PLANT SCIENCE TODAY, 2020
Vol 7(2): 164-171
https://doi.org/10.14719/pst.2020.7.2.703
ISSN 2348-1900 (online)

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RESEARCH ARTICLE

Genome wide characterization of WUSCHEL-related homeobox (WOX) gene family in *Apostasia shenzhenica*, a primeval orchid

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ARTICLE HISTORY
Received: 12 September 2019
Accepted: 19 February 2020
Published: 01 April 2020

KEYWORDS
Apostasia shenzhenica; WUSCHEL; WOX

GUEST EDITOR
Dr. Nyuk Ling Ma
Universiti Malaysia Terengganu

ABSTRACT

In the present study, we report identification and characterization of the plant-specific *WUSCHEL-related homeobox* (*WOX*) gene family in *Apostasia shenzhenica*, a primeval orchid. WOX proteins are DNA-binding *WUSCHEL-related homeobox (WOX)* encoding transcription factors that play critical role in zygote patterning, embryo development, organogenesis, florigenesis, stress responses etc. Ten putative *AsWOX* genes were predicted in the *A. shenzhenica* genome and were characterized by the presence of DNA-binding helix-loop-helix-turn-helix motif. AsWOX proteins were grouped into three clades, ancient, intermediate and WUS on the basis of sequence homology with *Arabidopsis thaliana* (AtWOX), *Oryza sativa* (OsWOX), *Phalaenopsis equestris* (PeWOX) and *Dendrobium catenatum* (DcWOX) and their phylogenetic relationship was established. Gene structure analysis revealed that three *AsWOX* genes had two introns, six genes had a single intron, and one gene was intron-less. Expression profiling in a variety of tissue (tubers, seeds and pollens) was analysed in light of the presence of specific cis-regulatory elements in the promoter region and their role in various developmental processes was discussed. Three dimensional structures were predicted for three selected AsWOX proteins representing the three clades. The present study provides insights to the role of AsWOX gene family in various vital developmental processes, establishes phylogenetic relationships with related plant species and provides a platform for functional validation of specific *AsWOX* genes.

Introduction

The *WUSCHEL-related homeobox* (*WOX*) gene family is involved in plant embryonic patterning, stem cell maintenance, organogenesis, florigenesis, somatic embryogenesis and stress responses (1-3). These genes encode plant specific DNA-binding homeobox transcription factors which are characterized by 60-66 amino acid (aa) residues long homeobox domain with embedded DNA-binding helix-loop-helix-turn-helix motif (4, 5). Based on sequence homology, WOX proteins can be classified into three clades, Ancient, Intermediate and WUS (1). The Ancient clade evolved earlier and can be found from algae to angiosperms. The Intermediate clade emerged after the origin of pteridophytes and is absent in algae and bryophytes. The WUS clade, on the other hand, is found only in angiosperms, indicating that it is the most advanced clade (1).

WUS gene was first identified in *Arabidopsis thaliana*, with roles in shoot and floral apices development by maintaining the stem cell potency (6). Later, several of WOX genes were functionally characterized in other organism ranging from algae to flowering plants, such as *Ostreococcus tauri*, *O. lucimarinus* and *Physcomitrella patens* (7), *Selaginella kraussiana* and *S. moellendorffii* (8), *Picea abies* (9), *Arabidopsis thaliana* (7), *Populus trichocarpa* (10), *Solanum lycopersicum* (11), *Monotropa hypopitys* (12), *Broussonetia papyrifera* (13), *Prunus persica*, *P. mume*, *Pyrus bretschneideri* and *Fragaria vesca* (14), *Vitis vinifera* (15), *Gossypium arboreum*, *G. raimondii* and *G. hirsutum* (16), *Cucumis sativus*, *C. melo* and *Citrullus lanatus* (17), *Oryza sativa*, *Zea mays* and *Sorghum bicolour* (10), *Ananas comosus* (18), *Phalaenopsis equestris* and *Dendrobium catenatum* (19), *Salix suchowensis* (20), *Camellia sinensis* (21), *Brassica napus*,

To cite this article: Ramkumar TR, Kanchan M, Sembi JK. Genome wide characterization of WUSCHEL-related homeobox (WOX) gene family in *Apostasia shenzhenica*, a primeval orchid. Plant Science Today. 2020;7(2):164-171. https://doi.org/10.14719/pst.2020.7.2.703

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Table 1. Characterization of AsWOX protein sequences

Gene	NCBI ID	AA	MW	pI	Ins	AI	GRAVY	Loc	SP	TMD
AsWOX12	PKA61831.1	204	22.48	9.41	72.46	75.05	-0.375	Nuclear	No	0
AsWOX11	PKA58526.1	148	15.83	9.5	67.26	72.43	-0.223	Nuclear	No	0
AsWOX9	PKA48738.1	349	37.31	6.31	62.41	74.76	-0.351	Nuclear	No	0
AsWOX13	PKA60098.1	245	27.74	8.2	46.17	58.49	-0.787	Nuclear	No	0
AsWOX2A	PKA63241.1	248	27.57	9.52	67.65	67.66	-0.697	Nuclear	No	0
AsWOX2B	PKA48554.1	258	27.26	5.99	66.67	50.08	-0.721	Nuclear	No	0
AsWOX3A	PKA49032.1	207	23.19	9.03	68.39	55.65	-0.757	Nuclear	No	0
AsWOX3B	PKA46270.1	215	23.86	7.11	60.11	66.84	-0.593	Nuclear	No	0
AsWUS	PKA53419.1	268	29.02	6.5	64.63	50.37	-0.724	Nuclear	No	0
AsWOX7	PKA52499.1	137	16.13	10.44	72.83	67.59	-0.958	Nuclear	No	0

Isoelectric point (pI), protein molecular weight (MW) in kDa, instability index (Ins), aliphatic index (AI) grand average of hydropathy (GRAVY), localization (Loc), signal Peptide (SP) transmembrane domain (TMD)

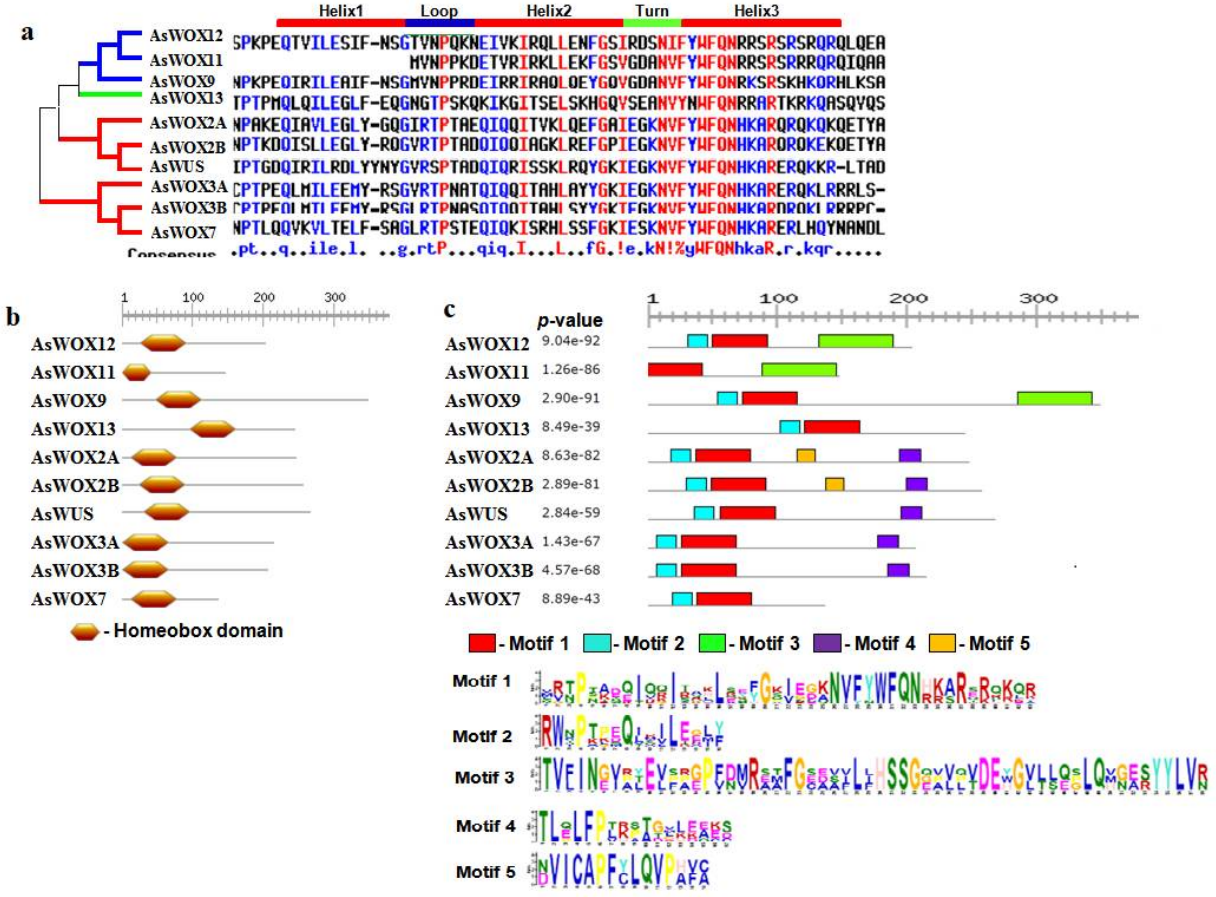

Fig. 1. Domain and motif analyses in AsWOX proteins: **a.** Multiple sequence alignment of AsWOXs, the DNA-binding helix-loop-helix-turn-helix region is marked; **b.** Representation of homeobox domain; **c.** Identified conserved motifs are marked in coloured boxes and sequence logo of these motifs showing degree of conservation at each amino acid position.

Brassica rapa and *Brassica oleracea* (22) and *Juglans regia* (23).

In post-genomics era, genome-wide characterizations of gene families are prevalent in crop plants, however, such studies are scarce in orchids as only a few orchid genomes have been sequenced so far (24-26). For the present work, *A. shenzhenica*, a primeval terrestrial orchid, was selected because of its evolutionarily significance as it represents the most primitive subfamily, Apostasioideae which has strong divergence from Orchidaceae. This taxa is characterized by actinomorphic flowers, indistinct labellum, rudimentary gynostemium, absence of pollinia and non-resupinating ovary which are contrasting to the general characteristics of orchids (27). Thus, it is important to study the plant in order to evaluate its evolutionary status and relationship. In this report, a genome-wide analysis was done to study physico-chemical characterization, structure prediction, phylogenetic relationships, analysis of *cis*-regulatory elements and spatio-temporal expression profiling of *AsWOX* gene family. The study would pave way for functional characterization of *WOX* genes and provide insights for their potential role in growth and development of orchids.

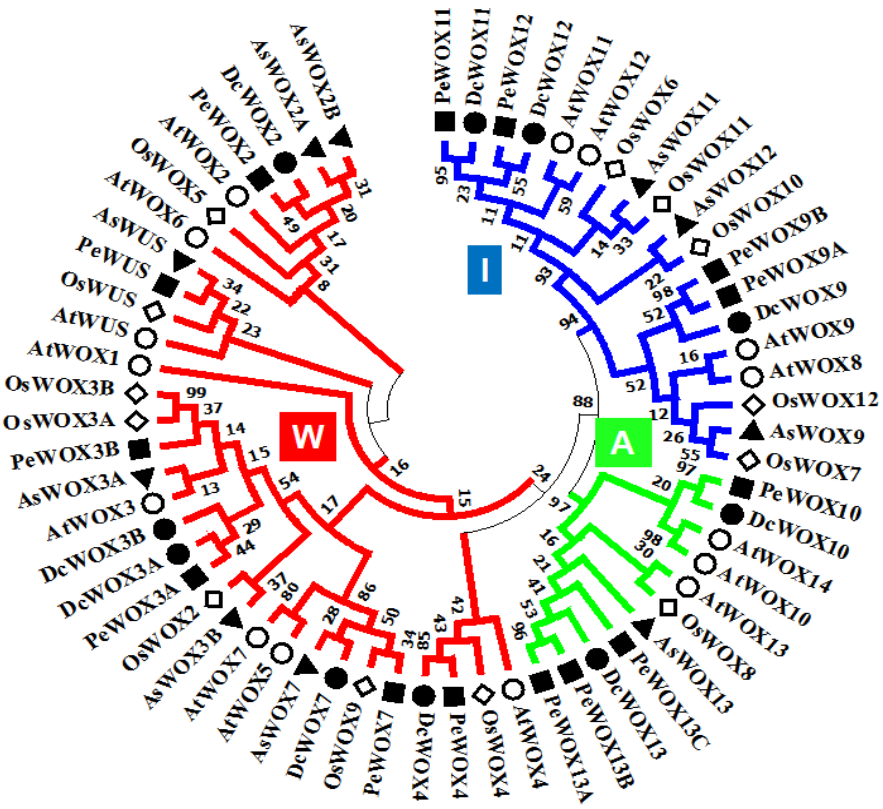

Fig. 2. Phylogenetic analysis of AsWOXs proteins: Phylogenetic tree with the sequences of AsWOX with PeWOX (*P. equestris*), DcWOX (*D. catenatum*), AtWOX (*A. thaliana*) and OsWOX (*O. sativa*) showing grouping into three distinct clades, ancient (A), intermediate (I) and WUS (W) marked in green, blue and red respectively.

Table 2. Gene scaffold and gene stretch region with NCBI ID of *AsWOX* genes

	Gene	Gene Scaffold	Gene Stretch
1	AsWOX12	fragScaff_scaffold_40	KZ451923.1:2177800-2179273
2	AsWOX11	fragScaff_scaffold_68	KZ451951.1:c777337-775757
3	AsWOX9	original_scaffold_327	KZ452209.1:176088-178201
4	AsWOX13	fragScaff_scaffold_59	KZ451942.1:c2215404-2208264
5	AsWOX2A	fragScaff_scaffold_26	KZ451909.1:307739-310274
6	AsWOX2B	original_scaffold_431	KZ452313.1:c950380-948698
7	AsWOX3A	fragScaff_scaffold_158	KZ452041.1:c426344-424274
8	AsWOX3B	original_scaffold_2507	KZ454389.1:c500918-499419
9	AsWUS	fragScaff_scaffold_115	KZ451998.1:c1564267-1563301
10	AsWOX7	fragScaff_scaffold_119	KZ452002.1:c793199-792786

The prefix 'c' in gene stretch region represents the presence of gene complementary strand

Materials and Methods

Identification of WOX family proteins

The WOX protein sequences of *Arabidopsis thaliana* (AtWOXs), *Oryza sativa* (OsWOXs**),** *Phalaenopsis equestris* (PeWOX) and *Dendrobium catenatum* (DcWOX) (1, 10, 19) were used as query sequences and Blastp was carried out against the NCBI derived *Apostasia shenzhenica* protein database; taxid:1088818). The retrieved AsWOX sequences were then analysed for the presence of WUSCHEL-related homeobox (pfam00046) domain using SMART server (28) and the domain architecture was constructed using Expasy - Prosite server (29). To locate the DNA-binding helix-loop-helix-turn-helix domain, multiple sequence alignment using MULTALIN tool (30) was done. The conserved motifs were identified using MEME suite server (31), with preset parameters (maximum number of motifs - 05, number of repetitions - any, optimum motif width - ≥6 and ≤ 200).

Phylogenetic analysis

Full length protein sequences (AsWOX, PeWOX, DcWOX, AtWOX and OsWOX) were initially aligned with MUSCLE program and the phylogenetic tree was then constructed using MEGA7 tool (32) by maximum-likelihood method at bootstrap value of 1000.

Physico chemical characterization

The AsWOX sequences were analysed using the Expasy-ProtParam server (33) to calculate the physico chemical properties such as molecular weight,

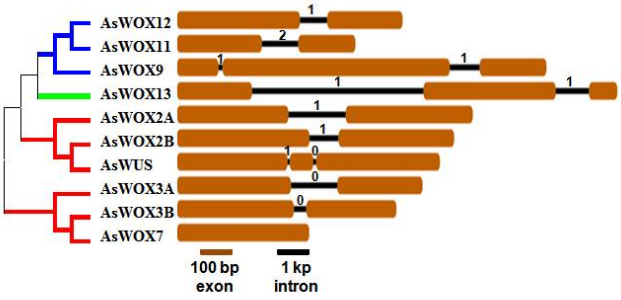

Fig. 3. Structural organisation of *AsWOX* genes: Exon-intron organization showing exons marked as boxes and introns as lines. Intronic phases 0 1 and 2 are also represented.

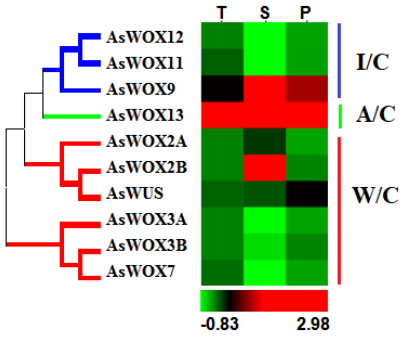

Fig. 4. Expression profile of *AsWOX* genes: Spatio-temporal expression of *AsWOX* genes shown in the Heat map in various tissues, tuber (T), seed (S), and pollen (P)

Table 3. Secondary structure and ligand binding sites in selected AsWOX proteins

Protein	Alpha helix	Random coil	Extended strand	Beta turn	Ligand	Ligand binding sites
WOX13	32.24 (79)	52.65 (129)	8.98 (22)	6.12 (15)	Nucleic acid	100, 101, 102, 103, 104, 106, 147, 150, 151, 154, 158
WOX9	22.92 (80)	61.60 (215)	11.17 (39)	4.30 (15)	Nucleic acid	106, 109, 110
WOX2B	20.16 (52)	62.79 (162)	11.63 (30)	5.43 (14)	Glycerol	82, 85

Values indicate: % of amino acids (No. of amino acids are shown in brackets)

aliphatic index, instability index, pI and grand average of hydropathicity (GRAVY). Online tools such as Signal P.4.0 (34) and TMHMM v.2.0 (35) were used to detect the signal peptide and transmembrane region CELLO v.2.5 (36), WoLF PSORT (37), TargetP-2.0 (38) and Plant-mPLoc (39) were used to predict the sub-cellular localization.

Gene structure and cis-regulatory elements analyses

For each AsWOX protein sequence, coding sequence (CDS) and gene sequence were retrieved from NCBI database. The gene structure with exon-intron display was drawn using Gene Structure Display Server 2.0 (40). The promoter regions were retrieved from 1.5 kb upstream sequences of the genes, from NCBI database and the presence of *cis*-regulatory elements was confirmed using PLACE server (41). Promoter elements were further analysed to identify common and specific promoter elements using Venn Diagram tool.

Gene duplication events and ortholog prediction

The sequence similarity index among *AsWOX* CDS sequences were obtained using MUSCLE tool (42), and the genes sharing ≥ 80% identity were considered duplicated. To predict the ortholog proteins, a local NCBI BLASTp search was performed with each candidate AsWOX protein sequences querying independently against the WOX protein sequences of target species i.e. *A. thaliana* (AtWOX), *O. sativa* (OsWOX), *P. equestris* (PeWOX) and *D. catenatum* (DcWOX), and the orthologs were identified.

Expression analysis

The *AsWOX* CDS sequences were used for BLASTn search against the high throughput RNA-seq data for various developmental stages like tuber (SRX2938654), seed (SRX2938653) and pollen (SRX2938652) of *A. shenzhenica* available from NCBI database (26). The hits were counted and the RPKM values (Reads per Kilobase per Million) were calculated using the formula RPKM = (C x 10^9) / (N x L), in which C represents number of hits for the candidate gene, N represents total mapped reads in the concerned RNA-seq experiment and L represents the length of gene in base-pairs (43). The heat maps to visualise the differential expression of were generated using Hierarchical Clustering Explorer 3.5 (44).

Molecular modelling

The secondary structure of AsWOX proteins was analysed using the online tool SOPMA secondary structure prediction (45), for the presence of alpha helices, random coils, beta turns and extended strands. For prediction of tertiary structure, I-Tasser, a molecular modelling tool (46) was used by simulating with top 10 closely related homologous templates in PDB (Protein Data Bank) with the help of BS-scores, TM-scores, IDEN coverage. The DNA-bindng site prediction was based on identification of analogs with similar binding sites with BS-score value of > 0.5.

Results and Discussion

Identification and characterization of WOX gene family proteins

WUS gene was the first *WOX* gene family member identified in *Arabidopsis thaliana* (*AtWUS*) and characterized to be involved in meristem maintenance in shoot and floral apices (6, 47) and later this gene was shown to regulate floral patterning as well (48). In general, *WOX* gene family members regulate zygote and embryonic patterning and development, organogenesis, florigenesis and participate in stress responses (1, 3). To identify WOX members in *Apostasia shenzhenica* (AsWOX), extensive BLASTp was carried out and a total of 10 AsWOX protein sequences were identified (Table 1). No splice variants for any of the *WOX* genes were identified. The size of the *WOX* gene family in *A. shenzhenica* (10 genes) was comparable with the

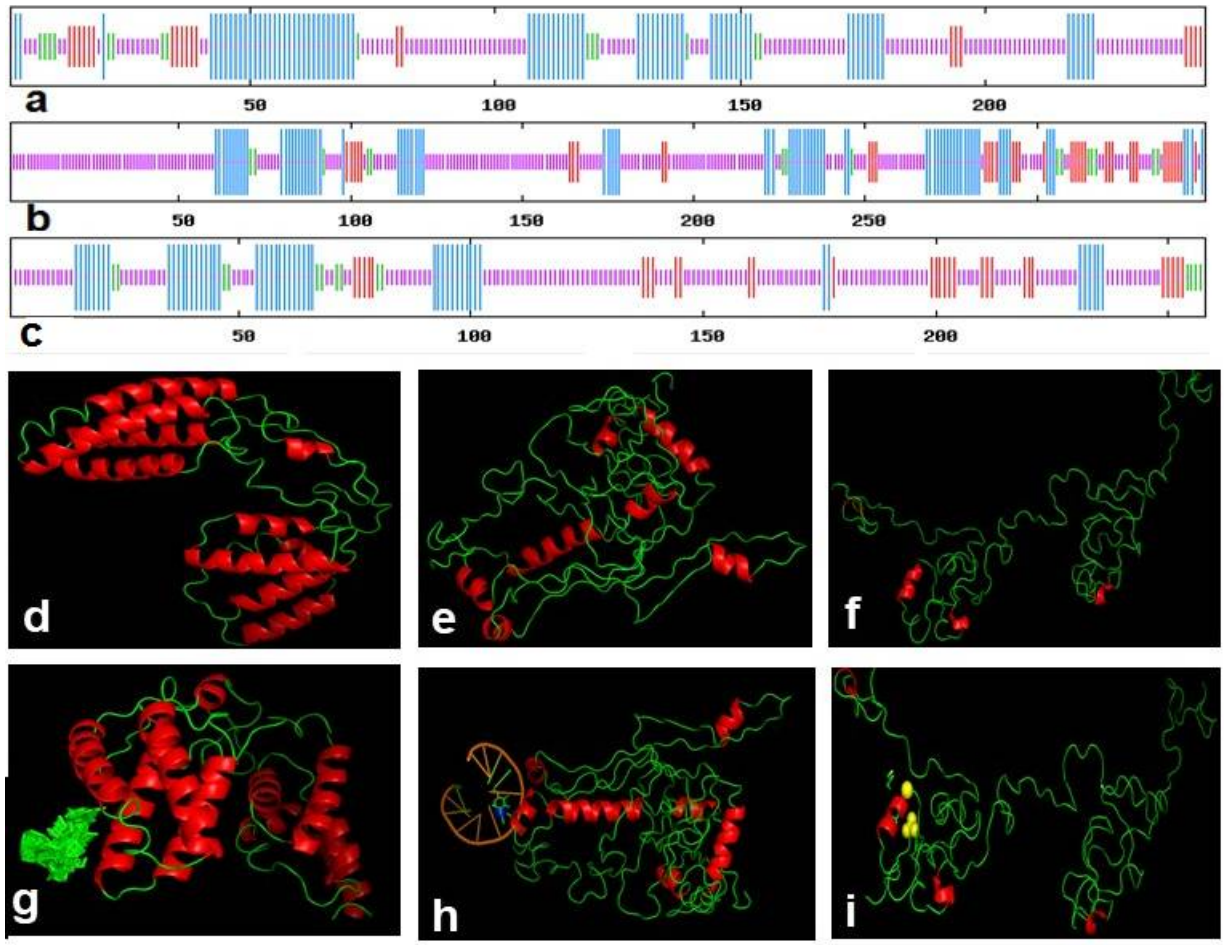

Fig. 5. Structural analysis of AsWOX13, AsWOX9, and AsWOX2B proteins: a,b,c. Secondary structures; **d,e,f.** Simulated three dimensional structures; **g,h,i.** Ligand-binding sites.

related orchid species like *Phalaenopsis equestris* (14 genes, including 3 duplicated genes) and *Dendrobium catenatum* (10 genes) (19). Multiple sequence alignment indicated that all AsWOXs carry DNA-binding helix-loop-helix-turn-helix region (Fig. 1a). Domain analysis showed that all AsWOX sequences consisted of WUSCHEL-related homeobox domain (helix-loop-helix-turn-helix region) (pfam00046) (Fig. 1b). MEME Suite server identified five conserved motifs, with reduction in evolutionary clade advancement (Fig. 1c).

Phylogenetic analysis for AsWOXs was carried out with those of *D. catenatum* (DcWOXs), *P. equestris* (PeWOXs), *A. thaliana* (AtWOXs) and *O. sativa* (OsWOXs), to establish the evolutionary relationships (Fig. 2). Tight clustering of AsWOX13 with PeWOX13A, PeWOX13B, PeWOX13C, DcWOX13 and AtWOX13 revealed that it is a member of ancient clade. Similarly, rest of the genes clustered in the other two clades, Intermediate and WUS, along with the respective sequences of P. *equestris, D. catenatum, O. sativa* and *A. thaliana* (Fig. 2).

The peptide length of AsWOXs varied from 137 aa (AsWOX7) to 349 aa (AsWOX9), with an average length of 228 aa (Table 1) which is in sync with reports of *Dendrobium catenatum* (19). The average molecular weight of AsWOX proteins was 25.03 kDa, highest being 37.31 kDa in AsWOX9 and lowest being 16.13 kDa in AsWOX7. The isoelectric point ranged from 5.99 (AsWOX2B) to 10.44 (AsWOX7) with an average of 8.2 and the aliphatic index ranged from 50.08 (AsWOX2B) to 75.05 (AsWOX12) with an average of 63.89. The grand average of hydropathy (GRAVY) value of all AsWOX proteins had a negative value suggesting their hydrophilic nature (Table 1). No signal peptide or transmembrane helix region was detected in any of the AsWOX protein sequences. These were localised in the nucleus as expected for DNA-binding transcription factors (Table 1) which is in conformity with earlier reports (49).

Gene characterization and duplication events and ortholog prediction

Genomic scaffold regions for each *AsWOXs* were identified from the NCBI database and listed (Table 2). The exon-intron gene structure analysis of *WOXs* showed that three *WOX* genes (*AsWUS*, *AsWOX9* and *AsWOX13*) carried two introns, while six genes (*AsWOX2A*, *AsWOX2B*, *AsWOX3A*, *AsWOX3B*, *AsWOX11* and *AsWOX12*) were mono-intronic genes and one gene (*AsWOX7*) was intron less (Fig. 3). Multiple sequence alignment-based sequence similarity index among *AsWOX* CDS sequences indicated that no gene duplication event occurred within *WOX* gene family in *A. shenzhenica* genome (Supplementary Table 1) which is in conformity with the results in a related orchid species, *D. catenatum* having 10 *WOX* genes (19).

The orthologs for each AsWOX were predicted against the sequences of taxonomically closest

species, *P. equestris* and *D. catenatum*, *A. thaliana* and *O. sativa* (Table 2).

Cis-regulatory elements prediction and Expression analysis

Detailed analysis revealed that the 1.5 kb upstream promoter sequences in all the *WOX* genes carried *cis*-regulatory elements including core promoter elements TATA-box (TATABOX5) and CAAT-box (CAATBOX1), and other elements that direct specific expressions such as, root-specific (ROOTMOTIFTAPOX1, OSE2ROOTNODULE), mesophyll-specific (CACTFTPPCA1), pollen-specific (POLLEN1LELAT52, GTGANTG10), dehydration-esponsive (MYCCONSENSUSAT), light-responsive (IBOXCORE, GT1CONSENSUS), Dof proteins binding domain (DOFCOREZM), WRKY proteins binding W-box (WRKY71OS) and wound activating W-box (WBOXNTERF3). In addition, the AGL15 binding element (CARGCW8GAT) and scaffold or matrix attachment region (1 MARTBOX) were predominant (Supplementary Table 2). This reflects the diverse role of *WOX* genes in plant development. In *A. shenzhenica*, RNA-seq data was available for three developmental stages i.e. tuber, seed and pollen. *AsWOX13*, the ancient clade member, showed high expression in all the three developmental stages (Fig. 4). The presence of pollen-specific (POLLEN1LELAT52, GTGANTG10) promoter elements in *AsWOX13* suggests a role in anther development, the expression profile of this gene also confirms the same (Fig. 4). This has also been reported in *A. thaliana*, where, the expression of *AtWOX13* was found to be higher at floral transition stage, in inflorescences, floral buds, gynoecium and relatively weak expression in fruits and leaves indicating towards its role in flower and embryo development (7, 51). It is reported that *WOX13* from *Physcomitrella patens* (*PpWOX13*) is involved in reprogramming of leaf cells and protoplast cells into stem cells (50). In *A. thaliana*, *AtWOX14*, a homologous gene of *AtWOX13* gene, is involved in gibberellin synthesis, vascular cell differentiation, floral transtition, anther development and lateral root development (7, 52, 53). The *WOX13* genes from *Ananas comosus* (*AcoWOX13*), *D. catenatum* (*DcWOX13*) and *P. equestris* (*PeWOX13A*, *PeWOX13B* and *PeWOX13C*) had similar expression pattern (18, 19). The *AsWOX9* gene (Fig. 2) had maximum expression in seed (Fig. 4). The promoter region of *AsWOX9* carried *cis*-regulatory elements AACACOREOSGLUB1, 2SSEEDPROTBANAPA, DPBFCOREDCDC3, for seed/embryo specific expression (Table 3). The *A. thaliana* genes *AtWOX9* and *AtWOX8* which are phylogenetically closest to *AsWOX9*, have been reported to play a role in zygote patterning, late embryo development and apical growth (54, 55). This suggests that the *AsWOX9* gene, with maximum expression in seed could have a role in embryo development. *AsWUS* showed significant expression in pollen (Fig. 4) which is comparable to the expression profile for *WUS* gene in *P. equestris* (19). Earlier reports in *A. thaliana*, indicated the role of AtWUS protein as a repressor in stem cell regulation and an activator in floral patterning (56). AtWUS also promotes vegetative-to-embryonic transition (57) and involves in somatic embryogenesis (58). Promoters of *AsWOX9* and *AsWUS* carried tuber-specific element (SP8BFIBSP8BIB) and water stress regulated element (MYB2AT). The *AsWUS* gene promoter carried element (EVENINGAT) for circadian rhythm as well. These observations suggest that *AsWUS* could possibly play a role in flower development. Another member, *AsWOX2B* gene (Fig. 2), had seed specific expression (Fig. 4). *AsWOX2B* can be related to the *AtWOX2* of *A. thaliana*, which expresses downstream to *AtWOX8* and was shown to be involved in zygote patterning and apical growth patterning regulation (54, 55). *AsWOX2B* with seed specific expression might be playing a similar role. All the other *WOX* genes showed weak expression in tuber, pollen and seed. Promoters of genes *AsWOX2A*, *AsWOX3A*, *AsWOX9* and *AsWOX12* carried elements that interacts with *AGAMOUS* gene target sequence WUSATAg of intron and that are light-regulated (IBOX). A coupling element (CGACGOSAMY3) for the G box element was found in the promoters of genes *AsWOX2A*, *AsWOX7*, *AsWOX11* and *AsWOX13*. Promoters of the genes, *AsWOX2B*, *AsWOX3B*, *AsWOX9* and *AsWOX13* carried (gibberellic acid) GA-responsive element (GAREAT) (Supplementary Table 3). These results support the involvement of *WOX* gene family in overall plant development.

Protein structure and Homology modeling

AsWOX13 of ancient clade, *AsWOX9* gene of intermediate clade and *AsWOX2B* gene of WUS clade were selected for protein structure simulation and homology modelling, based on high expression profile. These sequences were dominated with random coils ranging from 52.65% (AsWOX13) to 62.79% (AsWOX2B) and alpha helix regions ranged from 32.24% in AsWOX13 to 20.16% in AsWOX2B (Fig. 5a-c; Table 3). The three-dimensional structure simulation analysis predicted that AsWOX13 and AsWOX9 were nucleic acid binding proteins (Fig. 5d, e, g, h; Table 3), while AsWOX2B was predicted to bind with glycerol (Fig. 5f, i).

Conclusion

The present study characterizes the WOX gene family in *Apostasia shenzhanica*. Ten *AsWOX* genes were identified and grouped into three clades, ancient, intermediate and WUS based on homology modelling with related plants and establishes phylogenetic relationships amongst these. This study opens vistas for functional characterization of AsWOX gene family in various developmental pathways.

Acknowledgements

Authors are grateful to Department of Science and Technology (DST), Government of India for partial financial support under Promotion of University Research and Scientific Excellence (PURSE) grant scheme. TRR and JKS are thankful to Science and Engineering Research Board (SERB) for National-Postdoctoral Fellowship (File Number: PDF/2016/003098). MK is thankful to Department of Science and Technology (DST), for INSPIRE fellowship under INSPIRE programme for Junior Research Fellowship (DST/INSPIRE fellowship/2017/IF170940).

Authors' contribution

TRR and MK executed the experiments and drafted the manuscript. JKS designed the project, analysed the data and revised the manuscript.

Competing interest

The authors declared that they have no competing interests.

Supplementary files

Supplementary Table 1

Supplementary Table 2

Supplementary Table 3

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