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Research article

Identification and 3D gene expression patterns of WUSCEHEL-related homeobox (WOX) genes from *Panax ginseng*

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ABSTRACT

Wild ginseng (*Panax ginseng*) can survive in their natural habitat for hundreds of years, reflecting a remarkable plasticity. Plant stem cells (SCs) play a key role in the regenerative capacity and lifelong activity of these plants. WUSCHEL-RELATED HOMEOBOX (WOX) genes are master regulators of plant SC pluripotency, but their functions in medicinal plants have not been previously reported. To investigate whether these genes define different SC niches in ginseng, we cloned and analysed five *WOX* genes in ginseng (*PgWOXs*) and found that they might regulate root reconstruction. Then, the whole-mount RNA *in situ* hybridization was used to characterize the 3D gene expression pattern of *PgWOXs* in ginseng seedlings and cultured adventitious roots. *PgWOX4* was expressed in vascular cambium SCs; *PgWOX5* and *PgWOX11* were mainly expressed in the tips of seedling and adventitious roots, which are the energetic centre of the meristem; and *PgWOX13a* and *PgWOX13b* were detected in the parenchyma cells of the main root of seedlings and cultured adventitious roots, suggesting that they are important for maintaining the balance between SC differentiation and self-renewal in the phloem and xylem. This is the first report of SC regulation in medical herbs; we expect that *P. ginseng* can serve as a model herb for investigating the relationship between SCs and their herbal morphological features, which would be a new research direction to improve the yield and quality of the medicinal materials by regulating the herbal SCs.

1. Introduction

Chinese ginseng (*Panax ginseng* C. A. Meyer) is a medically important herb that has been widely used in East Asia for over 2000 years (Hemmerly, 1977). As a symbol of traditional Chinese medicine, Chinese ginseng has been used to restore stamina and enhance the capacity to cope with fatigue and physical stress (Gillis, 1997). Wild ginseng can survive in their natural habitat for hundreds of years, reflecting a remarkable plasticity (Li et al., 2015) that endows the detached and wounded root with regenerative capacity. During the life cycle, the main root of wild ginseng is often consumed by herbivores or destroyed by fungal erosion, and the arm root (fibre root of ginseng rhizome) can develop a new main root, although the mechanistic basis of this phenomenon is not well understood.

Lifelong activity and regenerative capacity in plants depend on the balance between pluripotent stem cells (SCs) and differentiated daughter cell populations (Heyman et al., 2014). Plant SCs exist in specific niches known as meristems and differentiate into various tissues and organs or form a new plant under specific environmental conditions (Aichinger et al., 2012). WUSCHEL-RELATED HOMEOBOX (WOX) genes are master regulators of plant SC pluripotency and may have established different SC niches (SCNs) during land plant evolution (Dolzblasz et al., 2016). The WOX family is divided into three separate clades, i.e., modern (WUS), intermediate, and ancient based on phylogenetic analysis of *Arabidopsis thaliana*, *Petunia hybrida*, tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) (Haecker et al., 2004). The ancient clade mainly includes land plants and green algae, while the intermediate and modern clades comprise ferns and seed plants (Lian et al., 2014; Yang et al., 2017). Previous studies have shown that specified WOX genes participate in seed plant root development and reestablishment. WOX4 promotes the differentiation and maintenance of the vascular procambium in *A. thaliana* (Ji et al., 2010), and controls

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Abbreviations

CDS	coding sequence
EdU	5-ethynyl-2′-deoxyuridine
IBA	indole-3-butyric acid
ISH	*in situ* hybridization
PBS(T)	phosphate-buffered saline (with 0.1% Triton X-100)
PIN	PIN-FORMED
qRT-PCR	quantitative real-time PCR
QC	quiescent centre
SCs	Stem cells
SCNs	stem cell niches
SCR	SCARECROW
SHR	SHORT ROOT
SRA	sequence read archive
RACE	rapid amplification of cDNA ends
RAM	root apical meristem
RNA-seq	RNA sequencing
WOX	WUSCHEL-related homeobox

cell division activity in the vascular cambium and secondary growth in *Populus* (Kucukoglu et al., 2017). WOX5 is expressed in the root quiescent centre (QC) and defines the root SCN (Kong et al., 2015; Sarkar et al., 2007), whereas WOX11 controls root hair development and branching (Cheng et al., 2016; Jiang et al., 2017; Sheng et al., 2017), and may directly respond to wound-induced AUXIN in and around the procambium to modulate adventitious root formation in poplar (Liu et al., 2014; Zhao et al., 2009). WOX13 is dynamically expressed during primary and lateral root initiation and development in *A. thaliana* (Deveaux et al., 2008). Although the roles of WOXs have been well-studied in *A. thaliana* and rice, little is known about their function in herbal plants.

To investigate the mechanisms underlying the longevity of ginseng, we analysed the sequences of genes encoding SC master regulators. Five WOX members were isolated and characterised from the root of *P. ginseng* using rapid amplification of cDNA ends (RACE) technology, which named as *PgWOX4*, *PgWOX5*, *PgWOX11*, *PgWOX13a* and *PgWOX13b*, respectively. Whole-mount *in situ* hybridization (*ISH*) was used to characterize the 3D gene expression pattern of *PgWOXs*. We found that *PgWOX4* was associated with the maintenance of vascular meristem organisation during secondary growth. *PgWOX5* and *PgWOX11* were mainly expressed in tips of seedling and adventitious roots; and *PgWOX13a* and *PgWOX13b* were highly expressed in the parenchyma cells of the main root. This is the first report of SC regulation in medical herbs and we expect that our findings could provide a novel insight into the improvement of the yield and quality of the medicinal materials including herbal shapes, external features, length, and bioactive compounds.

2. Materials and methods

2.1. Tissue culture and plant materials

Adventitious roots derived from the callus of 10-year-old mountain ginseng (*P. ginseng*) roots were cultured in MS medium supplemented with 3.0 mg L^{-1} indole-3-butyric acid (IBA) and 3% sucrose at 25 °C ± 1 °C in the dark. Adventitious roots were isolated from explants and subcultured in 1/2 MS liquid medium containing 3.0 mg L^{-1} IBA at 25 °C ± 2 °C in the dark on a rotary shaker (100 rpm). One generation was subcultured every 3 weeks, and 12 weeks later, stably and rapidly growing adventitious roots were used as experimental materials.

Two-year-old ginseng seedlings were mainly supplied by the Kangmei Pharmaceutical Company planting base and were identified as *P. ginseng* C. A. Meyer by Prof. Luqi Huang (China Academy of Chinese Medical Sciences), which were used in whole-mount *ISH*. Additionally, 2-, 4-, 6-, 8-, and 10-year-old cultivated ginseng samples were collected from Fu-Song in August 2016, which are belong to the landrace COMMON ginseng from population FS (Li et al., 2015). Then, the main roots of these samples were divided and immediately frozen in liquid nitrogen and stored at −80 °C, which were used as samples in qRT-PCR.

2.2. Total RNA isolation and cloning of WOX family genes in ginseng

P. ginseng root was collected from three individual samples. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentration and purity were evaluated by spectrophotometry at 260 and 280 nm.

We searched *A. thaliana WOX* cDNA sequences from the National Centre for Biotechnology Information (NCBI) database and *P. ginseng WOX* unigenes from RNA sequencing (RNA-seq) data (BioProject: PRJNA302556). After alignment using the ClustalW (v.1.83) program (Thompson et al., 1994), conserved sequences were used to design degenerate primers to amplify *P. ginseng WOX* genes (Table S1). First-strand cDNA was reverse-transcribed from 5 μg total RNA with an oligo $(dT)_{18}$ primer (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. The amplified PCR product was purified and cloned into a pMD19-T vector (Takara Bio, Dalian, China) for Sanger sequencing. The core sequence was used to design and synthesise 3′- and 5′-RACE primers (Table S2). The 3′- and 5′-ready cDNA were synthesised by reverse-transcribing 1 μg total RNA with 3′- and 5′-CDS primers, respectively, using the BD SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The 3′- and 5′-RACE PCR products were also purified and cloned into the pMD-19T vector, followed by sequencing. After aligning and assembling the sequences of the core fragment, and 3′- and 5′-RACE sequences, the full-length cDNA sequence of *PgWOX* was deduced and obtained by RT-PCR using open reading frame primers (Table S3).

2.3. Domain, conserved motifs and phylogenetic analysis

P. ginseng WOX4, WOX5, WOX11, WOX13a and WOX13b domains were identified using the Pfam database (http://pfam.sanger.ac.uk/). We used MEME online software to identify shared motifs among WOX protein sequences, and domain conservation was predicted using Pfam online software. Conserved motifs were analysed online using a MEME system with set parameters (minimum width = 6 amino acids, maximum width = 200 amino acids, maximum number of motifs = 20) (http://meme.nbcr.net/meme/cgi-bin/meme.cgi).

For the phylogenetic analysis of PgWOX family members, full-length amino acid sequences for *A. thaliana*, *Brassica rapa*, *Camelina sativa*, *Glycine max*, *Nicotiana sylvestris*, *Theobroma cacao*, *Vigna angularis*, and *Vitis vinifera* WOX were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/protein). Multiple sequence alignments were carried out with ClustalW for the Simple Modular Architecture Research Tool-defined WOX homeodomain sequences. All positions containing gaps were eliminated. Phylogenetic reconstructions for different protein family members were inferred by the neighbour-joining method in the P-distance model with 1000 bootstrap replications.

2.4. Transcriptome data analysis and gene expression heatmap

Raw RNA-seq data were downloaded from the NCBI sequence read archive (SRA) library (BioProject: PRJNA302556). Transcripts were assembled using Trinity v.2.5.0 and quantified as transcripts per million using RSEM (Li and Dewey, 2011). *WOX* expression was obtained from total expression data. Heatmap was generated using imageGP (www.ehbio.com/ImageGP).

2.5. qRT-PCR

Frozen tissue samples (100 mg) were ground into a fine powder in liquid nitrogen and total RNA was extracted using TRIzol reagent. The RNA was dissolved in 50 µl RNase-free Tris-EDTA (10 mM Tris, 1 mM EDTA [pH 8.0]) and stored at −80 °C. RNA concentration and purity were assessed by spectrophotometry at 260 and 280 nm, and 1 µg was reverse transcribed at 42 °C using TransScript Reverse Transcriptase (TransBionovo Co., Beijing, China) and oligo(dT)$_{18}$ primer. The cDNA was diluted 1:5 with H_2O prior to use in qRT-PCR.

The qPCR reactions were performed in duplicate for each condition using the KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Charlestown, MA, USA) on a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). Each 20 µl reaction contained 1 µl cDNA and 200 nM of each primer (Table S2). The cycling conditions were 1 cycle at 95 °C for 3 min, followed by 45 two-segment cycles of amplification (95 °C for 10 s and 60 °C for 30 s) where the fluorescence was automatically measured during PCR, and one three-segment cycle of product melting (95 °C for 5 s, 65 °C for 1 min, and 95 °C for 30 s). The

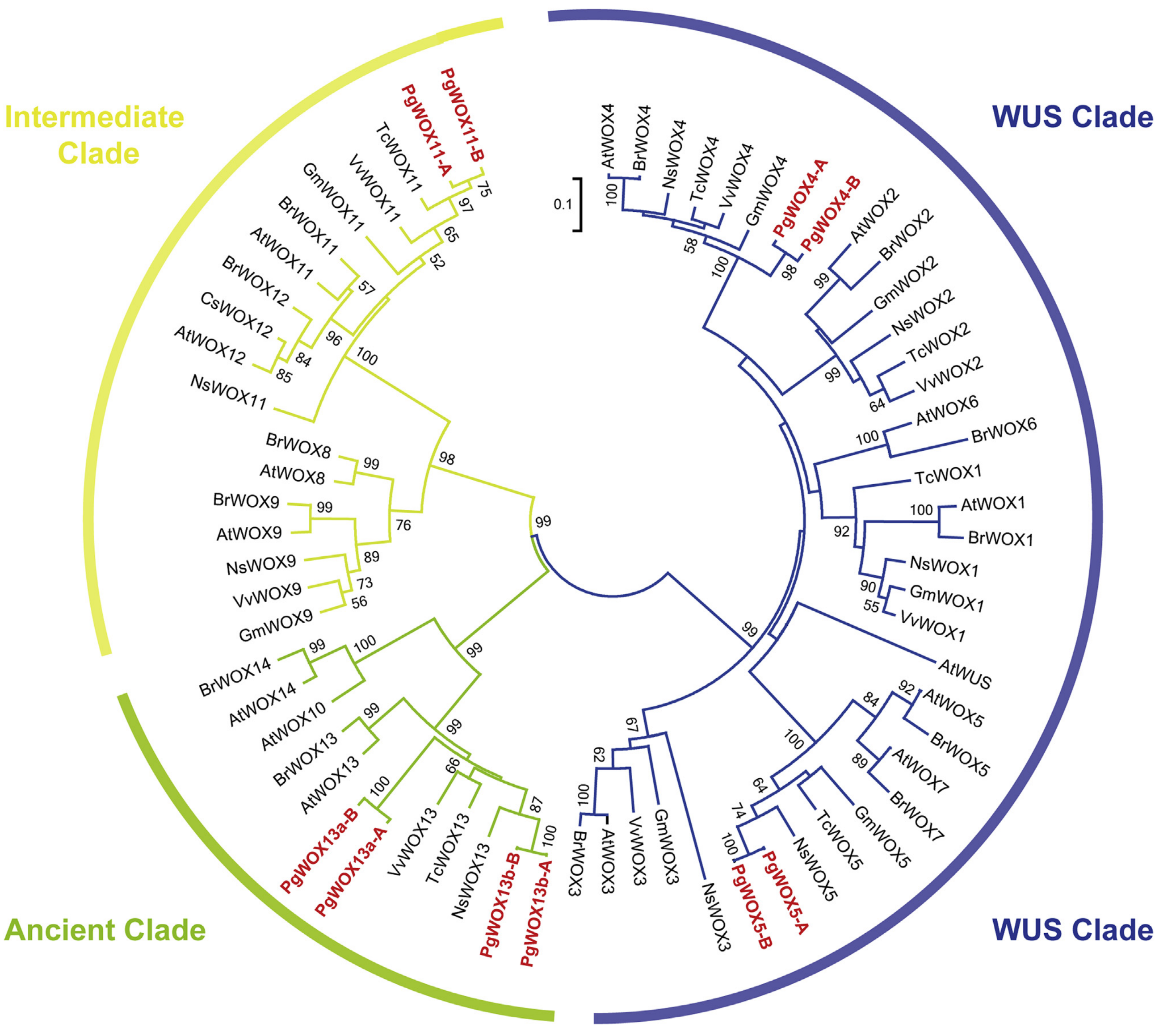

Fig. 1. Phylogenetic tree of WOXs in plants.
AtWUS, NP_565429.1; AtWOX1, AEE76034.1; AtWOX2, AED97173.1; AtWOX3, AEC08149.1; AtWOX4, AEE32127.1; AtWOX5, AEE75021.1; AtWOX6, Q9ZVF5.2; AtWOX7, AED90922.1; AtWOX8, AED95323.1; AtWOX9, AEC08902.1; AtWOX10, Q9LM83.1; AtWOX11, AEE73969.1; AtWOX12, AED92473.1; AtWOX13, AEE86529.1; AtWOX14, AEE30012.1; BrWOX1, XP_009135563.1; BrWOX2, XP_009120441.1; BrWOX3, XP_009140922.1; BrWXO4, XP_009107441.1; BrWOX5, XP_009107441.1; BrWOX6, XP_009118131.1; BrWOX7, XP_009122174.1; BrWOX8, XP_009128835.1; BrWOX9, XP_009132964.1; BrWOX11, XP_009102066.1; BrWOX12, XP_009121050.1; BrWOX13, XP_009138373.1; BrWOX14, XP_009149550.1; CsWOX12, XP_010492747.1; GmWOX1, XP_003530958.1; GmWOX2, XP_003540730.2; GmWOX3, XP_006602580.1; GmWOX4, XP_006601279.1; GmWOX5, XP_003552848.1; GmWOX9, XP_006583838.1; GmWOX11; XP_003553354.1; NsWOX1, XP_009804164.1; NsWOX2, XP_009758504.1; NsWOX3, XP_009793790.1; NsWOX4, XP_009776871.1; NsWOX5, XP_009783476.1; NsWOX9, XP_009793301.1; NsWOX11, XP_009797137.1; NsWOX13, XP_009801335.1; TcWOX1, EOY00990.1; TcWOX2, EOY34076.1; TcWOX4, EOY14402.1; TcWOX5, EOY17892.1; TcWOX11, EOY10442.1; TcWOX13, EOX90654.1; VaWOX8, XP_017435801.1; VvWOX1, XP_010663679.1; VvWOX2, XP_002281161.1; VvWOX3, XP_002281707.1; VvWOX4, XP_002284927.1; VvWOX9, XP_002273188.1; VvWOX11, XP_002269282.3; VvWOX13, XP_002279942.1.At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Cs, *Camelina sativa*; Gm, *Glycine max*; Ns, *Nicotiana sylvestris*; Tc, *Theobroma cacao*; Va, *Vigna angularis*; Vv, *Vitis vinifera*.

baseline adjustment method of the LightCycler 480 software was used to determine the cycle threshold (Ct) of each reaction. *Eukaryotic translation initiation factor 3G1* (*IF3G1*) served as internal control (Wang and Lu, 2016) and the expression levels of target genes were analysed with a relative quantification approach based on the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

2.6. Whole-mount RNA ISH and ISH

Plant materials were fixed overnight at 4 °C in 4% paraformadehyde in a solution composed of 1 × phosphate-buffered saline (PBS), 3% Nonidet P-40, 10% dimethylsulfoxide, 0.1% Tween-20, and 0.1% diethyl pyrocarbonate. After washing twice in PBS containing 0.1% Triton X-100 (PBST) for 20 min with rotation at room temperature, the samples were dehydrated in a graded series of 20%, 40%, 60%, 80% and 100% methanol in PBST with rocking at room temperature. The tissues was then dehydrated through a graded series of ethanol (methanol/ethanol, 80%/20%, 60%/40%, 40%/60%, and 20%/80%, followed by 100% ethanol), a xylene series (xylene/ethanol, 25%/75%, 50%/50%, and 75%/25%, followed by 100% xylene), and another ethanol series (ethanol/xylene, 25%/75%, 50%/50%, and 75%/25%), and were finally stored in 100% ethanol.

Whole-mount RNA *ISH* and normal *ISH* were respectively conducted as previously described (Hejátko et al., 2006; Kulikova et al., 2018) using digoxigenin-labeled probes. A linear fragment of the target genes was generated by PCR amplification using primers whose binding sites flanked the gene insert and retained RNA polymerase binding sites in the amplified region (Table S3). The similarity of antisense probes was predicted (Table S4). Digoxigenin-labeled antisense and sense RNA probes were synthesised in vitro using SP6 and T7 RNA polymerase (Roche), respectively. Probe hybridization and immunological detection were perfored according to the method described by García-Aguilar et al. (2005) and Theodosiou (2013). The hybridised probes were detected using anti-digoxigenin-alkaline phosphatase antibody and then visualised with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. Signals were observed and photographed under a light microscope (AX10; Carl Zeiss, Oberkochen, Germany).

2.7. EdU assay and microscopy

The EdU assay was used for *in situ* detection of cell proliferation to evaluate the activity of plant SCs. For the EdU assay, the ginseng adventitious root was incubated for 24 h in 10 μM EdU solution. The medium was then removed and 1 ml of 3.7% formaldehyde in PBS was added to each sample, followed by incubation for 15 min at room temperature. The samples were washed twice with 3% bovine serum albumin in PBS. After removing the wash solution, 0.5% Triton X-100 in PBS was added to each sample, followed by incubation at room temperature for 20 min. EdU detection and DNA staining were performed using the Click-iT EdU Imaging kits (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Images were acquired with two-photon laser-scanning confocal microscope (LSM880; Carl Zeiss) with a 20 × water immersion lens. Click-iT EdU cells were detected with Alexa Fluor 594 dye at excitation and emission wavelengths of 590 nm 615 nm, respectively; Hoechst 33342 dye bound to DNA was detected at wavelengths of 350 and 461 nm, respectively.

2.8. Data analysis

Experiment results were the mean of three biological repeats. The data were subjected to analyze using analysis of variance (ANOVA) to assess the significant differences between different years of cultivated ginseng samples using Duncan's multiple range tests ($p \leq 0.05$). Also, the values are expressed as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Identification and phylogenetic analysis of WOX genes in P. ginseng root

We analysed the transcriptome of 13 tissues from *P. ginseng* in the NCBI SRA library, including fruit flesh, fruit pedicel, fruit peduncle, leaf blade, leaflet pedicel, leaf peduncle, stem, rhizome, arm root, phloem and periderm in the main root, xylem in the main root, leg root, and fibre root (SRA: SRR2952867-SRR2953879). We used WOXs from *A. thaliana* as queries to search ginseng transcriptome databases (rhizome, arm root, phloem and periderm of the main root, xylem of the main root, leg root, and fibre root) using the BLASTp program; hits with an e-values of 1e-5 were considered significant. Based on our RNA-seq data, we identified five genes predicted to encode a homeodomain similar to that of the WOX family proteins. The cDNA of CDS sequences of the five *PgWOX* genes were further confirmed by rapid amplification of cDNA ends (RACE) and reverse transcription-polymerase chain reaction (RT-PCR) using total RNA from the root of ginseng. The above cloned *PgWOX* genes were registered in GenBank (Accession No. MK644616, MK644617, MK644618, MK644619, MK644620, MK644621, MK644622, MK644623, MK644624, MK644625). Thereafter, PSOSITE (http://prosite.expasy.org/) and InterProscan 56.0 (http://www.ebi.ac.uk/interpro/) were used to search for the Homeobox (HOX) domain in the obtained sequences and were confirmed (Fig. S1, Table S5).

The phylogenetic tree was carried out based on multiple sequence alignments for the above cloned ginseng WOX protein sequences and

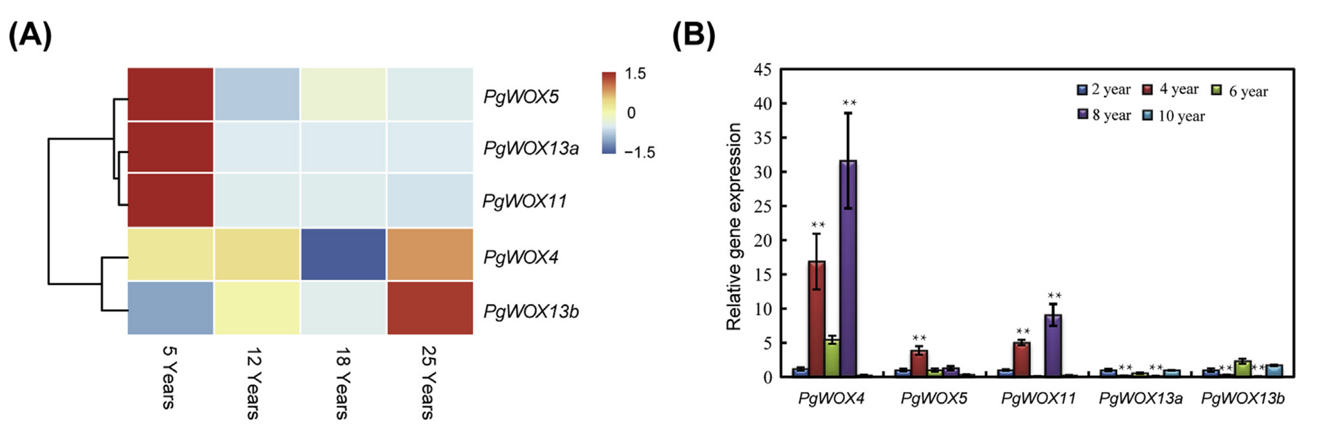

Fig. 2. Gene expression pattern of PgWOXs in different growing year of ginseng main roots.
(A) Heatmap of *PgWOXs* expression in ginseng main roots after 5, 12, 18, 25 growing years. (B) Expression of *PgWOXs* in ginseng main roots after 2, 4, 6, 8, and 10 growing years. ** indicated above the bar represent statistically significant difference at $p \leq 0.01$ (Duncan's multiple range test).

the full-length WOX protein sequences of *A. thaliana*, *B. rapa*, *C. sativa*, *G. max*, *N. sylvestris*, *T. cacao*, *V. angularis*, and *V. vinifera* (Fig. 1). According to evolutionary relatedness, we named the five candidate *PgWOX* genes as *PgWOX4*, *PgWOX5*, *PgWOX11*, *PgWOX13a*, *PgWOX13b*, respectively, and all of these *PgWOXs* included two types (A and B) for the Single Nucleotide Polymorphism (SNP) (Figs. S2–S6). As show in Fig. 1, PgWOXs were divided into three clades: PgWOX4-A, PgWOX4-B, PgWOX5-A, and PgWOX5-B belonged to the WUS clade; PgWOX11-A and PgWOX11-B to the intermediate clade; and PgWOX13a-A, PgWOX13a-B, PgWOX13b-A, and PgWOX13b-B to the ancient clade (Fig. 1). The evolution of WOX genes in plants from ancient to intermediate to *WUS* clades has been accompanied by the emergence of SCs (Ge et al., 2016). Green algae and mosses only harbour WOXs of the ancient clade; moss PpWOX13Ls plays a key role in apical SC formation and regeneration (Mukherjee et al., 2009). Our data demonstrate that ginseng has at least two different *WOX13* isoforms. Since ginseng is a tetraploid plant, the *WOX* family is diverse and is likely to have rapidly evolved, and could therefore be a useful tool for studying SC functions in ginseng.

3.2. Temporal expression pattern of PgWOXs

To investigate *PgWOX* temporal expression patterns, *PgWOX4*, *PgWOX5*, *PgWOX11*, *PgWOX13a*, and *PgWOX13b* unigenes in transcriptome database were firstly characterised in different year old *P. ginseng* roots (Fig. 2A). Most *PgWOXs* specifically expressed within the first 12 years of growth, except for *PgWOX13b* (Fig. 2A). For most cultured ginseng were under 10 year growth period, we then characterised the expression of *PgWOXs* in 2-, 4-, 6-, 8- and 10-year-old cultured ginseng roots by qRT-PCR (Fig. 2B) and found that *PgWOX4*, *PgWOX5*, and *PgWOX11* showed the opposite trend to *PgWOX13a* and *PgWOX13b*, suggesting that these genes have inter-correlated expression (Fig. 2). In *A. thaliana*, *WOX11/12* activate *WOX5/7* and *LBD16*, which initiate the division of the root founder cells and switch them into root primordium cells (Hu and Xu, 2016). Our data showed that the expression patterns of *PgWOX5* and *PgWOX11* were similar, which suggested that they might also interact with each other in *P. ginseng*. *WOX13* gene regulates fruit patterning in *A. thaliana* (Romera-Branchat et al., 2013), and plays important roles in RAM maintenance in *Arachis hypogaea* (Wang et al., 2015). Our data also demonstrated that the expression pattern of both *PgWOX13* genes in different year of *P. ginseng* root was opposite with that of *PgWOX4* and *PgWOX11*, which showed that *PgWOX13* may be correlated with the expression of *PgWOX4* and *PgWOX11*.

3.3. Whole-mount in situ hybridization (ISH) and normal ISH analysis of PgWOXs localisation in ginseng seedlings

Elucidating the functions of *PgWOXs* in ginseng growth requires the determination of mRNA localisation patterns. Although whole-mount *ISH* has been successfully implemented in *A. thaliana* and maize (Hejátko et al., 2006; Torres et al., 1995), there are no reports of its

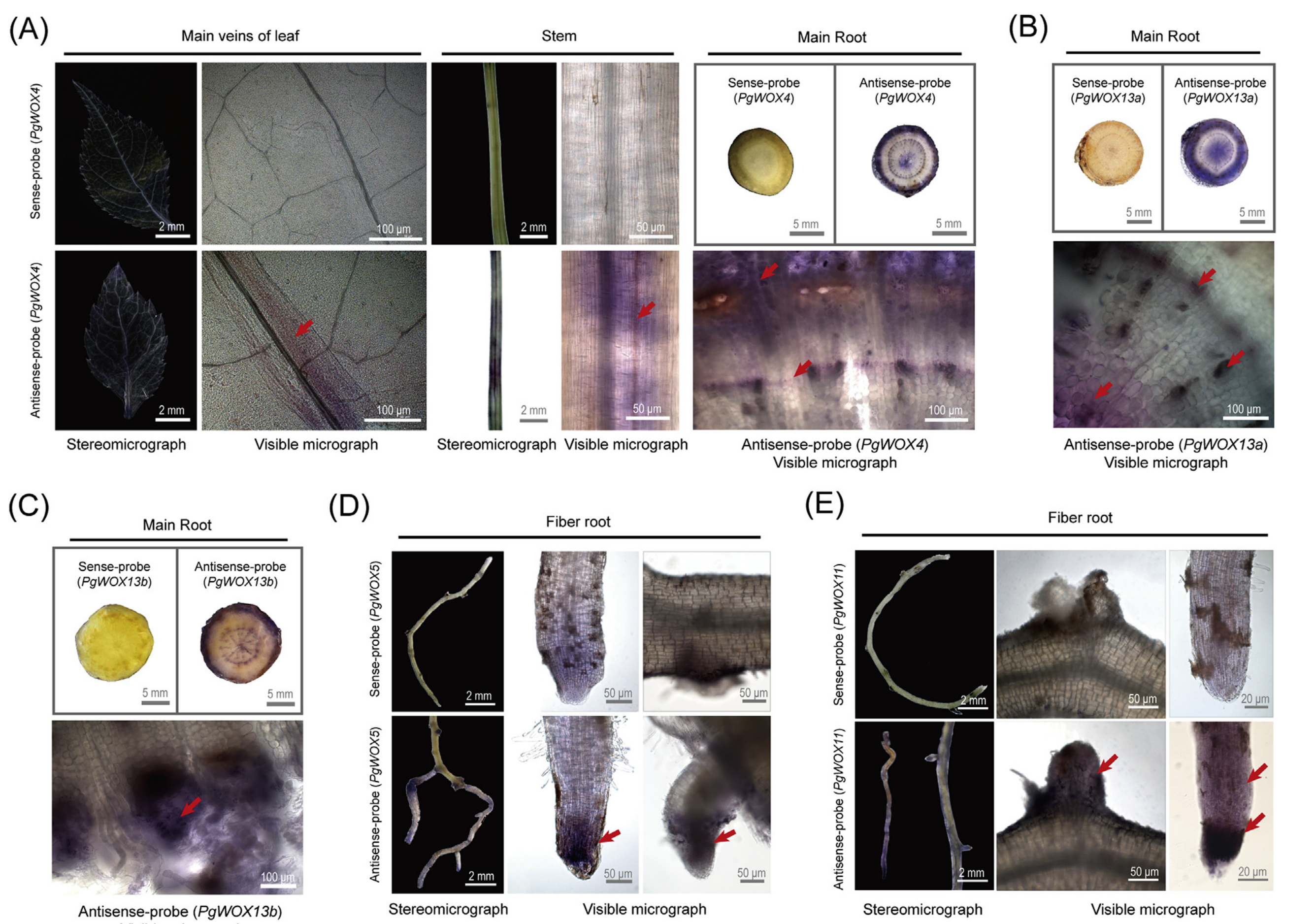

Fig. 3. Visualisation of 3D *PgWOX* expression in ginseng seedling by whole-mount RNA ISH.
(A) *PgWOX4* expression in leaf, stem, and main root of ginseng. (B) *PgWOX13a* expression in the main root of ginseng. (C) *PgWOX13b* expression in the main root of ginseng. (D) Expression of *PgWOX5* in fiber root of ginseng. (E) Expression of *PgWOX11* in fiber root of ginseng. The red arrows in (A–E) panels showed the cells stained by *ISH*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

application in medical plants. To identify the location of ginseng SCs and the role of *PgWOXs*, we performed whole-mount *ISH* and normal *ISH* in seedlings.

In *A. thaliana*, WOX4 regulates vascular SC proliferation in the development of vasculature in multiple tissues and is involved in meristem maintenance and cambium activity (Hirakawa et al., 2010; Ji et al., 2010; Ohmori et al., 2013). *ISH* analyses of ginseng seedling revealed *PgWOX4* expression in the developing vascular traces of the leaf, stem and main root (Fig. 3A). *PgWOX4* was strongly expressed in phloem ray, cortical parenchymal, and cambium cells of root, all of which have the re-differentiation potential in ginseng root (Fig. 3A). In contrast to *WOX4*, *WOX13* is dynamically expressed during primary and lateral root initiation and development, and affects the floral transition in *A. thaliana* (Deveaux et al., 2008). To clarify the functional differences between *PgWOX13a* and *PgWOX13b*, we visualised the transcripts by ISH. *PgWOX13a* was detected in xylem ray, wood-parenchymatous, cambium, and cortex parenchyma cells of the main root (Fig. 3B), implying that it is related to the development of this tissue. In contrast, *PgWOX13b* was highly expressed in the cortex, especially in parenchymal cells around resin ducts (Fig. 3C). In *A. thaliana*, the first step in the transition from regeneration-competent cells to root founder cells is activation of WOX11 expression by auxin, which directly activates WOX5 to induce root primordium initiation and organogenesis (Hu and Xu, 2016). *PgWOX5* and *PgWOX11* were both highly expressed in the fibre root tip(Fig. 3D and E). *PgWOX5* was specifically expressed in the root apical meristem (RAM) and primordium during adventitious rooting, while *PgWOX11* was mainly stained in the exodermis (Fig. S7). The fibre and adventitious roots are critical for root organogenesis during regeneration, thus, *PgWOX5/PgWOX11* could serve as meristem markers in these tissues.

3.4. SC characteristics of cultured ginseng adventitious roots

In vitro cultivation of plant cells, tissues and organs is one of the most powerful tools available for plant development research, production of valuable phytocompounds, and preservation of endangered plant species. The cultured adventitious roots of ginseng have a high transparency, and may therefore be an ideal model for studying plant SCs. To investigate the characteristics of ginseng SCs, we established adventitious root cultures.

The roots were divided into a 1 cm tips, branching part and brownish yellow old root to investigate the expression of different marker genes in root SCs (Fig. 4A). In addition to *PgWOXs*, other root SC marker genes, such as *SCARECROW* (*SCR*), *SHORT ROOT* (*SHR*), and *PIN-FORMED* (*PIN*) were detected (Fig. 4B). In *A. thaliana*, *WOX5* would be defined as a root QC marker (Sarkar et al., 2007); *SCR* is expressed in the QC, SCs in ground tissue (cortex and endodermis), and the endodermis (Di Laurenzio et al., 1996); and *SHR* is expressed in the stele, with the protein moving to the QC and surrounding cells to activate *SCR* expression along with *WOX5* for coordinated regulation of QC identity, thereby maintaining balance between root SC division and differentiation (Kong et al., 2015). *PIN* genes encode an AUXIN transport facilitator that contributes to inter- and intracellular AUXIN transport, while *PIN4* is usually expressed in the QC and plays a critical

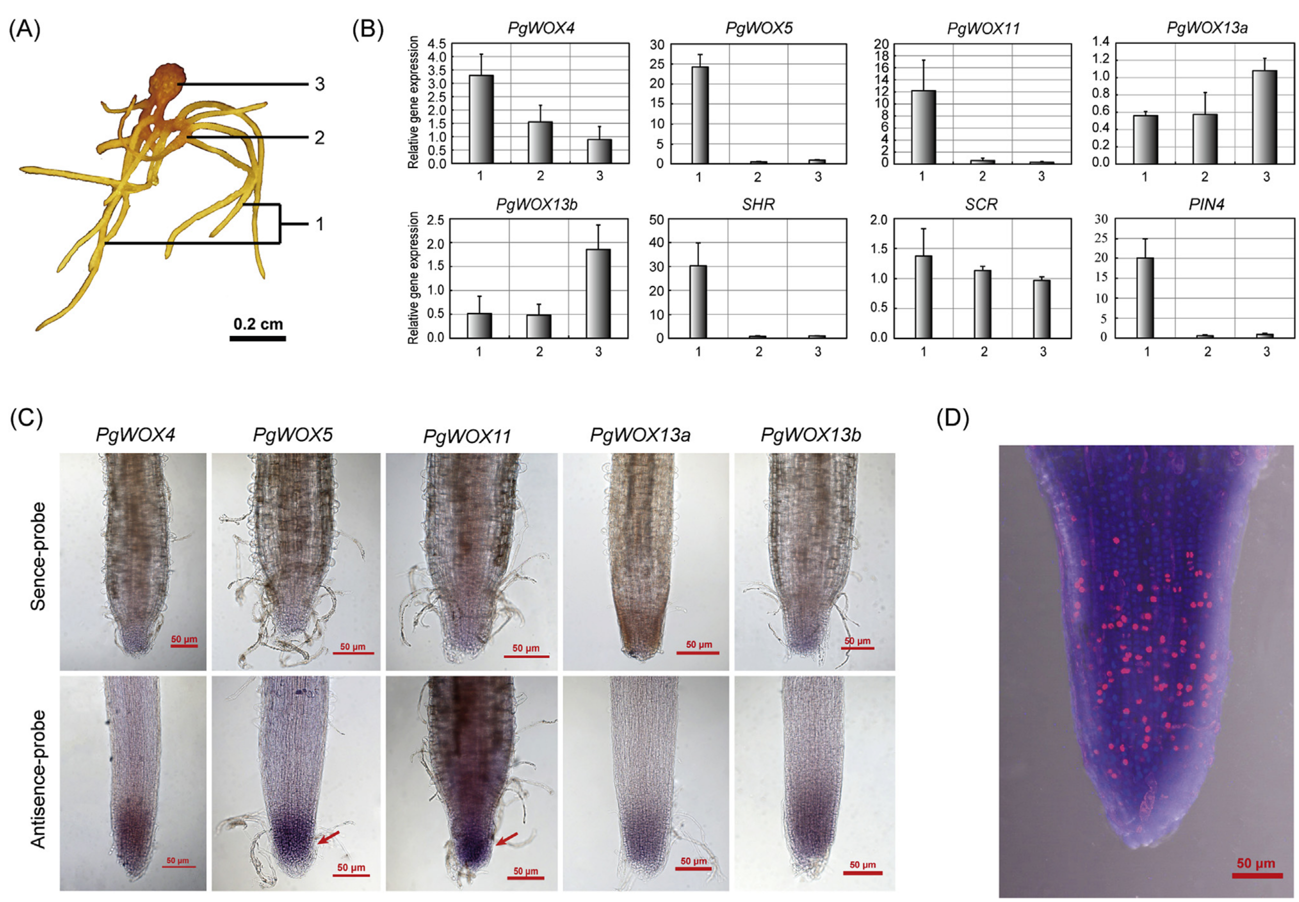

Fig. 4. Characteristics of SCs in cultured adventitious roots of ginseng.
(A) Different parts of adventitious roots of ginseng. The three regions of adventitious roots were root tip (1), root branch (2), old root with yellow color (3). (B) Gene expression related to plant root SCs. (C) Expression of five *PgWOXs* in root tips of cultured adventitious roots of ginseng. The red arrows showed the cells stained by *ISH*. (D) EdU assay for detection of proliferation cells in root tips of cultured adventitious roots of ginseng; labeled cells were detected by laser scanning confocal microscopy. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

role in root meristem maintenance (Moubayidin et al., 2010). *A. thaliana* mutants of *SHR* and *SCR* revealed that the SHR/SCR pathway regulates QC identity and SCN activity (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003). *PgWOX5*, *PgWOX11*, *SHR*, and *PIN4* were expressed over 10 fold higher in the 1 cm root tip than the basal levels, as determinate by qRT-PCR (Fig. 4B). Previous reports reveal that auxin activates the expression of *WOX11/12* in *A. thaliana*, and reprograms the regeneration-initial cells into the root founder cells (Liu and Xu, 2018; Sang et al., 2018)., IBA, one type of auxins, was used during the ginseng adventitious root culturing, which could significantly stimulate the expression of *PgWOX11* in adventitious root tip of *P. ginseng*. Additionally, *WOX11/12* could activate *WOX5/7* and *LBD16* in *A. thaliana*, which initiate the division of the root founder cells (Liu and Xu, 2018; Sang et al., 2018). Our data also suggested that *PgWOX5* might be activated by *PgWOX11*, for both of these genes were highly expressed in cultured adventitious root tips.

Since the cultured adventitious roots were highly transparent, the expression of SC-related genes (*PgWOX4*, *PgWOX5*, *PgWOX11*, *PgWOX13a*, and *PgWOX13b*) could be visualised by whole-mount *ISH* (Fig. 4C; Fig. S8). *PgWOX5* and *PgWOX11* were mainly detected in the tip of the adventitious root (Fig. 4C), with *PgWOX5* also accumulated in the lateral root primordium (Fig. S8). *PgWOX4* was concentrated in the vascular cambium and lateral root branching parenchyma cells, and both *PgWOX13a* and *PgWOX13b* were expressed in parenchyma cells around the vasculature of cultured adventitious roots (Fig. S8).

SC activity was assessed by measuring cell proliferation in the growing cone of cultured adventitious roots with the 5-ethynyl-2′-deoxyuridine (EdU) assay over 24 h (Kotogány et al., 2010). EdU incorporation in the adventitious root tip revealed an area with a short cell cycle length; these cells were located in the root tip meristem and appeared to have a high metabolic rate (Fig. 4D). This region overlapped the *PgWOX5* and *PgWOX11* expression area, suggesting that these genes are markers of SC activity in the adventitious root tip of *P. ginseng*.

4. Conclusions

Remarkable advances in the knowledge of plant regeneration have been made in the last years (Perez-Garcia and Moreno-Risueno, 2018), but it is still unknown any regenerative mechanisms in any herbal plants. Chinese ginseng (*P. ginseng*) is commonly used in Chinese medicine. This herbal plant, which is native to northeastern Asia, has a long growth period. The plant roots demonstrate a strong capacity of regeneration, likely due to the SC population. WOX family proteins, which share a conserved homeodomain, play key roles in SC regulation and could contribute to the regrowth of damaged or missing plant organs in ginseng.

To investigate the function of the five cloned *PgWOX* genes, we analysed their sequences, as well as their expression patterns, relative to cell proliferation patterns. The *WOX11/12* subclade was reported to be involved in adventitious rooting in *A. thaliana*, rice (Oryza sativa), and poplar (*Populus deltoids* × *Populus euramericana*) (Liu et al., 2014; Zhao et al., 2009; Xu et al., 2015). In *P. ginseng*, *PgWOX11* was mainly expressed in the fibre root and root tip of adventitious root, which suggested that *PgWOX11* might be also involved in adventitious rooting process. *WOX5* is expressed in the QC to regulate the columella stem cell identity in *A. thaliana* (Kong et al., 2015), and *PtoWOX5a* is involved in adventitious root development in poplar (Li et al., 2018). *PgWOX5* was expressed in the RAM of fibre and adventitious root, suggesting that both genes regulated SCs in growing root tips in *P. ginseng*. Expression pattern of *PgWOX5* was consistent with that of *PgWOX11*, suggesting that both genes were involved in the root development, but their regulatory mechanism in *P. ginseng* might be more complex. The conserved *WOX4* function is to promote differentiation and maintenance of the vascular procambium in *A. thaliana* (Ji et al., 2010), and PttWOX4 could regulate cambial cell division activity and secondary growth in *Populus* trees (Kucukoglu et al., 2017). *PgWOX4* is likely critical for the regulation of vascular SC proliferation, since it was detected in the vasculature of multiple tissues. *Arabidopsis WOX13* promotes the replum formation during fruit development (Romera-Branchat et al., 2013), while *AhWOX13* plays important roles in RAM maintenance (Wang et al., 2015). *PgWOX13a* and *PgWOX13b* was expressed in the parenchyma cells of the main root of ginseng seedlings and cultured adventitious roots, implying that they are important for maintaining the balance between SC differentiation and self-renewal in the phloem and xylem. Expression analyses of genes related to plant SCs in cultured adventitious root revealed that *PgWOX5*, *PgWOX11*, *SHR*, and *PIN4* were expressed in root tip, which was the high metabolic region of adventitious root meristem as well as the highest accumulation of EdU-positive cells. Interestingly, ginsenosides are mainly distributed in the periderm and the tip region of the *P. ginseng* root as compared to the central region (Taira and Ikeda, 2010), which suggests that ginsenosides might influence the RAM regulation network. In conclusion, this work is firstly reported *WOX* genes regulated SCs in herbal plants, and we believe that our work will facilitate to analyze the formation of the herbal morphological features and the development of a new research direction in medical herbs.

Declaration of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Juan Liu: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft. **Chao Jiang:** Data curation, Formal analysis, Methodology, Writing - original draft. **Tong Chen:** Data curation, Software. **Liangping Zha:** Formal analysis, Validation. **Jie Zhang:** Visualization. **Luqi Huang:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

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