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A *Rosa canina* WUSCHEL-related homeobox gene, *RcWOX1*, is involved in auxin-induced rhizoid formation

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Abstract Homeobox (HB) proteins are important transcription factors that regulate the developmental decisions of eukaryotes. WUSCHEL-related homeobox (WOX) transcription factors, known as a plant-specific HB family, play a key role in plant developmental processes. Our previous work has indicated that rhizoids are induced by auxin in rose (*Rosa* spp.), which acts as critical part of an efficient plant regeneration system. However, the function of *WOX* genes in auxin-induced rhizoid formation remains unclear. Here, we isolated and characterized a WUSCHEL-related homeobox gene from *Rosa canina*, *RcWOX1*, containing a typical homeodomain with 65 amino acid residues. Real-time reverse transcription PCR (qRT-PCR) analysis revealed that *RcWOX1* was expressed in the whole process of callus formation and in the early stage of rhizoid formation. Moreover, its expression was induced by auxin treatment. In *Arabidopsis* transgenic lines expressing the *RcWOX1pro::GUS* and *35S::GFP-RcWOX1*, RcWOX1 was specifically expressed in roots and localized to the nucleus. Overexpression of *RcWOX1* in *Arabidopsis* increased lateral root density and induced upregulation of *PIN1* and *PIN7* genes. Therefore, we postulated that RcWOX1 is a functional transcription factor that plays an essential role in auxin-induced rhizoid formation.

Keywords WUSCHEL-related homeobox (WOX) transcription factors · Rhizoids · *Rosa canina* · *RcWOX1*

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Introduction

Efficient vegetative propagation methods in plants include: planting stock, spore propagation and tissue culture (George et al. 2008). Plant tissue culture generally refers to the aseptic rapid culture of cells, tissues and organs under certain physical and chemical conditions in vitro (Elhiti and Stasolla 2011; Thorpe 1990). Therefore, tissue culture is a powerful tool in basic and applied researches as well as in commercial application. Plant cells used in tissue culture require the property of cell totipotency (Gautheret 2003; Vasil and Hildebrandt 1965). Cell totipotentiality is the ability of a cell to produce a new genetically identical cell and ultimately tissues, organs and complete individuals through cell division and differentiation processes (García-Gonzáles et al. 2010).

Homeobox (HB) transcription factors represent a large group of transcriptional factors, which is characterized by the presence of a conserved DNA-binding homeodomain (HD) folded into by a short stretch of 60–66 amino acids residues (Gehring et al. 1990, 1994; van der Graaff et al. 2009). HB transcription factors have also been identified in plants, and play multiple roles in plant growth and developmental processes. The plant HB superfamily can be divided into six families based on the phylogenetic relationship and domain structures, including homeodomain associated with a leucine zipper (HD-Zip), plant homeodomain associated with a finger domain (PHD finger), Bell, zinc finger

associated with a homeodomain (ZF-HD), WUSCHEL-related homeobox (WOX) and knotted-related homeobox (KNOX) (Ariel et al. 2007; Chan et al. 1998).

WOX family members are involved in many key developmental processes through their activities in promoting cell division and/or preventing premature cell differentiation in plants (van der Graaff et al. 2009; Yadav et al. 2010). Besides the homeodomain, the typically conserved structure of WOX proteins is the WUS-box motif (T-L-X-L-F-P-X-X, where X represents any amino acid) (Haecker et al. 2004), which distinguishes them from other HB protein families. Plant WOX proteins have been classified into three clades based on the phylogenetic analysis: WUS, intermediate and ancient clades (Lin et al. 2013; Nardmann et al. 2009). In *Arabidopsis*, the members of the WUS clade include WUS and WOX1-7 (Zhang et al. 2010), containing the two-amino-acid motif (T-L) at the beginning of the WUS-box motif (van der Graaff et al. 2009).

So far, the analysis of *WOX* gene expression and function shows that WOX family members play a wide variety of roles in plant development, such as embryonic patterning (Haecker et al. 2004), stem-cell maintenance (Breuninger et al. 2008) and stress response (Muehe et al. 2014). *WOX2* and *WOX6* genes were shown to be required for embryonic patterning during embryo development in *Arabidopsis* (Park et al. 2005; Wu et al. 2007). In *Picea abies*, *WOX2* expression is also tightly associated with somatic embryogenesis (Palovaara and Hakman 2008; 2009). In *Arabidopsis* and *Antirrhinum majus*, loss of *WUS* function promotes stem cells differentiation (Kieffer et al. 2006; Mayer et al. 1998). Root columella stem cells, which normally produce the gravity-sensing root cap cells, also undergo differentiation in the *Arabidopsis wox5* loss-of-function mutant (Kamiya et al. 2003). Interestingly, *WOX6* is also found to be involved in the response to cold stress in *Arabidopsis* (Zhu et al. 2004). However, the role of *WOX* genes in organogenesis during regeneration in *Rosa canina* remains uncharacterized.

In our previous work, we established a highly efficient regeneration system through organogenesis and somatic embryogenesis from cultured leaf explants of *R. canina* (Tian et al. 2008). Auxin was identified as a trigger of rhizoid formation, while cytokinin only modulates the process (Gao et al. 2013). However, the molecular mechanism of auxin-induced rhizoid formation is still unclear. Previous studies indicate that WOX family play an essential role in stem cell maintenance and lateral root formation (Breuninger et al. 2008; Liu et al. 2014; Tian et al. 2014), so they are potential key regulators during rhizoid formation. Here, we identified *RcWOX1*, which encoded a member of WOX family and was up-regulated rapidly and significantly by auxin. We further showed the lateral root initiation rate were remarkably increased in *RcWOX1* overexpression transgenic lines.

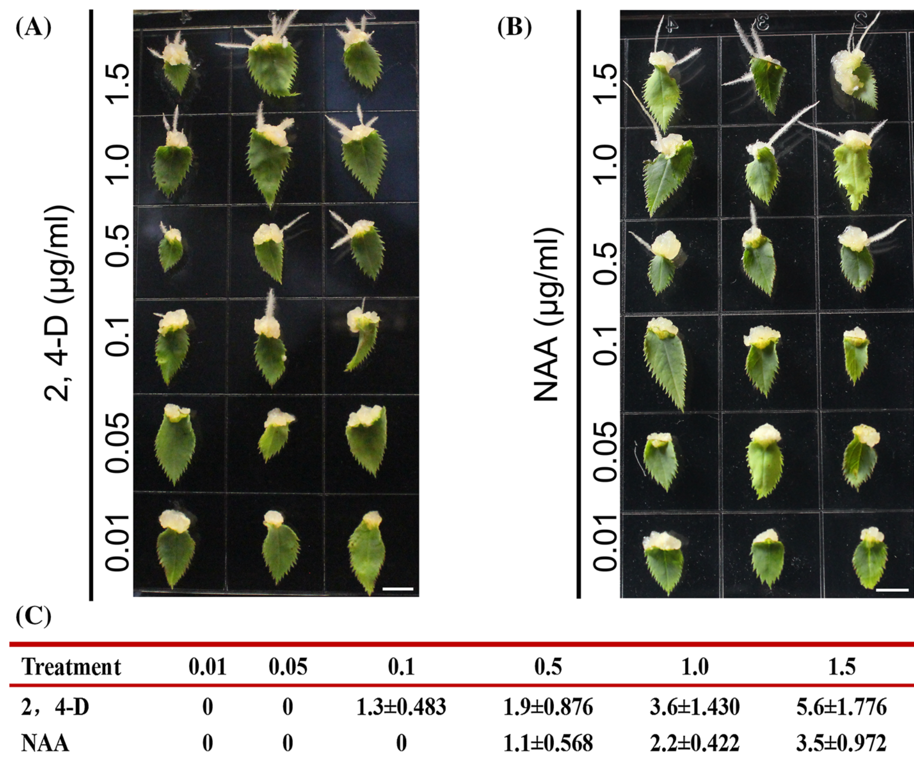
Results

Role of auxin in rhizoid formation

A new and efficient plant regeneration system based on the root-like organs named rhizoids has been achieved in several rose cultivars. Tian demonstrated that 2,4-D (1.5–3 μg/ml) is necessary for rhizoid formation (Tian et al. 2008). In order to study the role of auxin in detail during rhizoid initiation in *R. canina*, leaf explants were incubated on medium containing different concentrations of 2,4-D or NAA. We found that rhizoids can be induced by 0.1 μg/ml 2,4-D, while only callus could be distinguished at lower concentrations. However, rhizoids were not induced in the presence of 0.1 μg/ml NAA (Fig. S1). Further treatment of explants with different concentrations of NAA to identify the NAA induction threshold concentration revealed that 0.5 μg/ml NAA is sufficient to initiate rhizoid formation (Fig. 1a, b). Moreover, statistical analysis showed that rhizoid outputs induced by 2,4-D were higher than those by NAA at the same doses. Rhizoid induction with 1.5 μg/ml 2,4-D was 37.5 % higher than that with equivalent doses of NAA (Fig. 1c). The results suggest that a certain auxin concentration is required for rhizoid induction.

Isolation of *RcWOX1*

Previous study showed that the formation of rhizoids appears to be the result of callus cell dedifferentiation. A number of WOX family transcription factors are known critical regulators of apical meristem cell division and differentiation (Haecker et al. 2004). WOX5 has been demonstrated to be mainly expressed in the root and plays an essential role in root apical meristem maintenance. To identify the potential *WOX* genes involved in rhizoid formation, we aligned the *WOX5* gene from different species, including *Arabidopsis thaliana*, *Theobroma cacao, Pisum sativum* and *Raphanus sativus* and designed the degenerate primers in the conserved region (Fig. S2) and cloned the full-length cDNA of *WOX* gene from *R. canina* using RACE method. The full-length cDNA (accession number KJ699116) of *RcWOX1* is 692 bp. The coding sequence encodes 186 amino acids containing a typical HD with 65 residues, a WUS box motif (T-L-Q-L-F–P-I–N) and an ERF-associated amphiphilic repression (EAR) motif (L-D-L-R-L) (Fig. 2a). Phylogenetic and domain structure comparisons of RcWOX1 with *Arabidopsis* WOX protein members further revealed that RcWOX1 belongs to the WUS clade of the WOX protein family, with higher sequence similarity to WOX5 than other members of the WOX family in *Arabidopsis* (Fig. 2a). RcWOX1 has 66.13 % sequence identity to TcWOX5, 60.57 % to PsWOX5 and 56.04 % to AtWOX5 (Fig. 2b).



Treatment	0.01	0.05	0.1	0.5	1.0	1.5
2，4-D	0	0	1.3±0.483	1.9±0.876	3.6±1.430	5.6±1.776
NAA	0	0	0	1.1±0.568	2.2±0.422	3.5±0.972

Fig. 1 Effect of auxin on callus-rhizoid induction from leaf explants in *R. canina*. **a** Callus-rhizoid formation when grown on medium containing different concentrations of 2,4-D. **b** Callus-rhizoid formation when grown on medium containing different concentrations of NAA. *Scale bar* 5 mm in (**a, b**). **c** Statistical analysis of the number of rhizoids after 4 weeks of 2,4-D and NAA treatments. Rhizoid numbers were surveyed from 15 calluses. Values are presented as mean ± SD

Expression dynamics of *RcWOX1* during rhizoid induction

Given the high sequence similarity of RcWOX1 to WOX5 from other species, we further investigated whether RcWOX1 is involved in rhizoid formation. Rhizoid formation occurred one month after callus initiation upon cultivation on medium containing auxin. We first examined the *RcWOX1* expression dynamics within the first month after callus initiation. As shown in Fig. 3a, the expression level of *RcWOX1* gradually increased during the first 3 weeks after induction as compared with the control, and then slowly decreased (Fig. 3a), suggesting significant correlation with rhizoid formation. To determine whether auxin affects *RcWOX1* expression within a narrower time scale, 2-week callus were incubated in MS liquid medium containing 0.5 µg/ml 2,4-D for 0, 4, 8, 16 and 24 h. Our results showed that *RcWOX1* expression was significantly increased from 4 h after auxin treatment compared with the control (Fig. 3b). Given that auxin is required for rhizoid induction and the *RcWOX1* level is correlated with the rhizoid formation process, we speculate that auxin induces rhizoid formation of *R. canina* at least partially by regulating *RcWOX1* expression.

Root-specific expressed RcWOX1 is localized to nuclear

The efficient rose regeneration system is mainly divided into five stages: leaf explants, callus, rhizoids, protocorm-like bodies (PLBs) and regenerated seedlings. For analysis of the tissue expression patterns of *RcWOX1*, RT-PCR was performed to examine levels of the transcript in root, stem, leaf, seedlings, callus and rhizoid. Highest *RcWOX1* expression was evident in roots, rhizoids, callus and seedlings, but not the other tissues tested (Fig. 4a).

To further investigate the expression patterns of *RcWOX1* in plants, we cloned a 1,257 bp genomic fragment upstream of the gene and analyzed its *cis*-elements using PLACE (Higo et al. 1999). Among these, an auxin- and several cytokinin-responsive elements were located at positions −1,230/−1,225, −656/−652, −611/−607, −206/−202 and −147/−143. We additionally identified a HD-Zip (TAATGATTA) motif at positions −150/−142 (Fig. S3). Next, a *RcWOX1* promoter::β-glucuronidase (GUS) recombinant plasmid was constructed and transformed into *Arabidopsis* plants via an *Agrobacterium*-mediated method. As shown in Fig. S4, overall GUS staining revealed specific expression of *RcWOX1* in roots. Further detailed analysis disclosed strong GUS activity in the primary root tip (Fig. 4b), root hair zone (Fig. 4c) and lateral root tip (Fig. 4d) in transgenic plants, which was consistent with RT-PCR findings in Fig. 4a. To ascertain the subcellular localization of RcWOX1, the ORF was translationally fused to the C-terminus of the GFP reporter under control of the CaMV 35S promoter and transformed into *Arabidopsis*. The GFP signal was localized in the nuclei of

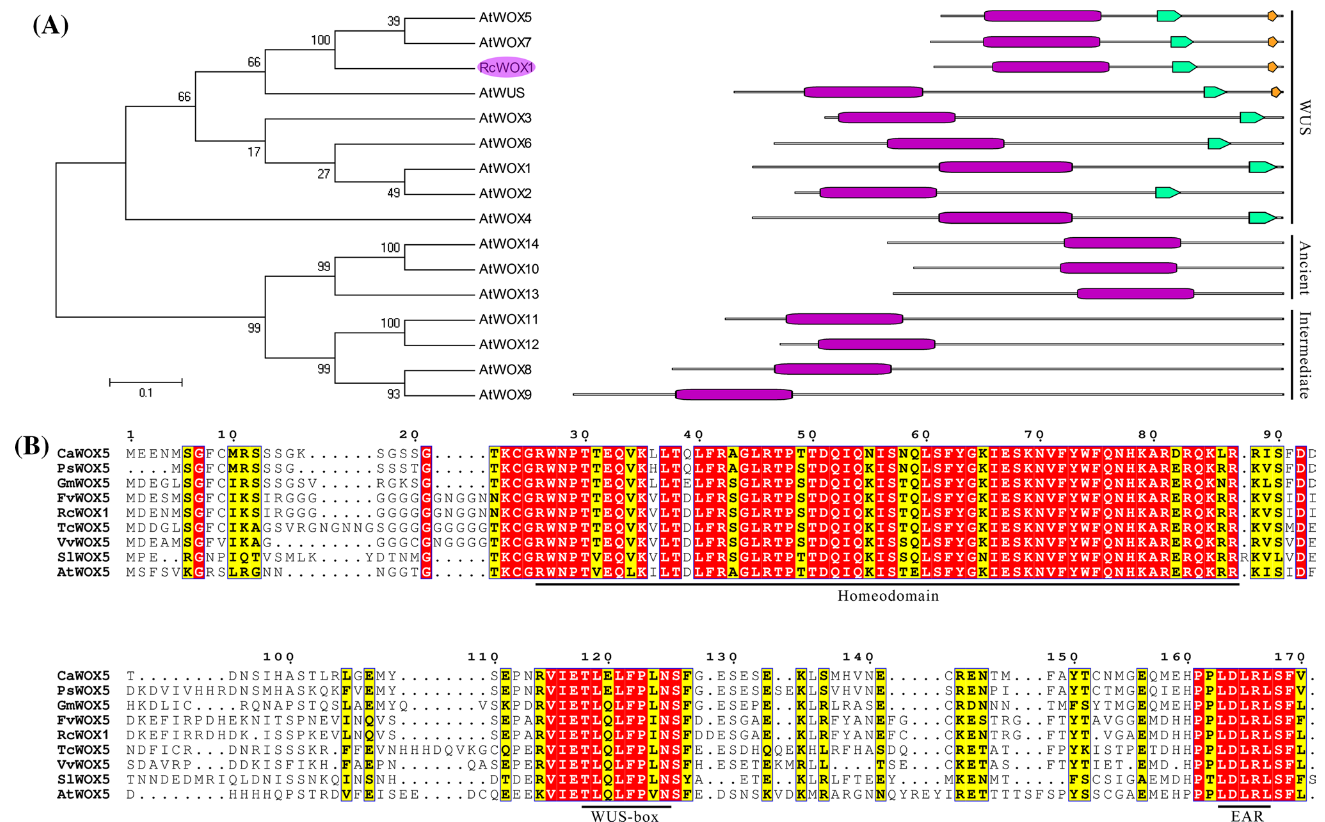


Fig. 2 Phylogenetic analysis of RcWOX1. **a** Phylogenetic relationships and schematic domain structures of RcWOX1 and *Arabidopsis* WOX proteins. *Purple block* represents HD domain; *Cyan block* represents WUS-box; *Orange block* represents EAR-like domain. **b** Sequence alignment of *R. canina* (RcWOX1) with its orthologues in *Cicer arietinum* (CaWOX5), *Fragaria vesca* (FvWOX5), *Glycine max* (GmWOX5), *P. sativum* (PsWOX5), *Solanum lycopersicum* (SlWOX5), *T. cacao* (TcWOX5), *Vitis vinifera* (VvWOX5) and *A. thaliana* (AtWOX5). The positions of the conserved HD, WUS-box and EAR-like domain are indicated by *black lines*

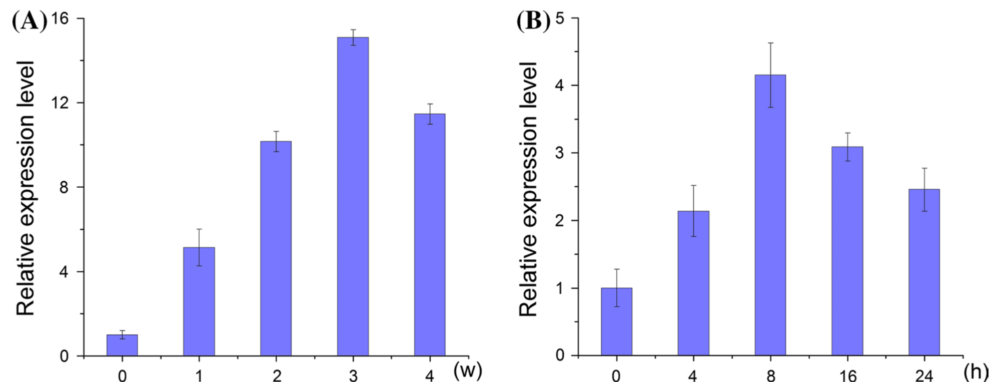


Fig. 3 Quantitative real-time PCR showing *RcWOX1* expression. **a** qRT-PCR analysis of *RcWOX1* expression during rhizoid formation 0, 1, 2, 3 and 4 weeks after callus initiation. **b** qRT-PCR analysis of *RcWOX1* expression in callus treated with 0.5 μg/ml 2,4-D for various time-periods

Arabidopsis root cells, clearly suggesting that RcWOX1 is a nuclear transcription factor (Fig. 4e).

Effect of ectopic expression of *35S::RcWOX1* on *Arabidopsis* root development

Gene expression data suggested that *RcWOX1* might be involved in root organogenesis. Accordingly, we further analyzed the phenotypes of *RcWOX1* overexpression in transgenic *Arabidopsis* plants. Two lines of transgenic plants with high *RcWOX1* expression were selected using RT-PCR for subsequent phenotypic analysis (Fig. 5a). Five days after germination, we observed no obvious differences between *RcWOX1*-overexpressing and wild-type lines (Fig. 5b). However, ten days after germination, the lateral root number of the OW7 and OW12 transgenic

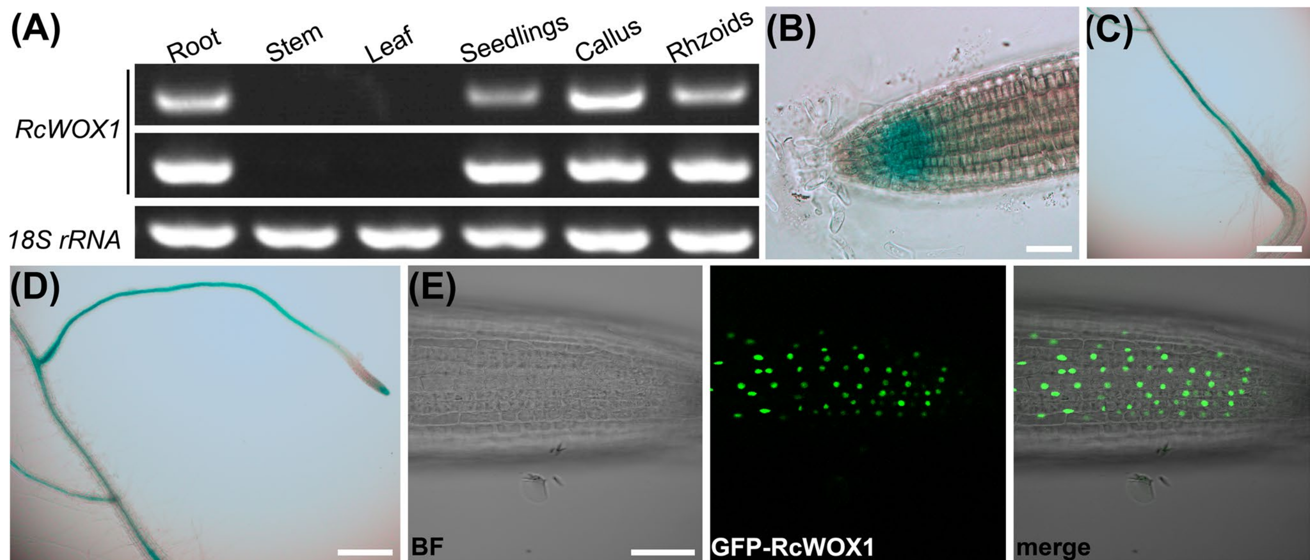


Fig. 4 Tissue expression patterns and subcellular localization of RcWOX1. **a** Analysis of the *RcWOX1* transcript in different organs using RT-PCR. *Top* target sequence was amplified by 25 cycles of semi-quantitative PCR. *Center* target sequence was amplified by 30 cycles of semi-quantitative PCR. *Bottom TUBULIN* (*TUB*) transcripts were detected as controls. (**b**–**d**) Histochemical localization of GUS activity in the 8-day-old *RcWOX1pro::GUS* transgenic *Arabidopsis* seedlings, primary root tip (**b**), root hair zone (**c**) and lateral root (**d**). **e** Subcellular localization of RcWOX1 in *Arabidopsis* root tip cells. *Scale bar* 50 µm in (**b**, **e**) and 500 µm in (**c**, **d**)

plants increased by 46 and 39.3 % as compared with the wild type, respectively (Fig. 5c; Fig. S5), indicating that *RcWOX1* significantly promotes organogenesis of lateral roots.

Expression of auxin-related genes in *RcWOX1* transgenic plants

The analysis of the promoter *cis*-elements of *RcWOX1* revealed that this gene may involve in auxin signal transduction. Moreover, *PIN1* expression was reduced in the embryos of the *wox8-1wox9-1* double mutant (Breuninger et al. 2008). To check whether auxin signal transduction is affected in the *RcWOX1* transgenic *Arabidopsis* plants, we analyzed the expression of the auxin efflux carrier genes, *PIN1*, *2*, *3*, *4* and *7*, which is involved in root development (Blilou et al. 2005), in 1-week-old seedlings using qRT-PCR. We found that *PIN1* and *PIN7* genes were slightly up-regulated in *35S::RcWOX1* transgenic seedlings as compared with the control group (Fig. 6). Based on these results, we propose that *RcWOX1* participates in lateral root organogenesis via regulating the auxin-signaling pathway.

Discussion

It has been established that WOX protein family is one member of plant HB transcription factor superfamily (van der Graaff et al. 2009) and is involved in various aspects of plant development. In the present study, we isolated the *RcWOX1* gene from *R. canina* and characterized its expression patterns during rhizoid formation. We also found that overexpression of *RcWOX1* had a significant effect on the formation of lateral root. Collectively, our results suggested that *RcWOX1* plays an essential role in rhizoid formation, a root-like structure, from leaf explants of *R. canina*.

Auxin is one of the earliest discovered plant growth promoting hormones (Davies 2010) that mainly regulates embryogenesis (Müller and Sheen 2008; Nolan et al. 2003), vascular differentiation (Aloni et al. 2006), organogenesis (Cheng et al. 2007; Pernisová et al. 2009) and tropic growth (Esmon et al. 2006; Takahashi et al. 2009). In *R. canina*, threshold concentrations of 2,4-D and NAA required to induce rhizoid formation were determined as 0.1 and 0.5 µg/ml, respectively, in the present study. Below the threshold, only callus could be distinguished and any organogenic response was not detected, while both callus and rhizoids were induced above these concentrations. This finding is consistent with data from a previous study on *Arabidopsis* which reported that induction of new root-like organs requires a specific concentration of auxin (Pernisová et al. 2009). Moreover, at the same dose levels, outputs induced by 2,4-D were significantly higher than that of NAA. Taken together, these results suggest that auxin is essential for the formation of callus and rhizoids, which is a prerequisite for the development of PLB.

A previous study showed that *WOX5* is specifically expressed in quiescent center cells and loss of *WOX5*

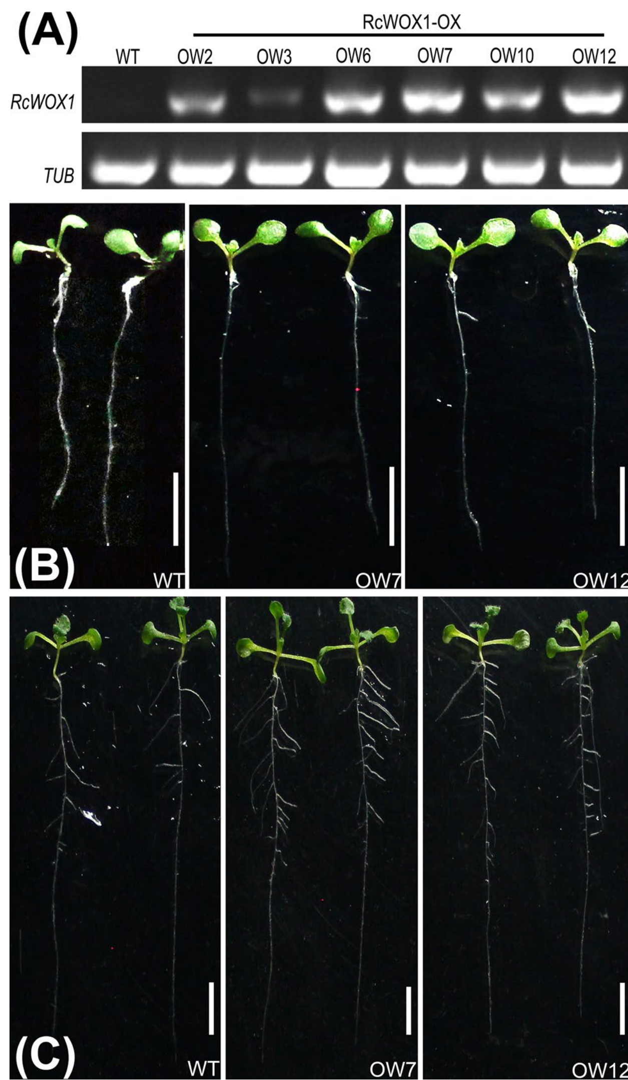


Fig. 5 Overexpression of *RcWOX1* in *Arabidopsis*. **a** Analysis of *RcWOX1* transcripts using RT-PCR in six transgenic lines. *TUBULIN* (*TUB*) was used as an internal control. **b** Comparison of 5-day-old seedlings of the wild type (WT) with the transgenic plants (OW7 and OW12). **c** Comparison of 10-day-old seedlings of the wild type (WT) with the transgenic plants (OW7 and OW12). *Scale bar* 5 mm in (**b**, **c**)

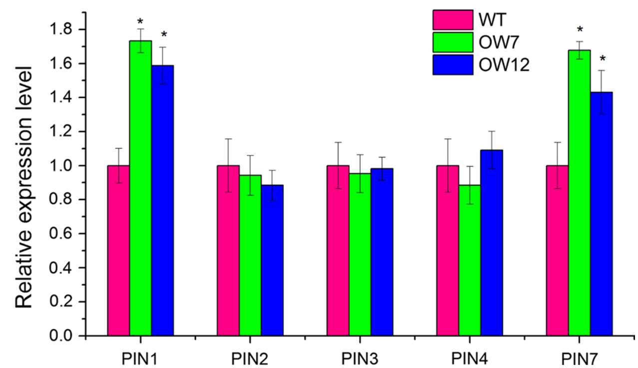


Fig. 6 Expression analysis of auxin efflux carrier genes in the *RcWOX1*-OX *Arabidopsis*. qRT-PCR analysis of *PIN1*, *2*, *3*, *4* and *7* genes in the wild-type and transgenic plants. *Ubiquitin* (*UBQ*) was used as an internal control. *Asterisks* indicate statistically significant differences (Student's *t* test; $P < 0.01$) between wide type and RcWOX1-OX. Values are presented as mean ± SD

function leads to terminal differentiation in distal stem cells, supporting an essential role of this gene in regulation of root stem cell differentiation and lateral root initiation (Sarkar et al. 2007). Our sequence analysis showed that RcWOX1 has high sequence similarity with WOX5. Moreover, qRT-PCR analysis revealed that *RcWOX1* is expressed predominantly at the rhizoid initiation stage, with high transcript abundance at the beginning of induction followed by a decrease during auxin-induced rhizoid formation. The induction effect of auxin on *RcWOX1* expression was also evident within a shorter time frame of 0–24 h of treatment. These data indicate that auxin-induced expression of *RcWOX1* is a possible prerequisite for initiation of rhizoid formation.

In *Arabidopsis*, *WOX* genes are involved in the regulation of *PIN1* and *DR5rev:GFP* expression during embryo development, indicating that WOX transcriptional machinery is essential for the establishment of localized auxin response (Breuninger et al. 2008). Thus, we speculated that *RcWOX1* plays a role in the regulation of auxin signal transduction. Our results showed altered expression of polar auxin transport genes, *PIN1* and *PIN7,* in *RcWOX1* overexpression transgenic plants. Moreover, analysis of the effect of *RcWOX1* overexpression on *Arabidopsis* lateral root formation revealed that lateral root densities are significantly increased in *RcWOX1*-overexpressing lines as compared with the control. Taken together, these data presented here suggested that *RcWOX1* is likely an important player in auxin-induced rhizoid formation in *R. canina.*

Rose is one of the most popular cut flowers,which has broad markets and high sales (Zlesak 2006). Due to dependence on the genotype, induction of somatic embryogenesis and regeneration in vitro are difficult, which seriously restricts commercial production and the international trade of roses. Therefore, establishment of an efficient regeneration system is a prerequisite for rose molecular breeding. Studies on the mechanism of regeneration will provide a theoretical basis for rapid propagation of rose in vitro. In this study, we found that *RcWOX1* appears to play a pivotal role in auxin-induced rhizoid formation, which is a critical step for *R. canina* regeneration through PLB. Our results reveal new insights into the molecular mechanism of *R. canina* regeneration system and provide a theoretical basis for efficient regeneration in vitro, which might have potential application in rapid propagation and engineering of rose.

Materials and methods

Plant materials

Stem segments from 1-year-old shoots of *R. canina* were harvested from the field and surface-sterilized. Single node segments were germinated in shoot multiplication medium (SMM) according to the protocol of Tian et al. (2008). Leaf explants from 6-week-old plantlets were used for all analyses in this study.

Auxin treatments

For long-term auxin treatment, leaf explants were cultured on rhizoid formation medium containing 0.01, 0.05, 0.1, 0.5, 1.0 and 1.5 μg/ml 2,4-D or NAA for 4 weeks. For short-term auxin treatment, callus cultured in the dark for 2 weeks were incubated in half-strength MS liquid medium supplemented with 0.5 μg/ml 2,4-D for 0, 4, 8, 16 and 24 h.

Cloning of the *RcWOX1* gene

Total RNA of rhizoids was extracted using the RN09-EASY spin Kit (Biomed, China). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, China). Degenerate primers P_for (5′-AAGTGYGGKCGDTGGAAYCCAAC-3′) and P_rev (5′-CCARTAGAAVACRTTCTTGCTC-3′) were designed for *RcWOX1* cloning based on the conserved regions of *WOX5* genes from *A. thaliana*, *T. cacao, P. sativum* and *R. sativus*. The amplification product was used to design 3′ RACE primers 3′-race_for (5′-TACTGAGCAGGTCAAAGTTTTGACG-3′) and 3′-race_rev (5′-GGCCACGCGTCGACTAGTAC-3′). The 5′ fragment was obtained using the Genome Walking Kit (Takara, Japan). The full-length of *RcWOX1* was amplified using PrimeSTAR HS DNA Polymerase (Takara, Japan) and cloned into the pEASY-Blunt Simple Cloning Vector (Trans, China) for sequencing. Sequence alignments were generated using ClustalW and ESPript program. Phylogenetic analysis was performed using the MEGA4 program. Accession numbers for the public sequences used in the phylogenetic analysis are listed in Table S1.

Quantitative real-time PCR

Total RNAs were extracted and 2 μl cDNAs were used for quantitative real-time PCR analysis on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) using SYBR® Premix Ex Taq™ II (Sangon Biotech, China). The PCR procedure was as follows: denature at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C, 30 s at 58 °C. The primers used for this quantitative real-time PCR were RcWOX1_for (5′-ACTTACACTGTTGGAGCAGAAATGG-3′) and RcWOX1_rev (5′-ACAAGTTTCATTTCTCTCCCCCTAC-3′); PIN1_for (5′-TACTCCGAGACCTTCCAACTACG-3′) and PIN1_rev (5′-TCCACCGCCACCACTTCC-3′); PIN2_for (5′-GGCGAAGAAAGCAGGAAGA-3′) and PIN2_rev (5′-GGTGGGTACGACGGAACA-3′); PIN3_for (5′-GAGGGAGAAGGAAGAAAGGGAAC-3′) and PIN3_rev (5′-CTTGGCTTGTAATGTTGGCATCAG-3′); PIN4_for (5′-CTGAACGATGGCTATACGGAGAAG-3′) and PIN4_rev (5′-CTTGGCTTGTAATGTTGGCATCAG-3′); PIN7_for (5′-CGGCTGATATTGATAATGGTGTGG-3′) and PIN7_rev (5′-GCAATGCAGCTTGAACAATGG-3′); *18S rRNA*_for (5′-ACTTACACTGTTGGAGCAGAAATGG-3′) and *18S rRNA*_ rev (5′- ACAAGTTTCATTTCTCTCCCCCTAC-3′); *UBQ10*_for (5′-CACACTCCACTTGGTCTTGCGT-3′) and *UBQ10*_rev (5′-TGGTCTTTCCGGTGAGAGTCTTCA-3′) were used as internal controls for normalization. The expression levels were analyzed using the relative $2^{-\Delta\Delta CT}$ method. All experiments were performed on three biological replicates.

Promoter analysis

Genomic DNA from leaf explants was isolated using the Plant Genomic DNA Purification Kit (Tiangen Biotech). The promoter sequence of *RcWOX1* was obtained using specific primers SP2 (5′-CATGATCTCGACGTATGAACTCCTTG-3′) and SP3 (5′-ACCACAAGGACATGGGAGTAAGCC-3′) coupled with primers (AP1–AP4) provided by the Genome Walking Kit. The promoter fragment was amplified using the specific primers *Hind*III_for (5′-GTAAAGCTTAGCCACATGACATAACAAACTCC-3′) and *Xba*I_rev (5′-CGCTCTAGATTCAGCTTCAAATGCCTAGAAAG-3′), and inserted into pBI121-GUS to produce the RcWOX1pro::GUS expression vector, which was transformed into *Arabidopsis* using the floral-dip method.

For RcWOX1pro::GUS activity analysis, the 6-day-old seedlings were incubated with GUS staining solution at 37 °C overnight and decolorized using 70 % ethanol. The GUS signal was visualized under a Zeiss LSM 510 META microscope.

Subcellular localization

The open reading frame (ORF) of *RcWOX1* was amplified with primers *Pst*I_for (5′-GTAGGATCCATGGATGAAAATATGTCAGGGTT-3′) and *BamH*I_rev (5′-CGCCTGCAGTCAAAGGAAGCTTAAGCGTAGATC-3′). After digestion with *BamH*I and *Pst*I, the fragment was inserted into pCambia 2300-GFP to obtain the pCambia 2300-GFP-RcWOX1 expression vector for *Arabidopsis* transformation. GFP fluorescence was visualized using confocal microscopy. Images were processed using ImageJ software and Adobe Photoshop CS3.

Phenotypic analysis

For lateral root density measurements, *Arabidopsis* seedlings were grown vertically on 1/2 MS medium. Seedlings were photographed 5 and 10 days after germination, respectively. For 10-day-old seedlings, the number of lateral roots was counted and subjected to statistical analysis using Origin8.0 software.

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References

Aloni R, Aloni E, Langhans M, Ullrich C (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot 97:883–893

Ariel FD, Manavella PA, Dezar CA, Chan RL (2007) The true story of the HD-Zip family. Trends Plant Sci 12:419–426

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433:39–44

Breuninger H, Rikirsch E, Hermann M, Ueda M, Laux T (2008) Differential expression of *WOX* genes mediates apical-basal axis formation in the *Arabidopsis* embryo. Dev Cell 14:867–876

Chan RL, Gago GM, Palena CM, Gonzalez DH (1998) Homeoboxes in plant development. BBA-Gene Struct Expr 1442:1–19

Cheng Y, Qin G, Dai X, Zhao Y (2007) NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in *Arabidopsis*. Proc Natl Acad Sci USA 104:18825–18829

Davies PJ (2010) The plant hormones: their nature, occurrence, and functions. In: Plant hormones. Springer, Netherlands, pp 1–12

Elhiti M, Stasolla C (2011) The use of zygotic embryos as explants for *in vitro* propagation: an overview. In Plant embryo culture. HumanaPress, pp 229–255

Esmon CA, Tinsley AG, Ljung K, Sandberg G, Hearne LB, Liscum E (2006) A gradient of auxin and auxin-dependent transcription precedes tropic growth responses. Proc Natl Acad Sci USA 103:236–241

Gao B, Fan L, Li X, Yang H, Liu F, Wang L, Xi L, Ma N, Zhao L (2013) *RcRR1*, a *Rosa canina* type-A response regulator gene, is involved in cytokinin-modulated rhizoid organogenesis. PLoS One 8:e72914

García-Gonzáles R, Quiroz K, Carrasco B, Caligari P (2010) Plant tissue culture: current status, opportunities and challenges. Cienc Investig Agrar 37:5–30

Gautheret RJ (2003) Plant tissue culture: the history. Plant tissue culture. Springer, Vienna, pp 105–113

Gehring W, Müller M, Affolter M, Percival-Smith A, Billeter M, Qian Y, Otting G, Wüthrich K (1990) The structure of the homeodomain and its functional implications. Trends Genet 6:325–328

Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D, Affolter M, Otting G, Wuthrich K (1994) Homeodomain-DNA recognition. Cell 78:211–224

George EF, Hall MA, De Klerk G-J (2008) Plant propagation by tissue culture: the background, vol 1. Springer, pp 1–28

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 131:657–668

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27:297–300

Kamiya N, Nagasaki H, Morikami A, Sato Y, Matsuoka M (2003) Isolation and characterization of a rice *WUSCHEL*-type homeobox gene that is specifically expressed in the central cells of a quiescent center in the root apical meristem. Plant J 35:429–441

Kieffer M, Stern Y, Cook H, Clerici E, Maulbetsch C, Laux T, Davies B (2006) Analysis of the transcription factor WUSCHEL and its functional homologue in *Antirrhinum* reveals a potential mechanism for their roles in meristem maintenance. Plant Cell 18:560–573

Kubo M, Kakimoto T (2000) The *CYTOKININ-HYPERSENSITIVE* genes of *Arabidopsis* negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development. Plant J 23:385–394

Lin H, Niu L, McHale NA, Ohme-Takagi M, Mysore KS, Tadege M (2013) Evolutionarily conserved repressive activity of WOX proteins mediates leaf blade outgrowth and floral organ development in plants. Proc Natl Acad Sci USA 110:366–371

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 26:1081–1093

Mayer KF, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T (1998) Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95:805–815

Muehe EM, Eisele JF, Daus B, Kappler A, Harter K, Chaban C (2014) Are rice (*Oryza sativa* L.) phosphate transporters regulated similarly by phosphate and arsenate?A comprehensive study. Plant Mol Biol 85:301–316

Müller B, Sheen J (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature 453:1094–1097

Nardmann J, Reisewitz P, Werr W (2009) Discrete shoot and root stem cell-promoting WUS/WOX5 functions are an evolutionary innovation of angiosperms. Mol Biol Evol 26:1745–1755

Nolan KE, Irwanto RR, Rose RJ (2003) Auxin up-regulates *MtSERK1* expression in both *Medicago truncatula* root-forming and embryogenic cultures. Plant Physiol 133:218–230

Palovaara J, Hakman I (2008) Conifer *WOX*-related homeodomain transcription factors, developmental consideration and expression dynamic of *WOX2* during *Picea abies* somatic embryogenesis. Plant Mol Biol 66:533–549

Palovaara J, Hakman I (2009) *WOX2* and polar auxin transport during spruce embryo pattern formation. Plant Signal Behav 4:153–155

Park SO, Zheng Z, Oppenheimer DG, Hauser BA (2005) The *PRETTY FEW SEEDS2* gene encodes an *Arabidopsis* homeodomain protein that regulates ovule development. Development 132:841–849

Pernisová M, Klíma P, Horák J, Válková M, Malbeck J, Souček P, Reichman P, Hoyerová K, Dubová J, Friml J (2009) Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc Natl Acad Sci USA 106:3609–3614

Sarkar AK, Luijten M, Miyashima S, Lenhard M, Hashimoto T, Nakajima K, Scheres B, Heidstra R, Laux T (2007) Conserved factors regulate signalling in *Arabidopsis thaliana* shoot and root stem cell organizers. Nature 446:811–814

Takahashi H, Miyazawa Y, Fujii N (2009) Hormonal interactions during root tropic growth: hydrotropism versus gravitropism. Plant Mol Biol 69:489–502

Thorpe T (1990) The current status of plant tissue culture. Dev Crop Sci 19:1–33

Tian C, Chen Y, Zhao X, Zhao L (2008) Plant regeneration through protocorm-like bodies induced from rhizoids using leaf explants of *Rosa* spp. Plant Cell Rep 27:823–831

Tian H, Jia Y, Niu T, Yu Q, Ding Z (2014) The key players of the primary root growth and development also function in lateral roots in *Arabidopsis*. Plant Cell Rep 33:745–753

van der Graaff E, Laux T, Rensing SA (2009) The WUS homeobox-containing (WOX) protein family. Genome Biol 10:248

Vasil V, Hildebrandt A (1965) Differentiation of tobacco plants from single, isolated cells in microcultures. Science 150:889–892

Wu X, Chory J, Weigel D (2007) Combinations of *WOX* activities regulate tissue proliferation during *Arabidopsis* embryonic development. Dev Biol 309:306–316

Yadav RK, Tavakkoli M, Reddy GV (2010) WUSCHEL mediates stem cell homeostasis by regulating stem cell number and patterns of cell division and differentiation of stem cell progenitors. Development 137:3581–3589

Zhang X, Zong J, Liu J, Yin J, Zhang D (2010) Genome-wide analysis of *WOX* gene family in rice, sorghum, maize, *Arabidopsis* and poplar. J Integr Plant Biol 52:1016–1026

Zhu J, Shi H, Lee BH, Damsz B, Cheng S, Stirm V, Zhu J-K, Hasegawa PM, Bressan RA (2004) An *Arabidopsis* homeodomain transcription factor gene, *HOS9*, mediates cold tolerance through a CBF-independent pathway. Proc Natl Acad Sci USA 101:9873–9878

Zlesak DC (2006) Rose. Flower breeding and genetics. Springer, Netherlands, pp 695–740