LETTER

Transcriptome framework of root regeneration reveals the conservation of the *LBD16*-mediated pathway in poplar cuttings

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Dear Editor,

The regeneration of adventitious roots (i.e., *de novo* root regeneration) from cuttings is a useful vegetative propagation method for trees.[1] Studies on the model plant *Arabidopsis thaliana* have revealed that the pathway involving auxin, the adventitious root founder cell marker gene *WUSCHEL-RELATED HOMEOBOX11* (*AtWOX11*), and the root primordium marker gene *LATERAL ORGAN BOUNDARIES DOMAIN16* (*AtLBD16*) promotes cell fate transition during adventitious root organogenesis from leaf cuttings.[2] Poplars are important tree species with rapid growth and large biomass, and have been widely used in studies on the mechanisms of adventitious root regeneration from stem cuttings.[3] The poplar *WOX11* gene has been confirmed to promote adventitious root regeneration.[4,5] Here we show a transcriptome framework of adventitious root regeneration in poplar cuttings and demonstrate the conserved role of *LBD16* in promoting adventitious root regeneration.

To gain an overview of adventitious root regeneration in poplar cuttings (Figures 1A–1D), we performed time-lapse RNA sequencing (RNA-seq) analyses of the wounded region of the stem from hybrid poplar 84K (*Populus alba* × *Populus glandulosa*) cultured on half-strength Murashige and Skoog (1/2 MS) medium with 2% w/v sucrose at time 0 (t_0), 1 day, 2 days, and 4 days after cutting. About 0.5-cm stem tissues at the wounding site as indicated in the boxed region in Figure 1B were collected for RNA-seq using the Illumina Novaseq 6000 platform. In our culture system, the root primordium and root apical meristem formed within 4 days (Figure 1B) and the mature root tip could be clearly observed at 6 days after cutting (Figure 1C). The RNA-seq data have been deposited in the Genome Sequence Archive (https://ngdc.cncb.ac.cn/gsa/) under the accession number CRA010051 and can be accessed using the online tool (http://xulinlab.cemps.ac.cn/).

Gene set enrichment analysis (GSEA)[6] of the RNA-seq data revealed changes in the expression of genes in multiple pathways during adventitious root regeneration. The transcript levels of photosynthesis-related genes rapidly decreased after wounding (Figure 1E). There was rapid up-regulation of genes involved in the response to wounding and oxidative stress, and of genes related to the ethylene-activated signaling pathway and the abscisic acid (ABA)-activated signaling pathway, indicating that these pathways might be involved in the protection of the damaged stem after wounding (Figure 1E).[1,7] Genes related to transporter activity, nitrate transmembrane transporter activity, and potassium ion transmembrane transporter activity were also rapidly up-regulated, indicative of resource allocation and sink establishment at the wounded region of the stem (Figure 1E).[1,7] Genes related to endoplasmic reticulum to cytosol auxin transport were up-regulated, consistent with the concept that auxin is the key hormone in promotion of rooting (Figure 1E).[2] Genes related to root development, meristem development, and meristem maintenance were also up-regulated at the wounded site of cuttings (Figure 1E). These data suggest that basic physiological processes might be similar in cuttings of diverse species.[1,2,7]

Analyses of key genes involved in cell fate transition during adventitious root organogenesis showed that *PagWOX11.1a/b* and *PagLBD16.2a/2b/3a* were up-regulated from 1 day after cutting and reached peak transcript levels at 2 days after cutting, indicating the formation of the adventitious root founder cell and the root primordium at around 2 days (Figure 1F). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis confirmed the expression patterns of *PagWOX11.1a/b* and *PagLBD16.3a/b* after cutting (Figures 1G, 1H). The transcript levels of many genes homologous to *Arabidopsis* genes that function in the stem cell niche activity and cell differentiation within the root apical meristem or lateral root organogenesis, e.g. *SHORT-ROOT* (*PagSHRa/b*), *PLETHORA1/4* (*PagPLT1a/1b/4.1b/4.2a/4.2b*), *LATERAL ROOT PRIMORDIUM1* (*PagLRP1b*), *STYLISH1* (*PagSTY1a*), *PUCHI* (*PagPUCHIa*), *RGF1 INSENSITIVE1* (*PagRGI1.1b/1.2a*), *ROOT MERISTEM GROWTH FACTOR8* (*PagRGF8b*), *SOMBRERO* (*PagSMBa*), *FEZ* (*PagFEZb*), and *TORNADO1* (*PagTRN1a*), peaked at 4 days after cutting, indicating the formation of the adventitious root apical meristem at around 4 days after cutting (Figure 1F). In addition, the genes related to the auxin signaling pathway (e.g. *AUXIN RESPONSE FACTOR*s, *PagARF*s), auxin transport (e.g. *AUXIN RESISTANT1*s, *PagAUX1*s; *PIN-FORMED*s, *PagPIN*s), and auxin biosynthesis (e.g. *YUCCA*s, *PagYUC*s) were upregulated during root regeneration (Figure 1F). These data suggest that the gene network (e.g. developmental pathways and auxin pathways, etc.) involved in root regeneration might be similar in cuttings of diverse species.[1,2,7]

Next, we focused on the role of *LBD16* in adventitious root regeneration in poplar. Phylogenetic analysis[8] of the class-IB *LBD* genes showed that there are two branches of *AtLBD16* homolog genes in the *Populus* genus and the *Salix* genus, i.e., the *LBD16.2* branch and the *LBD16.3* branch (Figure 1I), with both located in the subclass IIIA.[9] The genome of hybrid poplar 84K contains four *LBD16* genes, i.e., *PagLBD16.2a* (*Pag.A02G002381*), *PagLBD16.2b* (*Pag.B02G000427*), *PagLBD16.3a* (*Pag.A05G002243*), and *PagLBD16.3b* (*Pag.B05G002221*) (where *a* and *b* indicate chromosomes from *P. alba* and *P. glandulosa*, respectively) (Figures 1F–1J). The *Populus trichocarpa* genome contains two *LBD16* genes, i.e., *PtrLBD16.2* (*Ptr_Potri.002G041200*) and *PtrLBD16.3* (*Ptr_Potri.005G221900*) (Figures 1I and 1J). Overexpression of *PtrLBD16.3* ($35S_{pro}$*:PtrLBD16.3*) resulted in significantly enhanced adventitious root regeneration from 84K cuttings (Figures 1K–1M), indicating that the role of the LBD16 protein in promoting adventitious rooting might be evolutionarily conserved.

The *LBD16.2* genes in the *Populus* genus are usually located at a superlocus, which comprises the *LBD16.2* genes and the subclass-IIIB *LBD* genes (Figure 1J).[9] The *LBD16.3* genes in *Populus* genus are not related to this superlocus (Figure 1J). The *Arabidopsis* genome encodes a single *LBD16* gene, which is located in the superlocus.[9] There are many conserved regions in the promoters of *LBD16.2* and *LBD16.3* genes in the *Populus* genus, including auxin response elements (AuxREs)[9] and WOX-binding *cis* elements (WOXCEs) (Figure 1J).[10] The results of qRT-PCR analyses showed that treatment of the wounded region of cuttings with synthetic auxin 1-naphtalene acetic acid (NAA) up-regulated *PagLBD16.3a/b* (Figure 1N). *PagLBD16.3a/b* were also up-regulated in transgenic 84K overexpressing *PagWOX11.1a* ($35S_{pro}$*:PagWOX11.1a*) (Figure 1O). The chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA) data confirmed that the PagWOX11.1a protein might bind to a WOXCE in the *PagLBD16.3a* promoter (Figures 1P and 1Q). These findings suggest that auxin and *WOX11* might upregulate *LBD16* expression during adventitious rooting in poplar, similar to the *WOX11-LBD16* molecular pathway in *Arabidopsis*.[2]

In conclusion, the results of this study and those of other studies on poplar

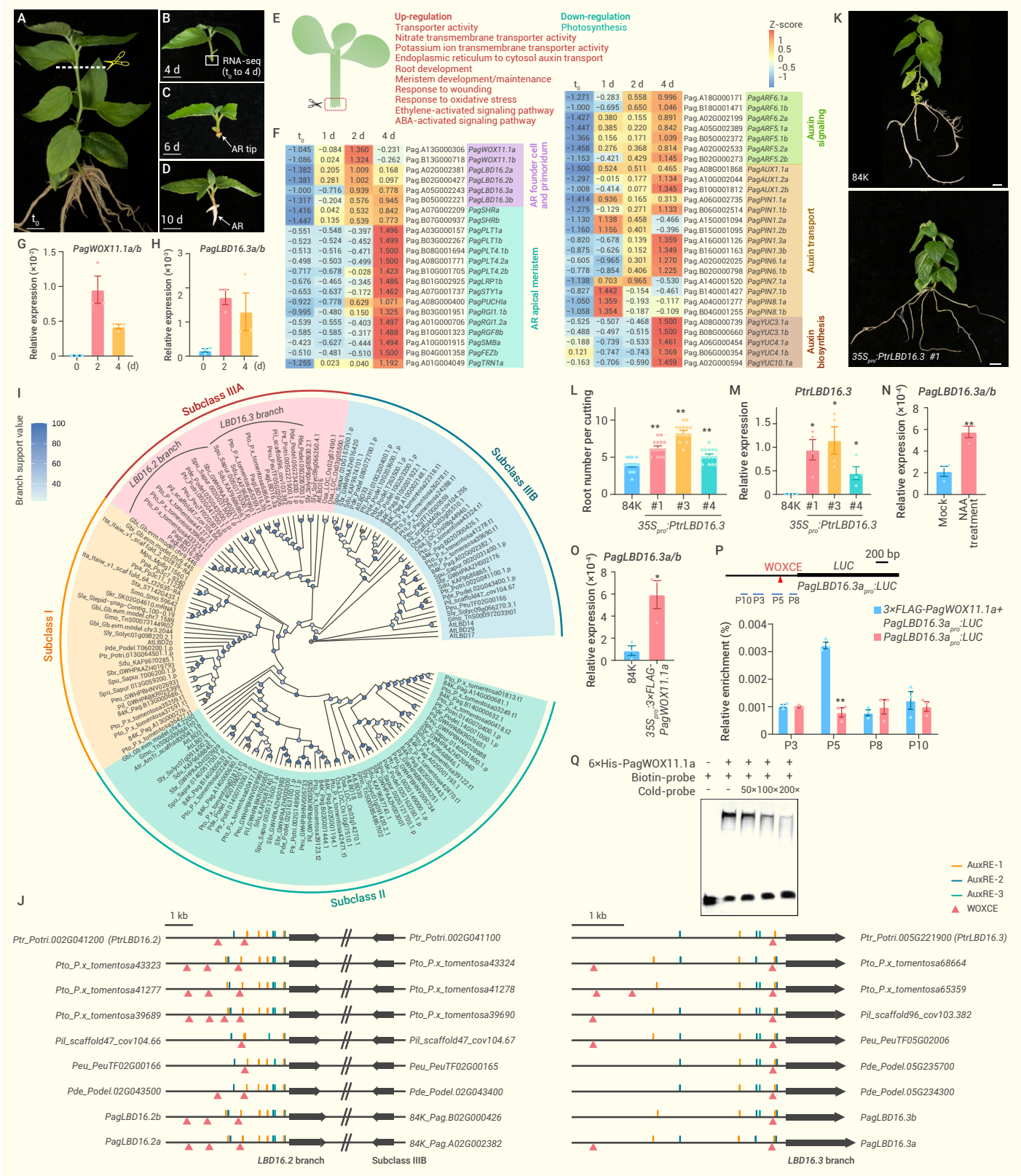

Figure 1. Adventitious root regeneration in poplar cuttings. (A–D) Adventitious root regeneration in cuttings of hybrid poplar 84K. Scissors indicate the excision site of the stem for cutting (A). AR, adventitious root. (E) Selected GSEA data on gene sets related to adventitious root regeneration in 84K cuttings (1 day *vs.* t_0, 2 days *vs.* t_0, and 4 days *vs.* t_0). (F) Relative transcript levels of genes related to root organogenesis and auxin pathways. (G, H) qRT-PCR analysis of *PagWOX11.1a/b* (G) and *PagLBD16.3a/b* (H) during adventitious root organogenesis in 84K cuttings. (I) Phylogenetic analysis of class-IB *LBD*s in *Populus* genus, *Salix* genus, and other plant species. (J) Conserved locations of *cis* elements (WOXCE and AuxRE) at *LBD16.2* and *LBD16.3* loci in *Populus* genus. AuxRE-1: TGTCTC; AuxRE-2: TGTCCC; AuxRE-3: TGTCGG; WOXCE: TTAATGG/C. (K, L) Phenotype (K) and statistical (L) analysis of rooting in $35S_{pro}$:*PtrLBD16.3* transgenic 84K poplar at 4 weeks after cutting (transgenic lines #1, #3, #4). (M) qRT-PCR analysis of *PtrLBD16.3* in the $35S_{pro}$:*PtrLBD16.3* lines. (N, O) qRT-PCR analysis of *PagLBD16.3a/b* at 1 day after cutting in 0.5 μM NAA treatment (N) or in the $35S_{pro}$:*3×FLAG-PagWOX11.1a* background (O). (P) ChIP analysis showing that PagWOX11.1a binds to *PagLBD16.3a* promoter in tobacco leaves cotransformed with $35S_{pro}$:*3×FLAG-PagWOX11.1a* and $PagLBD16.3a_{pro}$:*Luciferase* (*LUC*). PCR fragment positions and WOXCE were shown. (Q) EMSA of 6×His-PagWOX11.1a binding to *PagLBD16.3a* promoter fragment *in vitro*. 6×His-PagWOX11.1a protein was purified and incubated with a biotin-labeled probe, which was a sequence of the *PagLBD16.3a* promoter with WOXCE (CCATTAA), or the cold probe without biotin label. Scale bars, 1 cm (A–D, K).

WOX11 and *Arabidopsis*,[2,4,5] suggest that the network involving auxin, *WOX11*, and *LBD16* that promotes the regeneration of adventitious roots from cuttings might be evolutionarily conserved. Therefore, *LBD16* is a promising candidate as a molecular tool for promoting the rooting efficiency of cuttings from diverse species in the future.

The PCR primer and EMSA probe sequences used in this study are as follows: *PagLBD16.3a/b* and *PtrLBD16.3* (5'-GTTGTCACTATCGCCTAT-3' and 5'-GTGCTAGTTGAGCCTTCACT-3') , *PagWOX11.1a/b* (5'-CTCTAATCAAATGGCCTT-3' and 5'-CTGTGATGAATCCAGCAGT-3') , *PagACTIN* (5'-AAACTGTAATGGTCCTCCCTCCG-3' and 5'-GCATCATCACAATCACTCTCCGA-3'), P3 (5'-AAATGGGCATGGTGTACATAA-3' and 5'-TTAAGTCATCTTGAGTGATTA-3'), P5 (5'-CTTGGTAAGCAAAACTCCCA-3' and 5'-TCATGCACGGTTTTCAGGGA-3'), P8 (5'-CTCATTTTGTTTTGCACCAT-3' and 5'-ATGAGAGAAACAAAGAGAGG-3'), P10 (5'-AGAGAGGCATCATGCTTGT-3' and 5'-TGCTCCCCTGCCTAAAGAG-3'), and the EMSA probe (5'-CATATTTGCATAAACCCATTAATAAATGTTCCCTGAA-3'). The *PagWOX11.1a/b* qRT-PCR primers can detect both *PagWOX11.1a* and *PagWOX11.1b*. The *PagLBD16.3a/b* qRT-PCR primers can detect both *PagLBD16.3a* and *PagLBD16.3b*. Expression values in qRT-PCR were normalized to that of *PagACTIN*. ChIP results were normalized to the input control.

For phylogenetic analysis in Figure 1I, Class-IB *LBD* genes were identified using OrhoFinder v2.5.4. Protein sequences were aligned using MAFFT v7.515 with the parameters '--localpair --maxiterate 1000 --anysymbol'. We used IQ-TREE2[8] v2.2.0.3 with the parameters '-m MFP -bb 1000 -bnni' to find the best-fitting model and constructed the phylogenetic trees. *Ptr*, *Populus trichocarpa*; *Pag*, *Populus alba × P. glandulosa*; *Pde*, *Populus deltoides*; *Pto*, *Populus tomentosa*; *Peu*, *Populus euphratica*; *Pil*, *Populus ilicifolia*; *Spu*, *Salix purpurea*; *Sdu*, *Salix dunnii*; *Sbr*, *Salix brachista*; *At*, *Arabidopsis thaliana*; *Sly*, *Solanum lycopersicum*; *Osa*, *Oryza sativa*; *Atr*, *Amborella trichopoda*; *Gbi*, *Ginkgo biloba*; *Gmo*, *Gnetum montanum*; *Skr*, *Selaginella kraussiana*; *Sle*, *Selaginella lepidophylla*; *Smo*, *Selaginella moellendorffii*; *Sta*, *Selaginella tamariscina*; *Ita*, *Isoetes taiwanensis*; *Mpo*, *Marchantia polymorpha*; *Ppa*, *Physcomitrella patens*.

For statistical analysis data, individual values are indicated by circles (Figures 1G, 1H, and 1L–1P), and data are mean ± s.e.m. from three biological repeats (Figures 1G, 1H, and 1N–1P), 15 samples (Figure 1L), or four biological repeats (Figure 1M) (three technical repeats in each biological repeat, Figures 1G, 1H, and 1M–1P). * $P < 0.05$ and ** $P < 0.01$ in two-sided Mann-Whitney U-test (Figure 1L), Welch's t-test (Figure 1M), or Student's t-test (Figures 1N–1P) compared with non-transgenic wild-type 84K poplar (Figures 1L, 1M, and 1O), non-treated 84K poplar (mock, Figure 1N), or transformation with only $PagLBD16.3a_{pro}$:*LUC* (Figure 1P).

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AUTHOR CONTRIBUTIONS

GZ: Conceptualization (supporting), Funding acquisition (equal), Investigation (lead). GC: Investigation (lead). YZ: Investigation (lead). YE: Investigation (support). XL: Conceptualization (supporting), Funding acquisition (equal), Supervision (equal), Resources (equal). LX: Conceptualization (lead), Funding acquisition (equal), Writing – original draft (lead). JL: Conceptualization (supporting), Funding acquisition (equal), Supervision (equal), Resources (lead).

DECLARATION OF INTERESTS

The authors declare no competing interests.