Reprogramming of the cambium regulators during adventitious root development upon wounding of storage tap roots in radish (*Raphanus sativus* L.)

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**ABSTRACT**

Cambium contains a stem cell population that produces xylem and phloem tissues in a radial direction during the secondary growth stage. The growth of many storage roots, including in the radish, *Raphanus sativus* L., also depends on cambium. Interestingly, we observed numerous adventitious roots (ARs) emerging from the cambia of cut surfaces when the bases of radish storage tap roots were removed. Previous studies in Arabidopsis showed that the WOX11/12 pathway regulates AR initiation and meristem establishment in an auxin-dependent manner. Here, we provide evidence indicating the evolutionary conservation of the WOX11/12 pathway during the AR development in radishes. Additionally, we found that expression of two cambium regulators, *PXY* and *WOX4*, is induced in the cambium regions that are connected to emerging ARs via vascularization. Both AR formation and genes associated with this were induced by exogenous auxin. Our research suggests that some key cambium regulators might be reprogrammed to aid in the AR development in concert with the WOX11/12 pathway.

This article has an associated First Person interview with the first author of the paper.

**KEY WORDS:** Radish, Adventitious root, Cambium, Auxin, WOX11, PXY-WOX4

**INTRODUCTION**

The vascular cambium is a meristem organized in radial files between the xylem and the phloem. Cambial initial cells undergo periclinal (asymmetric) division to produce xylem mother cells towards the center of the plant axis and phloem mother cells towards the periphery. Xylem and phloem mother cells divide further and differentiate into cell types constituting secondary xylem and phloem (Miyashima et al., 2013).

In the cambium, signaling sets up the boundary between the xylem and the phloem. Small protein ligands called CLAVATA3/EMBRYO-SURROUNDING REGION (CLE) 41/44 or TDIF are secreted from the phloem sieve element and diffuse to the cambium where it binds a receptor-like kinase (RLK) called PHLOEM INTERCALATED WITH XYLEM (PXY) or TDR. *PXY/TDR* is specifically expressed in the procambium and cambium (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008). When PXY/TDR interacts with CLE41/44, it triggers two pathways in an independent manner. The first pathway regulates the expression of *WUSCHEL RELATED HOMEOBOX4 (WOX4)*, a gene homologous to *WUSCHEL (WUS)*, and its redundant gene *WOX14* (Etchells et al., 2013). This plays a big part in the proliferation of vascular stem cells by mediating the auxin responsiveness (Suer et al., 2011). The second pathway is involved in xylem inhibition redundantly with BRASSINOSTEROID-INSENSITIVE 2 (BIN2) (Kondo et al., 2014).

The radish, *Raphanus sativus* L., develops an edible storage taproot. It belongs to the Brassicaceae family, which includes *Arabidopsis thaliana* and species in *Brassica*. Evolutionary proximity of radish to Arabidopsis makes cross-species analyses based on genome information feasible. Its diploid genome is thought to be diverged from the *Brassica* via genome duplication (Mitsui et al., 2015). The growth of radish storage root is driven by high cambium activity in the taproot (Fig. S1A,B). Our previous research has shown that the cell division in the cambium is directly correlated with the girth and yield of storage roots (Jang et al., 2015).

In the presence of wounding or stress, it is a common strategy for plants to repair or regenerate damaged tissues or organs as a survival mechanism. Among many types of plant regeneration, the organogenesis of adventitious roots (ARs) from wounded or detached plant organs has been frequently used as a simple method for vegetative regeneration in agriculture.

Previous research has shown that free auxin accumulates in the wounded organ. Then, high auxin stimulates the transition of a regeneration competent cell to a root founder cell (Hu and Xu, 2016). During this process, auxin activates *WUSCHEL-RELATED HOMEOBOX11 (WOX11)*, a gene that is usually used as root founder cell marker. *WOX11* and its paralog, *WOX12*, activate a set of genes for the development of a root primordia (Liu et al., 2018; Sheng et al., 2017). One of them is *LATERNAL ORGAN BOUNDARIES DOMAIN16 (LBD16)*, which functions as a transcriptional regulator involved in the initiation of lateral and adventitious roots (Lee et al., 2017). Additionally, *WOX11* directly activates *WOX5* and *WOX7*, two essential genes for the establishment of a root apical meristem (RAM) (Hu and Xu, 2016).

In this report, we will provide evidence for the radish cambial cells as regeneration competent cells. We will show how the cambium of the taproot reshapes to connect with the AR procambium and reprograms key cambium genes such as *PXY* and *WOX4*, likely for vascularization. Additionally, we will provide support for the...
evolutionary importance of the WOX11/12 pathway by describing their expression patterns in developing ARs in radish.

RESULTS AND DISCUSSION

Cambium cells are competent for adventitious root formation

Tissues around cambia in cut stems of tomato and Eucalyptus have been shown to form AR (de Almeida et al., 2015; Sala et al., 2017). The cambium area has been identified before as an active area during AR and LR formation in woody plants (Chiatante et al., 2010, 2007; de Almeida et al., 2015). In addition, in Arabidopsis the primary roots undergoing the secondary growth, cambium cells could lead to the formation of root founder cells for lateral roots (Baesso et al., 2018). The radish storage taproot rapidly increases its biomass in a radial direction via cambial cell divisions (Jang et al., 2015; Fig. S1A,B). Based on these, we asked whether the cambium tissue serves as a preferential origin of ARs in the radish.

To induce the AR formation, the base of radish storage taproot was cut off transversely and the remaining root attached to stems and leaves was grown either in soil or in hydroponic media (Fig. S1C). After 2 weeks, we observed the emergence of numerous ARs from cut surfaces. As expected, ARs seemed to appear mostly along the cambium (Fig. 1A). We performed scanning electron microscopy (SEM) and confirmed that AR primordia arose along the cambium (Fig. 1B). Taking advantage of the feasibility of tracking the cell files in ARs, we analyzed how cells are organized in the cambium where ARs emerged (Fig. 1C; Fig. S1C). We noticed the thin layers of cambial cells in the taproot being connected with the AR via strands of small cells (Fig. 1C, indicated by red arrows). These indicated that the cambium in the root undergoing active secondary growth might be reprogrammed to form founder cells of ARs in response to root cutting.

Auxin responsive WOX11/12 pathway is conserved

Previous research has shown that the AR formation is regulated by the WOX11/12 pathway in Arabidopsis and Populus (Hu and Xu, 2016; Liu et al., 2014; Sheng et al., 2017). To see whether WOX11/12 pathway is also associated with the AR formation in radish, we identified the orthologous genes of WOX11 and WOX12 in radish and analyzed their expression levels after inducing the regeneration of ARs from cut radish taproots for 2 weeks in hydroponic culture. To find orthologs of WOX genes in radish and Arabidopsis, we searched all the WOX homologs in the radish genome database (http://www.radish-genome.org/) and aligned the predicted amino acid sequences using MUSCLE (Edgar, 2004; Fig. S2). We then created a phylogenetic tree using Neighbor-Joining method with bootstrapping 1000 times using MEGA-X (Kumar et al., 2018). Rs290750 and Rs389380 were most closely related to Arabidopsis WOX11 and 12, thus named as RsWOX11 and RsWOX12, respectively (Fig. 2A). When AR roots were induced, RsWOX11, RsWOX12, RsLBD16 and RsWOX5 were significantly upregulated, supporting evolutionary conservation of WOX11/12 pathway (Fig. 2F; Fig. S3).

To find the spatial distribution of WOX11 during AR formation in radish, we performed RNA in situ hybridization (Fig. 2D). RsWOX11 showed expression homogeneous in the AR primordia, which was similar to the expression pattern of Os-WOX11 in rice during crown root formation (Cheng et al., 2016). This result suggests that the WOX11 pathway is conserved, even between an eudicot and a monocot.

In Arabidopsis AR development, auxin was indicated to play a major role (Sheng et al., 2017). To find whether a similar process operates in radish, we proceeded to regenerate ARs in hydroponic culture for 1 week, by dividing into two groups: one supplemented with 1 μM of auxin (IAA) and the other without auxin (control). For this experiment we used a total of 15 biological replicates for each of the treatments (the control and the auxin group). The experiment was repeated three times. The auxin group had an incidence of AR 1.5 times higher than the control group: an average of 11.13 ARs with a standard error (s.e.) of 2.86 in the auxin group versus 4.53 ARs with a s.e. of 1.93 in the control group. Using Mann–Whitney U-test (P<0.01), we could verify that the increase of AR formation in the auxin treatment was significant. Representative results are visually shown by scanning electron microscopy (Fig. 2B,C). To check whether this exogenous auxin treatment promoted downstream signaling pathways, we selected and analyzed the expression of RsIAA27 (Rs048860) and RsARF5 (Rs414220) and detected the upregulation of RsARF5 by auxin treatment (Fig. 2G).

These data collectively suggest that auxin promotes the formation of AR primordia. PIN1 is involved in directing auxin flow for vascular formation in developing roots and leaves. We thus analyzed the spatial distribution of PIN1 protein in the cut taproot with emerging AR primordia (Fig. 2E; Fig. S6). As expected, PIN1 proteins were detected throughout the AR primordia as well as the area of vascular connection between the AR and the taproot. This indicates that reprogramming in auxin distribution in the cut taproot might affect the AR formation.

PXY-WOX4 may be reprogramed to aid the adventitious root formation

Founder cells of ARs seemed to be derived from the cambium. During this reprogramming the cut radish taproots no longer grew in the radial direction as shown in the images of cut radishes pictured after decapitation and after the hydroponic incubation with or without auxin treatment for 7 days (Fig. S4). To find the
relationships between changes in cambium activities and their regulators, we analyzed the expression patterns of RsPXY and RsWOX4. In the intact radish taproot undergoing active secondary growth, these genes showed expression specific to the cambium near emerging xylem vessels (Fig. 3A). When ARs developed from cut roots, we observed the significant reduction of RsWOX4 and RsPXY expression in qRT-PCR, which is consistent with the lack of the cambial activities in the cut roots generating ARs (Fig. 3B). PXY and WOX4 are known to drive the cambium cell proliferation in an auxin-dependent manner (Suer et al., 2011). We observed that auxin promotes AR development from the radish taproot cambium. If PXY and WOX4 are a part of AR development, expression of these genes is likely upregulated upon AR induction in response to auxin treatment (1 µM of IAA). Supporting this idea, when AR formation was induced by incubating cut roots with 1 µM of IAA, expression of both RsPXY and RsWOX4 significantly increased (Student’s t-test; P<0.01) (Fig. 3C). To find how the expression of RsPXY and RsWOX4 was reprogrammed during AR organogenesis, we performed RNA in situ hybridization again. RsPXY was expressed in a gap between the vascular cambium and the AR primordia, which is where the vascularization of the AR happened (Fig. 3A; Fig. S5). Similarly, RsWOX4 was present in areas where vascularization was taking place between the AR primordia and the vascular cambium (Fig. 3A; Fig. S5).

Expression of RsPXY in a gap between the vascular cambium and the AR primordia was further examined by analyzing its expression in the root generating mature AR. During vascularization, auxin maximum is established along vascular precursors via PIN1. ARF5 activated by auxin then promotes HB8 transcription and HB8 in turn activates PIN1 expression. This positive feedback regulation supporting canalization model enables the formation of vascular strands along the auxin flow (Baima et al., 1995, 2001; Scarpella et al., 2006). We thus included RsHB8 as a vascularization marker and analyzed its expression together with RsPXY. RsHB8 showed an irregular expression pattern with high expression in the areas of vascular initiation during the AR initiation, as expected. Interestingly, RsPXY showed the expression pattern very similar to RsHB8 (Fig. 3D). In the mature AR, RsPXY and RsHB8 were expressed along the AR procambium which connects with the vascular cambium of the cut taproot (Santuari et al., 2011). These expression changes indicate that PXY-WOX4 might function for the AR development when the secondary growth no longer happens in the cut taproot.

Our investigation in radish suggested the recruitment of PXY and WOX4 to the AR development. To find whether these regulators actually affect AR formation from the cut taproots, we counted the ARs in Arabidopsis pxy mutant. Col-0 (n=23) and pxy (n=24) plants were...
grown for 18 days in MS media, their primary roots were decapitated, and then the shoots without roots were grown in B5 media for 4 days (Fig. S7). The number of ARs from cut root surfaces decreased significantly from an average of 2.91 to 0.62 in the pxy (Fig. 3E).

In Arabidopsis, WOX11/12 promotes AR founder cells and subsequently activates LBD16 and WOX5, which coordinate the initiation of a root primordium and a root apical meristem (Hu and Xu, 2016). Our expression analyses indicate a similar program might operate in AR development from radish taproots and the cambium serve as foci for the AR initiation (Fig. 1). During AR development, we found the vascular connection being established between the cambium of a cut taproot and the AR primordium.

Auxin flow plays a key role in the formation of vascular connections during organogenesis, and HB8 and PIN1 promotes this process as parts of positive feedback regulation (Baima et al., 2001; Donner et al., 2010; Scarpella et al., 2006). Consistently, in radish we detected the expression of PIN1 and RsHB8 in the region where the vascular connection was made between cut taproot cambium and the AR primordium. Exogenous application of auxin to the cut taproot promoted the AR formation specifically from the cambium (Fig. 2). These collectively indicate that the stem cells in the cambium have a capacity to initiate AR formation in an auxin dependent manner. Our further investigation suggests that RsPXY and RsWOX4 that are known to be in charge of cambial cell division during secondary growth (Etchells et al., 2013; Suer et al., 2011) and regulation of xylem differentiation (Fisher and Turner, 2007; Kondo et al., 2014) are reprogramed to re-establish their expression in the junction between AR primordia and the cut taproot. In an RNA in situ hybridization, we observed RsPXY expression largely disappeared when the secondary growth of a taproot stopped upon the removal of the root base and then re-emerged intermittently where vascularization happened as AR primordia developed. At the point of AR growth, it showed homogeneous expression in the AR procambium that was connected to the vascular cambium (Fig. 3). RsHB8 also showed expression pattern similar to RsPXY, confirming the procambium-cambium connection (Baima et al., 1995; Santauri et al., 2011). Recruitment of RsPXY and RsWOX4 to AR development is also supported by their response to auxin. Exogenous auxin application to the cut taproot no longer promoted the secondary growth, however strongly induced the expression of RsPXY and RsWOX4 (Fig. 4). This process seems functionally important since pxy mutant showed significant reduction in the emergence of ARs from the cut taproots in Arabidopsis. We interpret this behavior as a plants’ survival strategy that allows them
to initiate organ regeneration upon wounding in a time- and cost-efficient way. Unraveling molecular mechanisms underlying how auxin redirects the vascular stem cell function from organ growth to organ regeneration will advance our understanding of the remarkably dynamic nature of plant development.

MATERIALS AND METHODS

Plant materials and growth conditions
Radishes for this experiment were grown in a growth room with a constant temperature of 22°C and a photoperiod of 16 h of light and 8 h of darkness. For hydroponic culture, we used Hoagland solution (50% v/v) after adjusting its pH to 7.

Tissue staining and imaging
Paraplast®-embedded tissue sections were prepared on glass slides following the same procedures for the tissue preparation for RNA in situ hybridization. For the Toluidine Blue staining, slides were placed in a slide rack and passed through a hydration series of Histoclear® for 10 min two times, 100% ethanol for 2 min two times, 90% ethanol for 2 min, 70% ethanol for 2 min, 50% ethanol for 2 min, and water for 2 min. Then, the tissues on the glass slides were dipped in Toluidine Blue solution (0.001 g in 200 ml) for 20 s and washed in distilled water three times. Finally, slides were processed in a reverse order of a hydration series for mounting. Images were taken with a Nikon eclipse Ni light microscope.

Real-time quantitative PCR
cDNAs were synthesized and diluted twofold, by mixing 20 µl of Milli-Q water and 20 µl of cDNA. qPCR reactions were set up with 10 µl per reaction as follows: 5 µl of iTaq™ SYBR Green® supermix (Bio-Rad, Cat No. 1708880), 0.5 µl forward primer and 0.5 µl reverse primer (10pm), 3 µl of Milli-Q water, and 1 µl of cDNA. Cycling conditions were as follows: 3 min at 95°C, 48 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 56°C, and extension for 30 s at 72°C. qPCR reactions and fluorescence detection were performed using a CFX96 Real-Time PCR machine (Bio-Rad). RsActin was used as a reference gene in order to assess gene expression levels of our genes of interest. To perform the qPCR reactions, specific primers (Table S3) were designed using Primer3 (Rozen and Skaletsky, 2000).

Scanning electron microscopy
Radish storage tap roots grown for 5 weeks post seed planting were cut transversely and upper parts of plants were grown in hydroponic media for 1 week. Root segments with emerging ARs from cut surface were fixed overnight at 4°C in 25% of Glutaraldehyde in 25 mM of phosphate buffer (pH 7). The next day, the fixative was poured off and 1% of osmium tetroxide solution was added. The vials were incubated at 4°C for 2 days. The samples were thoroughly rinsed in 25 mM of phosphate buffer (pH 7) and then dehydrated in an ethanol series. Specimens kept in 100% of ethanol were critical-point dried, coated with gold particles and then imaged under a scanning electron microscope (JSM-6390LV, JEOL).

RNA in situ hybridization
cDNA library preparation
For preparing the cDNA library, total RNA from 7-week-old radish roots was isolated using the QIAGEN RNeasy® Plant Mini kit (Qiagen Cat No./ID: 74903). For the cDNA library preparation, a reverse-transcription reaction was carried out using 1µg of the total RNA with the Invitrogen™ SuperScriptIII reverse transcriptase kit (Thermo Fisher Scientific, Cat No.180800930) following the manufacturer’s instruction.

Gene cloning
To clone our selected genes, gene-specific forward and reverse primers were designed and obtained (Table S1). Genes were PCR-amplified from the
cDNA library obtained in the previous step. The product was run through an electrophoresis on 1% agarose gel and purified by the QIAquick™ Gel Extraction Kit (Qiagen, Cat No. 28704). The purified product was inserted into the PENTER/D-TOP vector with the pENTER™/D-TOP™® Cloning Kit Invitrogen™ (Thermo Fisher Scientific, Cat No. K240020). Gene clones were verified by Sanger sequencing in NICEM at Seoul National University.

**RNA probe preparation**
Template DNAs for RNA probes were synthesized by PCR amplification of a part of genes cloned in the previous section. Primers used can be found in Table S2. After gel purification, the DNA template concentration should be at least 100 ng/µl. The transcription using T7 RNA polymerase was carried out with the DIG labeling kit (Sigma-Aldrich, Cat No. 1175025910). In vitro transcription mixture was prepared by adding 2 µl of acetylated BSA, 2 µl of transcription buffer, 2 µl of 10% Triton X-100, 2 µl of 10XNTP labeling mixture and 1 µl of RNase inhibitor with a final volume of 9 µl. To this mixture, 9 µl of purified PCR product and 2 µl of T7 RNA polymerase were added. The reaction was incubated at 37°C for 3 h and then DNA was removed from the mixture by adding 5 µl of DNase buffer, 22 µl of RNase Free water and 2 µl of RNase-free DNase. The mixture was incubated for 15 min. at 37°C. Finally, the probe was let to precipitate overnight at −80°C by adding 6 µl of 4 M LiCl, 1 µl of 0.5 M EDTA and 180 µl of 100% ethanol. The next day, RNA was precipitated by centrifugation at 13,000 rpm for 30 min. at 4°C. RNA pellet was washed with ethanol, dried, and dissolved in RNase-free water.

**Tissue preparation**
The radish root segments with AR regeneration were collected and fixed in 4% paraformaldehyde. The tissue was vacuum-infiltrated for 5 min three times, and incubated overnight at 4°C in fresh fixative. The next day, the fixative was removed and the tissue was rinsed four times with 1X Phosphate-Buffered Saline (PBS) for 15 min each time. Tissue was then dehydrated using the following ethanol series (25%, 50%, 75%, and three times in 100% of ethanol), and then infiltrated with a series of Histoclear® diluted in 100% of ethanol: 25%, 50%, 75%, and three times in 100% of ethanol), and then infiltrated with a series of Histoclear® diluted in 100% of ethanol: 25%, 50%, 75%, and three times in 100% of ethanol. Finally, a half of the tube was filled with ParaPlast® chips and left overnight at 58°C for infiltration. For the following 4 days the ParaPlast® was replaced twice a day and then the tissue was imbedded with ParaPlast® in a mold and let solidify. Tissue blocks were sliced using a RM 2255 microtome (Leica) in 15 µm of thickness and then mounted on a TRUBOND® (Thermo Fisher Scientific, Cat No. NC0270688).

**Slide pretreatment**
Slides were placed in a rack and passed through the following solution series in RNase free conditions. To begin with, slides were placed two times in Histoclear® for 10 min. Then the slides were passed through ethanol series (100%, 100%, 95%, 85%, 70%, 50%, 30% of ethanol diluted in 0.5% of NaCl) for 1 min in each solution. Afterwards slides were placed in a series of following solutions: 0.85% of NaCl for 2 min, 0.2 M of HCl for 20 min, RNase-free water for 5 min, 1×PBS for 2 min. Then, the tissue sections on slides were treated with Pronase enzyme (0.135 mg/ml, Sigma-Aldrich, Cat. No.P6911) for 28 min at 37°C. Reaction was stopped with 0.2% of glycine in 1×PBS, washed in 1×PBS for 2 min, and fixed in 4% of paraformaldehyde for 10 min. Slides were rinsed for 2 min in 1×PBS, then submerged in 0.85% of NaCl for 2 min and finally treated in an ethanol series in a reverse order of dehydration. Slides were dried in RNase free conditions for 1 h.

**Prehybridization and hybridization**
Slides were placed in the container that soaked with 50% of formamide. Each slide with tissue sections was coated with 250 µl of prehybridization solution (50% formamide, 1× salts, 1× Denhardt’s, 200 µg/ml of tRNA, 1.25 µl of RNase inhibitor, 1.5 µg/ml of DEP C water) and incubated in the 50% formamide chamber at 45°C for 24 h.

**Post-hybridization washes**
After the hybridization, slides were placed in a rack which stands in a jar with 0.2× of Saline Sodium Citrate Buffer (SSC) for 1 h at 55°C. The solution was later replaced and incubated for 1 h. After finishing the washes, slides were rinsed in NTE solution (10 mM of Tris pH8.0; 5 mM EDTA) and incubated for 30 min at 37°C in a solution of 10 µg/ml RNase A in 0.5 M NaCl; NTE. Slides were rinsed for 5 min in NTE and incubated for 1 h in 0.2XSSC at 55°C. Finally, slides were rinsed in 1×PBS.

**Signal detection**
Slides were placed in Blocking solution [100 mM Tris, 100 mM NaCl, 1% blocking reagent of DIG Nucleic Acid detection kit (Roche Cat No. 1175041910)] with gentle agitation for 45 min. Then the blocking buffer was replaced by buffer A (100 mM Tris, 100 mM NaCl, 1%BSA, 0.3% Triton X-100) and slides were incubated for another 45 min. Antibody conjugate from the DIG Nucleic Acid detection kit was spread on the slides in a 1:1000 ration in Buffer A. Slides were incubated for 2 h with high humidity. After this step, slides were washed in Buffer A three times for 20 min each time and then twice in Detection Buffer (100 mM Tris pH9.5, 100 mM NaCl, 50 mM MgCl2) for 5 min each time. Finally, slides were incu bated with 500 µl of color substrate (200 µl of NBT/ BCIP solution DIG Nucleic Acid detection kit in 10 ml of detection buffer and 100 µl of levamisole) at room temperature for 24 h in the dark condition.

**Immunolocalization with PIN1 antibody**
Paraplast-embedded tissue sections were prepared on glass slides following the same procedure as the tissue preparation for RNA in situ hybridization. For dewaxing, glass slides were incubated in Histoclear® for 15 min at room temperature, transferred to 100% ethanol for 10 min two times, and then air-dried for 15 min. Then, slides were treated in a following rehydration series: fresh 100% ethanol, 90% of ethanol diluted in ddH2O, two times with 70% ethanol diluted in 1×PBS, 50% and 25% of ethanol diluted in 1×PBS, and two times with 1×PBS. In each step, slides were incubated for 10 min at room temperature. 150 µl of blocking solution [2% BSA fraction V (Sigma-Aldrich A-3912) in 1×PBS] was spread on each slide and incubated in a humid chamber for 30 min at room temperature. After washing glass slides in 1×PBS, we applied 500 times diluted PIN1 antibody (Cat#: R2114-2, Rabbit polyclonal, Abiocode) in a blocking solution. After incubating overnight at 4°C, glass slides were washed in 1×PBS six times, for 10 min at room temperature each time. We applied glass slides with 200-fold diluted secondary antibody [Alexa Fluor™ 488 Fl(ab²) 2 fragment of goat anti-rabbit IgG, IgM (H+L), Invitrogen™] in a blocking solution. We incubated the glass slides in a humid chamber in the dark for 1 h at room temperature. And then glass slides were washed six times in 1×PBS, for 10 min at room temperature each time. The glass slides were mounted with ddH2O and signals were detected and imaged using Laser Scanning Confocal Microscope (Leica SP8) with the excitation/emission wavelength of 488 nm/505 to 530 nm.

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**Competition interests**
The authors declare no competing or financial interests.

**Author contributions**
Conceptualization: A.C.A.F., J.-Y.L.; Methodology: A.C.A.F., H.K., J.D.R., J.-Y.L.; Validation: A.C.A.F., J.D.R.; Formal analysis: A.C.A.F., H.K.; Investigation: A.C.A.F., H.K., J.D.R., J.-Y.L.; Resources: J.-Y.L.; Data curation: A.C.A.F.; Writing - original
Data availability
Data are available upon request.

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References

Baesso, B., Chiatante, D., Terzaghi, M., Zenga, D., Nieminen, K., Mahonen, A. P., Siligato, R., Helariutta, Y., Scippa, G. S. and Montagnoli, A. (2018). Transcription factors PRE3 and WOX11 are involved in the formation of new lateral roots from secondary growth taproot in A.thaliana. Plant Biol. 20, 426-432.

Baima, S., Nobili, F., Sessa, G., Lucchetti, S., Ruberti, I. and Morelli, G. (1995). The expression of the ATHB-8 homeobox gene is restricted to provascular cells in Arabidopsis thaliana. Development 121, 4171-4182.

Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M. M., Ruberti, I. and Morelli, G. (2001). The Arabidopsis ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristems. Plant Physiol. 126, 643-655.

Cheng, S., Zhou, D-X. and Zhao, Y. (2016). WUSCHEL-related homeobox gene WOX11 increases rice drought resistance by controlling root hair formation and root system development. Plant Signal. Behav. 11, e1130198.

Chiatante, D., Di Iorio, A. and Scippa, G. S. (2007). Modification of root architecture in woody plants is possible for the presence of two different mechanisms of lateral root production: the effect of slope in Spartium junceum L. seedlings. Plant Biol. 141, 502-510.

Chiatante, D., Beltotto, M., Onelli, E., Di Iorio, A., Montagnoli, A. and Scippa, S. G. (2010). New branch roots produced by vascular cambium derivatives in woody parental roots of Populus nigra L. Plant Biol. 144, 420-433.

de Almeida, M. R., de Bastiani, D., Gaeta, M. L., de Araújo Mariath, J. E., de Costa, F., Retallick, J., Nolan, L., Tai, H. H., Stromvik, M. V. and Fett-Neto, A. G. (2015). Comparative transcriptional analysis provides new insights into the molecular basis of adventitious rooting recalcitrance in Eucalyptus. Plant Sci. 239, 155-165.

Donner, T. J., Sherr and Scarpella, E. (2010). Auxin signal transduction in Arabidopsis vein formation. Plant Signal. Behav. 5, 70-72.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792-1797.

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.