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ORIGINAL ARTICLE

Genome-wide identification and expression profiling of WUSCHEL-related homeobox (WOX) genes during adventitious shoot regeneration of watermelon (*Citrullus lanatus*)

Na Zhang[1,2] · Xing Huang[1] · Yaning Bao[1] · Bo Wang[1] · Lijun Liu[1] · Lunjin Dai[1] · Jie Chen[1] · Xia An[1] · Yuhong Sun[2] · Dingxiang Peng[1]

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Abstract The WUSCHEL-related homeobox (WOX) gene family is a hotspot for diverse functions in development biology. Recently available whole-genome sequences allowed a more comprehensive analysis of WOX genes in watermelon (*Citrullus lanatus*). The results of this study provide a genomic framework for further research of watermelon WOX genes and contribute to understanding of the evolutionary mode of WOX genes in Cucurbitaceae crops. The qRT-PCR analysis demonstrated active expression of 11 WOX genes in watermelon tissues, which brings new evidence for WOX genes acting as conserved factors during watermelon development. Moreover, the distinct expression profiles of WOX genes during shoot initiation might lead to different shoot regeneration abilities. This work gives an overview of the differentially expressed WOX genes during shoot regeneration in watermelon. The interrelations of WOX genes, phytohormones and other transcription factors during the process will be the focus of future studies.

Keywords WOX family · Watermelon · Expression pattern · Shoot regeneration

Abbreviations

WOX	WUSCHEL-related homeobox
WUS	WUSCHEL
MS	Murashige and Skoog
6-BA	6-Benzylaminopurine
NAA	Naphthaleneacetic acid

Introduction

The WUSCHEL-related homeobox (WOX) gene family is a subgroup of the plant homeodomain (HB) transcription factors that regulate cell differentiation and cell fate. The

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✉ Yuhong Sun
sunyh68@163.com

✉ Dingxiang Peng
pdxiang@mail.hzau.edu.cn

Na Zhang
zn800329@163.com

Xing Huang
hxalong@gmail.com

Yaning Bao
baoyaning92@gmail.com

Bo Wang
wangbo@mail.hzau.edu.cn

Lijun Liu
liulijun@mail.hzau.edu.cn

Lunjin Dai
dailunjin@gmail.com

Jie Chen
lqlcj@126.com

Xia An
anxia111@126.com

[1] College of Plant Science and Technology, Huazhong Agricultural University, #1 Shizishan Street, Hongshan District, Wuhan 430070, Hubei, China

[2] Wuhan Institute of Agricultural Science, Wuhu Eco-Agricultural Park, Huangpi District, Wuhan 430345, Hubei, China

WOX genes contain a family featured helix-loop-helix-turn-helix structure compared with other HB transcription factors (Gehring et al. 1990; Mukherjee et al. 2009). Recent studies indicate that plant WOX genes contribute to various physiological and developmental processes. The 15 WOX members of the model eudicot plant *Arabidopsis thaliana* are grouped into three clades (van der Graaff et al. 2009) and several were shown to function in stem-cell maintenance (Aichinger et al. 2012), embryonic patterning (Haecker et al. 2004) and organ formation (Lin et al. 2013b; Sarkar et al. 2007). Arabidopsis *WUSCHEL* (*AtWUS*) was shown to be essential for shoot apical meristem maintenance and promotes vegetative-to-embryonic transition (Mayer et al. 1998; Zuo et al. 2002). Overexpression of *AtWOX1* disrupts normal meristem development (Zhang et al. 2011). *AtWOX2*, *8* and *9* are expressed during zygote development (Haecker et al. 2004; Ueda et al. 2011), and *AtWOX2* and 8 promote cotyledon boundary formation (Lie et al. 2012). *AtWOX3* (*PRS1*) recruits founder cells that form lateral domains of vegetative and floral organs (Shimizu et al. 2009). *AtWOX4* and *14* both function in regulating vascular meristem development (Etchells et al. 2013; Hirakawa et al. 2010; Ji et al. 2010; Wang et al. 2013a). The function of *AtWOX5* is to maintain the root apical meristem by mediating cellular auxin response (Sarkar et al. 2007; Tian et al. 2014). *AtWOX6* (PRETTY FEW SEEDS2) regulates ovule development (Park et al. 2005). *AtWOX11* and *12* are involved in root organogenesis (Liu et al. 2014b) and *AtWOX13* promotes replum formation in fruit (Romera-Branchat et al. 2013). The functional diversity of WOX genes makes it a research hotspot in development biology.

Watermelon (*Citrullus lanatus*) is an important cucurbit crop with high production about 90 million tons in the world (http://faostat.fao.org/). Most consumers enjoy watermelon for the important nutritional compounds it contains, including sugars, lycopene and healthy amino acids (Collins et al. 2007; Perkins-Veazie et al. 2006). Therefore, watermelon studies have mainly focused on fruit development (Grassi et al. 2013; Guo et al. 2011) and disease resistance (Ouibrahim et al. 2014; Wu et al. 2014), with the aim of improving quality and production. In contrast, there are no reports on the mechanisms regulating shoot regeneration, although regeneration systems and genetic transformation have been successfully applied in watermelon for decades (Choi et al. 1994; Compton et al. 2004; Wang et al. 2013b). Shoot regeneration is affected by the expression of WOX genes (Liu et al. 2014a; Sarkar et al. 2007), especially *WUS* (Ikeda et al. 2009; Leibfried et al. 2005; Mayer et al. 1998). The completed watermelon genome allows the genome-wide analysis of WOX genes (Guo et al. 2013).

In the present study, we successfully identified 11 WOX genes from the watermelon inbred line 97103 genome. Phylogenetic analysis of WOX proteins was conducted to investigate the evolutionary pattern in Arabidopsis, watermelon, cucumber (*Cucumis sativus*) and melon (*Cucumis melo*). We further examined the expression patterns of WOX genes in different watermelon tissues and the process of shoot regeneration. The results provide an overview for the transcription regulation of watermelon shoot regeneration and serve as a guideline for future study.

Materials and methods

Identification of watermelon WOX genes

The 15 sequences of Arabidopsis WOX genes were downloaded from The Arabidopsis Information Resource (http://www.arabidopsis.org) and used as query sequences using TBLASTN (Altschul et al. 1997) to search WOX genes in the watermelon genome database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi) with a cut-off *E* value of 10^{-5}. Obtained sequences were used as query to search the watermelon genome database again. Redundant sequences with different identification numbers and the same chromosome loci were removed. We also obtained information on chromosome localization for the watermelon WOX genes. The exon/intron structures were analyzed using the online gene structure display server (GSDS, http://gsds.cbi.pku.edu.cn) with coding sequences and genomic sequences (Guo et al. 2007).

Phylogenetic analysis of WOX proteins

A neighbor-joining (NJ) phylogenetic tree was constructed for WOX proteins using MEGA 5.0 software (Tamura et al. 2011). The most parsimonious tree with bootstrap values from 1000 trials was used. Amino acid sequences of WOX protein were aligned using Clustal X (Thompson et al. 1997). The Arabidopsis WOX proteins were from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) and were selected as the model system for phylogenetic comparisons. The WOX proteins in cucumber and melon were downloaded from National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). The WOX sequences of orange, soybean, tomato and grape were downloaded from Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp).

Plant materials and sampling

Plants of the diploid watermelon inbred line A7 were grown in a temperature-controlled greenhouse at the experimental farm of Wuhan Institute of Agricultural Sciences. Unexpanded leaves, stems, shoot buds and flower buds were collected from the top of flowering plants and roots were collected from the root tip part after 2 months of growth. All samples were performed in triplicate and frozen in liquid nitrogen immediately and stored at −75 °C until RNA isolation.

Regeneration studies were according to published protocols (Compton et al. 2004). Seeds were decoated and surface-sterilized in 10 % NaClO solution for 5 min. After several washings in sterile distilled water, seeds were implanted in culture tubes (25 × 150 mm) containing 20 mL of Murashige and Skoog (MS) medium for germination. Cotyledons from 7-day-old aseptic seedlings were cut into four types (Fig. 1) of segments (0.5 × 0.5 mm) and cultured on MS medium supplemented with 6-BA (1.0 mg/L, Sigma) and NAA (1.0 mg/L, Sigma) in a culture room under cool white fluorescent light with a 16/8 h (light/dark) cycle at 25 ± 2 °C during the day and 20 ± 2 °C at night. Each experiment was repeated three times with 24 cotyledon segments per treatment in each experiment. The adventitious shoot numbers of the four types of cotyledon segments were counted after 21 days and data analyzed using SPSS (Liu et al. 2003).

RNA was extracted from cotyledon segments grown under the same growth conditions. The samples for type-D segments were collected at 7, 14, 21 and 28 days (Fig. 1, D1–4) and the samples for the four types of segments were collected when incubated for 14 days. Approximately ten explants from the same plate were pooled for RNA extraction, which was repeated three times per treatment.

Quantitative real-time PCR (qRT-PCR) analysis

Watermelon samples at the different growth stages were collected and total RNA was isolated from each sample using a Tiangen®RNA prep Pure Plant Kit (Tiangen Biomart, Beijing). Reverse cDNA for each sample was generated using the GoScript™ Reverse Transcription System (Promega, USA), according to the manufacturer's instructions. An optical 96-well plate iQ5 multicolor real-time PCR system (Bio-RAD, USA) was used for qRT-PCR analysis. Each reaction contained 1 μL of cDNA template, 10 nM gene-specific primers, 10 μL of iTaq™ Universal SYBR® Green Supermix (Bio-RAD, USA) and 7 μL of ddH_2O in a final volume of 20 μL. The watermelon glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene was selected as the endogenous control (Kong et al. 2014). Gene-specific primers (Table 1) were designed using Primer 3 (http://primer3.ut.ee/) and commercially synthesized (Sunny Biotech, Shanghai). The thermal cycle used was as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Following amplification, a dissociation stage was carried out to detect any complex products. The qRT-PCR was performed in triplicate for each sample. Relative expression levels were calculated as reported previously (Livak and Schmittgen 2001).

Results

Identification of watermelon WOX genes

After removing the redundant sequences, 11 WOX genes were obtained and named according to both phylogenetic groups and closely related Arabidopsis homologs (Table 2). The 11 WOX genes were located on one or two

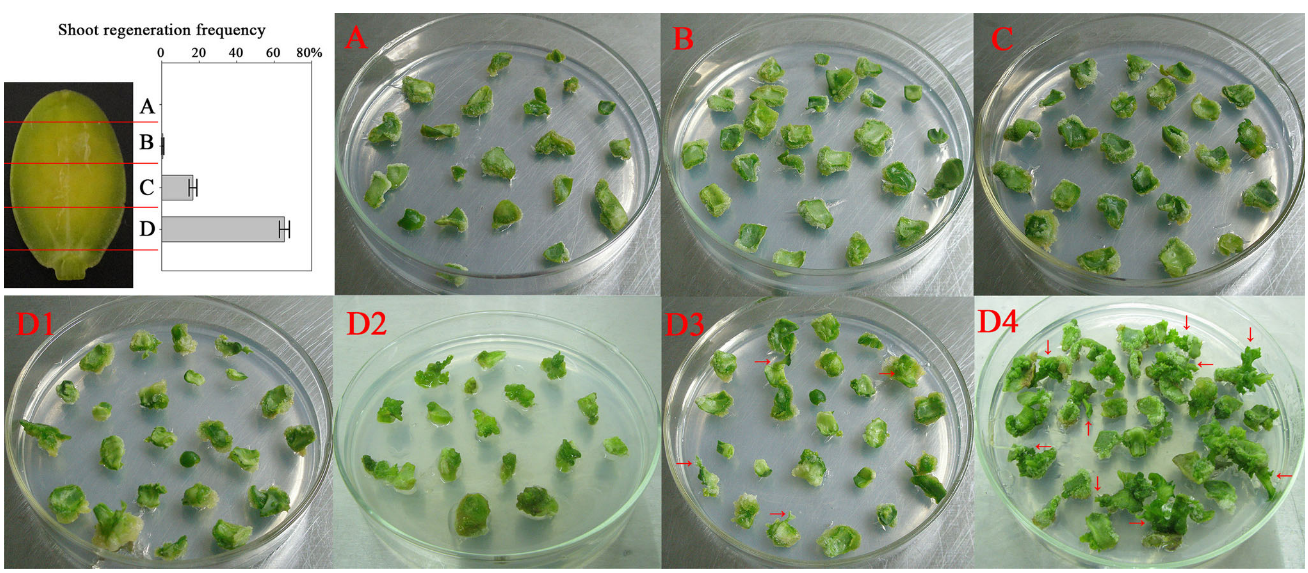

Fig. 1 Adventitious shoot-regeneration frequency and morphological observation for different cotyledon segments of watermelon. **A**, **B** Cotyledon segments of *A*, *B* and *C* when incubated for 21 days, respectively; **D1–4** cotyledon segments of *D* when incubated for 7, 14, 21 and 28 days, respectively. The *arrows* indicated adventitious shoots

ends of eight chromosomes (Fig. 2). The predicted nucleotide and protein sequences of all 11 watermelon WOX genes are provided in Supplementary material 1.

The exon/intron positions and phases of the 11 WOX genes in watermelon were further studied. The exon/intron structures were analyzed using GSDS with coding sequences and genomic sequences (Guo et al. 2007). Figure 3 provides a detailed illustration of the relative lengths of the introns and conservation of the corresponding exon sequences within each WOX gene. There were 1–3 introns each in the 11 WOX genes: 1 intron for *ClaWOX2*, *3*, *5*, *6*, and *10*; two for *ClaWUS*, *ClaWOX4*, *7* and *8*; and three for *ClaWOX1* and *9*.

Phylogenetic analysis of WOX proteins in Arabidopsis, Cucurbitaceae crops and other four eudicot plants

Phylogenetic analysis via the NJ method was conducted in order to determine the relationships among WOX proteins in Arabidopsis, three Cucurbitaceae crops (Table 2) and other four eudicot plants. The Arabidopsis WOX proteins were selected as the model system for phylogenetic comparisons. The Cucurbitaceae sequences were grouped together in each of the subfamilies, respectively. Previous studies showed that the Arabidopsis WOX proteins were divided into three subclades (van der Graaff et al. 2009; Zhang et al. 2010). As a result, WUS, ancient and intermediate clades were composed of 22, six and six Cucurbitaceae sequences, respectively (Fig. 4). The WUS clade contained seven sequences from watermelon, eight from cucumber and seven from melon. Two sequences from each Cucurbitaceae crop were clustered into ancient and intermediate clades.

The WOX family contains the helix-loop-helix-turn-helix domain (Kamiya et al. 2003) and most WOX proteins contain the complete or incomplete WUS box (Lin et al. 2013b). We conducted multiple sequence alignments of the 49 amino acid sequences to investigate features of WOXs (Fig. 5). The proximate Cucurbitaceae sequences were highly conserved. The homeodomain sequences have been shown to contain several conserved amino acids, e.g., Q, L and Y in helix1; P, I and L in helix2; I, N, V, W, F, Q, N, K, R and R in helix3; and G in turn. Interestingly, *CsaWOX5-like2* lacked the helix3 region and *ClaWOX10* lacked the helix1 region. Furthermore, we found that most WOX proteins in the WUS clade contained a complete WUS box (amino acids, TL × LFP) except *AtWOX7*, *CmeWOX5-like2*, *CsaWOX1-like*, *ClaWOX6* and *CmeWOX1*. Ancient and intermediate clade WOXs contained incomplete WUS boxes with only one conserved amino acid F.

Expression of WOX genes in watermelon tissues

To better understand where watermelon WOX genes are active, we analyzed their expression in different tissues from flowering-period plants using qRT-PCR (Fig. 6). All WOX genes were expressed at higher levels in leaf, bud and flower than in stem and extremely low levels in root and fruit. ClaWOX10 was expressed at a much higher level in leaf than other tissues. Additionally, *ClaWOX4*, *7* and *8* were more highly expressed in root, and *ClaWOX8* at much higher levels in fruit, than other WOXs.

Expression of WOX genes during adventitious shoot regeneration of watermelon

Cotyledons from the diploid watermelon inbred line A7 were cut into four types of segments (Fig. 1) and incubated on adventitious shoot induction medium, MS with 6-BA (1.0 mg/L) and NAA (1.0 mg/L). After 21 days, we counted adventitious shoot numbers on different types of cotyledon segments (Fig. 1a–c, d3). The result showed that

Table 1 Primers of watermelon GAPDH gene (endogenous control) and 11 WOX genes used for qRT-PCR

Gene	Forward primer	Reverse primer
ClaWUS	TACTTCTACTGCTTCTTCACCCA	TTTGGTGGGTATTTGTTGTGGAA
ClaWOX1	GAGTGTTGGAGGAATTGTATCGG	GTTTCTGCCGTTCTCTAGCTTTA
ClaWOX2	GCTGACCAAATACAACAGATAACG	TGGGATTGAAGTGAAGAAAGTGA
ClaWOX3	TGTCCAACTCCAGAACAAGTCAT	CGATCTCTAGCCTTGTGATTTTGAA
ClaWOX4	AACAACATAACCAACATCCCACC	CTGTCTGGAGGGAAGAAGAAGAG
ClaWOX5	CCAACTTCTTCTAACCACCAACA	GGTGATCATCATCTGGGAATCCT
ClaWOX6	AATAATGGAAAGAGTTGGTCGGG	TTTTGCCTTCTATTTTGCCGTAG
ClaWOX7	ATGAGGCTTGGGAATCTTTACTG	ATCCTTGATCTTCTGCTTGCTTG
ClaWOX8	TCACAGGAATGAGGTTGGGTAAT	TGATCTTTTGTTTGCTGGGAGTT
ClaWOX9	TTGCAAGAATATGGACAAGTGGG	TTTGGGAGAAGATTTGTCAGACG
ClaWOX10	TTTTCGATGTCAGGTCATATGGG	ATGAACTAACACTGTCTCTTGCC
GAPDH	TGGAAGAATCGGTAGGTTGG	CTGTCACTGTTTTTGGCGTC

Table 2 WOX genes in watermelon, Arabidopsis, cucumber and melon

Gene	Accession number	Species	Database
ClaWUS	Cla014280	Watermelon (*Citrullus lanatus*)	http://www.icugi.org/cgi-bin/ICuGI/index.cgi
ClaWOX1	Cla018458		
ClaWOX2	Cla008252		
ClaWOX3	Cla013275		
ClaWOX4	Cla022590		
ClaWOX5	Cla006578		
ClaWOX6	Cla018927		
ClaWOX7	Cla023456		
ClaWOX8	Cla008940		
ClaWOX9	Cla009659		
ClaWOX10	Cla017497		
AtWUS	AT2G17950	Arabidopsis (*Arabidopsis thaliana*)	http://www.arabidopsis.org/
AtWOX1	AT3G18010		
AtWOX2	AT5G59340		
AtWOX3	AT2G28610		
AtWOX4	AT1G46480		
AtWOX5	AT3G11260		
AtWOX6	AT2G01500		
AtWOX7	AT5G05770		
AtWOX8	AT5G45980		
AtWOX9	AT2G33880		
AtWOX10	AT1G20710		
AtWOX11	AT3G03660		
AtWOX12	AT5G17810		
AtWOX13	AT4G35550		
AtWOX14	AT1G20700		
CsaWUSCHEL-like	XM_004158825.1	Cucumber (*Cucumis sativus*)	http://www.ncbi.nlm.nih.gov
CsaWOX1-like	XM_004137578.1		
CsaWOX2-like	XM_004150141.1		
CsaWOX3-like	XM_004148196.1		
CsaWOX4-like	XM_004142824.1		
CsaWOX5-like1	XM_004138522.1		
CsaWOX5-like2	XM_004163604.1		
CsaWOX6-like	XM_004165984.1		
CsaWOX9-like	XM_004134628.1		
CsaWOX11-like	XM_004136702.1		
CsaWOX13-like1	XM_004145498.1		
CsaWOX13-like2	XM_004146124.1		
CmeWUSCHEL	XM_008442905.1	Melon (*Cucumis melo*)	http://www.ncbi.nlm.nih.gov
CmeWOX1	XM_008455465.1		
CmeWOX1like	XM_008465474.1		
CmeWOX3	XM_008465635.1		
CmeWOX4	XM_008446378.1		
CmeWOX5like1	XM_008443218.1		
CmeWOX5like2	XM_008459352.1		
CmeWOX8like	XM_008454753.1		
CmeWOX9like	XM_008441567.1		
CmeWOX11like	XM_008444910.1		
CmeWOX13like	XM_008450261.1		

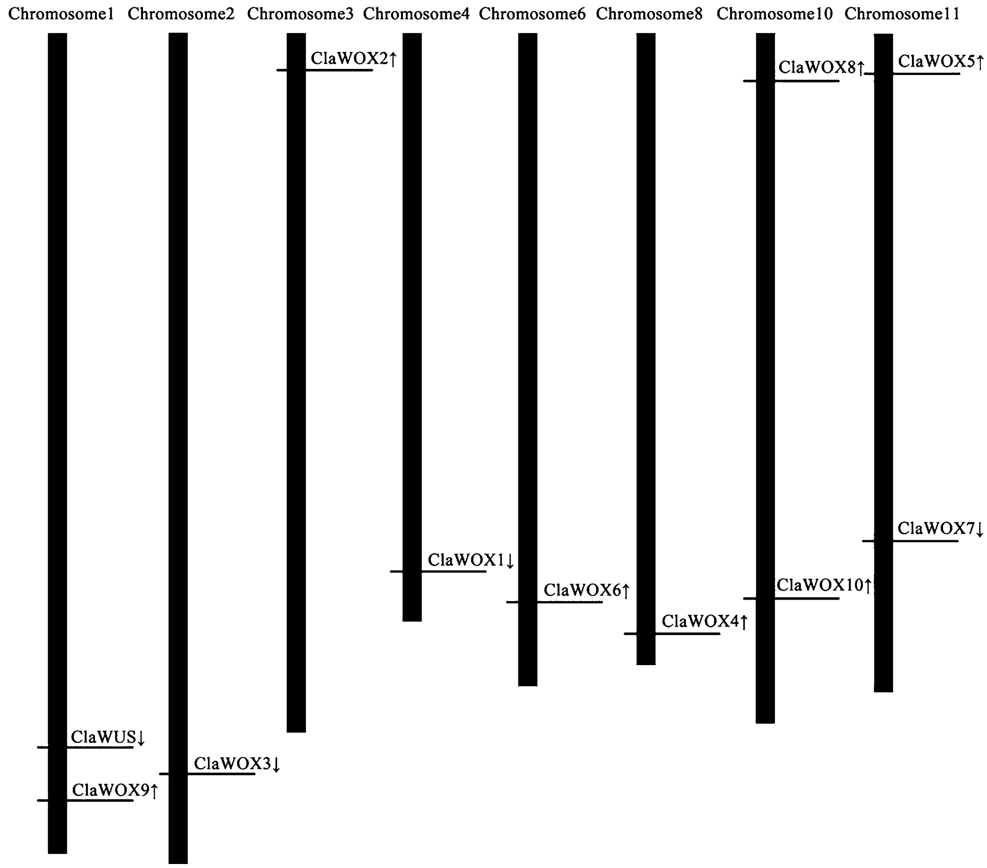

Fig. 2 Position of WOX genes on the watermelon chromosomes. The chromosome *number* is indicated at the *top* of each chromosome. The *arrows* next to these *numbers* show the direction of transcription

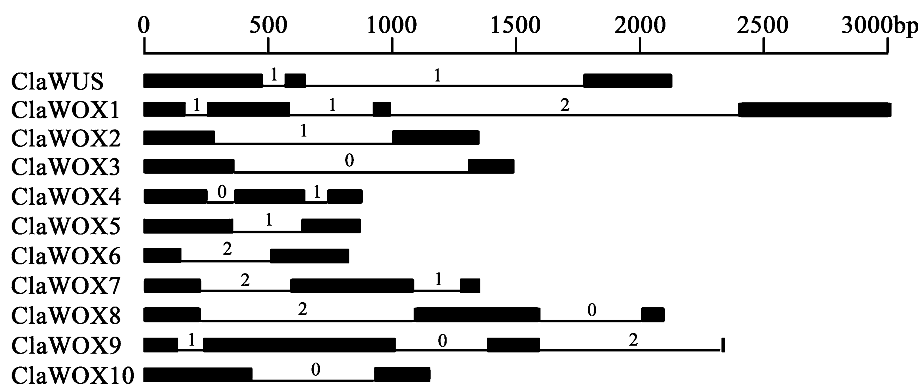

Fig. 3 Intron/exon configurations of WOX genes in watermelon. Introns and exons are drawn to scale with the full encoding regions of their respective genes. *Boxes* indicate the exon, and *lines* indicate the intron. *0* intron phase 0, *1* intron phase 1, *2* intron phase 2

proximal segments had a much higher shoot-regeneration frequency than distal parts of cotyledon (Fig. 1). Therefore, the proximal cotyledon segments (type-D) were selected for qRT-PCR analysis to reveal the expression modulation of WOX genes during watermelon shoot regeneration.

To generate a complete set of observations of WOX genes during shoot regeneration, proximal cotyledon segments (type-D) were sampled when cultured for 7, 14, 21 and 28 days (Fig. 1d1–4). The expression patterns of the 11 watermelon WOX genes showed that most of them were up-regulated at 14 and 28 days (Fig. 7), indicating that expression of WOX genes at 14 days might be related to shoot regeneration.

We conducted a further study to examine whether the up-regulation at 14 days of WOX genes affect shoot regeneration. The four types of cotyledon segments were cultured for 14 days and then sampled for qRT-PCR analysis. There were distinct expression patterns of WOX genes in the four cotyledon segment types (Fig. 8). All watermelon WOX genes were expressed at much higher levels in type-A segments. *ClaWUS*, *ClaWOX1*, *2*, *3*, *6* and *9* were expressed at much lower levels in type-B segments

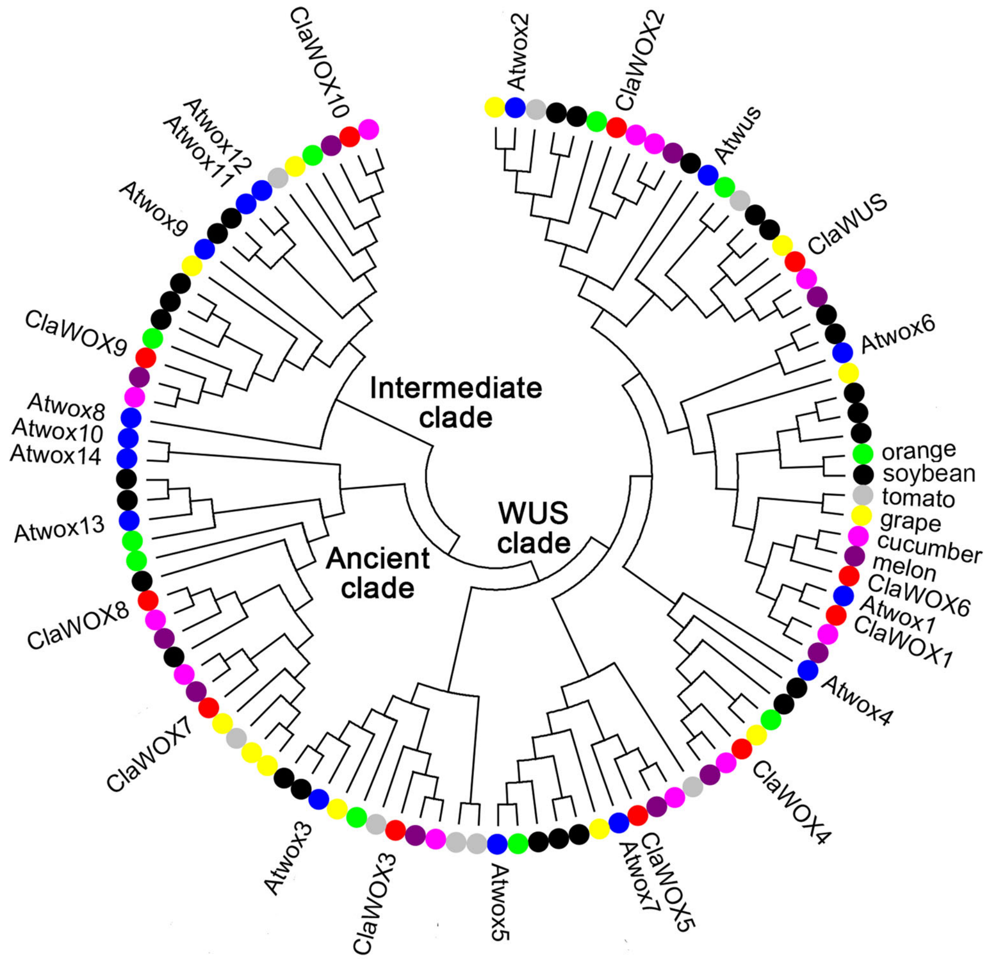

Fig. 4 Phylogenetic analysis of WOX genes in Arabidopsis, *orange*, soybean, tomato, grape, watermelon, cucumber and melon. Phylogenetic inference was conducted using MEGA 5.0 (Tamura et al. 2011). Branch width corresponds to support values. The *numbers* on the branches indicate the percentage of 1000 bootstrap replicates that support the node with only values >50 % reported. The Arabidopsis proteins are shown in *blue*, watermelon in *red*, cucumber in *pink* and melon in *black*. The three subclades are colored: WUS in *green*, ancient in *purple* and intermediate in *yellow*

than type-C and -D. Additionally, expression of WOX genes differed slightly between type-C and -D segments.

Discussion

Cucurbitaceae genomes possess smaller scales of WOX genes than Arabidopsis

We successfully identified 11 watermelon WOX genes, which supplement WOX family in the plant kingdom. Previous studies reported a large number of WOX genes in other plants, such as Arabidopsis, poplar, rice, maize and cucumber (Lian et al. 2014; van der Graaff et al. 2009)—on average there were >10 WOX genes in these plants. As we know, the gene family scale in genome was influenced by whole-genome duplication (WGD) (Huang et al. 2009). There were 11 WOX genes in watermelon and melon, while 12 in cucumber because of duplication in *AtWOX2* subgroup. We inferred that 11 WOX genes might be the smallest number needed for normal growth and development of Cucurbitaceae plants. There were 1–3 introns for each watermelon WOX gene, consistent with a previous report (Zhang et al. 2010); however, we did not find any watermelon WOX genes without an intron.

Conserved sequence of WOX proteins in Cucurbitaceae crops

In this study, phylogenetic analysis of WOX protein sequences revealed that Cucurbitaceae WOX proteins shared a higher similarity to each other than did those in Arabidopsis or other eudicot plants. The homeodomain of proximate Cucurbitaceae sequences showed high conservation, which indicated a close evolutionary history. Additionally, the ancient clade Cucurbitaceae WOXs were more distinct than those of Arabidopsis, which might indicate a longer evolutionary history than the other clades (Lian et al. 2014). The comparative analysis of homeodomain amino acids further supported this proposal. The conserved amino acids in the homeodomain and WUS box of WUS clade WOXs were consistent with previous studies (Lin et al. 2013b; Zhang et al. 2010). Moreover, the absence of helix1 in *ClaWOX10* and helix3 in *CsaWOX5-*

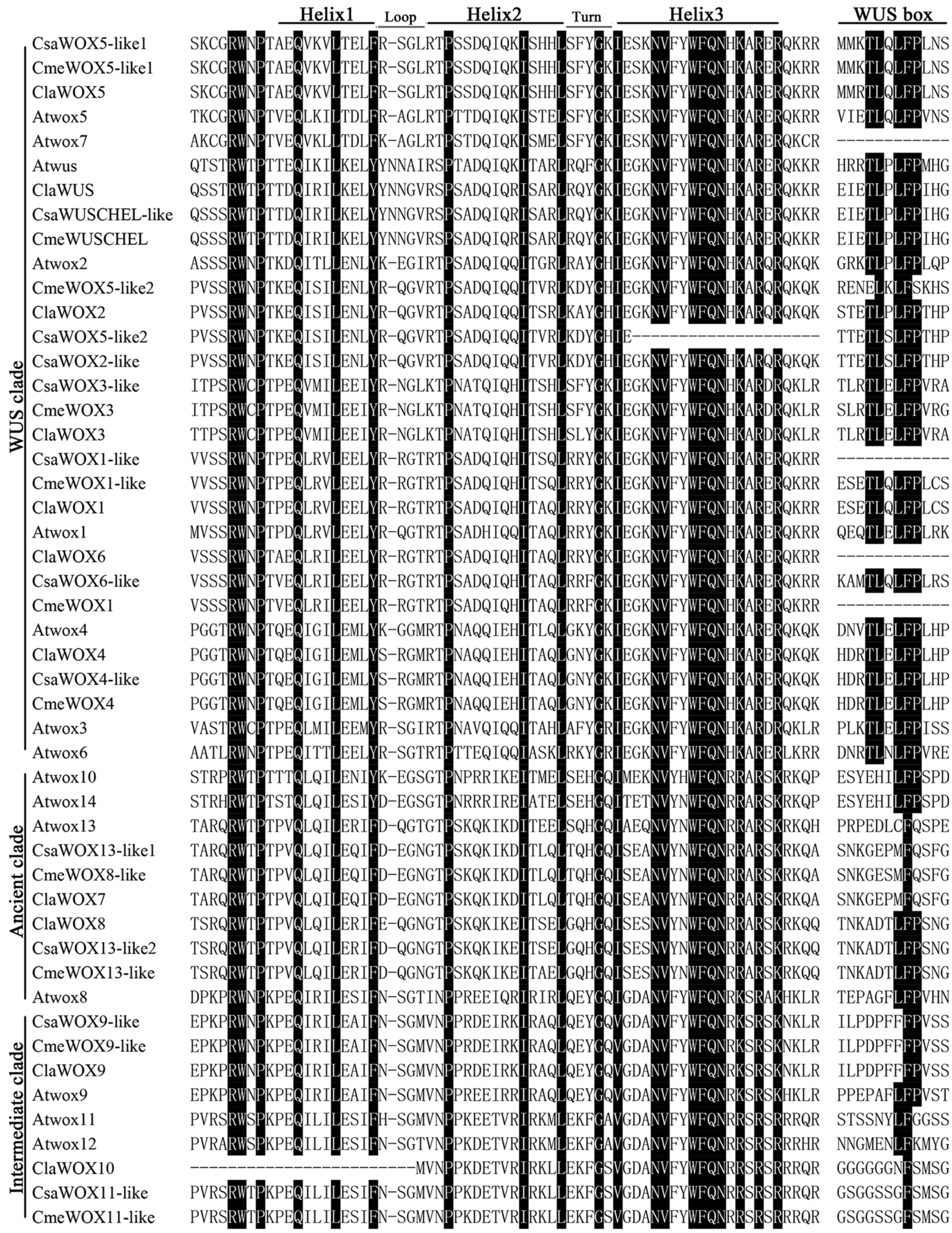

Fig. 5 Comparative analysis of the WOX homeodomain and WUS *box* sequences in Arabidopsis, watermelon, cucumber and melon. The homeodomain of WOX family contains the helix-loop-helix-turn-helix structure (Kamiya et al. 2003). The *shaded blocks* indicate highly conserved residues by the alignment of homeodomains and WUS boxes in the four angiosperms

like2 were interesting findings. The reasons for this phenomenon and their functions require further study. The absence of WUS box in *CsaWOX1-like*, *ClaWOX6* and *CmeWOX1* suggested that they might share the same function as *AtWOX7* (Lin et al. 2013a).

WOX genes were actively expressed in watermelon tissues

The expression patterns of WOX genes in watermelon tissues gave us a better understanding of their functions. Most watermelon WOX genes were actively expressed in root, leaf, stem, bud and flower, respectively. The higher expression of *ClaWOX4*, *7* and *8* compared to other WOXs in roots indicated that they might have the same function as the key regulation factor *AtWOX5* (Sarkar et al. 2007) in the root apical meristem. The high expression of *ClaWOX10* barely in leaf suggested it had an important role similar to *Medicago truncatula STENOFOLIA* (Zhang et al. 2014) in leaf development. However, there is still much work required to identify key WOX regulators during stem, bud and flower development: such as *AtWOX4* (Hirakawa et al. 2010; Ji et al. 2010) in the vascular meristem; *AtWUS* (Mayer et al. 1998; Schoof et al. 2000) in shoot apical meristem; and *AtWOX2*, *8* and *9* (Breuninger et al. 2008; Haecker et al. 2004; Wu et al. 2007) in embryonic

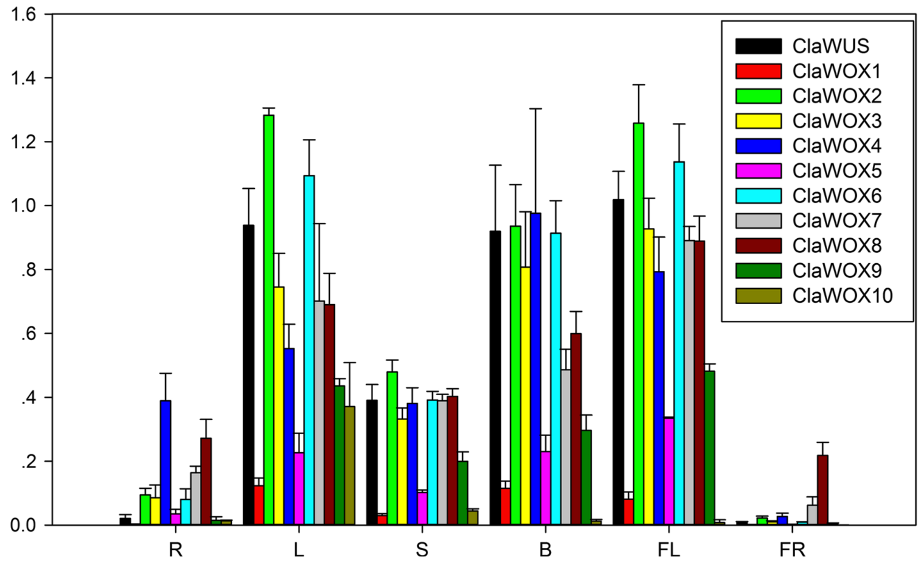

Fig. 6 Expression patterns of WOX genes in watermelon tissues. The *R*, *L*, *S*, *B*, *FL* and *FR* of *X*-axis represent root, leaf, stem, bud, flower and fruit, respectively

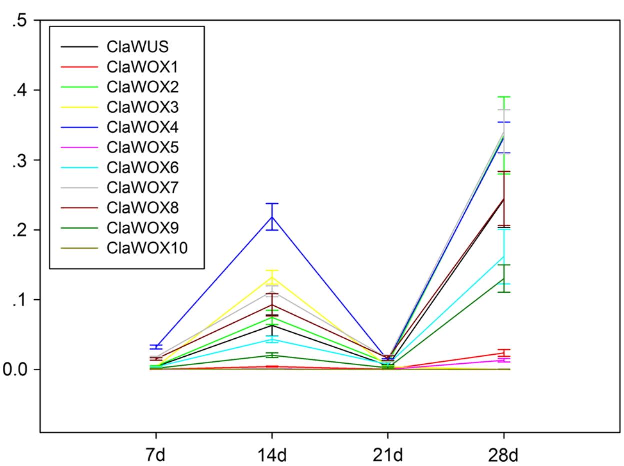

Fig. 7 Expression level of WOX genes in type-D of watermelon cotyledon segments at different development stages

development. Furthermore, *ClaWOX8* showed distinct expression in watermelon fruit compared to other WOXs. *AtWOX13* was reported to promote replum formation in fruit (Romera-Branchat et al. 2013). Previous studies on watermelon fruit mainly focused on functional genes that regulate metabolite profiles (Grassi et al. 2013; Guo et al. 2011). Studying the dynamics of WOX genes might bring a new perspective to understanding of the mechanisms of watermelon fruit and zygote development.

WOX genes were differentially expressed during adventitious shoot regeneration of watermelon

Watermelon cotyledons are regarded as the best resource of explants for adventitious shoot regeneration (Compton et al. 2004). We cultured cotyledon segments of diploid watermelon inbred line A7 to evaluate adventitious shoot regeneration ability of the genotype. Phytohormones and genotype are reportedly the most important factors affecting shoot regeneration (Akasaka-Kennedy et al. 2005; Landi and Mezzetti 2006; Pourhosseini et al. 2013; Sul and Korban 2005; Wang et al. 2013b). Surprisingly, only proximal cotyledon segments (type-D) showed a high shoot-regeneration frequency under the same culture conditions (Fig. 1). Few molecular studies have attempted to explain this phenomenon although it was discovered a decade ago (Compton 2000). WUS genes were reported to promote shoot regeneration (Chatfield et al. 2013; Li et al. 2011; Rashid et al. 2007) and thus we investigated the expression modulations of watermelon WOX genes during the shoot regeneration process. Most WOX genes were up-regulated when incubated for 14 days, indicating shoot meristem formation at this time point (Motte et al. 2014). The up-regulation of WOX genes at 28 days might be caused by shoot apical meristem maintenance (Mayer et al. 1998; Schoof et al. 2000).

We further analyzed the expression of WOX genes in the four types of cotyledon segments when cultured for 14 days. The results showed distinct expression patterns of WOX genes in type-A and -B segments, both of which generated few adventitious shoots. Overexpression of *WUSCHEL* could induce somatic embryogenesis and ectopic morphogenesis (Arroyo-Herrera et al. 2008; Bouchabke-Coussa et al. 2013; Xu et al. 2005). These processes might interrupt normal shoot regeneration in type-A segments. In type-B segments, *ClaWUS*, *ClaWOX1*, *2*, *3*, *6* and *9* might be expressed at too low levels to maintain the shoot apical meristem (Carles and Fletcher 2003; Schoof et al. 2000). Watermelon WOX genes had

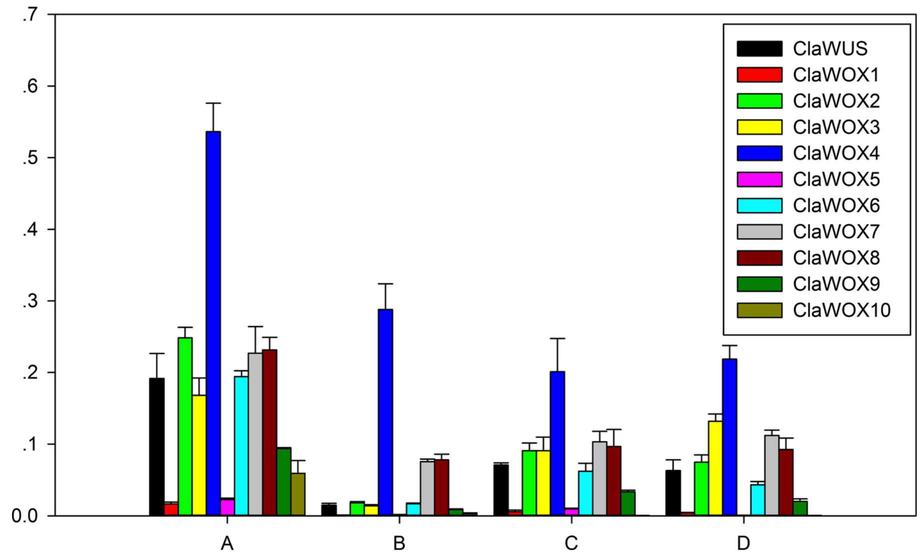

Fig. 8 Expression level of WOX genes in four types of cotyledon segments when incubated for 14 days

moderate and almost identical expression patterns in type-C and -D segments; however, there was a large difference between shoot-regeneration frequencies of these two types. This phenomenon might be caused by the higher expression of *ClaWOX3* in type-D compared to type-C, and more molecular evidence is needed to reveal the functions of WOX genes during shoot regeneration (Cary et al. 2002).

Conclusion

The results of this study provide a genomic framework for further research on watermelon WOX genes and contribute to understanding of the evolutionary mode for WOX genes in Cucurbitaceae crops. The qRT-PCR analysis demonstrates the active expression of the 11 WOX genes in watermelon tissues, and provides new evidence for WOX genes acting as conserved factors during watermelon development. Moreover, the distinct expression profiles of WOX genes during shoot initiation led to different shoot regeneration abilities. This work gives us an overview of how WOX genes regulate shoot regeneration. The interrelations of WOX genes, phytohormone and other transcription factors during this process will be the focus of future studies.

Author contribution statement Na Zhang, Dingxiang Peng and Xing Huang designed the experiment. Dingxiang Peng and Yuhong Sun provided guidance on the whole study. Na Zhang prepared materials and RNA samples. Xing Huang conducted qRT-PCR validation. Yaning Bao, Lijun Liu and Xia An helped in material preparation. Jie Chen and Lunjin Dai helped in RNA preparation. Na Zhang and Xing Huang carried out the data analysis and wrote the manuscript. Bo Wang revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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