Molecular Evolution of Auxin-Mediated Root Initiation in Plants

Jie Yu,[†,1] Yuyun Zhang,[†,1,2] Wu Liu,[1] Hua Wang,[1] Shaoting Wen,[1,3] Yijing Zhang,[1] and Lin Xu [*,1]

[1]National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China

[2]University of Chinese Academy of Sciences, Beijing, China

[3]College of Life and Environment Sciences, Shanghai Normal University, Shanghai, China

[†]These authors contributed equally to this work.

***Corresponding author:** E-mail: xulin01@sibs.ac.cn.

Associate editor: Heather Rowe

The RNA-seq data of *Ceratopteris richardii* with auxin treatment have been deposited at the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE134602. The *C. richardii* tissue-specific RNA-seq data have been deposited at GEO under the accession number GSE139160. The RNA-seq data of *Arabidopsis thaliana* have been deposited at GEO under the accession number GSE139165.

Abstract

The root originated independently in euphyllophytes (ferns and seed plants) and lycophytes; however, the molecular evolutionary route of root initiation remains elusive. By analyses of the fern *Ceratopteris richardii* and seed plants, here we show that the molecular pathway involving auxin, intermediate-clade *WUSCHEL-RELATED HOMEOBOX* (IC-*WOX*) genes, and WUSCHEL-clade *WOX* (WC-*WOX*) genes could be conserved in root initiation. We propose that the "auxin>IC-*WOX*>WC-*WOX*" module in root initiation might have arisen in the common ancestor of euphyllophytes during the second origin of roots, and that this module has further developed during the evolution of different root types in ferns and seed plants.

Key words: **root evolution, root initiation, WOX, auxin, *Ceratopteris richardii*.**

The appearance of roots during vascular plant evolution was a great step toward better adaptation to growth on land. The fossil evidence suggests that there were independent root origin events in euphyllophytes (i.e., ferns and seed plants) and lycophytes, the two surviving vascular plant lineages on earth (Raven and Edwards 2001; Pires and Dolan 2012; Kenrich and Strullu-Derrien 2014; Hetherington and Dolan 2018; Liu and Xu 2018). The first root-origin event was in the lycophyte lineage by the Early Devonian, resulting in the bifurcating roots in extant lycophytes, such as *Selaginella* (spike mosses) (Williams 1937; Webster 1969; Raven and Edwards 2001; Banks 2009; Pires and Dolan 2012; Sanders and Langdale 2013; Fujinami et al. 2017; Hetherington and Dolan 2017, 2018; Augstein and Carlsbecker 2018; Liu and Xu 2018; Fang et al. 2019; Mello et al. 2019). The second root-origin event was in the common ancestor of the euphyllophyte lineage by the Middle Devonian, giving rise to many types of roots in extant ferns and seed plants. For example, the fern *Ceratopteris richardii* has adventitious roots and endodermis-derived lateral roots (Howe 1931; Hou and Blancaflor 2009); and the seed plant *Arabidopsis thaliana* produces the primary root, adventitious roots, pericycle-derived acropetal lateral roots, and adventitious lateral roots (Ge et al. 2019; Motte et al. 2019).

Auxin is the major hormone that triggers root initiation in seed plants (Zimmerman and Wilcoxon 1935). In *A. thaliana*, all types of roots are initiated under the guidance of auxin (Casimiro et al. 2001; Friml et al. 2003; Okushima et al. 2007; Berckmans et al. 2011; Liu, Sheng, et al. 2014; Xuan et al. 2015, 2016; Sheng et al. 2017; Brumos et al. 2018). However, the role of auxin in root initiation in ferns is largely undetermined (Allsopp and Szweykowska 1960; Hou and Hill 2004; de Vries et al. 2016; Motte and Beeckman 2019), and it is not clear whether there is an evolutionarily conserved molecular pathway for root initiation in euphyllophytes (Huang and Schiefelbein 2015; Motte and Beeckman 2019).

To test whether auxin triggers root initiation in the fern *C. richardii*, we first analyzed root initiation phenotypes upon auxin or auxin-inhibitor treatment compared with the mock control. In the mock control, during vegetative growth (sporophyte seedlings with fewer than nine leaves), the adventitious roots were stem-borne roots that were produced from the shoot with the pattern of one root per node beneath each leaf (fig. 1*A* and *D*), typical of normal growth (Hou and Blancaflor 2009). This pattern occurs because usually only one hypodermal cell at the node beneath a leaf changes its cell fate to become the root founder cell (also known as the root mother cell). The division of the root founder cell results in the formation of the tetrahedral root apical cell with four division planes, forming three proximal merophytes and a distal merophyte (i.e., the root cap initial cell) (fig. 1*F* and *G*) (Hou and Hill 2002; Hou and Blancaflor 2009). Treatment of *C. richardii* with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram led to the formation of significantly more adventitious roots from the shoot (fig. 1*B*–*D*; supplementary fig. 1*A*, Supplementary Material

Letter

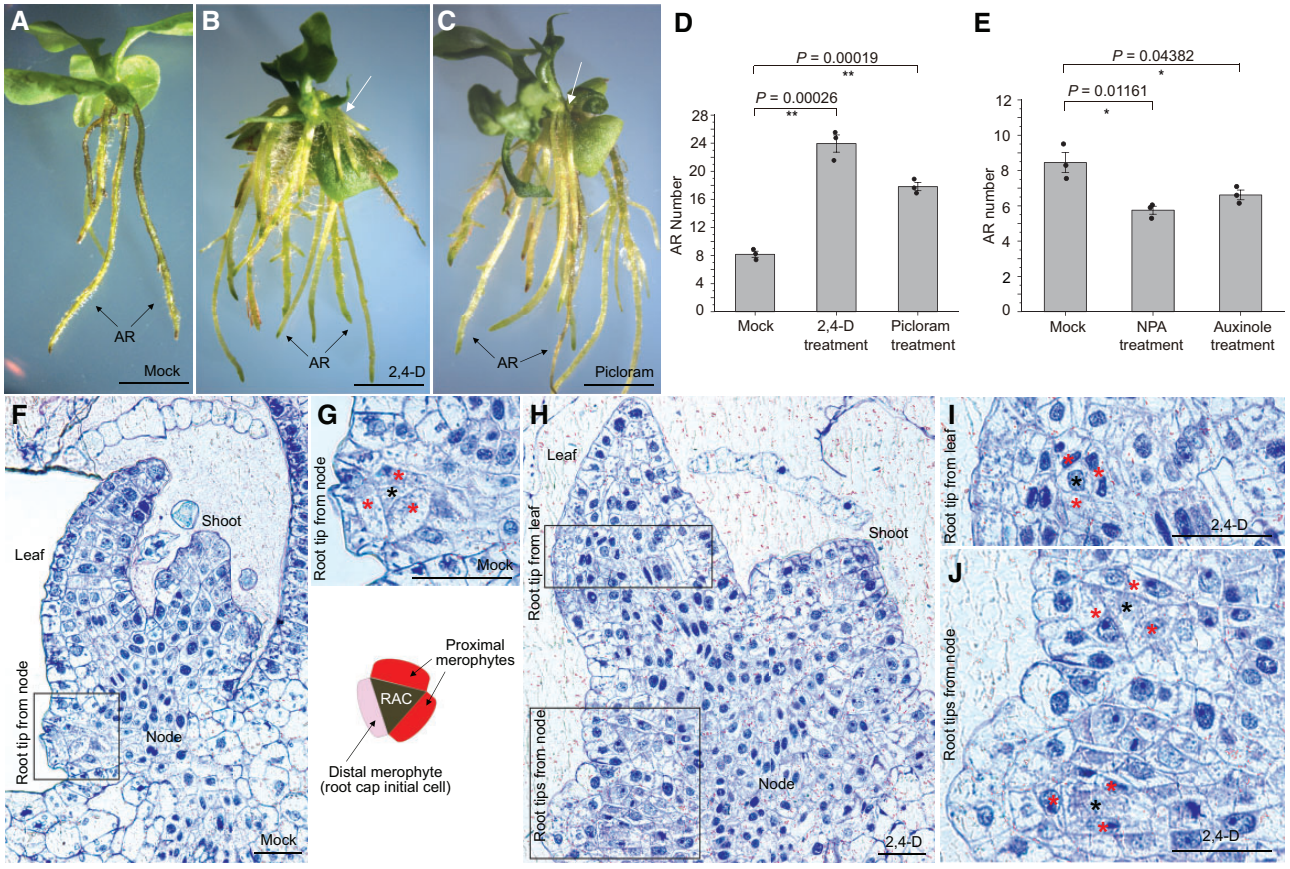

FIG. 1. Auxin promotes root initiation in *Ceratopteris richardii*. Phenotypic (*A*–*C*) and statistical (*D*, *E*) analysis of adventitious root formation in *C. richardii*. Mock, control without auxin or auxin inhibitor treatment. White arrows in (*B*, *C*) indicate many roots formed from a node. For 2,4-D or picloram treatment (*B*–*D*), plants were first grown on 1/2 MS medium for 30 days after sowing spores (DASS) and the first root tip could be observed at this time. Sporophytes were moved to 1/2 MS medium containing 0.5 μM 2,4-D or 5 μM picloram for 5 days and then on 1/2 MS medium for 12 days, because constant 2,4-D or picloram treatment might cause severe and overall developmental defects. For NPA or auxinole treatment (*E*), plants were first grown on 1/2 MS medium for 30 DASS, then on 1/2 MS medium containing 10 μM NPA or 15 μM auxinole for 17 days. Statistical analyses were performed on counts of adventitious roots obtained using Nikon SMZ1500 microscopy. Error bars show SEM from three biological repeats ($n = 20$ seedlings in *D*, *E*). *$P < 0.05$ and **$P < 0.01$ in two-tailed Student's *t*-tests (*D*, *E*). Individual values from each biological repeat (black dots) and means (bars) are shown (*D*, *E*). AR, adventitious root (also see supplementary fig. 1, Supplementary Material online). Thin sections of *C. richardii* shoots without (mock, *F*, *G*) or with (*H*–*J*) 2,4-D treatment. For auxin treatment, 30-DASS seedlings were grown on 1/2 MS medium containing 2 μM 2,4-D for 5 days. (*G*) and (*I*, *J*) are close-ups of boxed regions in (*F*) and (*H*), respectively. Diagram in (*G*) shows structure of root tip. Black asterisks indicate root apical cells; red asterisks show merophytes divided from division plane of root apical cell (*G*, *I*, *J*). RAC, root apical cell. Note that a section view could only present one or two from three proximal merophytes surrounding the tetrahedral root apical cell (*G*, *I*, *J*). Scale bars, 5 mm in (*A*–*C*) and 50 μm in (*F*–*J*).

online). Thin sections showed that several root tips were formed at the node (fig. 1*H* and *J*) and some root tips were even formed ectopically in the leaf (fig. 1*H* and *I*) after auxin treatment. When seedlings were treated with napthylphthalamic acid (NPA, a polar auxin transport inhibitor) or auxinole (Hayashi et al. 2012) (an auxin receptor inhibitor), significantly fewer adventitious roots formed (fig. 1*E*; supplementary fig. 1*B*–*D*, Supplementary Material online). We do not exclude the possibility that NPA or auxinole treatment may cause abnormal leaf development (supplementary fig. 1*B*–*D*, Supplementary Material online), which could also affect root initiation. Overall, these data suggest that auxin plays an essential role and is sufficient to trigger adventitious root initiation in *C. richardii*.

To confirm that auxin treatment triggers rooting in *C. richardii*, we performed the RNA-seq analysis. By comparing the transcriptomes of the *C. richardii* seedling upon 2,4-D treatment at time 0 (t_0, the control before treatment), 1 day and 2 days with those from the shoot tip, the leaf tip, and the root tip, we found that the transcriptional profile after auxin treatment was similar to that of the root tip, suggesting that the auxin signaling pathway can upregulate the rooting program (fig. 2*A*). In addition, transcriptional upregulation in the *C. richardii* seedling upon 2,4-D treatment showed a similar trend with that during adventitious root initiation from leaf explants in *A. thaliana* (Chen et al. 2014; Liu, Sheng, et al. 2014), indicating that the molecular program of adventitious root initiation might require some similar genes or pathways in euphyllophytes (fig. 2*B*).

To reveal the molecular pathway of auxin-mediated root initiation in *C. richardii*, we analyzed transcriptional changes at 1 day or 2 days after 2,4-D treatment compared with t_0

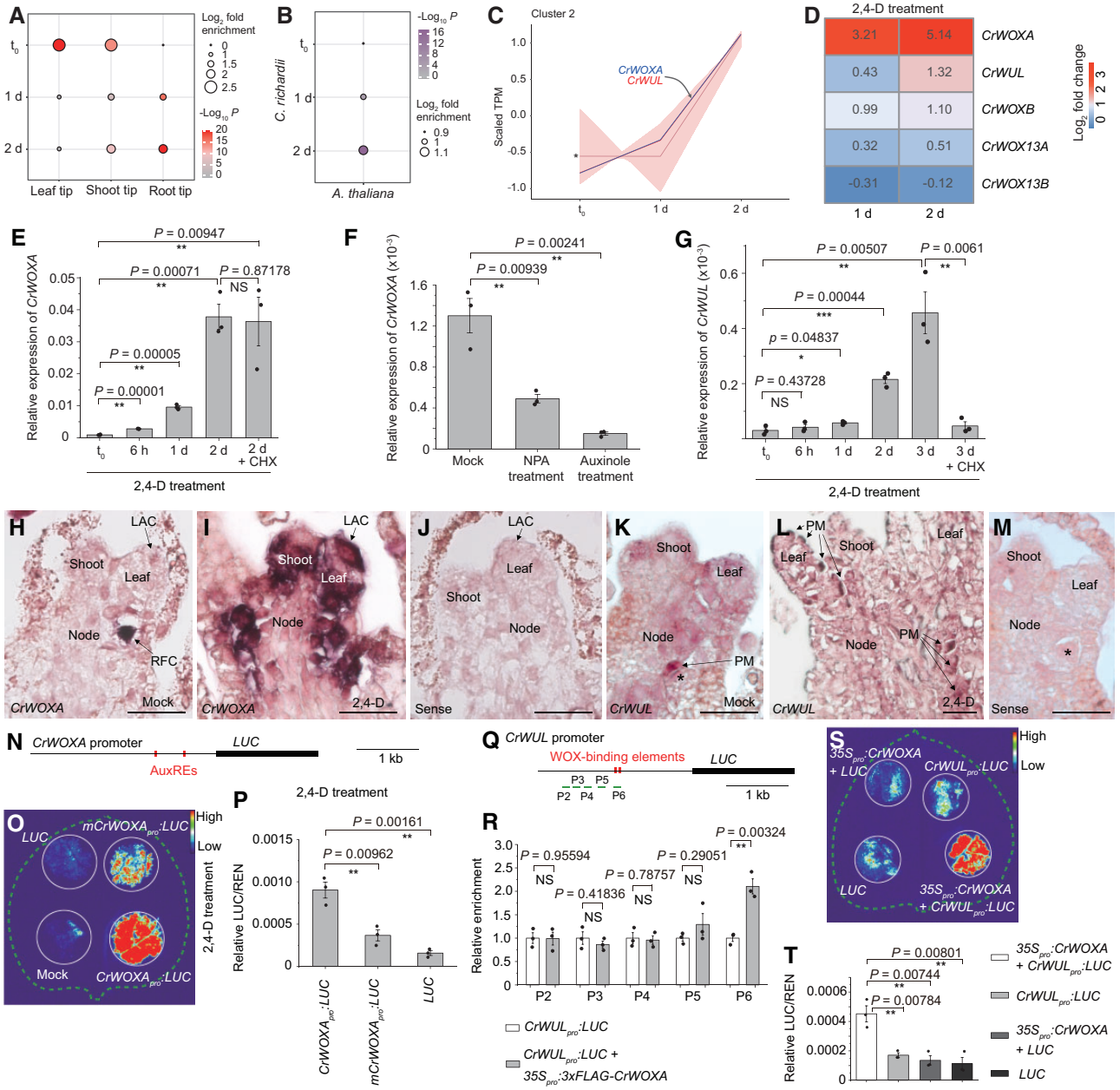

Fig. 2. Molecular pathway in *Ceratopteris richardii* root initiation. RNA-seq analysis showing transcriptome comparison of 30-day after sowing spores (DASS) *C. richardii* seedlings at t_0, 1 day or 2 days upon 2 μM 2,4-D treatment with shoot tips, leaf tips, and root tips from *C. richardii* (*A*) or the wounded petiole region from *Arabidopsis thaliana* leaf explants (*B*). The overlapping level of two gene sets is represented by bubble size, and the significance of association between two gene sets is color coded. Shoot tips, leaf tips, and root tips were collected from 50-DASS *C. richardii* seedlings, and tissue-specific genes were used for comparison (*A*). Detached leaf explants from 12-day-old *A. thaliana* seedlings were collected at t_0 and 2 days cultured on B5 medium with sucrose at 22 °C under dark conditions, and the wounded region (including the partial petiole and some surrounding tissues), which comprises the cells to form adventitious roots, was separated and collected for RNA extraction (*B*). Upregulated genes (2 days vs. t_0) during adventitious rooting from *A. thaliana* leaf explants were used for transcriptome comparison (*B*). (*C*) RNA-seq data showing cluster-2 genes in 30-DASS *C. richardii* seedlings upon 2 μM 2,4-D treatment for 1 day or 2 days (see supplementary fig. 2 and supplementary table 1, Supplementary Material online). *CrWOXA* and *CrWUL* were identified in cluster 2. Asterisks indicate average values. (*D*) RNA-seq analysis of five *WOX* genes in 30-DASS *C. richardii* seedlings upon 2 μM 2,4-D treatment for 1 day or 2 days compared with t_0. qRT-PCR analysis of *CrWOXA* (*E*, *F*) or *CrWUL* (*G*) transcript levels. *Ceratopteris richardii* seedlings at 30 DASS were treated with 2 μM 2,4-D or 2 μM 2,4-D together with 10 μM CHX for 0 to 3 days (*E*, *G*), or with 10 μM NPA or 15 μM auxinole for 3 days (*F*) (also see supplementary fig. 3, Supplementary Material online). In situ hybridization analyses of *CrWOXA* (*H*–*J*) or *CrWUL* (K–M) in *C. richardii* shoots without (mock, *H*, *K*) or with (*I*, *L*) 2,4-D treatment. *Ceratopteris richardii* seedlings at 30 DASS were moved to 1/2 MS medium containing 2 μM 2,4-D for 1 day (*CrWOXA*, *I*) or 3.5 days (*CrWUL*, *L*). (*J*) and (*M*) are sense controls for *CrWOXA* and *CrWUL*, respectively. Black asterisks indicate root apical cells (*K*, *M*). (*N*) Schematic of *CrWOXA* gene structure showing AuxREs. Fluorescence (*O*) and relative ratio of firefly LUC to *Renilla* luciferase (REN) activity (*P*) in transient expression assay of tobacco leaves transformed with *LUC* (without promoter), $CrWOXA_{pro}$:*LUC* or $mCrWOXA_{pro}$:*LUC* together with 2 μM 2,4-D. Mock (no transformation) served as negative control. (*Q*) Schematic of *CrWUL* gene structure, showing putative CrWOXA-binding *cis* elements. Horizontal lines below gene

from the RNA-seq data, and seven clusters of genes (i.e., clusters 1–7) were identified (fig. 2C; supplementary fig. 2 and supplementary table 1, Supplementary Material online). Two *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes, the intermediate-clade *WOX* (IC-*WOX*) gene *CrWOXA*, and the WUSCHEL-clade *WOX* (WC-*WOX*) gene *WUS lineage* (*CrWUL*), which are related to root initiation (Nardmann and Werr 2012; Liu and Xu 2018), were among the upregulated genes in cluster 2 (fig. 2C and *D*). The ancient-clade *WOX* (AC-*WOX*) genes (i.e., *CrWOX13A* and *CrWOX13B*) could not be upregulated by auxin treatment (fig. 2*D*).

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses confirmed that *CrWOXA* was gradually upregulated from 6 h of 2,4-D treatment (fig. 2*E*). This upregulation still occurred after treatment with cycloheximide (CHX), a protein synthesis inhibitor, indicating that *CrWOXA* could be a direct target of the auxin signaling pathway (fig. 2*E*; supplementary fig. 3, Supplementary Material online). Treatment with NPA or auxinole significantly inhibited *CrWOXA* expression (fig. 2*F*). The qRT-PCR results also showed that *CrWUL* was upregulated after 1 day of 2,4-D treatment (fig. 2*G*). This upregulation was inhibited by CHX, indicating that *CrWUL* activation by auxin requires one or more translated proteins in addition to the auxin signaling pathway (fig. 2*G*). Overall, the RNA-seq and qRT-PCR data show that auxin induces the successive expression of *CrWOXA* and *CrWUL*.

Both *CrWOXA* from the fern *C. richardii* and *AtWOX11* from the seed plant *A. thaliana* are IC-*WOX* genes (Haecker et al. 2004; Nardmann and Werr 2012; Liu and Xu 2018). *AtWOX11* is directly upregulated by the auxin signaling pathway and is specifically expressed in the root founder cell during adventitious root formation from detached leaves or during adventitious lateral root initiation from the primary root in *A. thaliana* (Liu, Sheng, et al. 2014; Sheng et al. 2017; Ge et al. 2019). In *C. richardii*, *CrWOXA* is specifically expressed in the root founder cells during either adventitious or lateral root initiation (Nardmann and Werr 2012). In situ hybridization analyses showed that, during adventitious root initiation in the mock control under our growth conditions, *CrWOXA* was expressed only in one hypodermal cell in the node beneath the leaf base, indicative of the formation of an adventitious root founder cell (fig. 2*H* and *J*). Upon 2,4-D treatment for 1 day, *CrWOXA* expression was induced in many cells in the node, leaf, and even ectopically in the leaf apical cell, suggesting that those cells had undergone the transition to become root founder cells (fig. 2*I*).

CrWUL from *C. richardii* and *AtWOX5* from *A. thaliana* are both WC-*WOX* genes (Haecker et al. 2004; Nardmann and Werr 2012; Liu and Xu 2018). During adventitious root initiation from detached leaves of *A. thaliana*, *AtWOX11* directly activates *AtWOX5* expression during the fate transition from root founder cell to the root primordium with cell division (Hu and Xu 2016). In *C. richardii*, *CrWUL* is specifically expressed in the proximal merophytes after the division of the root founder cell to form the root tip (Nardmann and Werr 2012). The in situ hybridization data showed that upon 2,4-D treatment for 3.5 days, *CrWUL* was induced in the proximal merophytes of many root tips within the shoot, whereas only one root tip formed in each node beneath a leaf in the mock control under our growth conditions (fig. 2*K*–*M*).

Two auxin response elements (AuxREs) were identified in the *CrWOXA* promoter (fig. 2*N*; supplementary table 2, Supplementary Material online). We generated two fusion constructs, $CrWOXA_{pro}$:*Luciferase* (*LUC*) and $mCrWOXA_{pro}$:*LUC*, in which the *LUC* reporter gene was fused downstream of the *CrWOXA* promoter and the *CrWOXA* promoter with mutated AuxREs (TGTCTC to TTTTTT), respectively. When these constructs were transiently expressed in tobacco (*Nicotiana tabacum*) leaves, auxin was able to activate luciferase signals via the AuxREs of the intact *CrWOXA* promoter, but this activation was much weaker when using the mutated promoter (fig. 2*O* and *P*). Therefore, the auxin signaling pathway might directly promote *CrWOXA* expression through the AuxREs in the *CrWOXA* promoter during the establishment of the root founder cell in *C. richardii*, similar to the activation of *AtWOX11* by auxin through AuxREs in adventitious root founder cells in *A. thaliana* (Liu, Sheng, et al. 2014).

Putative WOX-binding elements were also identified in the *CrWUL* promoter (fig. 2*Q*; supplementary table 2, Supplementary Material online). A chromatin immunoprecipitation (ChIP) analysis in tobacco leaves showed that CrWOXA could directly bind to the WOX-binding *cis* elements in the *CrWUL* promoter (fig. 2*R*). In a transient expression assay in which $35S_{pro}$:*CrWOXA* was coexpressed with $CrWUL_{pro}$:*LUC*, CrWOXA could activate the luciferase response in tobacco leaves (fig. 2*S* and *T*). Therefore, CrWOXA might directly activate *CrWUL* expression during the division of the root founder cell to the root tip in *C. richardii*, similar to the direct activation of *AtWOX5* by AtWOX11 during the root founder cell division to form the root primordium during adventitious root initiation in *A. thaliana* (Hu and Xu 2016).

Fig. 2 Continued

show positions of PCR fragments used in ChIP analysis in (*R*). (*R*) ChIP analysis showing that CrWOXA binds to *CrWUL* promoter in tobacco leaves cotransformed with $35S_{pro}$:3×*FLAG-CrWOXA* and $CrWUL_{pro}$:*LUC*. Transformation with only $CrWUL_{pro}$:*LUC* served as negative control. ChIP results were normalized to input control. Values from leaf tissue transformed with only $CrWUL_{pro}$:*LUC* were arbitrarily fixed at 1.0. Fluorescence (*S*) and relative ratio of firefly LUC to REN activity (*T*) in transient expression assay of tobacco leaves cotransformed with $35S_{pro}$:*CrWOXA* and $CrWUL_{pro}$:*LUC* or *LUC* (without promoter). Transformation with only $CrWUL_{pro}$:*LUC* or *LUC* (without promoter) served as negative controls. Error bars show SEM from three biological repeats ($n = 3$ technical repeats in each biological repeat in *E*–*G*, *P*, *R*, *T*). *$P < 0.05$ and **$P < 0.01$ in two-tailed Student's *t*-tests (*E*–*G*, *P*, *R*, *T*). Individual values (black dots) and means (bars) are shown (*E*–*G*, *P*, *R*, *T*). PM, proximal merophyte; LAC, leaf apical cell; RFC, root founder cell. Scale bars, 50 μm in (*H*–*M*).

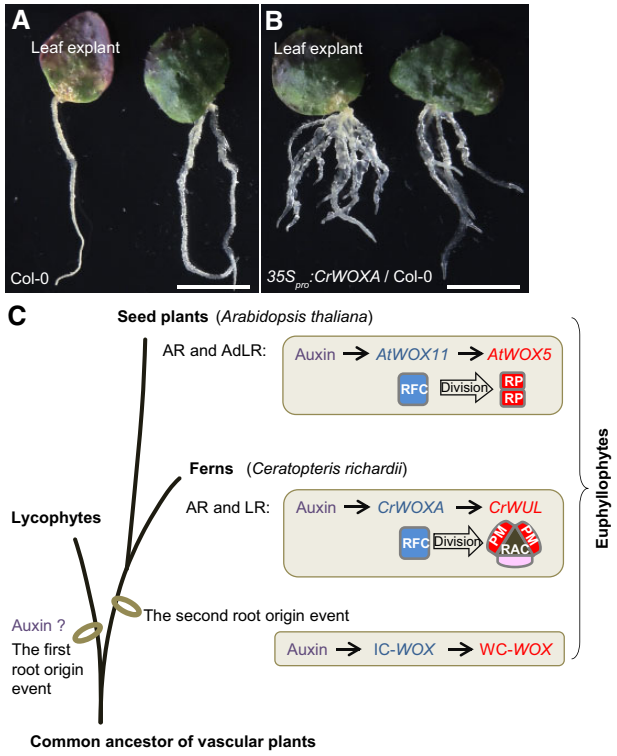

Fig. 3. *CrWOXA* overexpression in *Arabidopsis thaliana*. Phenotypic analysis of adventitious rooting from wild-type Col-0 (*A*) and 35S$_{pro}$:*CrWOXA* (*B*) leaf explants at 14 days of culture on B5 medium. *Arabidopsis thaliana* seeds were grown on 1/2 MS medium with sucrose at 22 °C under a 16-h light/8-h dark photoperiod. Detached leaf explants from 12-day-old seedlings were cultured on B5 medium without sucrose at 22 °C under 24-h light conditions for 14 days. (*C*) Model of root evolution in euphyllophytes, where "auxin>IC-*WOX*>WC-*WOX*" molecular module arose in common ancestor of ferns and seed plants during second origin of roots. Two independent transgenic lines were tested and showed similar results (*B*). Scale bars, 5 mm in (*A*, *B*). AdLR, adventitious lateral root; AR, adventitious root; LR, lateral root; PM, proximal merophyte; RAC, root apical cell; RFC, root founder cell; RP, root primordium.

To confirm the role of *CrWOXA* in root initiation, we carried out a genetic analysis. Overexpression of *CrWOXA* (35S$_{pro}$:*CrWOXA*) enhanced root formation from detached leaves of *A. thaliana* (fig. 3*A* and *B*). These data, together with the fact that *WOX11* is involved in rooting in many seed plants (Zhao et al. 2009; Liu, Sheng, et al. 2014; Liu, Wang, et al. 2014), suggest that the role of IC-*WOX* genes in promoting root initiation is conserved in euphyllophytes.

In summary, our results show that the conserved "auxin>IC-*WOX*>WC-*WOX*" molecular pathway functions during adventitious root initiation in the fern *C. richardii* and the seed plant *A. thaliana*. During the WOX family evolution, IC-*WOX* might have arisen in the common ancestor of lycophytes and euphyllophytes, whereas WC-*WOX* might be present only in euphyllophytes (Nardmann and Werr 2012). Here, we propose the following model of auxin-mediated root evolution (fig. 3*C*). During the second origin of roots in the common ancestor of euphyllophytes, the prototype of the auxin-mediated root initiation was established. This involves two steps of cell fate transition: In the first step, auxin directly activates IC-*WOX* gene expression to establish the root founder cell; in the second step, IC-WOX activates WC-*WOX* expression during root founder cell division to form the root tip. During the evolution of ferns, the "auxin>IC-*WOX*>WC-*WOX*" molecular pathway was adopted to promote adventitious root and endodermis-derived lateral root initiation. This is supported by the observations that *CrWOXA* is expressed in both adventitious and lateral root founder cells (Nardmann and Werr 2012) and endodermis-derived lateral root formation is also dependent on auxin in *C. richardii* (supplementary fig. 4, Supplementary Material online). During the evolution of seed plants, the "auxin>IC-*WOX*>WC-*WOX*" molecular pathway was adopted to promote adventitious root and adventitious lateral root initiation (Liu, Sheng, et al. 2014; Sheng et al. 2017; Ge et al. 2019).

However, the initiation of pericycle-derived acropetal lateral roots in the seed plant *A. thaliana* does not require IC-*WOX* genes (Sheng et al. 2017; Ge et al. 2019). Instead, *AUXIN RESPONSE FACTOR7* (*ARF7*) and *ARF19*, which arose with the appearance of seed plants (Finet et al. 2013; Kato et al. 2018), are specifically required for pericycle-derived acropetal lateral root initiation (Okushima et al. 2005). In addition, several pairs of pericycle cells are required for lateral root initiation in *A. thaliana*, whereas only one endodermal cell is sufficient to divide into a lateral root in *C. richardii*. Therefore, the molecular mechanisms involved in the initiation of pericycle-derived acropetal lateral roots in seed plants might differ from those involved in the initiation of endodermis-derived lateral roots in ferns.

Materials and Methods

Plant Materials and Culture Conditions

Spores of *C. richardii* (RNWT1 strain) were spread and germinated on 1/2 Murashige & Skoog (MS) medium without sucrose at 26 °C under a 16-h light/8-h dark photoperiod, and sterilized water was added once a week. The first root tip could be observed from the sporophyte at ~30 days after sowing spores. For chemical treatments, 2,4-D (Sigma, Cat. 573760, dissolved in DMSO), NPA (Sigma, Cat. 33371, dissolved in DMSO), auxinole (Hayashi et al. 2012, dissolved in DMSO), picloram (Sigma, Cat. P5575, dissolved in water), and CHX (Sigma, Cat. C7698, dissolved in ethanol) were added to 1/2 MS medium. Plants were grown vertically on 1/2 MS medium for chemical treatments. Mock controls were performed using the corresponding solvent. Detailed concentrations for chemical treatments were described in figure legends.

Seeds of *A. thaliana* were spread and germinated on 1/2 MS medium with sucrose at 22 °C. To generate 35S$_{pro}$:*CrWOXA* transgenic *A. thaliana* plants, the cDNA encoding CrWOXA was inserted into the pMON530 vector and then introduced into the *A. thaliana* wild-type Columbia-0 (Col-0) by *Agrobacterium tumefaciens*-mediated transformation.

ChIP, qRT-PCR, In Situ Hybridization, and Thin Sectioning

ChIP and qRT-PCR analyses were carried out as previously described (He et al. 2012). The PCR results are presented as relative transcript levels, normalized against that of *CrACTIN*. For ChIP, 3×*FLAG-CrWOXA* fused cDNA was cloned into the pMON530 vector. The anti-FLAG antibody (F1804, Sigma) was used in the ChIP analysis. In situ hybridization and thin sectioning were performed as previously described (Zeng et al. 2016; Hu et al. 2017).

Tobacco Assay

For the tobacco assay, the promoters of *CrWOXA*, *mCrWOXA*, or *CrWUL* (listed in supplementary table 2, Supplementary Material online) were each cloned into the pGREENII-0800 vector. The dual LUC assay in tobacco leaves was performed using the Dual-Luciferase Reporter Assay System (Promega). The primers used for molecular cloning and PCR are listed in supplementary table 3, Supplementary Material online.

RNA-seq

For de novo transcriptome assembly of *C. richardii*, raw reads from five tissues (leaf tip, leaf blade, shoot tip, root tip, and mature root region) were quality-trimmed using Trim Galore v0.5.0, and clean reads were input into Trinity v2.8.4 (Grabherr et al. 2011) separately for de novo assembly. The resulting transcriptomes were filtered and combined using CD-HIT v4.6.8 (Li and Godzik 2006) with sequence identity threshold at 0.8 and sequence minimum length at 500 bp to reduce sequence redundancy and avoid short contigs. TransDecoder v5.5.0 (http://github.com/TransDecoder/TransDecoder/wiki) was used to predict coding regions. OrthoFinder v2.3.3 (Emms and Kelly 2015) was used to detect orthogroups for all orthologous genes between *A. thaliana* and *C. richardii*.

For differential expression analysis, paired-end reads from *C. richardii* seedlings with auxin treatment were mapped to Trinity transcriptome using Bowtie2 v2.3.4.3 (Langmead and Salzberg 2012). The paired-end reads of *A. thaliana* leaf explants were mapped to *A. thaliana* genome (TAIR10) using STAR v2.7.2b (Dobin et al. 2013). Transcript abundance was determined using RSEM v1.3.2 (Li and Dewey 2011). All genes were filtered by the sum of TPM >1 to remove genes with low transcript levels, and the final 40,554 filtered genes for *C. richardii* and 20,170 filtered genes for *A. thaliana* were used for further analysis. To analyze time-series gene expression profiles, the filtered genes for *C. richardii* (t_0, 1 day, 2 days) were subjected to k-means clustering.

Tissue-specific genes (root tip, shoot tip, and leaf tip) of *C. richardii* were detected by TissueEnrich v1.5.1 (Jain and Tuteja 2019). *Ceratopteris richardii* genes (TPM $>$ 1) which have higher expression (>5-fold change) in one tissue than in the other two tissues were defined as tissue-specific genes (listed in supplementary table 1, Supplementary Material online). Enrichment analysis was used to determine whether *C. richardii* genes upregulated by auxin treatment were associated with tissue-specific genes in *C. richardii* (root tip, shoot tip, and leaf tip) or orthologous genes upregulated during root initiation from *A. thaliana* leaf explants (2 days vs. t_0, $\log_2$[fold change] ≥ 1.5). Fisher's exact tests were performed to determine the significant dependence (*P*-values). Enriched fold change was calculated using the following formula:

$$\text{Fold enrichment} = \frac{k/n}{K/N},$$

where N is the total number of genes as background; K is the total number of pre-defined gene sets, which were *C. richardii* tissue-specific gene sets or *A. thaliana*-upregulated gene sets; n is the number of input gene sets, which are genes have relatively high expression levels in each time point (t_0, 1 day or 2 days) in *C. richardii* based on threshold of $\log_2$[fold change] ≥ 1; k is the number of overlapped genes between predefined gene sets and input gene sets.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

We thank B. Hu for assistance with *CrWOXA* overexpression in *Arabidopsis thaliana* and Z. Qiu for chemical synthesis of auxinole. This work was supported by grants from the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB27030103), the National Natural Science Foundation of China (31630007), the Key Research Program of CAS (QYZDB-SSWSMC010), Youth Innovation Promotion Association CAS (2014241), and the National Key Laboratory of Plant Molecular Genetics.

Author Contributions

J.Y. and L.X. designed the research. J.Y., Yu.Z. and Yi.Z. performed the RNA-seq analyses. J.Y., W.L., H.W., and S.W. performed other experiments. J.Y. and L.X. analyzed the data. L.X. wrote the article.

References

Allsopp A, Szweykowska A. 1960. Foliar abnormalities, including repeated branching and root formation, induced by kinetin in attached leaves of *Marsilea*. *Nature* 186(4727):813–814.

Augstein F, Carlsbecker A. 2018. Getting to the roots: a developmental genetic view of root anatomy and function from *Arabidopsis* to lycophytes. *Front Plant Sci*. 9:1–15.

Banks JA. 2009. Selaginella and 400 million years of separation. *Annu Rev Plant Biol*. 60(1):223–238.

Berckmans B, Vassileva V, Schmid SPC, Maes S, Parizot B, Naramoto S, Magyar Z, Lessa Alvim Kamei C, Koncz C, Bögre L, et al. 2011. Auxin-dependent cell cycle reactivation through transcriptional regulation of *Arabidopsis* E2Fa by lateral organ boundary proteins. *Plant Cell* 23(10):3671–3683.

Brumos J, Robles LM, Yun J, Vu TC, Jackson S, Alonso JM, Stepanova AN. 2018. Local auxin biosynthesis is a key regulator of plant development. *Dev Cell*. 47:1–13.

Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inze D, Sandberg G, Casero PJ, et al. 2001. Auxin transport

promotes *Arabidopsis* lateral root initiation. *Plant Cell* 13(4):843–852.
Chen X, Qu Y, Sheng L, Liu J, Huang H, Xu L. 2014. A simple method suitable to study de novo root organogenesis. *Front Plant Sci*. 5:1–6.
de Vries J, Fischer AM, Roettger M, Rommel S, Schluepmann H, Bräutigam A, Carlsbecker A, Gould SB. 2016. Cytokinin-induced promotion of root meristem size in the fern *Azolla* supports a shoot-like origin of euphyllophyte roots. *New Phytol*. 209(2):705–720.
Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29(1):15–21.
Emms DM, Kelly S. 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome Biol*. 16(1):157.
Fang T, Motte H, Parizot B, Beeckman T. 2019. Root branching is not induced by auxins in *Selaginella moellendorffii*. *Front Plant Sci*. 10:154.
Finet C, Berne-Dedieu A, Scutt CP, Marlétaz F. 2013. Evolution of the ARF gene family in land plants: old domains, new tricks. *Mol Biol Evol*. 30(1):45–56.
Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G. 2003. Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* 426(6963):147–153.
Fujinami R, Yamada T, Nakajima A, Takagi S, Idogawa A, Kawakami E, Tsutsumi M, Imaichi R. 2017. Root apical meristem diversity in extant lycophytes and implications for root origins. *New Phytol*. 215(3):1210–1220.
Ge Y, Fang X, Liu W, Sheng L, Xu L. 2019. Adventitious lateral rooting: the plasticity of root system architecture. *Physiol Plant*. 165(1):39–43.
Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol*. 29(7):644–652.
Haecker A, Groß-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T. 2004. Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development* 131(3):657–668.
Hayashi KI, Neve J, Hirose M, Kuboki A, Shimada Y, Kepinski S, Nozaki H. 2012. Rational design of an auxin antagonist of the SCF TIR1 auxin receptor complex. *ACS Chem Biol*. 7(3):590–598.
He C, Chen X, Huang H, Xu L. 2012. Reprogramming of H3K27me3 is critical for acquisition of pluripotency from cultured *Arabidopsis* tissues. *PLoS Genet*. 8(8):e1002911.
Hetherington AJ, Dolan L. 2017. The evolution of lycopsid rooting structures: conservatism and disparity. *New Phytol*. 215(2):538–544.
Hetherington AJ, Dolan L. 2018. Stepwise and independent origins of roots among land plants. *Nature* 561(7722):235–238.
Hou G, Blancaflor EB. 2009. Fern root development. *Root Dev*. 37:192–208.
Hou G, Hill JP. 2002. Heteroblastic root development in *Ceratopteris richardii* (Parkeriaceae). *Int J Plant Sci*. 163(3):341–351.
Hou GC, Hill JP. 2004. Developmental anatomy of the fifth shoot-borne root in young sporophytes of *Ceratopteris richardii*. *Planta* 219(2):212–220.
Howe MD. 1931. Origin of leaf, and adventitious and secondary roots of *Ceratopteris thalictroides*. *Bot Gaz*. 92(3):326–329.
Hu B, Zhang G, Liu W, Shi J, Wang H, Qi M, Li J, Qin P, Ruan Y, Huang H, et al. 2017. Divergent regeneration-competent cells adopt a common mechanism for callus initiation in angiosperms. *Regeneration* 4(3):132–139.
Hu X, Xu L. 2016. Transcription factors WOX11/12 directly activate *WOX5/7* to promote root primordia initiation and organogenesis. *Plant Physiol*. 172(4):2363–2373.
Huang L, Schiefelbein J. 2015. Conserved gene expression programs in developing roots from diverse plants. *Plant Cell* 27(8):2119–2132.
Jain A, Tuteja G. 2019. TissueEnrich: tissue-specific gene enrichment analysis. *Bioinformatics* 35(11):1966–1967.
Kato H, Melkonian M, Weijers D, Rothfels C, Mutte SK, Wong G-S. 2018. Origin and evolution of the nuclear auxin response system. *Elife* 7:1–25.
Kenrich P, Strullu-Derrien C. 2014. The origin and early evolution of roots. *Plant Physiol*. 166:570–580.
Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 9(4):357–359.
Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12(1):323.
Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22(13):1658–1659.
Liu B, Wang L, Zhang J, Li J, Zheng H, Chen J, Lu M. 2014. WUSCHEL-related Homeobox genes in *Populus tomentosa*: diversified expression patterns and a functional similarity in adventitious root formation. *BMC Genomics* 15(1):296.
Liu J, Sheng L, Xu Y, Li J, Yang Z, Huang H, Xu L. 2014. *WOX11* and *12* are involved in the first-step cell fate transition during de novo root organogenesis in *Arabidopsis*. *Plant Cell* 26(3):1081–1093.
Liu W, Xu L. 2018. Recruitment of IC-*WOX* genes in root evolution. *Trends Plant Sci*. 23(6):490–496.
Mello A, Efroni I, Rahni R, Birnbaum KD. 2019. The *Selaginella* rhizophore has a unique transcriptional identity compared with root and shoot meristems. *New Phytol*. 222(2):882–894.
Motte H, Beeckman T. 2019. The evolution of root branching: increasing the level of plasticity. *J Exp Bot*. 70:771–784.
Motte H, Vanneste S, Beeckman T. 2019. Molecular and environmental regulation of root development. *Annu Rev Plant Biol*. 70(1):465–488.
Nardmann J, Werr W. 2012. The invention of WUS-like stem cell-promoting functions in plants predates leptosporangiate ferns. *Plant Mol Biol*. 78(1–2):123–134.
Okushima Y, Fukaki H, Onoda M, Theologis A, Tasaka M. 2007. ARF7 and ARF19 regulate lateral root formation via direct activation of *LBD/ASL* genes in *Arabidopsis*. *Plant Cell* 19(1):118–130.
Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, et al. 2005. Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in *Arabidopsis thaliana*: unique and overlapping functions of *ARF7* and *ARF19*. *Plant Cell* 17(2):444–463.
Pires ND, Dolan L. 2012. Morphological evolution in land plants: new designs with old genes. *Philos Trans R Soc B*. 367(1588):508–518.
Raven JA, Edwards D. 2001. Roots: evolutionary origins and biogeochemical significance. *J Exp Bot*. 52(Suppl 1):381–401.
Sanders HL, Langdale JA. 2013. Conserved transport mechanisms but distinct auxin responses govern shoot patterning in *Selaginella kraussiana*. *New Phytol*. 198(2):419–428.
Sheng L, Hu X, Du Y, Zhang G, Huang H, Scheres B, Xu L. 2017. Non-canonical *WOX11*-mediated root branching contributes to plasticity in *Arabidopsis* root system architecture. *Development* 144(17):3126–3133.
Webster TR. 1969. An investigation of angle-meristem development in excised stem segments of *Selaginella martensii*. *Can J Bot*. 47(5):717–722.
Williams S. 1937. Correlation phenomena and hormones in *Selaginella*. *Nature* 139(3527):966.
Xuan W, Audenaert D, Parizot B, Möller BK, Njo MF, De Rybel B, De Rop G, Van Isterdael G, Mähönen AP, Vanneste S, et al. 2015. Root cap-derived auxin pre-patterns the longitudinal axis of the *Arabidopsis* root. *Curr Biol*. 25(10):1381–1388.
Xuan W, Band LR, Kumpf RP, Van Damme D, Parizot B, De Rop G, Opdenacker D, Moller BK, Skorzinski N, Njo MF, et al. 2016. Cyclic programmed cell death stimulates hormone signaling and root development in *Arabidopsis*. *Science* 351(6271):384–387.
Zeng M, Hu B, Li J, Zhang G, Ruan Y, Huang H, Wang H, Xu L. 2016. Stem cell lineage in body layer specialization and vascular patterning of rice root and leaf. *Sci Bull*. 61(11):847–858.
Zhao Y, Hu Y, Dai M, Huang L, Zhou D-X. 2009. The WUSCHEL-related homeobox gene *WOX11* is required to activate shoot-borne crown root development in rice. *Plant Cell* 21(3):736–748.
Zimmerman PW, Wilcoxon F. 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. *Contrib Boyce Thompson Inst*. 7:209–229.