ORIGINAL ARTICLE

Genome-wide identification and expression profiling of *WUSCHEL-related homeobox* (*WOX*) genes confer their roles in somatic embryogenesis, growth and abiotic stresses in banana

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Abstract

Plant-specific WUSCHEL-related homeobox (WOX) transcription factors are known to be involved in plant developmental processes, especially in embryogenesis. In this study, a total of thirteen *WOX* members were identified in the banana (*Musa acuminata*) genome (*MaWOX*) and characterized for *in-silico* analysis. Phylogenetic analysis revealed that these genes were divided into three clades (ancient, intermediate and modern) which reflected the evolutionary history of WOX families. Furthermore, modern clade members have shown higher variations in gene structural features and carried unique conserved motifs (motif 3 and motif 4) when compared to the members of other clades. The differential expression of all 13 *MaWOX* was observed in early (embryogenic cell suspension (ECS), multiplying ECS, germinating embryos, young leaflet and node of germinated plantlets) and late (unripe fruit peel and pulp, ripe fruit peel and pulp) developmental stages of banana cultivar Grand Naine. The maximum expression of *MaWOX6* (18 fold) and *MaWOX13* (120 fold) was found during somatic embryogenesis and in unripe fruit pulp, respectively. Moreover, numerous *cis*-elements responsive to drought, cold, ethylene, methyl jasmonate (MeJA), abscisic acid (ABA) and gibberellic acid (GA) were observed in all *MaWOX* promoter regions. The subsequent expression analysis under various abiotic stresses (cold, drought and salt) revealed maximum expression of the *MaWOX3* (830 fold)*, MaWOX8a* (30 fold) and *MaWOX11b* (105 fold) in salt stress. It gives evidence about their possible role in salt stress tolerance in banana. Hence, the present study provides precise information on the *MaWOX* gene family and their expression in various tissues and stressful environmental conditions that may help to develop climate-resilient banana plants.

Keywords Abiotic stress · Banana · Somatic embryogenesis · Genome-wide identification · Gene expression

Introduction

Wuschel related homeobox (WOX) gene family belongs to the homeodomain (HD) superfamily which encodes one of the largest groups of transcription factors containing 60–66 amino acids conserved DNA binding domain (Alvarez et al. 2018). The homeobox (HB) transcription factors have been classified into 14 classes in plants which are HD-ZIP I-IV, KNOTTED-1 like homeobox (KNOX), plant homeodomain (PHD), DDT, nodulin homeobox gene (NDX), luminide-pendens (LD), BEL, wuschel related homeobox (WOX) and plant interactor homeobox (PINTOX), SAWADEE and plant zinc finger (PLINC) (Mukherjee et al. 2009; Alvarez et al. 2018). Evolutionary studies indicate that the *WOX* gene family of HB transcription factors have diverged into three clades. A total of 15 WOX proteins of the dicot model plant *Arabidopsis thaliana* have been classified into modern or Wuschel (WOX 1–7 and WUS proteins), intermediate (WOX 8, 9, 11 and 12 proteins), and ancient (WOX 10, 13 and 14 proteins) clades according to amino acid sequences of HD (Lian et al. 2014; Zhang et al. 2014).

Recent studies elucidate that the *WOX* genes have been shown to play a pivotal role in key developmental processes such as stem cell maintenance, embryo patterning and organ development in plants (Schoof et al. 2000; Ueda et al. 2011; Lin et al. 2013b). In *A. thaliana*, *WUS* (*AtWUS*) plays an

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important role in the maintenance of root and shoot apical meristem as well as vegetative to embryonic transition (Mayer et al. 1995; He et al. 2019). It also acts as a repressor in stem cell regulation and an activator in floral patterning (Ikeda et al. 2009). The overexpression of *AtWOX1* was reported to develop abnormal meristem which subsequently formed small leaves, low fertility and dwarf *A. thaliana* plants (Graaff et al. 2009; Zhang et al. 2011). *AtWOX3* plays a vital role in lateral organ formation by recruiting organ founder cells (Shimizu et al. 2009; Alvarez et al. 2018). *AtWOX2*, *8* and *9* are reported to be expressed during early embryonic development stages in plants (Alvarez et al. 2018; Wang et al. 2019). *AtWOX4* and *AtWOX14* are involved in cambial meristem differentiation and maintenance (Hirakawa et al. 2010; Liu et al. 2014). *AtWOX5* and *Populus trichocarpa WOX5a (PtWOX5a)* are known for their role in auxin-responsive regulation of stem cell differentiation in root apical meristem (Lopez-Moya et al. 2017; He et al. 2019). Four *PtoWOX* genes, when ectopically expressed in transgenic poplars; were reported to promote adventitious root formation (Liu et al. 2014). In rice, *OsWOX11* has been shown to modulate root development imparting enhanced drought resistance and is also involved in crown root emergence activation by cytokinin-auxin signaling integration (Zhao et al. 2009; Cheng et al. 2016). Therefore, the functional diversification of *WOX* genes makes them a research hotspot in plant developmental biology.

Bananas are perennial monocot herbs belonging to the Musaceae family and known as a staple food/fruit crop worldwide. Banana is considered the fourth most important global food crop while India is ranked first in the production of bananas with an average output of about 32 million tonnes per year (FAOSTAT 2020, http://faostat.fao.org). Total banana production in the world is reported as about 108.7 million tonnes in 2020 (FAOSTAT 2020, http://faostat.fao.org). Bananas are cultivated in the provinces of humid subtropics, humid tropics, and semiarid tropics, and some banana genotypes can grow up to an elevation of 2000 mean sea level (MSL). The selected cultivar in the present study i.e., Grand Naine is the most commonly grown commercially cultivated variety of banana worldwide. It is considered a member of the Cavendish banana cultivar group (AAA genome) and contributes not only to household food and nutritional security but also for income generation as a cash crop. Grand Naine is a high yielding variety amongst others in India and occupies the first position in production share with 63% (Devarajan et al. 2021). The major constraints for banana production are largely dominated by biotic and abiotic stresses (Tushemereirwe et al. 2004; Tripathi et al. 2008). Abiotic stresses such as salinity, extreme temperature, drought, flooding, low temperature, ultraviolet rays, nutritional imbalances, and oxidative stress results in yield losses. The productivity of the banana crop was significantly impacted by the drought and has been estimated in the range from 20 to 65%, particularly for bananas from east Africa with the AAA genome (van Asten et al. 2011). In case of biotic stresses, various viral, bacterial and fungal pathogens like banana streak virus (BSV), banana bunchy top virus (BBTV), *Ralstonia solanacearum* (bugtok and moko disease), *Fusarium oxysporum* f. sp. *cubense* (Fusarium wilt), and *Xanthomonas campestris* pv. *musacearum* (Xcm) (Xanthomonas wilt) etc. significantly affects the overall yield of banana cultivation (Tripathi et al. 2019).

The availability of the whole-genome sequence of banana enabled researchers to study the genome-wide evolution and divergence among the different members of various gene families (D'Hont et al. 2012; Davey et al. 2013). In the present study, we have identified 13 *WOX* (*MaWOX*) genes in the banana genome and characterized them using various bioinformatics tools. The basic local alignment search tool (BLAST) was used to categorize *MaWOX* genes in different groups. The structural features, conserved motifs and physicochemical properties were predicted using online tools like HMMER (http://www.ebi.ac.uk/Tools/hmmer), MEME (http://meme-suite.org/) and ExPASy (https://www.expasy.org/), respectively. The phylogenetic analysis was carried out to understand the evolutionary relationship of *MaWOX* with other plant species. Further, the differential expression analysis of all 13 *MaWOX* genes during somatic embryo's developmental stages, vegetative and fruit tissues have revealed their potential role in the growth and development of banana plants. The expression response of these genes was also performed in tissue culture raised banana plants under different abiotic stress conditions (cold, salt and drought). Further, promoter analysis was performed as the promoter's architecture and the presence of different *cis*-regulatory elements have also emerged as the critical factor that influences the tissue-specific expression level of a gene in terms of its transcript abundance in response to developmental phase or stress condition (Wang et al. 2019). *WOX* gene family has been explored in many plant species including arabidopsis, rice, poplar, tea, watermelon, wheat, *Brassica* and cotton (Zhang et al. 2015; Yang et al. 2017; Wang et al. 2019). In the present study, potential role of *cis*-elements in the promoter regions of predicted *WOX* genes in banana was analysed. However, studies highlighting the role of *WOX* gene family in banana are still lacking. Hence, intending to investigate the crucial role of this gene family, we have conducted genome-wide studies of *WOX* gene family in banana.

Materials and methods

Plant materials, growth conditions and stress treatments

To examine the tissue-specific expression of *MaWOX* genes, tissues from different development stages of the banana

cultivar (cv.) Grand Naine were collected from the experimental field of National Agri-Food Biotechnology Institute (NABI), Mohali, Punjab, India (310 m above sea level; Latitude 30° 47′ North; Longitude 76° 41′ East). The early development stages included in-vitro cultures viz., embryogenic cell suspension (ECS), multiplying ECS, germinating embryos, young leaf and nodal portion of germinated plantlet. The ECS was developed following the previously established protocol in our lab (Shivani et al. 2017; Shivani and Tiwari 2019). In short, immature male flowers were inoculated on callus induction medium containing 2, 4-dichlorophenoxyacetic acid (2, 4-D) under the dark condition at 27 °C. The embryogenic callus was isolated and inoculated in 2, 4-D (18 μM) and zeatin (0.2 μM) containing suspension medium and kept in dark at 27 °C with 90 rotations per minute agitation to generate ECS. The ECS was cultured on different semi-solid media for embryos multiplication, regeneration, germination and plantlets development. The fruit tissues (unripe peel, unripe pulp, ripe peel and ripe pulp) collected from the research field were considered as the late development stages.

For the gene expression analysis, tissue culture raised banana plants were treated under different abiotic stress conditions (cold, salt and drought). For cold stress, plants were shifted at 10 °C in the plant tissue culture chamber (Percival, USA). For the drought and salt stress treatments, plants were transferred to 10% (w/v) PEG-4000 and 150 μM NaCl solutions, respectively. The young leaves from control (untreated) and different treatments were harvested at 6 h (hour), 12 h, 24 h and 48 h. All treatments were performed with three independent biological replicates, and the samples were frozen in liquid nitrogen and stored at − 80 °C.

Identification of banana *WOX* (*MaWOX*) genes

The complete genome sequence of *Musa acuminata* (DH-Pahang v2) was downloaded from the banana genome hub (Droc et al. 2013; http://banana-genome.cirad.fr/). The WOX protein sequences of *A. thaliana, Oryza sativa* and *Zea mays* were downloaded from The Arabidopsis Information Resource (TAIR) (Swarbreck et al. 2007; http://www.arabidopsis.org), Oryzabase (Kurata and Yamazaki 2006; https://shigen.nig.ac.jp/rice/oryzabase/) and MaizeGDB (Lawrence et al. 2004; https://www.maizegdb.org/) databases. The BLASTP search was performed in the banana genome using WOX protein sequences from the selected plant species (Arabidopsis, rice and maize) as queries (E-value $< 10^{-10}$). The conserved domain database search (Marchler-Bauer et al. 2010) was used to confirm the presence of the homeobox domain (PF00046) in the extracted genes from the banana genome. Expasy program (Gasteiger et al. 2003) was used to calculate parameters like molecular weight, peptide length and isoelectric point in the identified protein sequences. Each *MaWOX* gene has been given a unique name based on the BLAST results and their similarity with *AtWOX* genes (Table 1).

Phylogenetic analysis of MaWOX proteins

The WOX proteins from monocots (*Oryza sativa* and *Zea mays*) as well as from dicots (*A. thaliana*, *Citrullus lanatus,* and *Cucumis sativus*) were considered to analyse the evolutionary relationship with MaWOX proteins. Sequences of WOX proteins were extracted from respective genome databases Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/) for rice, MaizeGDB (https://www.maizegdb.org/) for *Z. mays*, TAIR (https://www.arabi

Table 1 Homeodomain positions in each *MaWOX* gene

WOX gene	NCBI-ID	Gene ID	Start (amino acid position)	End (amino acid position)
MaWOX1	Uncharacterized protein	Ma09_g04070.1	5	66
MaWOX2	Putative WUSCHEL-related homeobox 2	Ma10_g26500.1	15	76
MaWOX3	Uncharacterized protein	Ma02_g17830.1	5	66
MaWOX4	WUSCHEL-related homeobox 4	Ma08_g19830.1	34	94
MaWOX6	Uncharacterized protein	Ma08_g01860.1	31	96
MaWOX8a	WUSCHEL-related homeobox 8	Ma01_g11240.1	118	179
MaWOX8b	Uncharacterized protein	Ma09_g06490.1	47	108
MaWOX9a	WUSCHEL-related homeobox 9	Ma07_g14800.1	21	84
MaWOX9b	Uncharacterized protein	Ma08_g27800.1	48	109
MaWOX11a	WUSCHEL-related homeobox 11	Ma06_g17450.1	18	79
MaWOX11b	WUSCHEL-related homeobox 11	Ma10_g12990.1	18	79
MaWOX13	WUSCHEL-related homeobox 13	Ma04_g32050.1	75	136
MaWUS	Putative Protein WUSCHEL	Ma07_g07440.1	43	103

dopsis.org/) for *A. thaliana*, Dicots PLAZA 3.0 (Proost et al. 2015; https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/) for *C. lanatus* and *Cucumis sativus* v1.0. (https://genome.jgi.doe.gov/portal/cucumber/cucumber.home.html) for *C. sativus*. Multiple sequence alignment was done using Clustal X (Larkin et al. 2007; http://www.clustal.org/) and a neighbor-joining tree with bootstrap value 1000 was built using MEGA X software (Kumar et al. 2018; https://www.megasoftware.net/). The final tree was improved manually using iTOL tool (Letunic and Bork 2021; https://itol.embl.de/).

Gene structure, conserved motif and domain analyses

The coding and genomic sequences of the *MaWOX* genes were extracted from the banana genome hub (http://banana-genome.cirad.fr/) and uploaded on Gene Structure Display Server 2.0 (Hu et al. 2015; http://gsds.gao-lab.org/) to understand the exon–intron patterns and position of homeodomain in each *MaWOX* gene. The location of the homeodomain was extracted using the HMMER web server (Finn et al. 2011; http://www.ebi.ac.uk/Tools/hmmer). Further, MEME (http://meme-suite.org/) software was used to analyze the motifs present in *WOX* genes (Bailey et al. 2015). Motifs with E-value $< 10^{-10}$ were retained for analysis.

Subcellular localization and chromosomal distribution of *MaWOX* genes

The genomic coordinates of all *MaWOX* genes were extracted by running BLASTN against the banana genome database (http://banana-genome.cirad.fr/). The chromosomal localization of the genes was displayed using the MapGene2Chrom (http://mg2c.iask.in) tool (Jiangtao et al. 2015). The syntenic relationship of *WOX* genes in *A. thaliana* and *M. acuminata* was drawn using circa (http://circos.ca/) software (Musliner et al. 1993). The subcellular localization of MaWOX proteins is predicted using Plant-mPLOC software (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (Chou and Shen 2010).

Cis-acting element analysis of *MaWOX* promoters

The 2000 bp regions upstream of the *MaWOX*s coding region were downloaded from the NCBI database. Subsequently, *cis*-acting regulatory elements in the promoter sequences of *MaWOX* gene family were analyzed using Plant-CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002; Wang et al. 2019).

RNA isolation and cDNA synthesis

The total RNA was isolated from different tissues using the Spectrum™ Plant Total RNA Kit (Sigma–Aldrich, USA) and given DNase (Thermo Scientific, USA) treatment to remove DNA contamination. The integrity and size of RNA were analyzed by agarose gel electrophoresis. DNase treated RNA was quantified and cDNA was synthesized as per the user manual of cDNA synthesis kit (Thermo Scientific, USA).

Expression pattern analysis of *MaWOX* genes by quantitative real-time PCR (qRT-PCR)

The qRT-PCR was executed using ABI 7500 sequence detector fast real-time PCR machine (Applied Biosystems, USA). *Actin1* (GenBank Accession No. AF246288) was used as a housekeeping gene to normalize the variable expression of *MaWOX* genes in different tissues (Shivani et al. 2017). Primers were checked by amplification with conventional end-point PCR. A melting curve study was carried out using conventional qRT-PCR. The total volume of each reaction was kept 10 µl and components were as follows: (1) 1X SYBR Green Master mix (Applied Biosystems, USA), (2) 5 pmol of primers (forward and reverse), (3) 0.5 µl template (cDNA), and (4) sterile distilled H2O. Further, real-time PCR experiment was performed following the given parameters step (1) 95 °C 3 min, step (2) (95 °C 10 s, 55 °C 10 s, 72 °C 30 s) × 40 cycles, step (3) 95 °C 10 s, step (4) 65 °C 5 s and step (5) 95 °C 5 s following the thermal dissociation curve. The data were calculated in the form of fold change in expression with respect to control plants using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). While $\Delta\Delta Ct$ is defined as Ct target − Ct actin at time$_x$ − (Ct target − Ct actin) at time 0. Data was expressed in the form of a heat map using TBtools software (Chen et al. 2020). All the experiments were executed taking three biological replicates and were comprised of three technical replicates of each sample. For statistical significance, Statistical analysis was performed using Student's unpaired t-test and statistical significance was checked at $*P \leq 0.01$; $**P \leq 0.001$; $***P \leq 0.0001$.

Results

Genome-wide identification, chromosomal location and physicochemical property analysis of *MaWOX* genes

Total 13 *MaWOX* candidate genes were found in the banana genome by considering WOX proteins from *A. thaliana*,

Oryza sativa and *Zea mays* as query sequences in BLASTP search. The putative *MaWOX* genes which were common from all the species were considered for downstream analysis. The homeobox domain (PS50071) was identified in all 13 retrieved sequences using PROSITE, HMMER and NCBI-CDD based analyses (Table 1). All the identified genes have the complete open reading frame and are encoding WOX protein-like features. Hence, they have been designated as putative members of the *WOX* gene family in banana. Physicochemical properties of the predicted MaWOX proteins were depicted in Table 2. The length of the proteins varied between 173 amino acids (MaWOX9a) to 404 amino acids (MaWOX8b). The molecular weight of proteins ranged from 19.86 kDa (MaWOX4) to 43.56 kDa (MaWOX8b), while the isoelectric point from 5.22 (MaWOX13) to 9.54 (MaWOX4). It has been observed that all the predicted MaWOX proteins were localized in the nucleus highlighting their role as transcription factors. Furthermore, the chromosomal distribution of *MaWOX* genes has shown that all *MaWOX* genes are scattered on 8 chromosomes, except chromosome numbers 3, 5 and 11. The highest numbers of genes were positioned on chromosome 8 (3 genes), followed by chromosomes 7, 9 and 10 with two genes, while the rest of the chromosomes harbor one gene only. Fine mapping of *MaWOX* genes on the respective chromosome is given in Fig. 1.

Phylogenetic analysis of MaWOX proteins

Phylogenetic analysis was performed to establish the evolutionary correlation of *MaWOX* genes with other plant species. We created neighbor-joining phylogenetic tree using a total of 83 WOX proteins from *A. thaliana* (15), *Z. mays* (20), *O. sativa* (13), *C. lanatus* (11), *C. melo* (12), *C. sativus* (11) and *M. acuminata* (13). The phylogenetic evaluation shows that all the banana WOX proteins are divided into three clades i.e., ancient, intermediate and modern clades (Fig. 2a). The total number of WOX sequences in ancient and intermediate clades were 14 and 24, respectively. Modern clade occupied 45 members, which is the highest as compared to others. The ancient clade harbors WOX13 and WOX14, while the intermediate clade has WOX8, WOX9a, WOX11a and WOX12 proteins. Further, the modern clade disseminated into subclades based on the presence of specific WOX proteins such as WOX2, WOX3, WOX4, WOX5 and Wuschel. Each subclade in modern clade has at least one member of each of the seven plants (*A. thaliana*, *Z. mays*, *O. sativa*, *C. lanatus, C. melo, C. sativus* and *M. acuminata*), indicating their potential role throughout evolution. Furthermore, it was observed that each clade was comprised of both monocots and dicots. Moreover, synteny analysis using circa software showed that the orthologues of all the 13 *MaWOX* genes were present in *A. thaliana*. For synteny analysis, 13 *WOX* gene sequences from *M. acuminata* genome and 15 *WOX* gene sequences from *A. thaliana* have been studied. Among these 13 orthologous pairs of *M. acuminata*—*A. thaliana* gene sequences mapped on *A. thaliana* genome, where one on each Machr2, Machr4, Machr6; two on each Machr7, Machr9, Machr10 and four on Machr8 (Fig. 2b).

## Gene structure and conserved motif analysis

Exon-introns and conserved motifs have been analyzed to understand the evolution of the *MaWOX* gene family. The intron–exon association of *MaWOX* genes is represented in Fig. 3. All the *MaWOX* genes in banana contain exons as well as introns mainly in their coding regions. A maximum of three exons and two introns are present in

Table 2 Basic physico-chemical properties of *MaWOX* genes

WOX gene	Protein ID	Length (amino acid residues)	Molecular weight (Kda)	Isoelectric point (pI)	Aliphatic index	GRAVY
MaWOX8a	Ma01_p11240.1	295	32.49	5.96	57.86	− 0.773
MaWOX3	Ma02_p17830.1	194	21.57	8.23	59.38	− 0.695
MaWOX13	Ma04_p32050.1	211	23.11	5.22	66.68	− 0.618
MaWOX11a	Ma06_p17450.1	249	26.82	7.72	66.99	− 0.329
MaWUS	Ma07_p07440.1	283	31.82	6.36	57.28	− 0.822
MaWOX9a	Ma07_p14800.1	173	19.90	8.96	54.8	− 0.957
MaWOX6	Ma08_p01860.1	216	24.95	8.28	61.02	− 0.847
MaWOX4	Ma08_p19830.1	175	19.86	9.54	71.37	− 0.713
MaWOX9b	Ma08_p27800.1	394	41.91	7.00	69.62	− 0.303
MaWOX1	Ma09_p04070.1	189	21.48	8.43	57.88	− 0.59
MaWOX8b	Ma09_p06490.1	404	43.56	7.26	64.08	− 0.359
MaWOX11b	Ma10_p12990.1	228	24.79	7.82	69.65	− 0.401
MaWOX2	Ma10_p26500.1	235	25.92	8.64	54.77	− 0.75
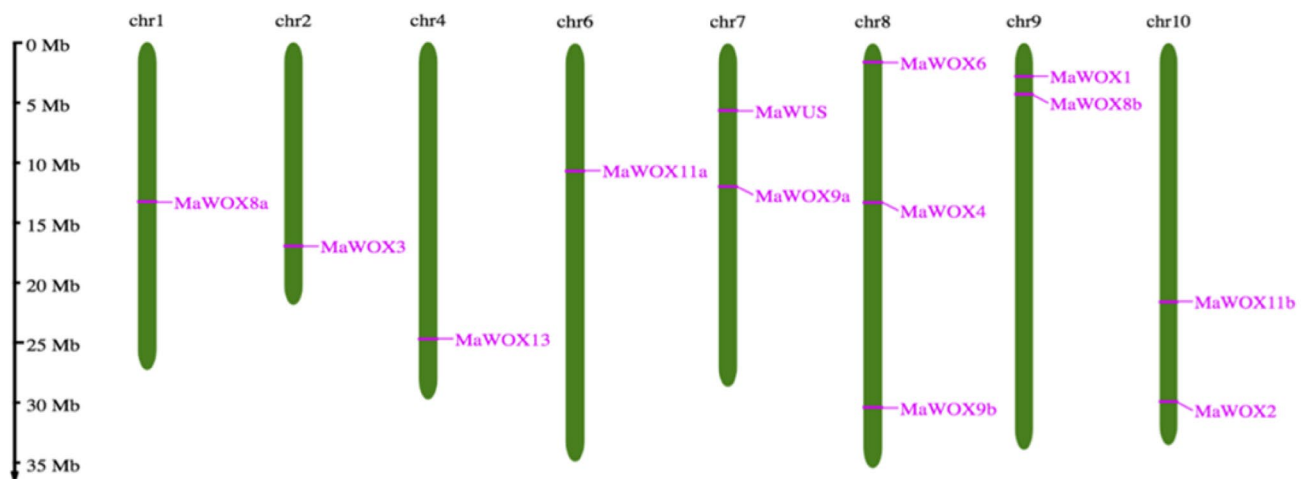
Fig. 1 Position of *WOX* genes on the banana chromosomes. The chromosome number is indicated at the top of each chromosome. The arrows show the position of *WOX* genes on the chromosome
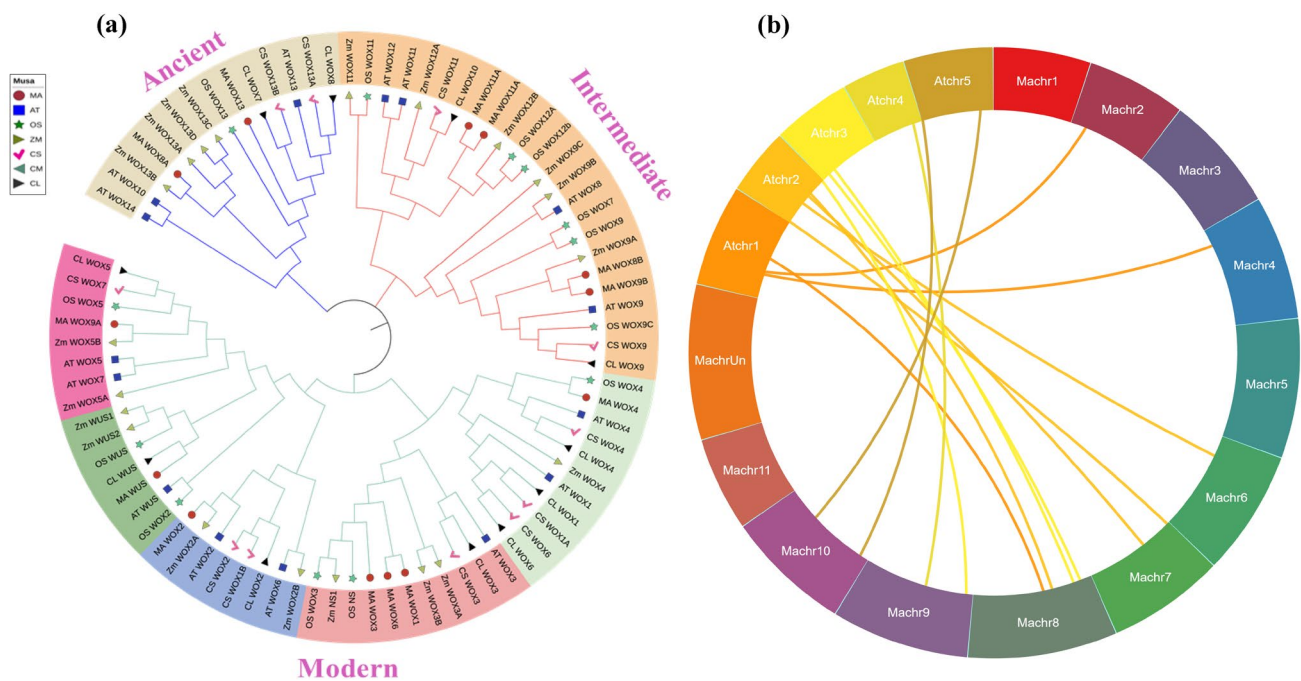

Fig. 2 Phylogenetic tree and synteny analysis. **a** Phylogenetic analysis of WOX proteins in *A. thaliana* (AT), *Z. mays* (ZM), *O. sativa* (OS), *C. lanatus* (CL), *C. melo* (CM), *C. sativus* (CS) and *M. acuminata* (MA). **b** Synteny analysis of *Arabidopsis thaliana* and *Musa acumi-* *nata* by CIRCOS Plot. Lines are ending with orthologs in *M. acuminata* and *A. thaliana.* Machr represents *M. acuminata* chromosomes while Atchr represents *A. thaliana* chromosomes

MaWOX genes. Total four conserved motifs were identified in each sequence and those were extracted using the MEME tool. Motifs 1 and 2 encoding for homeodomain are highly conserved in all MaWOXs protein sequences. A comprehensive list of predicted motifs in each MaWOX putative protein along with the sequence logo is represented in Fig. 4a, b.

Prediction of *cis*-regulatory elements

The *cis*-elements in the promoter regions of 13 *MaWOX* genes were analyzed to further understand the regulatory network control of the *MaWOX* family genes. In addition to the core *cis*-elements such as TATA-box and CAAT-box, several other *cis*-elements were distributed into three categories viz., plant growth and development, stress
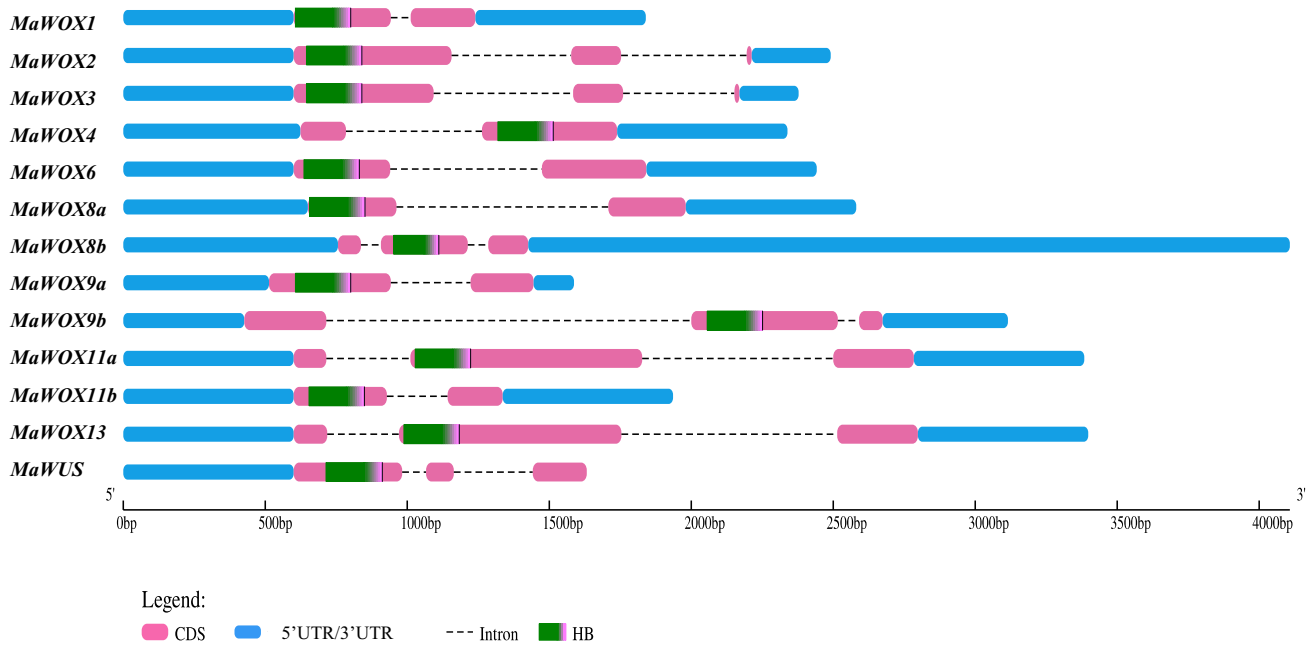
Fig. 3 Intron/exon configurations of *WOX* genes in banana. Boxes in pink color indicate the exon, blue colour boxes indicate 5´ and 3´UTR region and green color boxes, represent the homeobox domain in the coding region, and dashed lines indicate the intron
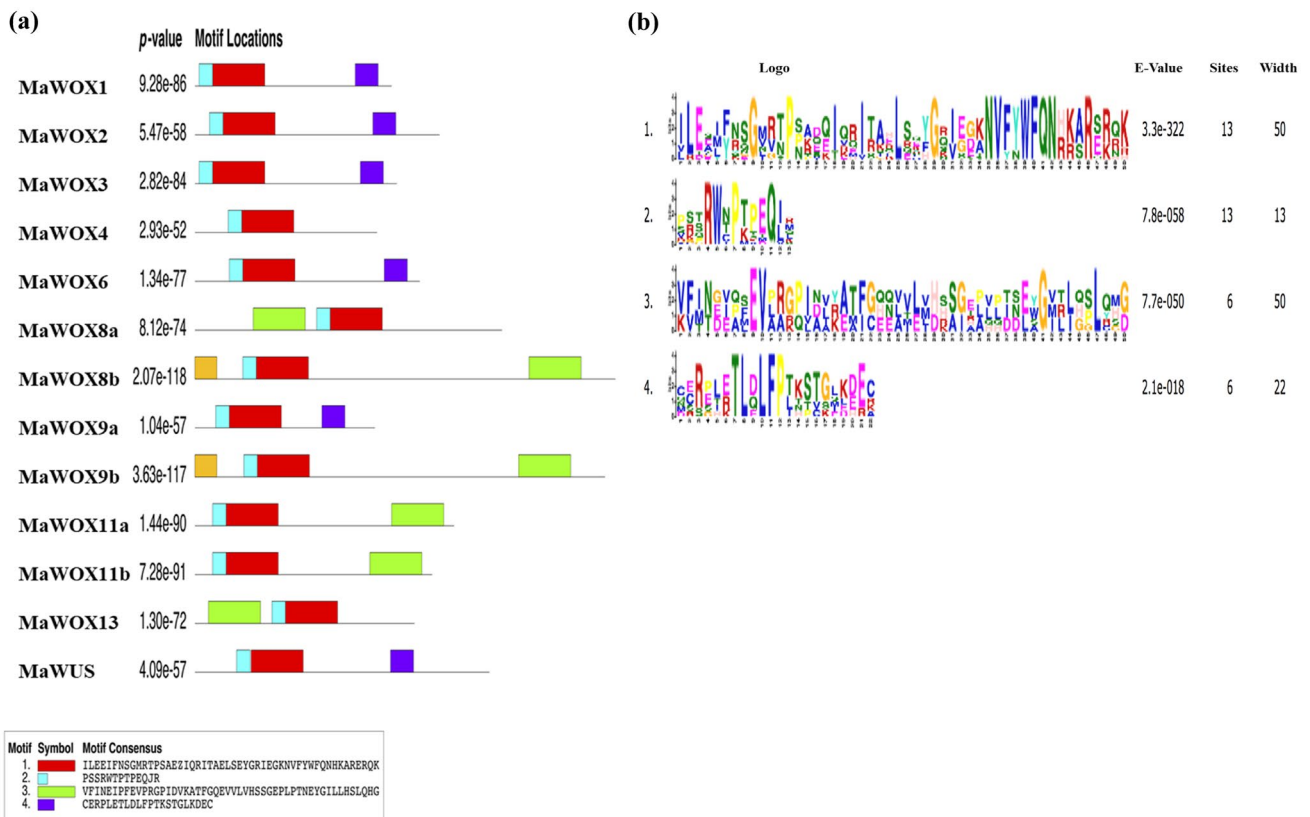
Fig. 4 MaWOX protein sequence analysis. **a** Representation of motif present in all MaWOX in banana. **b** Sequence logos of HD domains generated from all MaWOX. The *x*-axis represents the relative positions of the HD motifs and the *y*-axis represents the information content as measured in bits. 1,2,3 and 4 depict the different conserved motifs present in MaWOX protein sequences

responsiveness and hormone responsiveness (Fig. 5). In the first category (plant growth and development), *cis*-elements like light responsive (Box 4 and G-box), meristem specific expression (CAT-box), zein metabolism regulation (O2-site) and endosperm specific expression (GCN4-motif) were identified. In the second category (stress responsiveness), five types of *cis*-elements such as ARE for anaerobic induction, MBS and MYB for drought inducibility, MYC for low-temperature responsiveness and GC-motif for anoxic specific inducibility were identified. In the third category (hormone responsive), eight types of *cis*-elements involved in MeJA responsiveness (CGTCA and TGACG motifs), abscisic acid responsiveness (ABRE), salicylic acid responsiveness (TCA element), auxin responsiveness (AuxRR-core and TGA element) and gibberellin responsiveness (P-box and GARE motif) were observed. Therefore, considering the presence of the large number of *cis*-elements involved in different abiotic stresses, subsequent gene expression analysis was validated following cold, drought and salt stress treatments in banana plantlets.

Expression of the *MaWOX* genes in different tissues

The *MaWOX* genes were found to be differentially expressed in various tissues of banana. The expression of *MaWOX* genes was normalized with the expression of *Actin1* gene (GenBank Accession No. AF246288). The expression in the early developing tissues viz., ECS, multiplying ECS, germinating embryos, young leaflet and node of germinated plantlets revealed that *MaWOX6, MaWUS, MaWOX9a, MaWOX11a* and *MaWOX13* were highly expressed, while *MaWOX6* has shown maximum (18 fold) expression (Figs. 6a and S1). The expression in late-stage developing tissues (unripe fruit peel and pulp, ripe fruit peel and pulp)

showed that four *MaWOX* genes (*MaWOX3, MaWOX6, MaWOX8 and MaWOX13*) were highly expressed whereas *MaWOX13* showed 120 fold higher expression in unripe pulp tissue (Figs. 6b and S1). The *WOX4* and *WOX1* genes were not expressed or expressed at a low level in banana plant tissues. The differential expression of most of the *WOX* genes belonging to modern, intermediate and ancient clades have been noticed while comparing their expression with *WOX1* and *WOX4* genes of the modern clade. These results suggest that the different *WOX* genes might be playing a crucial role at various stages of plant growth and development.

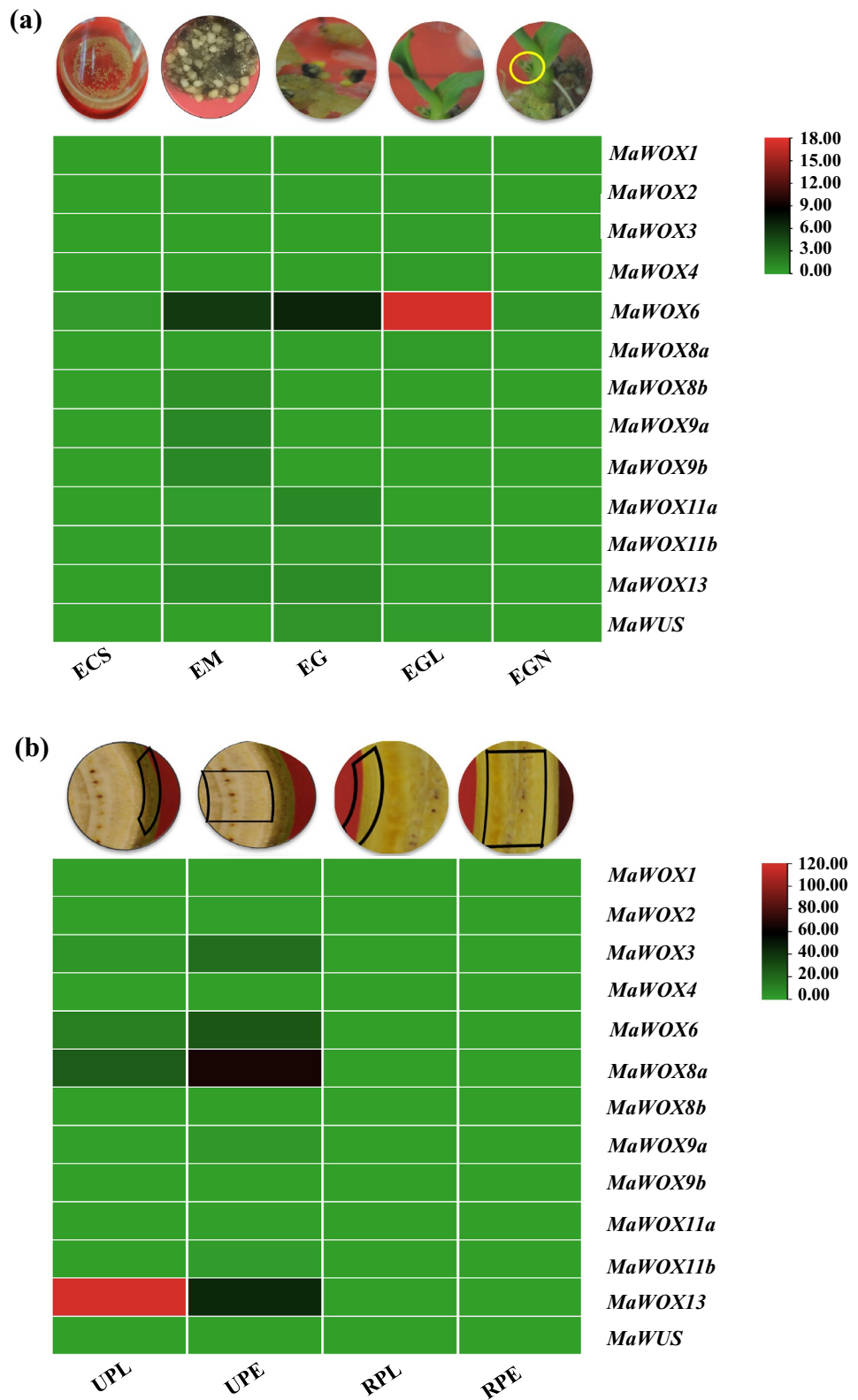## Expression analysis of *MaWOX* genes under abiotic stress conditions

To investigate the effects of drought, salt and cold stresses, we analyzed the expression profiles of the *MaWOX* genes at four-time points (0 h, 6 h, 12 h, 24 h and 48 h) after stress exposure. The expression of *MaWOX* at different time points was compared with the untreated (0 h) condition. Under drought stress, the expression of *MaWOX9a* was upregulated 35 fold at 6 h; then expression declined gradually to ~ sevenfold at 48 h (Figs. 7a and S2). Other *MaWOX* genes viz., *MaWOX1 and MaWOX3* were also expressed ≥ fivefold at 6 h and 12 h. In response to cold stress, only *MaWOX3* and *MaWOX8a* have shown expression 2 and fourfold, respectively at 6 h (Figs. 7b and S2). In salt stress treatment, expression of *MaWOX1, MaWOX4, MaWUS, MaWOX13* was significantly induced (≥ 11 fold) at 12 h compared to the untreated control. We noted expression of the *MaWOX3, MaWOX11b* and *MaWOX8a* upregulated by 830, 105 and 30 folds, respectively at 12 h as compared to control (Figs. 7c and S2). These observations have suggested that some of the *MaWOX* genes might have an important role

Fig. 5 Analysis of the *cis*-elements in the *MaWOX* genes. The numbers of different *cis*-elements in the *MaWOX* genes are indicated by numbers and different colors in the grid

	Hormone responsive element								Stress responsive element					Plant growth development element								
	ABRE	TGACG motif	CGTCA motif	TGA element	TCA element	P-box	GARE motif	AuxRR-core	MBS	ARE	MYB	MYC	GC-motif	BOX 4	CAAT-box	TCT-motif	GCN4-motif	TATA box	As-1	G-box	O2-site	CAT-box
MaWOX1	1	2	1	-	1	-	-	-	-	-	-	1	-	2	7	-	-	9	3	2	1	-
MaWOX2	-	1	-	1	-	1	-	-	-	-	-	1	-	-	10	-	-	17	1	2	-	1
MaWOX3	-	-	1	-	-	-	-	-	-	-	1	-	-	-	16	1	-	4	-	2	-	-
MaWOX4	-	1	-	-	1	1	-	-	2	-	4	1	-	1	18	1	-	16	1	1	-	-
MaWOX6	3	3	2	-	-	-	1	-	-	1	-	-	-	1	11	-	-	24	3	3	-	-
MaWOX8a	3	3	3	-	-	1	-	1	2	-	4	-	-	-	9	-	-	28	3	-	-	-
MaWOX8b	3	-	2	1	1	2	-	-	1	-	1	2	1	-	16	-	-	2	-	4	-	-
MaWOX11a	4	1	2	-	-	1	-	-	-	1	2	3	-	-	15	-	-	3	-	3	-	-
MaWOX11b	1	1	1	1	1	1	-	-	1	-	2	-	-	-	6	-	-	3	1	2	1	-
MaWOX9a	-	-	-	1	-	-	-	-	1	1	1	1	2	-	11	-	-	1	-	1	-	3
MaWOX9b	-	1	2	-	1	-	-	-	-	-	-	1	-	-	17	-	-	24	-	1	-	1
MaWOX13	-	-	2	-	1	1	-	-	1	-	1	1	-	4	-	1	-	28	2	10	-	-
MaWUS	2	3	-	-	1	-	-	1	2	-	1	4	-	1	25	-	1	23	2	2	-	-

Fig. 6 The *MaWOX* genes expression patterns in **a** early and **b** late developmental stages of tissues. Early developmental stages viz., ECS (Embryogenic cell suspension), EM (multiplying ECS), EG (germinating embryos), EGL (Embryo germinated young leaflet), EGN (Embryo germinated young node). Late developmental stages viz., UPL (Unripe pulp), UPE (Unripe peel), RPL (Ripe peel) and RPE (Ripe pulp). The heat map was generated by TBtools software. Columns and rows in the heat map represent tissues and *MaWOX* genes, respectively. Color scale indicates relative fold change in gene expression calculated by qRT-PCR analysis using three independent replicates of each tissue. The colour towards the red side shows the higher expression of *MaWOX* genes

Fig. 7 Expression analysis of *MaWOX* genes during the exposure of various stress treatments in banana plants. **a** Drought stress (DS), **b** cold stress (CS) and **c** salt stress (SS) treatments. The heat map was generated by TBtools software. Columns and rows in the heat map represent stress treatments with time-points of tissue collection and *MaWOX* genes, respectively. Color scale indicates relative fold change in gene expression calculated by qRT-PCR analysis using three independent replicates of each treatment. The colour towards the red side shows the higher expression of *MaWOX* genes

Fig. 7 (continued)

(c)
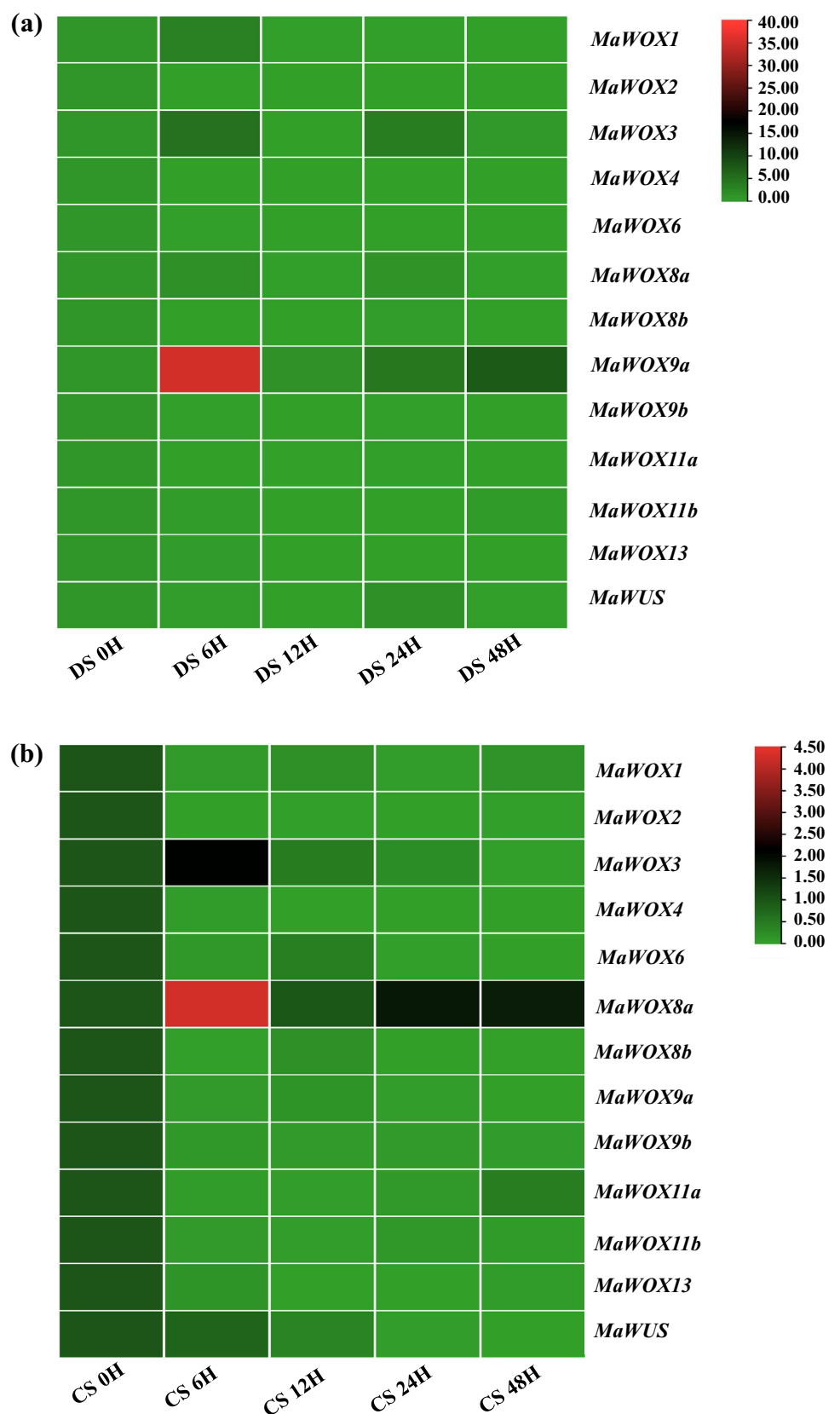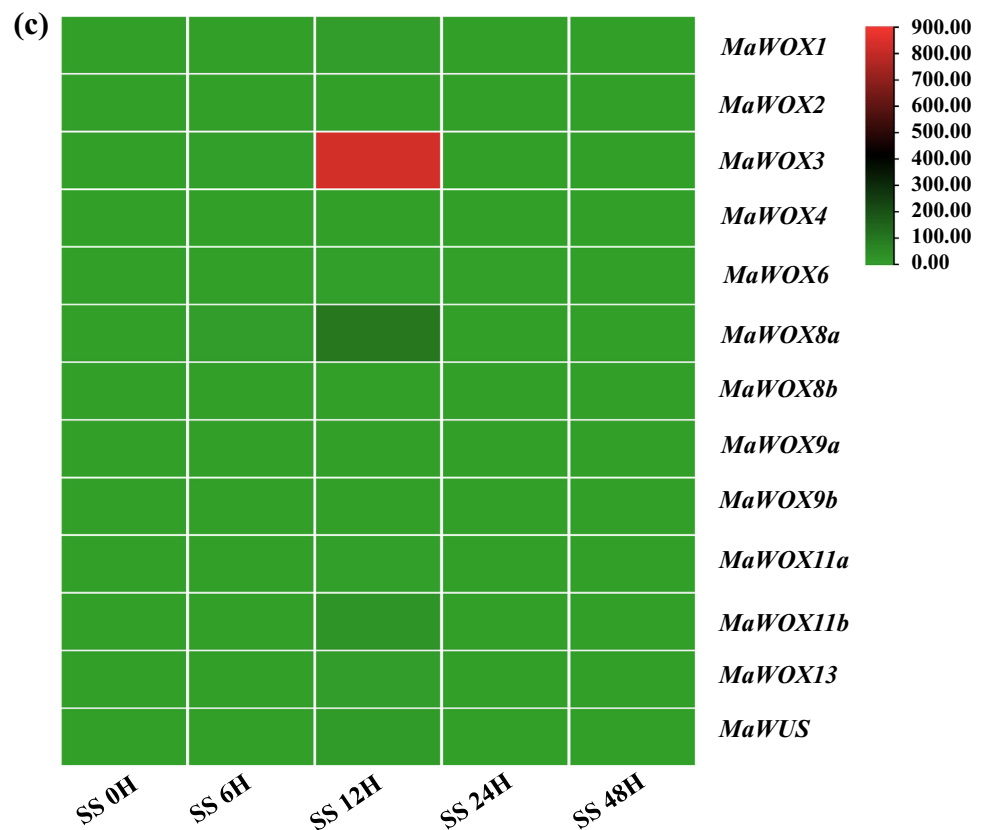
in the regulation of abiotic stresses, particularly in the salt stress condition at the early stage of treatment.

Discussion

The *WOX* gene family is crucial for plants as its members control cell division and differentiation, which in turn affects plant growth and development (Breuninger et al. 2008; Costanzo et al. 2014; Dolzblasz et al. 2016). Many plants have been the subject of prior research on the WOX gene family, including cotton, tobacco and maize (Zhang et al., 2010; Yang et al. 2017; Zhou et al. 2018). However, there were no reports on the identification of this gene family in banana. Our study is focused on studying the role of *MaWOX* genes in development processes as well as in abiotic stress condition (drought, salt, and cold) in *Musa acuminata* cv. Grand Naine. The expression of these genes in different tissues as well as under different stress conditions revealed their promising role in the growth, development and abiotic stress management in banana plants (Alvarez et al. 2018). The 13 *WOX* genes found in the banana genome are concomitant with previous reports in different plant species. For instance, 15, 12, 13, 21 and 11 *WOX* genes were reported in the genome of

A. thaliana, *P. trichocarpa*, *O. sativa*, *Z. mays* and *Sorghum bicolor*, respectively (Nardmann and Werr 2006; Zhang et al. 2010). The chromosomal localization suggested that all the *MaWOX* genes are distributed on 8 chromosomes, except chromosome numbers 3, 5 and 11. In the watermelon (*C. lanatus*), *CtWOX2* gene which showed similarity with *MaWOX2* found to be presented on chromosome 3.

The phylogenetic analysis with a total of 83 WOX proteins from *A. thaliana, Z. mays, O. sativa, C. lunatus* and *C. sativa* indicated that MaWOX proteins can be divided into three separate clades namely ancient, intermediate and modern clades. This pattern showed similarity to other plant species (Graaff et al. 2009). The banana derived WOX protein sequences presented in ancient (MaWOX13 and MaWOX8a), intermediate (MaWOX8b, MaWOX9b, MaWOX11a, and MaWOX11b) and modern (MaWOX6, MaWOX3, MaWOX1, MaWOX2, MaWOX4, MaWOX9a and MaWUS) clades showed consistent patterns of distributions as found previously in other plants (Lian et al. 2014). For example, *A. thaliana* derived AtWOX13, AtWOX10 and AtWOX14 (ancient clade), AtWOX11, AtWOX12, (intermediate clade) and AtWOX1, AtWOX2, AtWUS, AtWOX4, AtWOX5, AtWOX6 and AtWOX7 (WUS clade) proteins also showed the similar patterns of distribution in three clades.

The exon–intron distribution analysis predicted that there were 1–2 introns present in each *MaWOX* gene, that is similar to the arabidopsis, maize and rice (Zhang et al. 2010). Both the ancient and intermediate clades of *MaWOX* family genes have similar numbers of intron, as also reported previously in the *C. sinensis* plant, however, variations were noted in the genes belonging to the modern clade (Liu and Xu, 2018; Wang et al. 2019). The differences between the sequence of MaWOXs in the modern and other clades were further supported by the conserved motif analysis. Out of four (1–4), motifs 1 and 2 were found to be common in all MaWOX proteins. These two motifs are responsible for encoding the WOX homeodomain that contained helix-loop-helix-turn-helix structure consisting of fourteen amino acid residues i.e., Leu in helix 1, Pro, Ile, Ile and Leu in helix 2, Asn, Val, Trp, Phe, Gln, Asn and Arg in helix 3, Gly in loop and turn, were found to be presented in each MaWOX protein with high-level conservation. The third conserved motif (motif 3) was observed in the protein sequences belonging to the ancient and intermediate clades. This observation is also similar to WOX family members in the intermediate clade of walnut which is responsible for root development (Liu et al. 2014; Chang et al. 2020). All the members of the modern clade are having motif 4 (WUS-box motif), except in MaWOX4 which might be responsible for transcriptional inhibition and play a crucial role in flower and leaf development (Lin et al. 2013a).

The *cis*-regulatory elements analysis in MaWOXs promoter regions found to be responsive for hormonal (AuxRR-core, ABA, GA, Auxin Me-JA responsive), plant growth development (GCN4-motif, O2 site, CAT-box) and stress (ARE, MBS and GC-motif) responses in banana. The presence of a higher number of stress-responsive elements might be responsible for higher expression of *MaWOX8a*, *MaWOX11b* and *MaWOX3* during abiotic stress treatments. Similar observations have been reported previously in other plants such as *C. sinensis* and *G. hirsutum* (Wang et al. 2019).

Previous reports were mainly focused on characterizing role of *WOX* genes in plant growth, especially during embryogenesis (Zuo et al. 2002; Breuninger et al. 2008; Zhang et al. 2014). Previous study has shown that the expression of *WOX* genes in cotton was low under different developmental stages emphasising that they are mainly involved in embryogenesis and hence their expression was restricted (Yang et al. 2017). However, the present study demonstrated that *MaWOXs* are not only involved in growth and development but also responded to various abiotic stress conditions. For instance, *MaWOX13, MaWOX6, MaWOX3* and *MaWOX8* showed higher expression in at least four tissues i.e., ECS, regenerated cells, unripe pulp and unripe peel. The higher expression of four *MaWOX* genes i.e., *MaWOX9a, MaWOX13, MaWOX6* and *MaWOX3* in unripe peel, ripe peel and ripe pulp tissues suggested their expression is regulated during

fruit development stages in banana. Other *MaWOX* genes (*MaWOX1, MaWOX4, MaWOX11a*) showed either low (≤ twofold) or no expression in any of tested tissues. These observations indicated that the various *MaWOX* genes may have diverse role at different growth and development stages of banana. A previous study of *MaWOX* genes indicated that *MaWOX11* in transgenic rice plants enhances drought resistance by regulating root hair development (Cheng et al. 2016). *GhWOX13* gene is specifically expressed at a high level in cotton fiber as they influence cotton fiber development. Expression analysis showed that *GhWOX13a* and *GhWOX1b* genes were induced by multiple stresses, indicating that these genes might be involved during multiple stress (cold, heat and salt) conditions (Yang et al. 2017). In the present study, different time points of abiotic stress (drought, salt and cold) showed significant variation in *MaWOX* genes expression. Under drought stress, *MaWOX1*, *MaWOX9a* and *MaWOX3* upregulated their expression at 6 h and 12 h, while in salinity stress all the *MaWOX* genes upregulated their expression at 12 h in which *MaWOX8a, MaWOX11b* and *MaWOX3* depicted the highest expression. Banana is a highly salt sensitive crop and salt stress negatively impacts each physiological process that harmed its growth and development. As high soluble salt contents in soil causes accelerated breakdown of banana root system and ultimately compromise in the overall productivity (Turner et al. 2013; Sreedharan et al. 2015). However, most of the *MaWOX* genes were showing low expression in case of cold stress conditions. Henceforth, this study paved the way for functional validation of *MaWOX* genes that have shown promising expression responses at different developmental stages and under abiotic stress conditions.

Conclusion

Previous research has shown that the *WOX* gene family members play important roles in the regulation of plant growth and development. The results of the present study provide a genomic framework of *MaWOX* genes and contribute in understanding its evolutionary aspects as well as expression studies at different tissues developmental stages in banana. A total of 13 *MaWOXs* genes were identified in banana genome (DH-Pahang v2). *Cis*-acting element analysis of promoter region of *MaWOX* genes revealed their role in the regulation and synthesis of various plant hormones that are responsible for plant growth and stress response at different stages. The expression studies demonstrated in different banana tissues provided possible dimensions of *MaWOX* genes acting as conserved factors during the banana embryogenecity and various stress responses. Further, *MaWOX13, MaWOX6, MaWOX3, MaWOX8* and *MaWOX9a* showed higher expression (≥ 20 fold) in developing tissues suggesting their role during

growth and fruit development. While in salinity stress, *MaWOX8a, MaWOX11b* and *MaWOX3* depicted the highest expression (≥ 100 fold). The results have provided useful information for further studies on functional aspects of *MaWOXs*, particularly for the *MaWOX8a, MaWOX11b* and *MaWOX3* that may have the potential ability to respond under the salt stress conditions. Therefore the functional characterization of selected *MaWOX* genes (*MaWOX8a, MaWOX11b* and *MaWOX3)* could expand our understanding of their role in salt stress regulation resulting in amelioration of the incidence and consequences of salt stress in banana plants.

Declarations

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