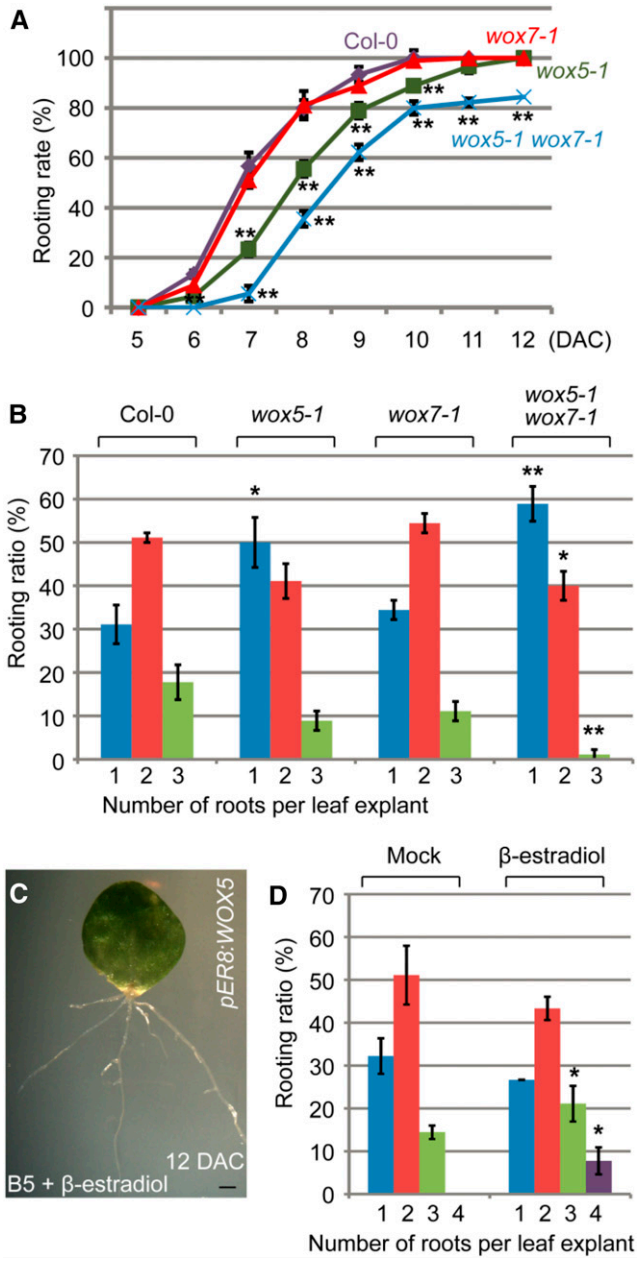


**Figure 2.** *WOX5/7* are involved in adventitious rooting. A, Rooting rate of leaf explants (percentage of leaf explants with regenerated adventitious roots) from wild-type Col-0, *wox5-1* single mutant, *wox7-1* single mutant, and *wox5-1 wox7-1* double mutant on B5 medium. B, Quantitative analyses of ratio of adventitious root number per 15-DAC leaf explant from Col-0, *wox5-1*, *wox7-1*, and *wox5-1 wox7-1* cultured on B5 medium. C, Leaf explant from *pER8:WOX5* at 12 DAC on B5 medium containing 10 μM β-estradiol. D, Ratio of adventitious root number per 15-DAC leaf explant from *pER8:WOX5* cultured on B5 medium without (mock) or with 10 μM β-estradiol. Bars in A, B, and D show SD from three biological repeats ($n = 30$ per repeat). $^*P < 0.05$ and $^{**}P < 0.01$ in two-sample *t* tests, compared with Col-0 (A and B) or mock (D). Bar = 1 mm (C).

### *WOX11/12* Promote *WOX5/7* Expression for Root Primordium Initiation

To test the genetic relationship between *WOX11/12* and *WOX5/7*, we first performed quantitative reverse transcription-PCR (qRT-PCR) analyses to determine the transcript levels of *WOX5/7* in $35S_{pro}$*:WOX11* and $35S_{pro}$*:WOX12* transgenic lines, which overexpressed

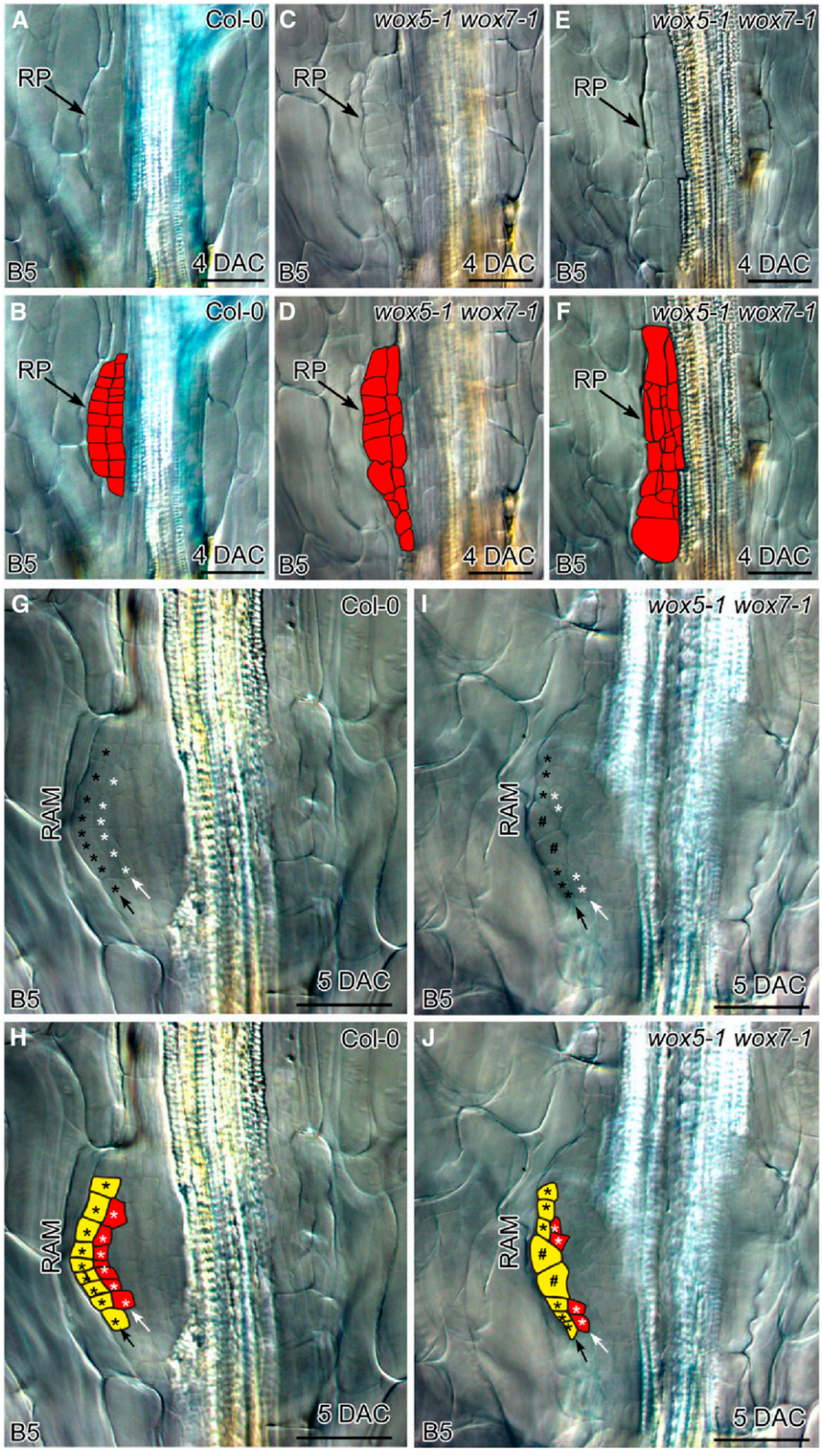


**Figure 3.** *WOX5/7* are involved in cell division and tissue organization during adventitious root primordium formation. A and B, Adventitious root primordium in 4-DAC wild-type Col-0 leaf explant showing two layers of cells constituting the typical dome-shaped primordium. Note that cells in the primordium were approximately the same size, suggesting that cell division is coordinated between the two layers. C and D, Adventitious root primordium in 4-DAC *wox5-1 wox7-1* leaf explant, showing mildly defective root primordium organization. Note that cell sizes differed within the primordium, indicating that cell division between the two layers was not properly coordinated. E and F, Adventitious root primordium in 4-DAC *wox5-1 wox7-1* leaf explant, showing strongly defective root primordium organization. Note severely disordered cell division and complete loss of dome shape. G and H, Newly formed RAM in 5-DAC wild-type Col-0 leaf explant. Black arrow shows outermost layer that will develop into the root cap. White arrow shows the second layer, which will harbor the QC of the stem cell niche at its center. I and J, Newly formed RAM in 5-DAC *wox5-1 wox7-1* leaf explant. Note enlarged cells in the center of the outermost layer (indicated by #) and absence of stem cell niche in the second layer. B, D, F, H, and J are schematics of each DIC image in A, C, E, G, and I, respectively. We analyzed 54 leaf explants from *wox5-1 wox7-1* at 4 DAC; 29 showed mildly defective root primordia as shown in C and D, and 25 showed strongly defective root primordia as shown in E and F. We analyzed 30 leaf explants from *wox5-1 wox7-1* at 5 DAC; 26 showed defective RAM organization as shown in I and J and four showed arrested development at the primordium stage with no RAM formation. RP, root primordium. Bars = 50 $\mu$m (A–J).

*WOX11* and *WOX12*, respectively. Transcripts of *WOX5* and *WOX7* could not be detected in wild-type leaves, but both *WOX5* and *WOX7* were detected in leaves of the *WOX11*- and *WOX12*-overexpression lines (Fig. 4, A and B), suggesting that *WOX11/12* can activate the transcription of *WOX5/7*.

Next, we analyzed the regulation of *WOX5/7* by *WOX11/12* during adventitious rooting from leaf explants. Expression of *WOX5/7* could be detected in 4-DAC wild-type leaf explants by qRT-PCR. The transcript levels of *WOX5/7* were reduced in 4-DAC leaf explants from the *wox11-2 wox12-1* double mutant (Fig. 4, C and D), which shows a mild defect in adventitious rooting (Liu et al., 2014). *WOX5/7* expression levels were severely reduced in 4-DAC leaf explants from the $35S_{pro}$*:WOX11-SRDX* lines (Fig. 4, C and D; Liu et al., 2014), in which a repression domain SRDX (Hiratsu et al., 2003) is fused to the WOX11 protein to block the *WOX11* pathway and adventitious rooting (Liu et al., 2014). Using the $WOX5_{pro}$*:GUS* reporter line, we observed that the *WOX5*-marked root primordium usually formed at the midvein of the leaf explants near the wound site (Fig. 4, E and F). In contrast, in the $35S_{pro}$*:WOX11* leaf explants, *WOX5*-marked root primordia formed in many regions, including lateral veins (Fig. 4, G and H). Occasionally, *WOX5* expression could be observed in mesophyll cells in the $35S_{pro}$*:WOX11* leaf explants (Fig. 4G). In the $35S_{pro}$*:WOX11-SRDX* background, no *WOX5*-marked root primordia formed from the leaf explants (Fig. 4, I and J). Similar results were obtained using the $WOX7_{pro}$*:GUS* reporter line (Supplemental Fig. S5). These data suggest that *WOX11/12* play a role in activating *WOX5/7* expression during de novo root organogenesis.

Leaf explants of $35S_{pro}$*:WOX11* and $35S_{pro}$*:WOX12* had enhanced rooting abilities and produced many more adventitious roots from the wound site than did leaf explants of wild type (Fig. 5A; Supplemental Fig. S6A; for comparison, see Fig. 1A; Liu et al., 2014). We introduced $35S_{pro}$*:WOX11* and $35S_{pro}$*:WOX12* into the

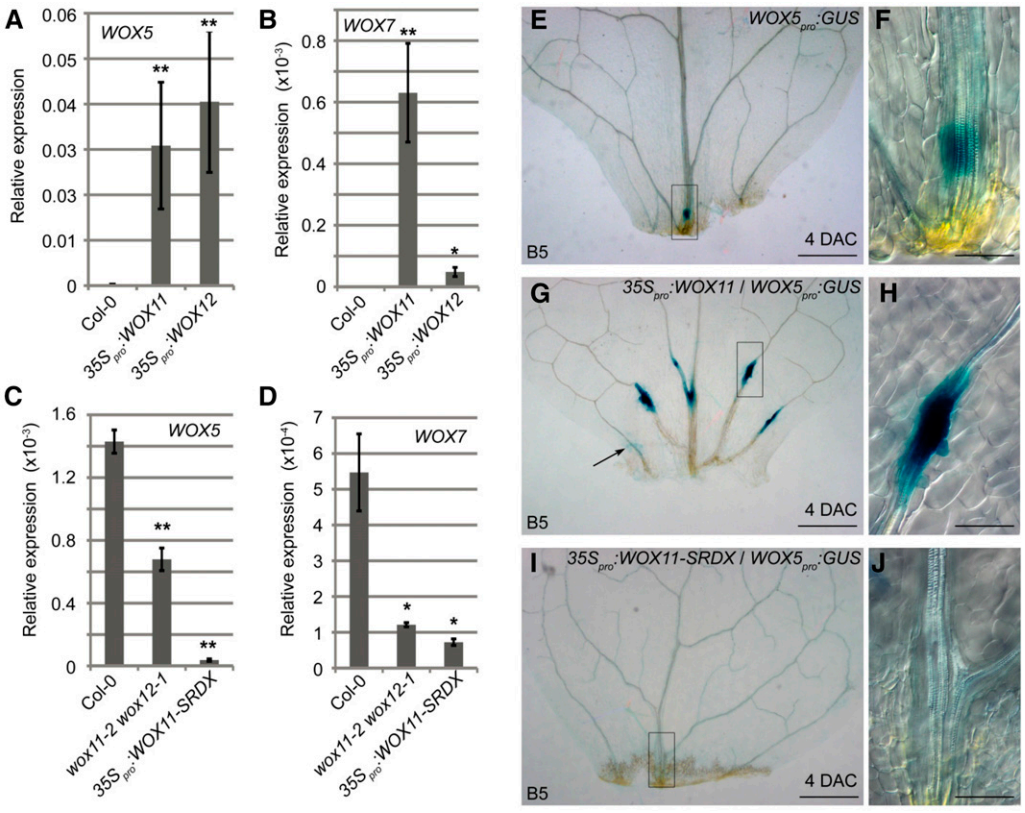


**Figure 4.** *WOX11/12* up-regulate *WOX5/7* expression. A and B, qRT-PCR analyses showing transcripts of *WOX5* (A) and *WOX7* (B) ectopically expressed in leaves of $35S_{pro}$*:WOX11* and $35S_{pro}$*:WOX12*. C and D, qRT-PCR analyses of transcripts of *WOX5* (C) and *WOX7* (D) in 4-DAC leaf explants from the wild-type Col-0, *wox11-2 wox12-1*, and $35S_{pro}$*:WOX11-SRDX* on B5 medium. Note that *WOX5* expression in $35S_{pro}$*:WOX11-SRDX* was previously reported (Liu et al., 2014). E to J, GUS staining of leaf explants from $WOX5_{pro}$*:GUS* in wild type (E and F), $35S_{pro}$*:WOX11* (G and H), and $35S_{pro}$*:WOX11-SRDX* (I and J) backgrounds at 4 DAC on B5 medium. F, H, and J are close-ups of the boxed regions in E, G, and I, respectively. Arrow in G indicate GUS signal in mesophyll cells. Bars in A to D show SE from three biological repetitions. Each biological repetition was performed with three technical repetitions. $*P < 0.05$ and $**P < 0.01$ (two-sample *t* test, compared with Col-0). Bars = 500 $\mu$m (E, G, and I) and 100 $\mu$m (F, H, and J).

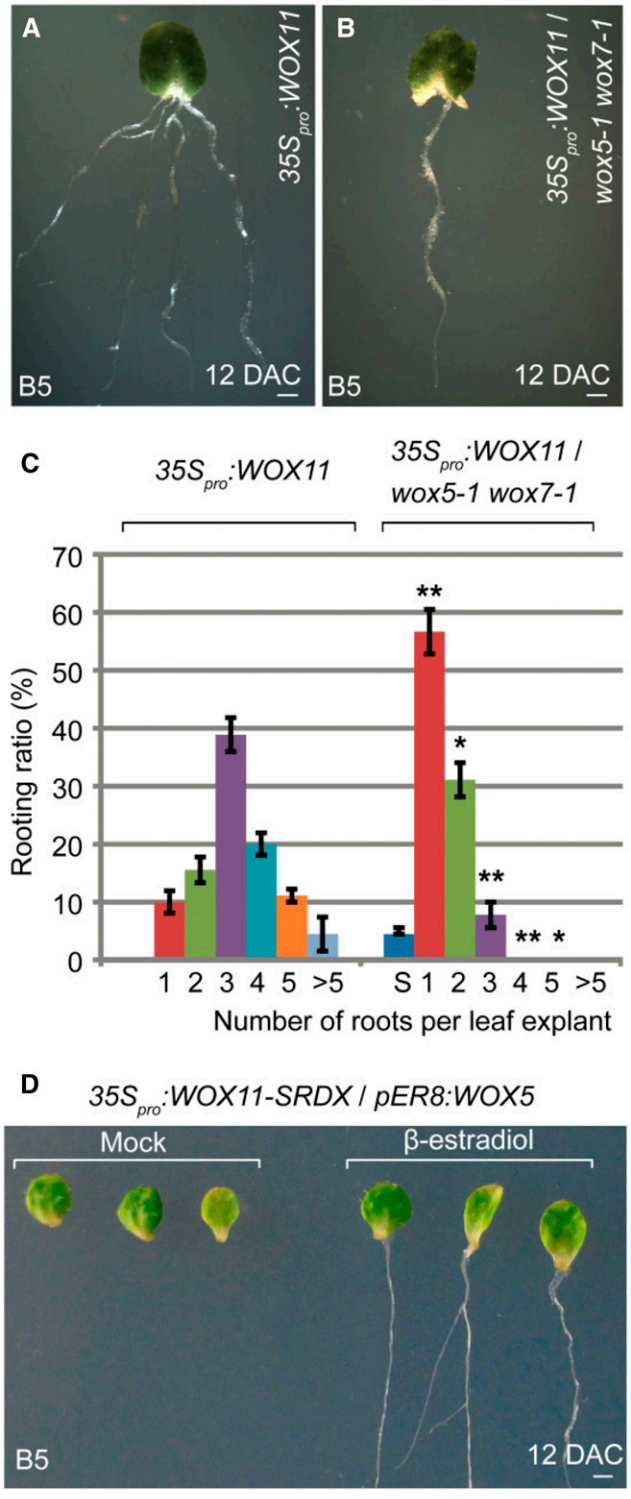


**Figure 5.** Genetic analysis of *WOX11/12* and *WOX5/7*. A and B, Leaf explants from *35S$_{pro}$:WOX11* (A) and *35S$_{pro}$:WOX11/wox5-1 wox7-1* (B) at 12 DAC on B5 medium. C, Ratio of adventitious root number per 15-DAC leaf explants from *35S$_{pro}$:WOX11* and *35S$_{pro}$:WOX11/wox5-1 wox7-1* on B5 medium. S, a small portion of leaf explants turned yellow and became senescence. Bars show SD from three biological repeats ($n$ = 30 per repeat). *$P < 0.05$ and **$P < 0.01$ (two-sample $t$ test, compared with *35S$_{pro}$:WOX11*). D, Leaf explants from *35S$_{pro}$:WOX11-SRDX/pER8:WOX5* at 12 DAC cultured on B5 medium without (left) or with (right) 10 $\mu$M $\beta$-estradiol. None of the 29 leaf explants cultured on B5 medium without $\beta$-estradiol formed adventitious roots. Seven of 29 leaf explants cultured on B5 medium with 10 $\mu$M $\beta$-estradiol regenerated adventitious roots. Bars = 1 mm (A, B, and D).

*wox5-1 wox7-1* double mutant background. The rooting abilities of *35S$_{pro}$:WOX11* and *35S$_{pro}$:WOX12* were reduced in the *wox5-1 wox7-1* background compared with those in the wild-type background (Fig. 5, A–C; Supplemental Fig. S6). These data suggest that the rooting-promotion abilities of *WOX11/12* at least partly depend on the function of *WOX5/7*.

Rooting was severely blocked in *35S$_{pro}$:WOX11-SRDX* leaf explants (Fig. 5D; Liu et al., 2014). When we introduced a $\beta$-estradiol-induced *WOX5*-overexpression vector, *pER8:WOX5*, into the *35S$_{pro}$:WOX11-SRDX* background, the overexpression of *WOX5* partly rescued the rooting defect caused by *WOX11-SRDX* (Fig. 5D). This result suggested that *WOX5* is a functional downstream gene controlled by the *WOX11* pathway.

Based on these genetic data, together with the expression analyses of *WOX11/12* and *WOX5/7*, we propose that *WOX11/12* promote *WOX5/7* expression for the fate transition of root founder cells to root primordium cells.

### WOX11/12 Directly Activate *WOX5/7* Expression

We conducted a chromatin immunoprecipitation (ChIP) experiment to test whether WOX11/12 proteins bind to the *WOX5/7* loci. Using the anti-FLAG antibody, we detected that the 3×FLAG-WOX11-GR and 3×FLAG-WOX12 fusion proteins directly bound to the predicted WOX-binding cis elements (TTAATGG; Lohmann et al., 2001; Leibfried et al., 2005; Zhao et al., 2009) on the promoters of *WOX5* and *WOX7* (Fig. 6A).

Next, we tested the direct activation of *WOX5/7* by WOX11/12 using a transient expression system in tobacco (*Nicotiana tabacum*) leaves (Hellens et al., 2005). We coexpressed *WOX11* or *WOX12* together with *WOX5$_{pro}$:LUC* or *WOX7$_{pro}$:LUC*, in which the luciferase reporter gene is fused downstream of the *WOX5* or *WOX7* promoter. The results showed that WOX11/12 activated the luciferase response in tobacco leaves (Fig. 6, B and C), confirming that WOX11/12 directly activate *WOX5/7* expression in planta.

To obtain genetic evidence that the WOX11/12-binding elements on the *WOX5* locus are required for activation of *WOX5*, we constructed *mWOX5$_{pro}$:GUS* reporter lines in which the WOX11/12-binding elements in the *WOX5* promoter were mutated. GUS signals were barely detected in adventitious root primordia from leaf explants of two independent *mWOX5$_{pro}$:GUS* lines (Fig. 6, D–F). In contrast, GUS signals could be clearly observed in the tip of the primary root (Fig. 6G). These results indicate that the WOX11/12-binding elements are specifically required for activation of *WOX5* during the adventitious root primordium initiation, and

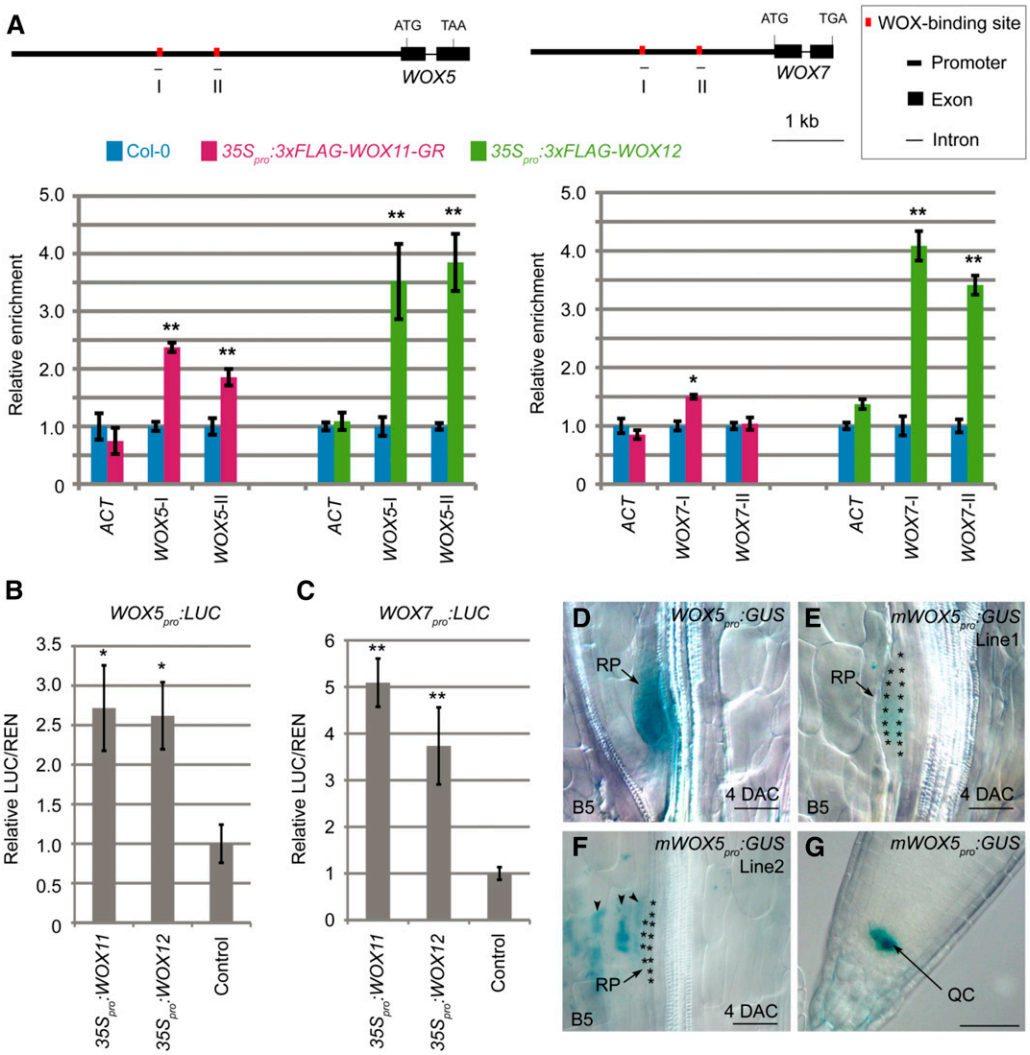


**Figure 6.** WOX11/12 directly regulate *WOX5/7* expression. A, ChIP analysis showing enrichment of 3×FLAG-WOX11-GR and 3×FLAG-WOX12 in promoters of *WOX5* and *WOX7*. Schematics of *WOX5* and *WOX7* gene structures are shown above ChIP data. Horizontal lines below genes show positions of PCR fragments in ChIP analysis. Bars show SD from three PCR repetitions. Results were confirmed with two independent biological repetitions. Values in Col-0 were arbitrarily fixed at 1.0. *ACTIN* (*ACT*) locus served as the negative control. $*P < 0.05$ and $**P < 0.01$ (two-sample *t* test, compared with Col-0 control). B and C, Relative ratio of firefly luciferase (LUC) to Renilla luciferase (REN) activity in tobacco leaves cotransformed with $35S_{pro}$:*WOX11* or $35S_{pro}$:*WOX12* and $WOX5_{pro}$:*LUC* (B), and cotransformed with $35S_{pro}$:*WOX11* or $35S_{pro}$:*WOX12* and $WOX7_{pro}$:*LUC* (C). Sole transformation with $WOX5_{pro}$:*LUC* (B) or $WOX7_{pro}$:*LUC* (C) served as the control. Bars show SE from three biological repeats. Each biological repetition was performed with three experimental repetitions. $*P < 0.05$ and $**P < 0.01$ (two-sample *t* test, compared with control). D to F, GUS staining of 4-DAC leaf explants from $WOX5_{pro}$:*GUS* (D) and two independent $mWOX5_{pro}$:*GUS* lines (E and F) on B5 medium. Asterisks in E and F indicate dome-shaped root primordia. $WOX5_{pro}$:*GUS* (D) served as the control. GUS signal could be occasionally observed in mesophyll in $mWOX5_{pro}$:*GUS* (arrowheads in F). G, GUS staining of the primary root tip from $mWOX5_{pro}$:*GUS* (line 1). Bars = 50 $\mu$m (D–G).

they are not involved in maintenance of *WOX5* expression in the stem cell niche within the primary root tip.

### Auxin Is Required for *WOX5/7* Activation

Auxin is the major hormone controlling cell fate transition during adventitious rooting (Greenwood et al., 2001; De Klerk, 2002; Ahkami et al., 2009; Correa et al., 2012; Liu et al., 2014; Xu and Huang, 2014). Therefore, we tested whether auxin is required for activation of *WOX5/7* by WOX11/12. We cultured leaf explants on B5 medium containing naphthylphthalamic acid (NPA; a polar auxin transport inhibitor), which has been shown to block rooting by inhibition of auxin transport from mesophyll cells to competent cells near the wound (Liu et al., 2014). Using $35S_{pro}$:*WOX11*/$WOX5_{pro}$:*GUS* and $35S_{pro}$:*WOX11*/$WOX7_{pro}$:*GUS*, we

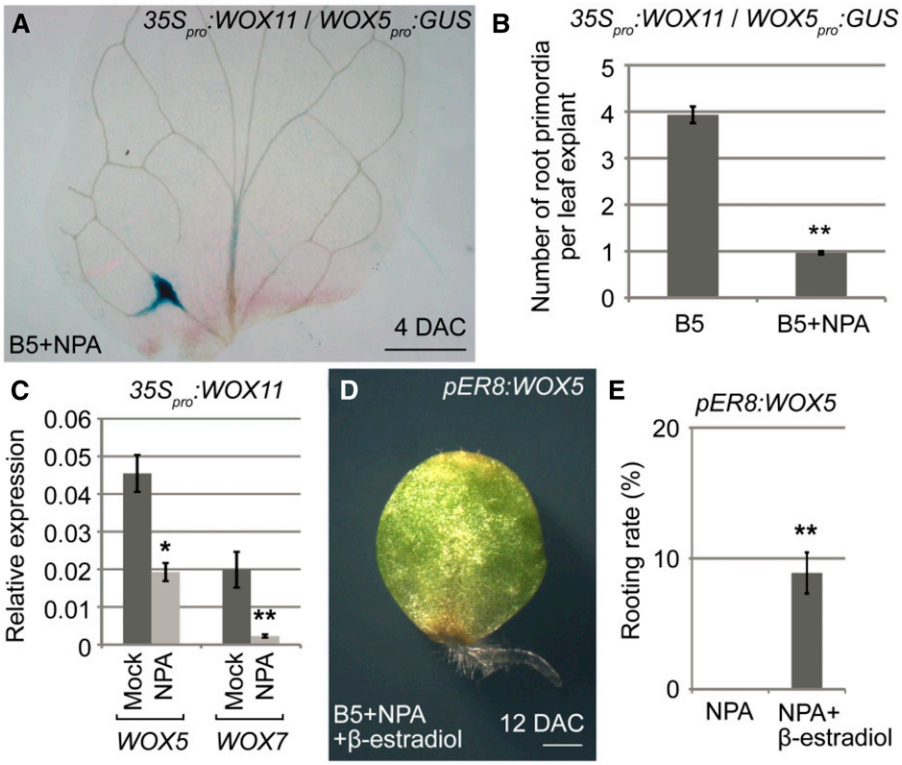


**Figure 7.** *WOX5/7* activation requires auxin. A, GUS staining of *35S$_{pro}$:WOX11/WOX5$_{pro}$:GUS* in leaf explant at 4 DAC on B5 medium containing 1 $\mu$M NPA. For comparison, see Figure 4G. B, Quantitative analyses of *WOX5*-marked root primordia in *35S$_{pro}$:WOX11/WOX5$_{pro}$:GUS* leaf explants at 4 DAC on B5 medium without or with 1 $\mu$M NPA. Bars show SE from three biological repeats. Each repeat comprised 10 leaf explants. $^{**}P < 0.01$ (two-sample *t* test). C, qRT-PCR analyses of transcript levels of *WOX5* and *WOX7* in *35S$_{pro}$:WOX11* leaf explants at 4 DAC on B5 medium without (mock) or with 1 $\mu$M NPA. Bars show SE from three biological repetitions. Each biological repetition was performed with three technical repetitions. $^{*}P < 0.05$ and $^{**}P < 0.01$ (two-sample *t* test, compared with mock). D, Leaf explants from *pER8:WOX5* at 12 DAC on B5 medium containing 1 $\mu$M NPA and 10 $\mu$M $\beta$-estradiol. Note that leaf explants on medium containing NPA could not regenerate adventitious roots (Liu et al., 2014), but overexpression of WOX5 partially rescued this rooting defect. E, Statistical analysis of rooting rates shown in D. Bars show SD from three biological repeats ($n = 30$ per repeat). $^{**}P < 0.01$ (two-sample *t* test). Bar = 500 $\mu$m (A) and 1 mm (D).

found that the formation of *WOX5*- or *WOX7*-marked root primordia was severely blocked, even when these GUS reporter constructs were expressed in the *WOX11*-overexpression genetic background (Fig. 7, A and B; Supplemental Fig. S7; for comparison, see Fig. 4G and Supplemental Fig. S5B). Consistent with the GUS observations, qRT-PCR analyses of *35S$_{pro}$:WOX11* leaf explants showed that the transcript levels of *WOX5* and *WOX7* were lower in leaf explants treated with NPA (Fig. 7C). In addition, overexpression of *WOX5* partially rescued the rooting defects of leaf explants cultured on B5 medium containing NPA (Fig. 7, D and E). Together, these data suggest that auxin acts upstream of *WOX5/7*, and auxin polar transport to the wounded region is required for activation of *WOX5/7* by WOX11/12.

## DISCUSSION

Plants have powerful abilities in regeneration of roots (Xu et al., 2006; Sena et al., 2009; Liu et al., 2014; Efroni et al., 2016). De novo root organogenesis from leaf explants requires consecutive cell fate transition steps to finally form an adventitious root. Here, we borrowed the cellular framework concept of lateral root formation (Péret et al., 2009; Lavenus et al., 2013; Goh et al., 2016) to summarize the consecutive steps in adventitious rooting (Fig. 8, model). The first step of cell fate transition is priming, which results in the formation of adventitious root founder cells. *WOX11/12* serve as molecular markers for founder cells. However, wound signals that trigger the priming step are not clear (León et al., 2001; Maffei et al., 2007; Iwase et al., 2011; Xu and Huang, 2014; Chen et al., 2016a; Chen et al., 2016b). The second step of cell fate transition is initiation, which results in the formation of the dome-shaped root primordium via cell division. The expression levels of *WOX11/12* decrease and those of *WOX5/7* increase as the root founder cells transition into the root primordium. Currently, it is unclear how *WOX11/12* expression is repressed in the root primordium cells (Fig. 8, X factor with a question mark). *WOX11* is normally expressed in the *wox5-1 wox7-1* background, suggesting that *WOX5/7* might not serve as the X factor

(Supplemental Fig. S8). The third step is patterning, which results in the formation of the preliminary RAM. In this step, the expression of *WOX5/7* is gradually restricted toward the stem cell niche, suggesting that the stem cell niche is forming at this stage. The fourth step is emergence, when the newly formed adventitious root tip emerges from the leaf explant. In this step, a mature RAM with a well-organized stem cell niche forms and undergoes rapid cell division, cell differentiation, and elongation.

Based on the above cellular and molecular framework of de novo root organogenesis, the founder-cell markers *WOX11/12* act at the very beginning of adventitious rooting and therefore could serve as a valve for all of the following events in adventitious rooting. In $35S_{pro}$:*WOX11-SRDX*, no root primordium was observed in 4-DAC leaf explants (Supplemental Fig. S9), suggesting that there was a complete block of root primordium initiation. In this study, we revealed that *WOX5/7* are direct downstream targets of WOX11/12 and that this molecular regulation delivers the fate transition from root founder cells to root primordium cells.

The results of our previous study suggested that *WOX11/12* can also activate the expression of *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29* (Liu et al., 2014). Therefore, *LBD*s could be another downstream pathway regulated by *WOX11/12* (Fig. 8). *LBD* genes are required for rooting and are likely involved in regulating cell division and cell wall metabolism (Lee et al., 2009; Berckmans et al., 2011; Feng et al., 2012a, 2012b; Goh et al., 2012; Lee et al., 2013). Although cell organization within the root primordium was defective in the *wox5-1 wox7-1* double mutant, cell division still occurred. One possibility is that the *LBD* pathway still functions in the *wox5-1 wox7-1* double mutant background and controls cell division to form abnormally organized root primordia.

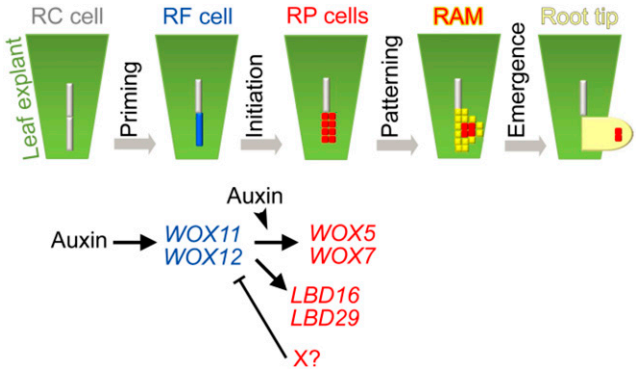


**Figure 8.** Model of de novo root organogenesis from leaf explant. De novo regeneration of adventitious root comprises priming, initiation, patterning, and emergence steps. Founder-cell-specific *WOX11/12* function as a valve to activate downstream gene expression, such as the *WOX5/7* and *LBD* pathways, which function in root primordium formation. Activation of *WOX11/12* and *WOX5/7* requires auxin. An unknown factor (X with question mark) might shut down *WOX11/12* expression in the root primordium. RC cell, regeneration-competent cell; RF cell, root founder cell; RP, root primordium.

Although both *LBD*s and *WOX5/7* function downstream of *WOX11/12*, they have different expression patterns. We observed that *LBD16* was expressed in dividing root founder cells and the two-layer root primordium cells, like *WOX5/7*, but it was not expressed in the newly formed RAM (Supplemental Fig. S10). Therefore, unlike the *WOX5/7* pathway, the *LBD* pathway does not continue to function after root primordium initiation.

In the tip of a normal root, *WOX5* and *WOX7* are expressed in the stem cell niche, which comprises the QC and initial cells (Scheres, 2007); *WOX5* is specifically expressed in the QC located in the center of the stem cell niche (Sarkar et al., 2007) and *WOX7* is specifically expressed in the endodermis-cortical initial cell, which is adjacent to the QC (Cui et al., 2011). The stem cell niche is the source of all tissues within the root (Scheres, 2007; Aichinger et al., 2012). In this study, we show that *WOX5/7* are ubiquitously expressed throughout the whole adventitious root primordium. Therefore, we hypothesize that the adventitious root primordium consists of precursor cells of QC and initials and is able to differentiate into a RAM. In addition, *WOX5/7* may also have a role in primary root and lateral root development, suggesting that the function of *WOX5/7* is not only restricted in adventitious root formation (Supplemental Fig. S11; Sarkar et al., 2007; Scheres, 2007; Chu et al., 2013; Tian et al., 2014; Ji et al., 2015; Pi et al., 2015; Zhou et al., 2015; Kong et al., 2016).

Overall, our study shows that the molecular mechanism of the second step of cell fate transition from root founder cells to root primordium initiation involves the direct activation of *WOX5/WOX7* by WOX11/12 during de novo root organogenesis.

## MATERIALS AND METHODS

### Plant Materials and Culture Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 was used as the wild type in this study. To produce $WOX12_{pro}$:*GUS* and $WOX7_{pro}$:*GUS* transgenic plants, 5.3-kb *WOX12* and 3.4-kb *WOX7* promoters were PCR amplified and each inserted into the pBI101 vector. $35S_{pro}$:*3×FLAG-WOX12* was constructed by insertion of the cDNA encoding the 3×FLAG-WOX12 protein into the pMON530 vector. For $mWOX5_{pro}$:*GUS*, a 4.6-kb mutated *WOX5* promoter with mutations at the WOX-binding sites was inserted into the pBI101 vector. To construct *pER8:WOX5*, a cDNA encoding the full-length WOX5 protein was PCR amplified and inserted into the pER8 vector (Zuo et al., 2000). Transgenic plants were obtained by *Agrobacterium tumefaciens*-mediated transformation of each of these constructs into Col-0. The *wox5-1* (SALK_038262; Sarkar et al., 2007) and *wox7-1* (SALK_065801; Kong et al., 2016) mutants and the *wox11-2 wox12-1* double mutant (Liu et al., 2014) were described previously. The $WOX11_{pro}$:*GUS*, $WOX5_{pro}$:*GUS*, $35S_{pro}$:*WOX11*, $35S_{pro}$:*WOX12*, and $35S_{pro}$:*WOX11-SRDX* transgenic plants were described previously (He et al., 2012; Liu et al., 2014). The primers used for plasmid construction are listed in Supplemental Table S1.

Culture conditions were as described previously (Chen et al., 2014). Briefly, Arabidopsis seedlings were first cultured on 0.5× Murashige and Skoog medium at 22°C under a 16-h-light/8-h-dark photoperiod. Leaf explants from 12-d-old seedlings were cultured on B5 medium with Suc in the dark for de novo regeneration of adventitious roots.

## RT-PCR, qRT-PCR, and ChIP Analyses

The extraction of RNAs and reverse transcription was conducted as described previously (He et al., 2012). The gene-specific primers used for PCR and real-time PCR are listed in Supplemental Table S1. The qRT-PCR results are shown as relative transcript levels, which were normalized against that produced using *ACT*-specific primers.

The ChIP experiment was carried out as described previously (He et al., 2012; Li et al., 2012). Briefly, 15-d-old seedlings of $35S_{pro}$:3×*FLAG-WOX11-GR* and the control Col-0 were treated with 10 $\mu$M dexamethasone for 4 h and leaves were harvested for chromatin extraction. Chromatin was also extracted from leaves of 15-d-old seedlings of $35S_{pro}$:3×*FLAG-WOX12* and the control Col-0. The anti-FLAG antibody (Sigma-Aldrich, F1804) was used for immunoprecipitation. The ChIP results were normalized against the input control. The primers used for real-time PCR are listed in Supplemental Table S1.

## Dual Luciferase Assay

To construct $WOX5_{pro}$:*LUC* and $WOX7_{pro}$:*LUC*, the promoter of *WOX5* or *WOX7* was inserted into the pGreenII-0800 vector (Hellens et al., 2005). The dual luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega).

## Histology

GUS staining was performed as previously described (Chen et al., 2014; Zeng et al., 2016). Differential interference contrast observations were performed using a Nikon ECLIPSE 80i microscope (Nikon) as described previously (Chen et al., 2014).

## Accession Numbers

Sequence data are listed in the Arabidopsis Genome Initiative under the following accession numbers: *WOX11* (AT3G03660), *WOX12* (AT5G17810), *WOX5* (AT3G11260), and *WOX7* (AT5G05770).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Identification of *wox5-1 wox7-1* double mutant.

**Supplemental Figure S2.** Expression patterns of *WOX11* and *WOX5* during adventitious rooting.

**Supplemental Figure S3.** *WOX5/7* expression in adventitious root tip.

**Supplemental Figure S4.** *WOX5/7* are involved in stem cell niche formation in the adventitious root rip.

**Supplemental Figure S5.** *WOX11* up-regulates *WOX7* expression.

**Supplemental Figure S6.** Function of *WOX12* is dependent on *WOX5/7*.

**Supplemental Figure S7.** *WOX7* expression is dependent on auxin.

**Supplemental Figure S8.** *WOX11* expression in *wox5-1 wox7-1*.

**Supplemental Figure S9.** Primordium defect in $35S_{pro}$:*WOX11-SRDX*.

**Supplemental Figure S10.** *LBD16* expression in de novo root organogenesis.

**Supplemental Figure S11.** *WOX5/7* in primary root and lateral root development.

**Supplemental Table S1.** List of primers used in this study.

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