Letter

Temporarily Withdrawn: Molecular evolution of auxin-mediated root initiation in plants

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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.

The appearance of roots during vascular plant evolution was a great step towards 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). 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 2002; 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).

Auxin is the major hormone that triggers root initiation in seed plants (Zimmerman and F. 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 et al. 2014; Xuan et al. 2015; Xuan et al. 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 (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. 1A,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. 1F,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. 1B–D). Thin sections showed that many root tips were formed at the node (Fig. 1H,J) and even ectopically in the leaf (Fig. 1H,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. 1E). Overall, these data suggest that auxin plays an essential role and is sufficient to trigger adventitious root initiation in *C. richardii*.

To reveal the molecular pathway of auxin-mediated root initiation in *C. richardii*, we performed RNA-seq to analyze genes that were upregulated at 1 d and 2 d of 2,4-D treatment compared with the control (that is, t_0). The upregulated genes included 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*) (Fig. 1K), which are related to root initiation (Nardmann and Werr 2012; Liu and Xu 2018). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses confirmed that *CrWOXA* was gradually upregulated from 6 h of 2,4-D treatment (Fig. 1L). 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. 1L). Treatment with NPA or auxinole significantly inhibited *CrWOXA* expression (Fig. 1M). The qRT-PCR results also showed that *CrWUL* was upregulated after 1 d of 2,4-D treatment (Fig. 1N). 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. 1N). 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. 2003; 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 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. 1O,Q). Upon 2,4-D treatment for 1 d, *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. 1P).

CrWUL from *C. richardii* and *AtWOX5* from *A. thaliana* are both WC-*WOX* genes (Haecker et al. 2003; 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 d, *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. 1R–T).

Two auxin response elements (AuxREs) were identified in the *CrWOXA* promoter (Fig. 2A; Supplementary Table 1). We generated two fusion constructs, $CrWOXA_{pro}$*:LUC* and $mCrWOXA_{pro}$*:LUC*, in which the luciferase 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. 2B,C). 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 et al. 2014).

Putative WOX-binding elements were also identified in the *CrWUL* promoter (Fig. 2D;

Supplementary Table 1). 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. 2E). 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. 2F,G). 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).

To confirm the role of *CrWOXA* in root initiation, we carried out a genetic analysis. Overexpression of *CrWOXA* ($35S_{pro}$*:CrWOXA*) significantly enhanced root formation from detached leaves (Fig. 3A,B) and seedlings (Fig. 3C,D) of *A. thaliana*, suggesting 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*. Since adventitious roots may be a root type that appeared early during euphyllophyte evolution (Liu and Xu 2018), we propose the following model of auxin-mediated root evolution (Fig. 3E). 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. 1). 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 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*, while 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.

The fossil evidence suggests that roots originated independently in lycophytes and euphyllopytes (Raven and Edwards 2001; Pires and Dolan 2012; Hetherington and Dolan 2018; Liu and Xu 2018). Auxin is known to be involved in root initiation in lycophytes (Williams 1937; Webster 1969; Mello et al. 2019), but its molecular mechanism is unknown. In addition, the molecular pathways involved in root apical meristem development show some similarities between lycophytes and euphyllophytes although their root structures are different (Banks 2009; Sanders and Langdale 2013; Huang and Schiefelbein 2015; Fujinami et al. 2017; Hetherington and Dolan 2017; Augstein and Carlsbecker 2018; Fang et al. 2019). An understanding of the molecular pathways involved in auxin-mediated root initiation in lycophytes will shed light on root origin events at the molecular level in vascular plant evolution.

Materials and Methods

Plant materials and culture conditions

C. richardii (RNWT1 strain) was grown on 1/2 MS medium without sucrose at 26 °C under a 16-h light/8-h dark photoperiod. *A. thaliana* seedlings were grown on 1/2 MS medium with sucrose at 22 °C. To generate *$35S_{pro}$:CrWOXA* transgenic 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 1) 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 2.

RNA-seq

For *de novo* transcriptome assembly, raw reads were quality-trimmed using Trim Galore v0.5.0, and clean reads from five tissues 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 default parameters. For differential expression analysis, paired-end reads were mapped to Trinity transcriptome using Bowtie2 v2.3.4.3 (Langmead and Salzberg 2012). Transcript abundance was determined using RSEM v1.3.2 (Li and Dewey 2011). Differentially expressed transcripts were identified by EBSeq (Leng et al. 2013) at a threshold of $|\log_2[\text{fold change}]| > 1.5$. The RNA-seq data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession code GSE134602.

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Author contributions

J.Y. and L.X. designed the research. J.Y., Yuyun Zhang and Yijing Zhang 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.

Competing interests

The authors declare no competing interests.

Figures

Fig. 1

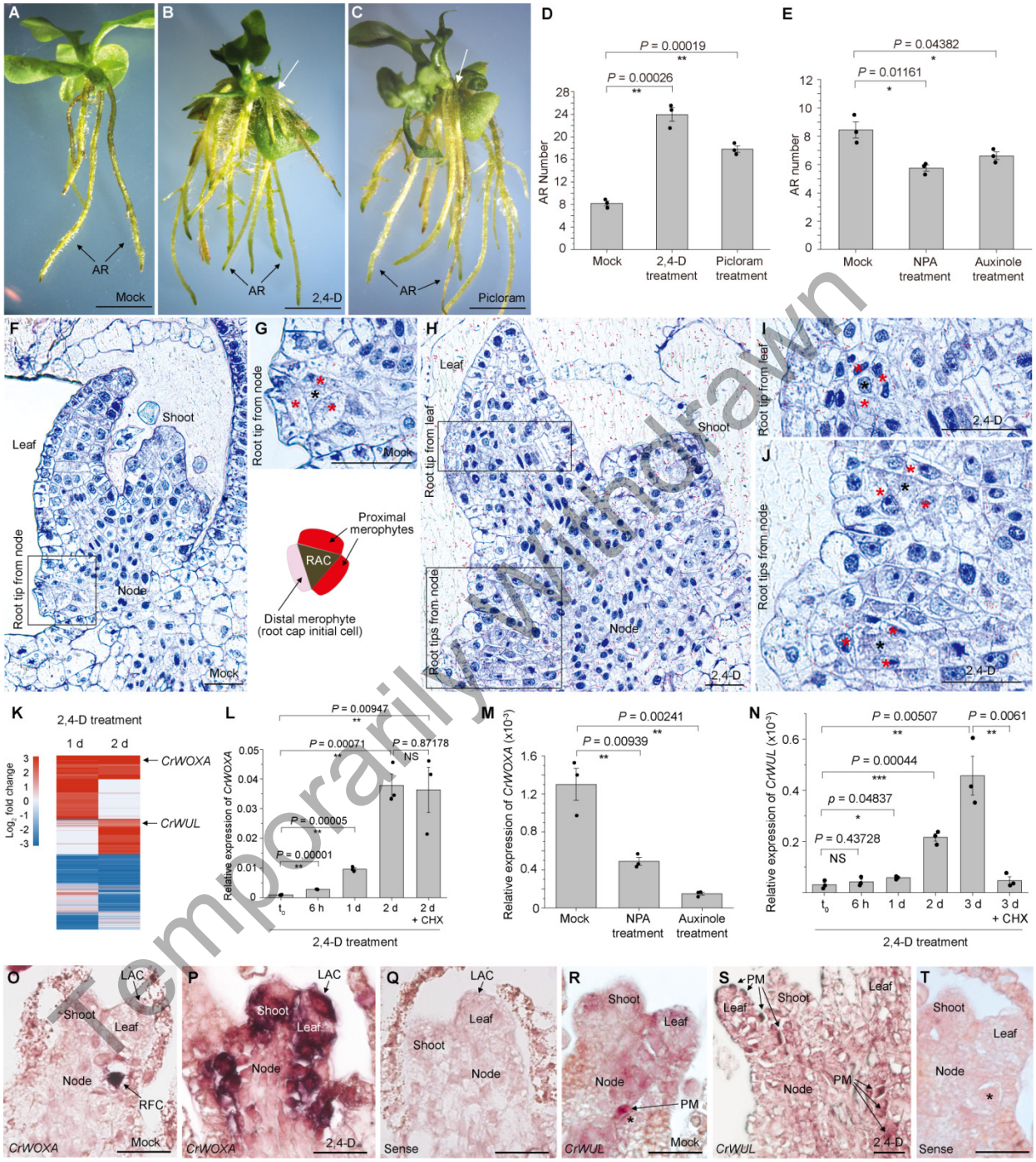

Fig. 1 Auxin promotes root initiation in *C. richardii*.

A–E, 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 treatments (B–D), plants were

first grown on 1/2 MS medium for 30 d after spore germination (DASG), then on 1/2 MS medium containing 0.5 μM 2,4-D or 5 μM picloram for 5 d, then on 1/2 MS medium for 12 d. For NPA or auxinole treatments (E), plants were first grown on 1/2 MS medium for 30 DASG, then on 1/2 MS medium containing 10 μM NPA or 15 μM auxinole for 17 d.

F–J, Thin sections of *C. richardii* shoots without (mock, F,G) or with (H–J) 2,4-D treatment. For auxin treatment, 30-DASG seedlings were grown on 1/2 MS medium containing 2 μM 2,4-D for 5 d. (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).

K, RNA-seq analysis of upregulated genes in 30-DASG *C. richardii* seedlings upon 2 μM 2,4-D treatment for 1 or 2 d.

L–N, qRT-PCR analysis of *CrWOXA* (L,M) or *CrWUL* (N) transcript levels. *C. richardii* seedlings at 30 DASG were treated with 2 μM 2,4-D or 2 μM 2,4-D together with 10 μM CHX for 0 to 3 d (L,N), or with 10 μM NPA or 15 μM auxinole for 3 d (M).

O–T, *In situ* hybridization analyses of *CrWOXA* (O–Q) or *CrWUL* (R–T) in *C. richardii* shoots without (mock, O,R) or with (P,S) 2,4-D treatment. *C. richardii* seedlings at 30 DASG were moved to 1/2 MS medium containing 2 μM 2,4-D for 1 d (*CrWOXA*, P) or 3.5 d (*CrWUL*, S). (Q) and (T) are sense controls for *CrWOXA* and *CrWUL*, respectively. Black asterisks indicate root apical cells (R,T).

Error bars show s.e.m. from three biological repeats (n = 20 seedlings in D,E; n = 3 technical repeats in L–N). *P < 0.05 and **P < 0.01 in two-tailed Student's t-tests (D,E,L–N). Individual values (black dots) and means (bars) are shown (D,E,L–N). AR, adventitious root; RAC, root apical cell; LAC, leaf apical cell; RFC, root founder cell. Scale bars, 5 mm in (A–C) and 50 μm in (F–J) and (O–T).

Fig. 2

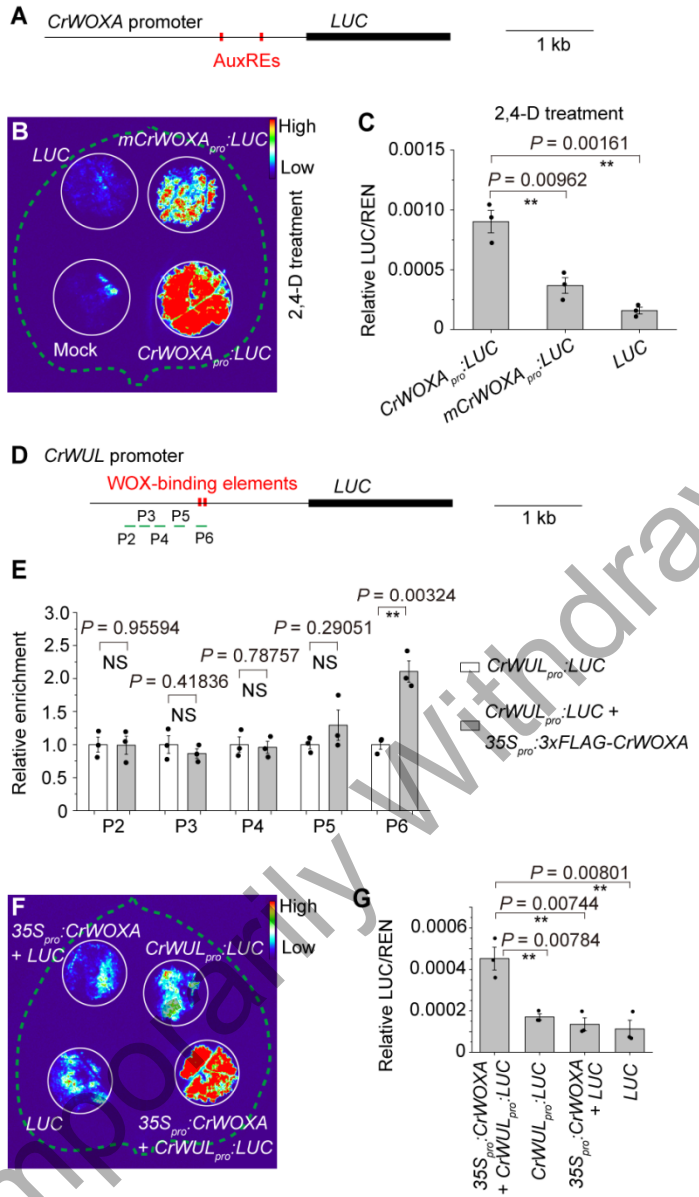

Fig. 2 Molecular interaction among auxin, *CrWOXA*, and *CrWUL*.

A, Schematic of *CrWOXA* gene structure showing AuxREs.

B,C, Fluorescence (B) and relative ratio of firefly LUC to Renilla luciferase (REN) activity (C) 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.

D, Schematic of *CrWUL* gene structure, showing putative CrWOXA-binding *cis* elements. Horizontal lines below gene show positions of PCR fragments used in ChIP analysis in (E).

E, 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.

F,G Fluorescence (F) and relative ratio of firefly LUC to REN activity (G) 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 s.e.m. from three biological repeats (n = 3 technical repeats) (C,E,G). $^{**}P <$ 0.01 in two-tailed Student's t-tests (C,E,G). Individual values (black dots) and means (bars) are shown (C,E,G).

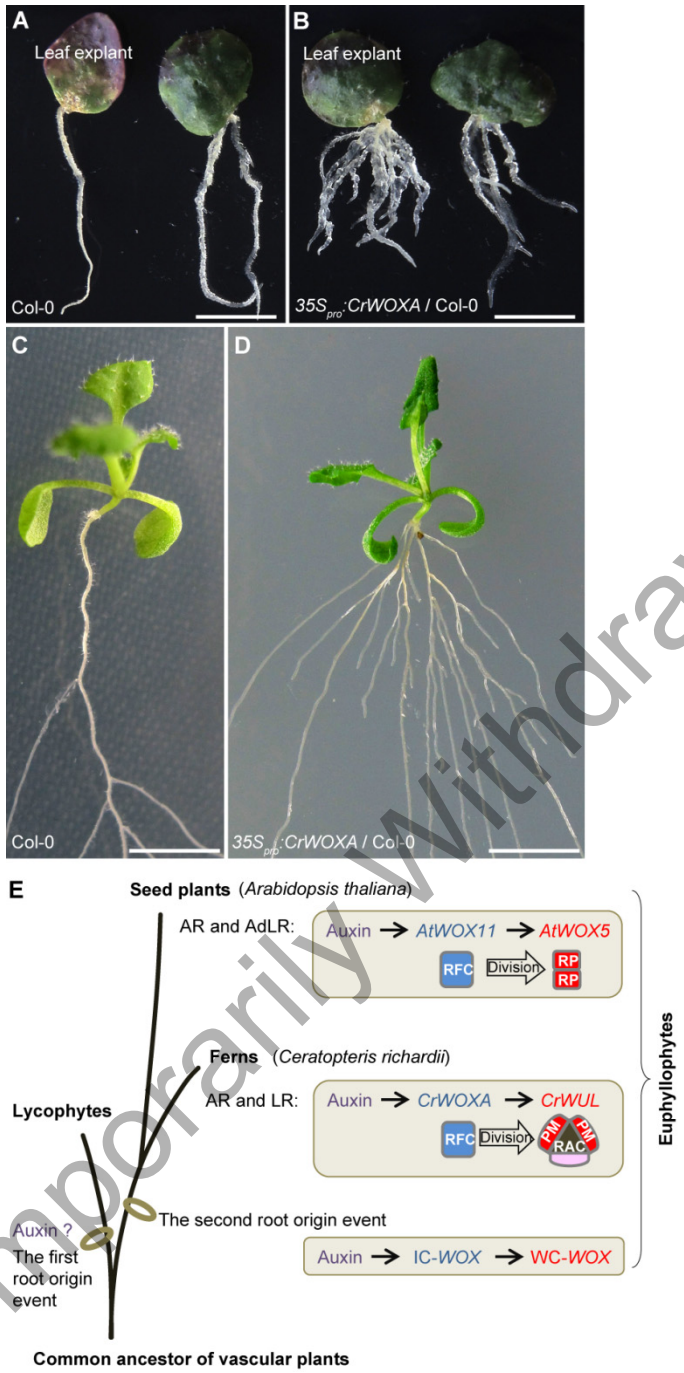

Fig. 3 *CrWOXA* overexpression in *A. thaliana*.

A,B, Phenotypic analysis of adventitious rooting from wild-type Col-0 (A) and *35S$_{pro}$:CrWOXA* (B) leaf explants at 14 d of culture on B5 medium. *A. 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 d.

C,D, Phenotypic analysis of root formation from wild-type Col-0 (C) and *35S$_{pro}$:CrWOXA* (D)

hypocotyls and roots. *A. thaliana* seedlings were grown on 1/2 MS medium with sucrose at 22 °C under a 16-h light/8-h dark photoperiod for 12 d.

E, 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,D). Scale bars, 5 mm in (A–D). AR, adventitious root; LR, lateral root; AdLR, adventitious lateral root; RFC, root founder cell; RP, root primordium; RAC, root apical cell; PM, proximal merophyte.