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# Genome-wide identification of WOX family members in rose and functional analysis of *RcWUS1* in embryogenic transformation

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## Abstract

WUSCHEL-related homeobox (WOX) is a class of plant-specific transcription factors and plays vital roles in plant development and evolution. Here, we analyzed WOX family genes of three *Rosa* species and explored their potential functions in *Rosa*. A total of 351 *WOX* genes were identified from *Rosa chinensis* 'Old Blush' (208), *Rosa wichuraiana* 'Basye's Thornless' (21) and *Rosa rugosa* (122). The *WOX* genes were found to significantly expand in *Rosa* compared to *Arabidopsis*. Phylogenetic analysis showed that *Rosa WOXs* genes were classified into an ancient clade, an intermediate clade, and a WUS clade. Collinearity analysis suggested that gene duplication and purifying selection might be important driving forces in the evolution of *WOXs*. Expression patterns of *WOX*s found that higher levels of *RcWUS1* expression were detected at the shoot apex somatic embryos. Furthermore, we found that *RcWUS1* was a transcriptional repressor located in the nucleus. Overexpression of *RcWUS1* enhanced the regeneration efficiency of somatic embryos. In summary, our results indicated the functional potential of *RcWUS1* in embryogenic transformation, which can be further utilized to improve the genetic transformation efficiency in rose.

## Plain Language Summary

Roses are the most important ornamental plants worldwide. The low transformation efficiency and time-consuming regeneration limit the application of gene editing technology in roses. Here, a total of 351 WUSCHEL-related homeobox (WOX) genes were identified from three *Rosa* species. The WOX genes were found to be significantly expanded in *Rosa*. Expression patterns of WOXs genes found that a higher level of *RcWUS1* expression was detected at the shoot apex somatic embryos.

**Abbreviations:** CDS, coding sequence; EC, embryogenic callus; GFP, green fluorescent protein; HB, homeobox; HD, homeodomain; Ka, nonsynonymous substitution rate; Ks, synonymous substitution rate; NEC, non-embryonic callus; NJ, neighbor-joining; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WOX, WUSCHEL-related homeobox; *WOX5*, *WUSCHEL-RELATED HOMEOBOX 5*; *WUS*, *WUSCHEL*.

Xue Bai and Qi Fu contributed equally to this work.

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Moreover, we found that *RcWUS1* was a transcriptional repressor located in the nucleus. Overexpression of *RcWUS1* enhanced the regeneration efficiency of somatic embryos. Therefore, our results provide a foundation for the use of *RcWUS1* in the genetic transformation of *Rosa*.

## 1 | INTRODUCTION

Roses are popular as cut flowers, garden plants, and potted flowers worldwide and have ornamental and high economic value. Modern roses are primarily bred from 8 to 20 *Rosa* species out of the approximately 200 wild species, from which over 35,000 complex hybrid rose cultivars have been hybridized and cultivated (Bendahmane et al., 2013; Raymond et al., 2018). Most cut roses are characterized by recurrent flowering, flower shape, and vase life. However, disease resistance and fragrance seem to have been largely lost during the breeding process. Rose is a woody plant with complex ploidy, which severely restricts the application of traditional cross-breeding techniques (Wang et al., 2023). Genetic transformation technology can rapidly cultivate new plant varieties at the genetic level (Su et al., 2023). Despite the successful cases that have been reported in rose, the low transformation efficiency and time-consuming regeneration limit the application of genetic engineering and gene editing technology (Liu et al., 2021; Shen et al., 2016).

The WUSCHEL-related homeobox (WOX) is a plant-specific homeobox transcription factor characterized by a homeodomain (HD) containing 60–66 amino acids (Alvarez et al., 2018). The *WUSCHEL* (WUS)-box motif consists of eight conserved residues at the C-terminus of the HD (van der Graaff et al., 2009). In general, WOX members are divided into three clades based on amino acid sequence homology: the ancient clade, intermediate clade, and modern/WUS clade (Deveaux et al., 2008). *WOXs* have been reported in many species, including 15 members in *Arabidopsis thaliana*, 14 in *Triticum aestivum*, and 13 in *Musa acuminata* (Chaudhary et al., 2022; Li et al., 2020; Zhang et al., 2010).

Recent studies have shown that *WOXs* play critical roles in plant cell division, embryo formation, maintenance of stem cell stability in meristematic tissues, and organ initiation (Chaudhary et al., 2022; Dolzblasz et al., 2016; Haecker et al., 2004). *WUS* is a regeneration-related gene that is expressed in the shoot apical meristem to maintain stem cell function (Laux et al., 1996; Pan et al., 2019). Overexpression of *WUS* in tobacco enhances regeneration and transformation efficiencies in vitro and promotes somatic embryogenesis (Heidmann et al., 2011; Zhou et al., 2018). Similarly, the overexpression of *WOX5* (*WUSCHEL-RELATED HOMEOBOX 5*), the homolog of *WUS*, in wheat reduces genotype dependence and improves transformation efficiency (Wang et al., 2022). Substantial evidence has shown that *WOXs* are also involved in plant regeneration. *AtWOX2* is expressed in the apical region of the embryo and associated with embryo formation (Haecker et al., 2004). *AtWOX9* is expressed in stem tip and root tip meristematic tissues (Ueda et al., 2011; Wu et al., 2005). Overexpression of *LlWOX9* and *LlWOX11* promotes bulbil formation, while silencing these genes inhibits bulbil formation (He et al., 2022). These results indicate that *WOXs* are involved in plant regeneration. Nevertheless, the genome-wide identification and function of *WOXs* are still lacking in *Rosa*, which required a comprehensive investigation.

In the present study, we identified 351 *WOXs* in three *Rosa* species and analyzed their basic traits. Furthermore, we evaluated the function of *RcWUS1* in somatic embryonic transformation. Our findings provide new perspectives for the functional studies of *WOXs* in *Rosa* and lay the foundation for their application in the genetic transformation of rose.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials and growth conditions

*Rosa chinensis* 'Old Blush' used for expression analysis were grown under controlled conditions in a greenhouse of the Flower Research Institute, Yunnan Agriculture Academic Science, Kunming, China (25.08° N, 102.45° E). *Rosa hybrida* 'Samantha' used for somatic embryo transformation experiments were cultured at (22 ± 1)°C under a 16 h light/8 h dark photoperiod of aseptic conditions.

### 2.2 | Identification and structural domain of WOX proteins

We used two methods to identify WOX genes of *R. chinensis* 'Old Blush', *R. rugosa*, and *R. wichuraiana* 'Basye's Thornless' plants. First, the 15 WOX sequences of *A. thaliana* (http://www.arabidopsis.org) were downloaded as reference sequences, and a local BLAST (version: 2.6.0+; parameters; *e*-value: $1e^{-6}$) search was performed using the genome database (https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/; https://www.Rosaceae.org/). Second, the Hidden Markov Model (HMM) of the WOX family (PF00046) was downloaded and searched with HMM

(version: 3.0; parameters: domE: $1e^{-6}$) (Chaudhary et al., 2022; Shafique Khan et al., 2021). The protein sequences were subjected to an NCBI CD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/) for structural domain screening, which resulted in the identification of *WOXs* in the three *Rosa* species. Conserved structural domains of the *WOXs* were analyzed with SMART and MEGA7.0, after which WebLogo (https://weblogo.threeplusone.com/create.cgi) was used to construct logo plots.

### 2.3 | Phylogenetic tree construction and gene nomenclature

To explore evolutionary relationships among *R. chinensis* 'Old Blush', *R. rugosa*, and *R. wichuraiana* 'Basye's Thornless', we performed phylogenetic analysis using MEGA7.0 and the neighbor-joining (NJ) method with a bootstrap set to 1000 (Yang et al., 2017). They were subsequently used to construct evolutionary trees with *Arabidopsis* for naming *WOXs*. Eventually, the WOX genes of the three *Rosa* species were combined to construct phylogenetic trees to determine the evolutionary relationships between the WOX genes of these plants.

### 2.4 | Gene amplification and collinearity analysis

TBtools (version: 2.016) was applied to obtain the loci of *WOXs* from GFF3. The genome and GFF3 cell line were constructed to extract collinearity information and highlight regions related to the collinearity of the target genes to construct a collinearity map of the *WOXs*. The simple nonsynonymous substitution rate (Ka)/synonymous substitution rate (Ks) calculator program was used to estimate the Ks and Ka.

### 2.5 | *cis*-Element analysis

Promoter sequences (2000 bp of DNA sequence upstream of the translational start codon) were obtained from the genome database of *R. chinensis* by TBtools and subsequently submitted to the PlantCARE website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify *cis*-acting elements (Lescot et al., 2002).

### 2.6 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from material using an EASYspin Plus Polysaccharide Polyphenol/Complex Plant RNA Extraction Kit (Centrifuge Column Type) (Wuhan Junod Biotechnology Co., Ltd.). SYBR Premix Ex TaqTM II (Takara) was used for reverse transcription-quantitative polymerase chain reaction analysis. All primers used in this study are listed in Table S1. *RhUBI2* was used as an internal control. Relative expression was calculated by the delta-delta Ct method. Each treatment was accompanied by three replicates.

**Core Ideas**

- 351 WOX genes were identified from *Rosa*.
- The WOX genes were significantly expanded in *Rosa*.
- *RcWUS1* was specifically and intensively expressed in meristems.
- *RcWUS1* overexpression can significantly improved the regeneration efficiency of somatic embryos.

### 2.7 | In situ hybridization analysis

The method of in situ hybridization used was described previously (Coen et al., 1990). The *RcWUS1* probe was used for full-length complementary DNA. Root tips (0.5 cm), apical meristems, somatic embryos, and calli of *R. chinensis* 'Old Blush' were processed and hybridized with digoxigenin-labeled sense and antisense probes. Signals were visualized by microscopy.

### 2.8 | Subcellular localization analysis

The *RcWUS1* coding sequence (CDS) was fused with the green fluorescent protein (GFP) sequence to construct the pSuper: *RcWUS1*-GFP plasmid, which was subsequently cotransformed with the pSuper: NF-YA 4-mCherry vector (nuclear indicator control) (Zhang et al., 2019) into *Agrobacterium tumefaciens* strain GV3101. After injection into *Nicotiana benthamiana*, the samples were cultured for 1 day in the dark and for 2 days in the light. Then, subcellular localization in leaves was observed under a laser confocal microscope.

### 2.9 | Transcriptional activity assay

The CDS of *RcWUS1* was inserted into the pBD vector and transformed into the Agrobacterium strain GV3101 (carrying the pSoup plasmid) for culture (Sainsbury et al., 2009). pBD/pBD-VP16/pBD-*RcWUS1*, pGAL4-LUC, and P19 were mixed at an equal ratio of 1:1:1, and *N. benthamiana* leaves

were injected. After 3 days (1 day of darkness and 2 days of light), tobacco leaves were removed, sprayed with a reaction solution containing 50 mg $L^{-1}$ of luminol substrate D-betaine, and then the luciferase activity images were captured using a CCD machine. All parameters were described previously (Jing et al., 2023).

### 2.10 | Transformation of somatic embryo

To conduct rose transformation, somatic embryo induction was carried out with germ seedling leaflets as explants. The induction method and transformation procedure followed the protocols reported in a previous report (Liu et al., 2021). GFP-carrying Super-1300 and Super-*RcWUS1* were introduced into somatic embryos using *A. tumefaciens* strain EHA 105 (Jing et al., 2023; Zhang et al., 2019), which was cultured overnight in Luria-Bertani (LB) supplemented with 50 mg·$L^{-1}$ kanamycin and 25 mg·$L^{-1}$ rifampicin (28°C, 200 r·$min^{-1}$), centrifuged at 5000×g for 8–10 min, and resuspended in Embryo Proliferation Solution (EPS) containing 200 μmol·$L^{-1}$ acetosyringone (Sigma-Aldrich); the $OD_{600}$ was adjusted to 0.4–0.6. The bacterial suspension was shaken at 28°C (200 r·$min^{-1}$) for 2 h, after which the somatic embryos were immersed in the bacterial suspension and incubated at 28°C in a shaker for 40 min (200 r·$min^{-1}$). The excess bacterial solution on the somatic embryos was removed by blotting with sterile paper. The somatic embryos were coincubated in cocultivation medium (CM) for 3 days at 24 ± 1°C in the dark. After dark culture, the somatic embryos were transferred to Selection and Proliferation Medium (SPM), and the transformation and regeneration efficiencies were assessed after 14 days.

### 2.11 | Statistical analysis

All experiments were performed with at least three biological replicates. All statistical analyses were performed using GraphPad Prism. All experimental data were tested using Student's *t* test and one-way or two-way ANOVA, as described in the corresponding legends. Asterisks indicate significant differences (ns indicates no significant difference; $p > 0.05$; $*p \leq 0.05$; $**p \leq 0.01$; $****p \leq 0.0001$).

## 3 | RESULTS

### 3.1 | Bioinformatics analysis of the WOX family in three *Rosa* species

A total of 351 WOX genes were identified in the genomes of *R. chinensis*, *R. rugosa*, and *R. wichuraiana* 'Basye's Thornless', with 208, 21, and 122 members, respectively. However, there are only 15 WOX genes in *Arabidopsis* genomes. Moreover, using conserved domain analysis and NCBI CD-Search, we found that all the 351 members contain the HD (PF00046) (Figure 1A). Some WOXs contain WUS-boxes (Figure 1B), and *WUS* and *WOX9* contain EAR motifs (Figure 1C), suggesting that the WOX family is highly evolutionarily conserved among the three *Rosa* species. However, the CDS lengths of *WOX*s varied from 273 to 5202 bp. The number of introns and exons varied from 0 to 19 (Tables S2–S4), implying that there might be some differences in the biofunctions of *Rosa* WOX family members. Our results indicated that the *WOXs* were significantly expanded in *Rosa*.

### 3.2 | Phylogenetic and collinearity analysis

To explore evolutionary relationships between the three *Rosa* species, we constructed phylogenetic trees with MEGA 7 using the NJ method. *WOXs* were named based on their phylogenetic relationships, respectively (Figures S1–S3). Rc, Rr, Rw, and At were used as prefixes for the names of *WOXs* from *R. chinensis*, *R. rugosa*, *R. wichuraiana* 'Basye's Thornless', and *A. thaliana* (Yang et al., 2017). The phylogenetic tree showed that the *WOXs* were divided into three clades (the ancient clade, intermediate clade, and WUS clade) (Figure 1D). The WUS clade comprises 323 members, which is significantly higher than the 34 numbers in the ancient clade and the nine numbers in the intermediate clade.

The collinearity of the WOX genes was analyzed for each of the three *Rosa* species. A total of 25 fragmentary duplicate genes and six tandemly duplicated genes were identified in *R. chinensis* 'Old Blush', three pairs of collinear genes were found in *R. rugosa*, and two fragment duplication genes were found in *R. wichuraiana* 'Basye's Thornless' (Figure 1E–G). The Ka/Ks ratios of these homologous pairs were all less than 1 (Table S5), suggesting that the WOX gene family of the three *Rosa* species might have undergone strong selective pressure for purification (Hurley et al., 2005; Vandepoele et al., 2009).

### 3.3 | *cis*-Element analysis of WOX genes in rose

To further investigate the potential function of *RcWOXs* involved in the plant regulatory network, the *cis*-regulatory elements of 36 *RcWOXs* promoters were analyzed using PlantCARE. The results indicated that the elements were generally categorized into three types: abiotic and biotic stress, plant hormone responses, and plant growth and development. According to previous reports (Han et al., 2021; Lv et al., 2023; Ren et al., 2022), many *cis*-elements were associated

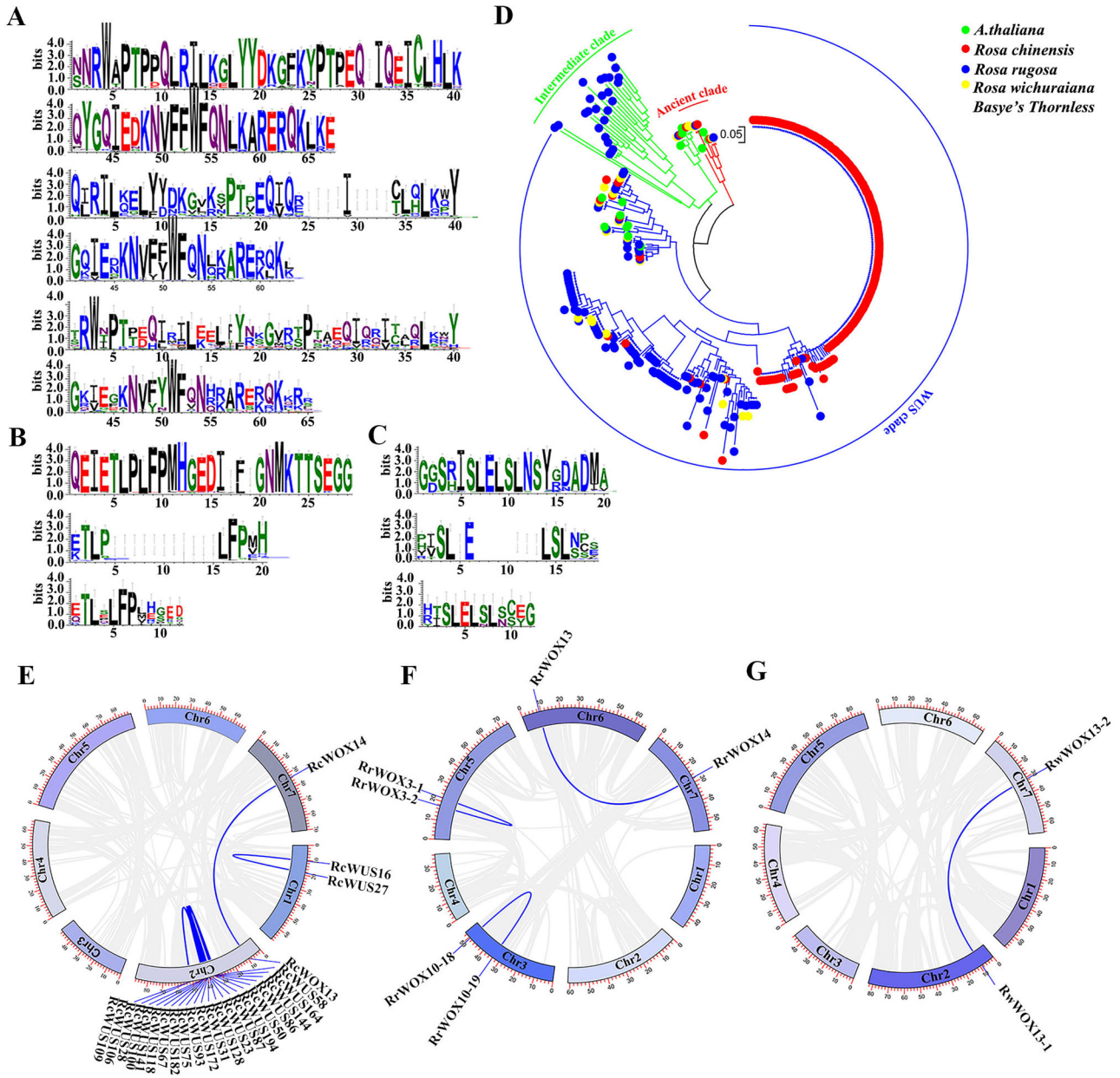


**FIGURE 1** Identification of WUSCHEL-related homeobox (*WOXs*) in three *Rosa* species. (A–C) Sequence logos showing the highly conserved homeobox (HB)-domains, TLXLFPXX structural sequence, and EAR motif in *R. chinensis*, *R. rugosa* and *R. wichuraiana* 'Basye's Thornless'. (D) The evolutionary relationship of *Rosa* species to *A. thaliana*. In the evolutionary tree, the differently colored dots represent the different species, as shown in the illustration; green indicates *A. thaliana*, red indicates *R. chinensis*, blue indicates *R. rugosa* and yellow indicates *R. wichuraiana* 'Basye's Thornless'. The branches of the evolutionary tree are represented in three different colors: red for the ancient branch, green for the intermediate branch and blue for the *WUSCHEL* (WUS) branch. (E–G) Syntenic relationships between *R. chinensis*, *R. rugosa* and *R. wichuraiana* 'Basye's Thornless'.

with abiotic and biotic stresses, phytohormone responses, and plant growth and development (Figure 2).

In the categories of abiotic and biotic stress, 10 *cis*-regulatory elements were identified, most of which responded to the MYB, MYC, and STRE stress-related motifs. Among the plant hormone response elements, the ABRE element, which is involved in the ABA response, was widely present in all 36 genes and was the most numerous. Additionally, there was a substantial number of elements related to the MeJA response, including CGTCA-motif and TGACG-motif, as well as elements related to SA and oxidation response the activation sequence-1 (as-1)-like. Notably, only *RcWUS1* contained the *cis*-regulatory elements TGA-element and AuxRR-core, which respond to auxin.

In the context of plant growth and development, the *cis*-elements primarily included ATCT motifs, G-boxes, GATA

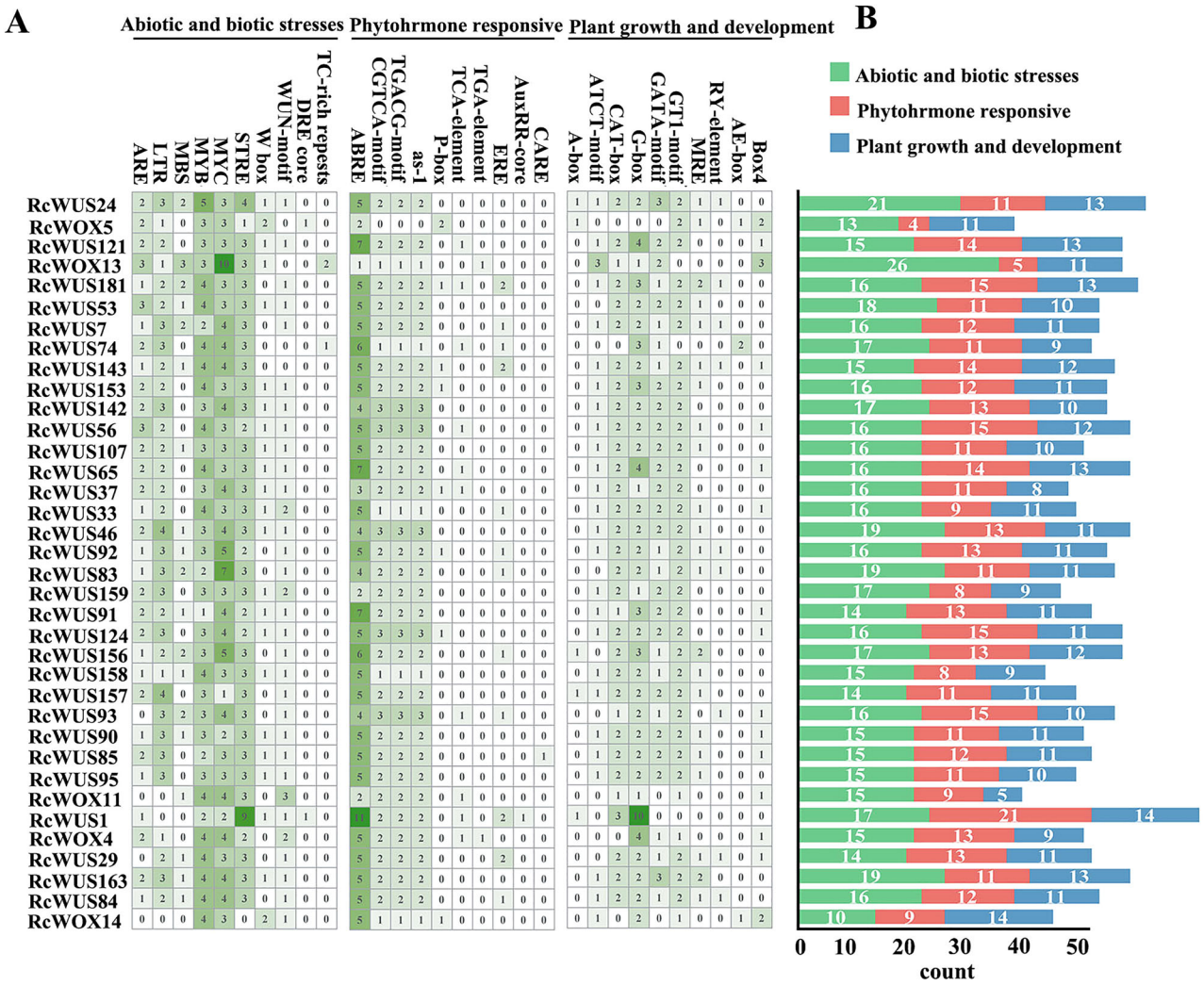


**FIGURE 2** *cis*-Element number analysis of *RcWOXs*. (A) The different intensity colors and numbers of grids indicated the numbers of different *cis*-elements in *RcWOX* promoters. (B) The different colored histograms represent the sum of the *cis*-acting elements in each category.

motifs, and GT1 motifs, all of which are involved in light responses. The proportion of *cis*-regulatory elements responding to meristem expression was also relatively high within *RcWOXs*, with *RcWUS1* being the most abundant. The analysis of *cis*-regulatory elements further suggested that *RcWUS1* might play an active role in the development of somatic embryos and terminal buds.

## 3.4 | Expression pattern and subcellular localization of *RcWUS1*

To investigate whether *RcWUS* takes roles in the development of embryogenic callus (EC), we checked the expression patterns of *RcWUS1*, *RcWUS24*, *RcWUS65*, and *RcWUS163*, whose promoters contain *cis*-regulatory elements related to meristematic tissue and plant development (Figure 3A). The results of RT-qPCR showed that the expression levels of *RcWUS1* were highest in EC than that of other tissues of *R. hybrida* 'Samantha' (Figure 3B). *RcWUS24* was higher expressed in EC and petals. *RcWUS65* and *RcWUS*163 exhibited the strongest expression levels in petals, with almost no expression in somatic embryos and callus tissue (Figure 3B). RNA in situ hybridization further showed that the *RcWUS1* transcripts accumulated at a greater level in the root cap, especially in the edge of the somatic embryo, compared to non-embryonic callus (NEC) (Figure 3B). These data collectively suggested *RcWUS1* might be vital for the development of EC in rose.

To study the subcellular localization of *RcWUS1*, we fused the *RcWUS1* with a GFP tag and obtained the *pSuper:RcWUS1-GFP* plasmid. We introduced them into the leaves of *N. benthamiana* and confirmed that *RcWUS1* localized to the nucleus in *N. benthamiana* (Figure 4A), indicating that *RcWUS1* might function as transcription factors in the nucleus.

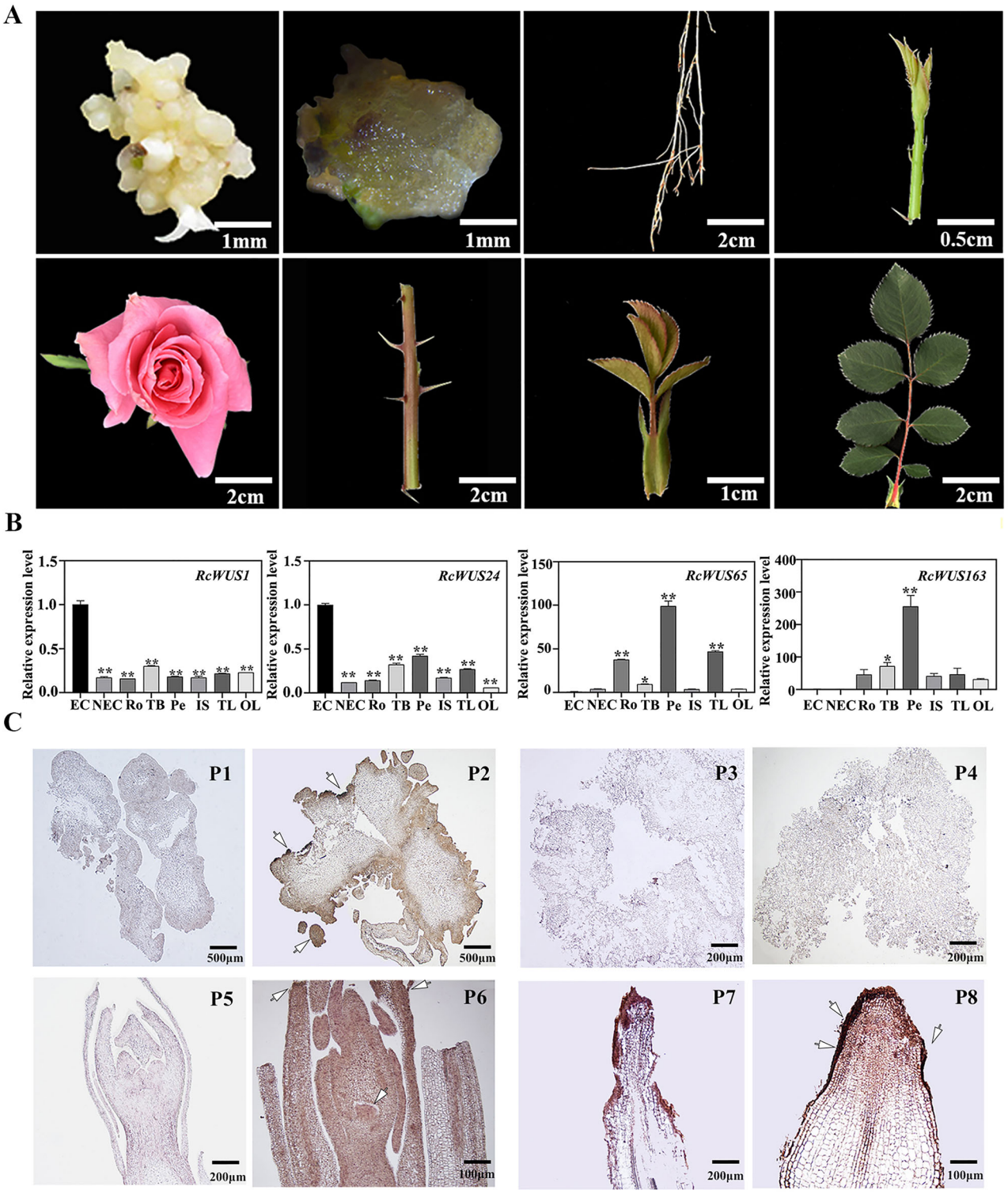


**FIGURE 3** Expression patterns of *RcWUS1*. (A) The different tissues analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in *R. hybrida* 'Samantha'. (B) The expression level of four genes by RT-qPCR. (C) In situ hybridization of *RcWUS1* in somatic embryo (P1–P2), callus (P3–P4), terminal buds (P5–P6), and root tip (P7–P8) tissues, the left side of each tissue is the control group, and the right side is the experimental group, respectively. The more pronounced the brownish-yellow signal is, the more *RcWUS1* is expressed. SE, somatic embryos; Ca, calli, Ro, roots; TB, terminal buds; Pe, petals; IS, immature stems; TL, tender leaves; OL, old leaves. *RhUBI2* was used as the internal control; the mean ± standard deviation of three biological replicates are given. (*$p \leq 0.05$, **$p \leq 0.01$; one-way analysis of variance [ANOVA]).EC, embryogenic callus; NEC, non-embryonic callus.

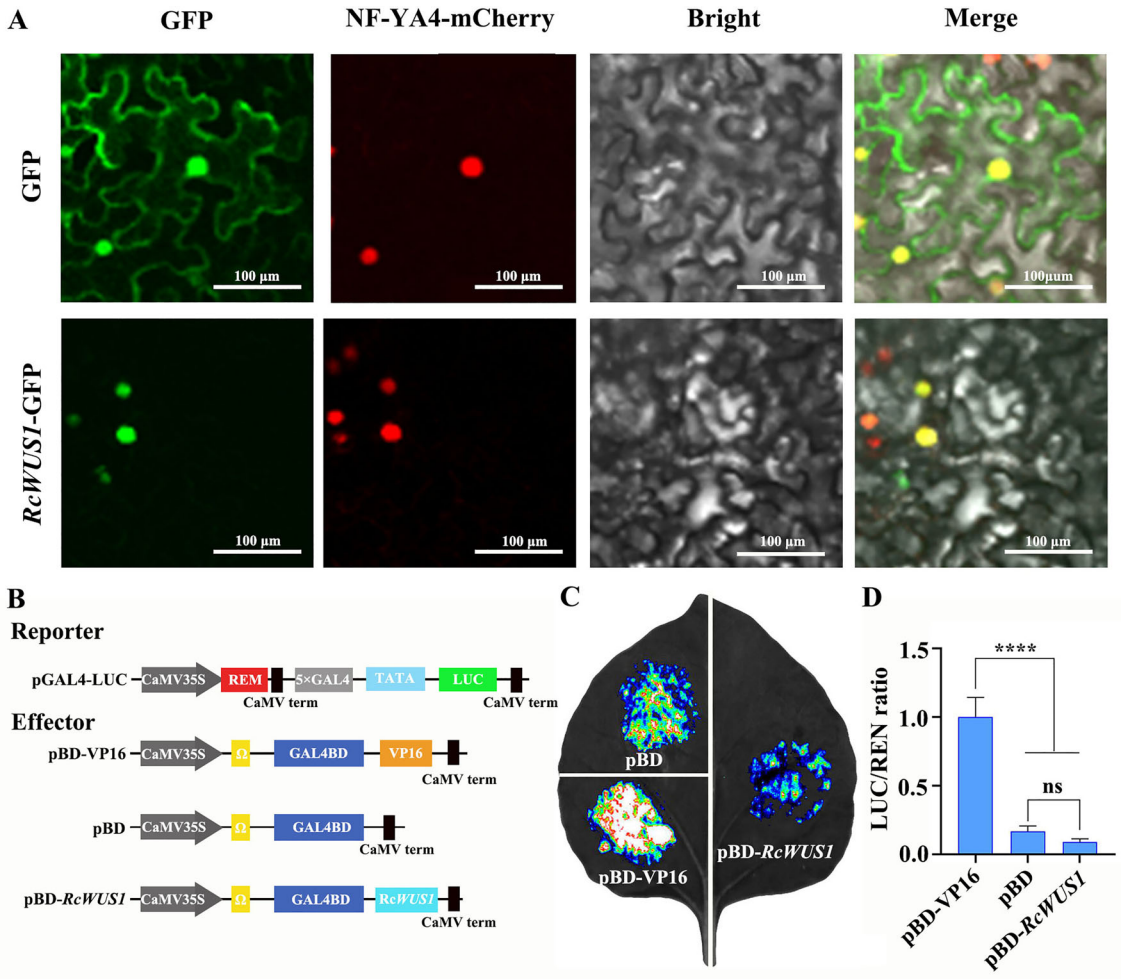


**FIGURE 4** *RcWUS1* is a transcriptional repressor that localizes in the nucleus. (A) Subcellular localization of *RcWUS1* in *N. benthamiana* leaves. *RcWUS1*-GFP was coinfiltrated with NF-YA4-mCherry (a nuclear marker) into *N. benthamiana* leaves. The fluorescence signal was visualized by confocal microscopy. (B) Diagrams of reporter and effector constructs. (C) Live imaging of luciferin (LUC) and renilla luciferase (REN) activity. (D) Quantitative analysis. Data are shown as means ± SDs ($n = 3$) (****$p \leq 0.0001$, ns indicates no significant difference, $p > 0.05$; one-way analysis of variance [ANOVA]). GFP, green fluorescent protein.

To further investigate the transcriptional activity of *RcWUS1*, the CDS of *RcWUS1* was inserted into the pBD vector, resulting in a recombinant vector (pBD-*RcWUS1*) that served as an effector. pGAL4-LUC was used as the reporter, pBD as the negative control, and pBD-VP16 as the positive control. We introduced these constructs into *N. benthamiana* leaves, simultaneously injecting the positive and negative controls along with the target gene into the same leaf. The results showed that the fluorescence signal from the positive control pBD-VP16 was the strongest, followed by the negative control pBD, while the fluorescence signal from pBD-*RcWUS1* was the weakest. This indicates that pBD-*RcWUS1* can inhibit the transcriptional activity of VP16, functioning as a transcriptional repressor with transcriptional repression activity (Figure 4B–D).

### 3.5 | *RcWUS1* overexpression enhances the regeneration efficiency of somatic embryos in rose

In the genetic transformation of roses, the regeneration of somatic embryos remains a major technical challenge, which severely restricts the improvement of rose varieties. To explore the role of *RcWUS1* in the regeneration of somatic embryos, we cloned the *RcWUS1* CDS and constructed the pSuper1300:*RcWUS1*-GFP plasmid. Densely growing somatic embryos with good granularity were selected as the infection recipients. After cocultivation, the somatic embryos were transferred to a selection and proliferation medium for somatic embryos, and the phenotypes were observed after 14 days.

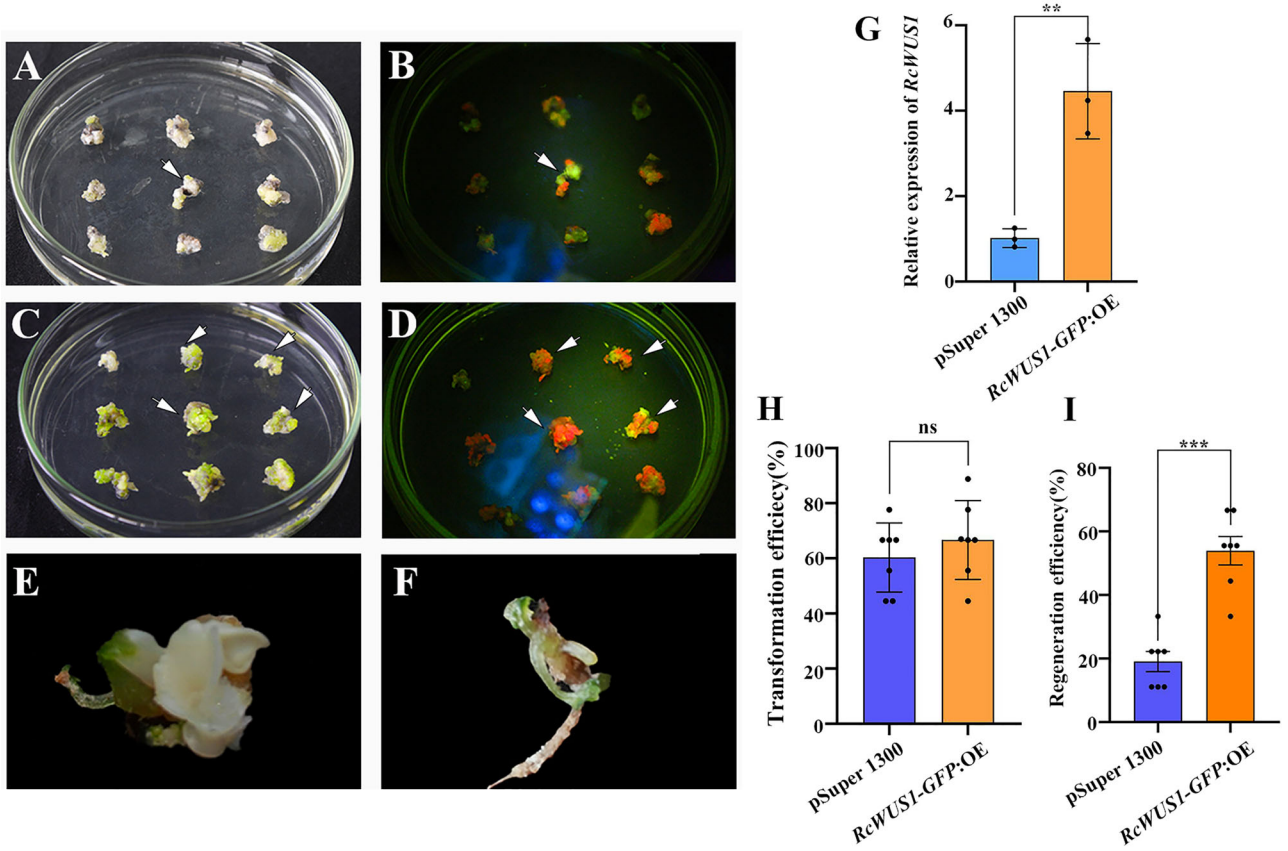


**FIGURE 5** Phenotypes and fluorescence of pSuper 1300 and *RcWUS1-GFP*:OE transgenic somatic embryos. (A) Growth state of control group (pSuper 1300). (B) Fluorescence microscopy of control group. (C) Growth state of experimental group (*RcWUS1*-GFP):OE after transformation for 14 days. (D) Fluorescence microscopy of experimental group. (E) Positive transformant. (F) Transgenic plantlets. (G) Relative expression of *RcWUS1* in somatic embryos after overexpressing *RcWUS1* (**$p \leq 0.01$, *t* test). (H) The transformation rate of *RcWUS1* in somatic embryos after overexpressing *RcWUS1* (ns indicates no significant difference, $p > 0.05$; *t* test). (I) The regeneration rate of *RcWUS1* in somatic embryos after overexpressing *RcWUS1* (***$p \leq 0.001$; *t* test). GFP, green fluorescent protein.

We identified positive transformants by fluorescence microscopy. Positive transformants were easily identified by their bright green fluorescence (Figure 5A–D). When the transgenic plants grew up (Figure 5E,F), we performed RT-qPCR using their cDNA from transgenic lines to identify positive lines. The results showed that the abundance of the *RcWUS1* transcript was substantially increased threefold after overexpression in pSuper1300:*RcWUS1*-GFP lines compared to control line pSuper1300 (Figure 5G). The transformation rate of the control group pSuper1300 after infection was 60.34%, while that of the experimental group *RcWUS1*-GFP:OE was 66.69%, indicating only a 6.35% increase, with no significant difference (Figure 5H, Figure S4). However, in the *RcWUS1*-GFP:OE treatment, the somatic embryos exhibited vigorous growth with many observable bud points. Data analysis indicated that the regeneration efficiency was 66.69%, significantly higher than that of the control (31.22%) (Figure 5I). These results indicate that *RcWUS1* can improve the regeneration efficiency of somatic embryos in roses to some extent, playing an important role in somatic embryo development.

## 4 | DISCUSSION

WOX genes are widely involved in plant growth and development during evolution (Breuninger et al., 2008; Ma et al., 2019). Many studies on *WOXs* have been reported for multiple plant species (Lv et al., 2023; Shi et al., 2021; Yang et al., 2017; Zhang et al., 2010). In this study, we identified 351 *WOX* genes in three *Rosa* species. However, 40, 18, and 15 WOX genes were reported in *P. domestica*, *M. domestica*, and *F. vesca* genomes, respectively. In addition, only 15 members of the *WOX* family in *Arabidopsis thaliana* (Lv et al., 2023). Our results indicated that the *WOX* genes were significantly expanded in *Rosa*. Similar to other plants, the *WOXs* of *Rosa* species are naturally divided into three clades, namely, the ancient clade, intermediate clade, and

WUS clade (Akbulut et al., 2022; Muhammad Tajo et al., 2022). However, the numbers of WOXs were significantly different among *R. chinensis* 'Old Blush', *R. rugosa*, and *R. wichuraiana* 'Basye's Thornless' in our study (Figure 1D). *R. rugosa* and *R. wichuraiana* 'Basye's Thornless' belong to wild *Rosa* plants, and Old Blush is the cultivar of Chinese old garden roses (Feng et al., 2022). The underlying reason might be that gene sequence duplication served as the main driving force for the evolution of WOX families among the three *Rosa* species. Protein structure analysis suggested the presence of unique conserved motifs within the same subclade (Figure 1A), thus revealing that they might have similar functions. The identified *WOXs* of *Rosa* species from this study are naturally divided into three clades (the ancient clade, intermediate clade, and WUS clade), which is in accordance with those found in other species (Akbulut et al., 2022; Muhammad Tajo et al., 2022). WOX genes can be found in multiple species and are widely involved in plant growth and development during evolution (Breuninger et al., 2008; Lv et al., 2023; Ma et al., 2019; Shi et al., 2021; Yang et al., 2017; Zhang et al., 2010). The segmental and tandem duplication found in the *Rosa WOXs* hinted that gene duplication might be involved in the *WOXs* evolution in *Rosa* (Lian et al., 2014). However, results from Ka/Ks in the three *Rosa* species supported that purifying selection might be the capital driving force in the evolution of *WOXs* in *Rosa* species (Cao et al., 2017). This view is also supported by our analysis of Ka/Ks in the three *Rosa* species (Table S4).

*WUS* is an HD transcription factor that regulates cell fate during cell dedifferentiation by maintaining pluripotent stem cells, including those of the shoot meristem, somatic embryo, and adventitious shoot (Gallois et al., 2004; Honda et al., 2018). *RcWUS1* is a novel transcriptional repressor that we discovered in this study. Contrary to other known WUS that are expressed only in a small population of cells (Jha et al., 2020), *RcWUS1* was distributed throughout the terminal buds and more highly expressed in the root apex, especially in the region of the root tip. Additionally, expression of *RcWUS1* in EC was greater and was only detected at the margin compared with that in NEC. This implied that *RcWUS1* can function similarly as other *WOXs* (e.g., *WOX5*) in the maintenance of shoot and root stem cell functions (Wang et al., 2022). Similarly, we found that expression of *RcWUS1* was concentrated in the root apex, especially in the region of the root tip (Figure 3C, P7–P8). Compared with that in NEC, expression of *RcWUS1* in embryonic callus was greater, but it was detected only at the margin (Figure 3C, P1–P4).

At present, there are several genetic and stable transformation methods for rose plants via *A. tumefaciens*-mediated infection (Liu et al., 2021). However, there are still several difficulties, especially in terms of genetic transformation efficiency and shoot regeneration. For example, approximately 70% of somatic embryos turn brown or die during subsequent cultivation, resulting in very low efficiency and difficulty in obtaining transgenic plants (Liu et al., 2021). Burgeoning evidence shows that overexpression of *WUS* not only improves transformation efficiency but also induces shoot-like organs or embryo-like structures (Gordon-Kamm et al., 2019; Zuo et al., 2002). For instance, *WUS2* overexpression increases transformation efficiency from 1.7% to 34.9% in maize (inbred PHN46) (Lowe et al., 2016), and estradiol induction via *AtWUS* leads to swelling of shoot tips and the generation of green shoots in tobacco (Rashid et al., 2007). Similar results were also obtained in this study, as *RcWUS1* overexpression can enhance the shoot regeneration efficiency in rose plants by 35.47% but did not affect the transformation efficiency. This indicated that the application of *RcWUS1* in the future improvement of the genetic transformation efficiency in *Rosa*.

## 5 | CONCLUSIONS

This is the first study to identify *WOXs* in three *Rosa* species. A total of 351 *WOXs* were identified and classified into three clades according to phylogenetic analysis. We found duplication of *WOXs* in *Rosa*, including segmental duplication and tandem duplication. Furthermore, *cis*-element analysis revealed that *WOXs* were involved in plant growth and might be associated with meristems. RT-qPCR and in situ hybridization showed that *RcWUS1* was highly expressed in the meristem. Subcellular localization and transcriptional activity analysis further revealed that *RcWUS1* was a transcriptional repressor localized to the nucleus. Overexpression of *RcWUS1* promoted regeneration in somatic embryos. Therefore, the results of this study provide a foundation for the use of WOX genes in the genetic transformation of *Rosa*.

## AUTHOR CONTRIBUTIONS

**Xue Bai**: Data curation; methodology; validation; writing—original draft. **Qi Fu**: Methodology; writing—original draft. **Weikun Jing**: Writing—original draft. **Hao Zhang**: Resources. **Hongying Jian**: Resources. **Xianqin Qiu**: Resources. **Hongjie Li**: Validation. **Qigang Wang**: Resources. **Shunting Yang**: Data curation. **Yiping Zhang**: Resources. **Huichun Wang**: Resources. **Lihua Wang**: Resources. **Kaixue Tang**: Supervision. **Ying Bao**: Validation. **Huijun Yan**: Methodology; resources; supervision; writing—review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data will be made available upon request.

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## SUPPORTING INFORMATION

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