Chinese Herbal Medicines 15 (2023) 57–62

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

Auxin induces lateral root formation in *Bupleurum*: A heme oxygenase dependent approach

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ARTICLE INFO

Article history:
Received 5 November 2021
Revised 8 January 2022
Accepted 18 March 2022
Available online 23 May 2022

Keywords:
auxin
Bupleurum L.
heme oxygenase
lateral root formation

ABSTRACT

Objective: The content of saikosaponins in genus *Bupleurum* is increased with numbers of lateral root, but the genetic mechanisms are largely unknown. This study aims to identify the heme oxygenase (HO) gene family members of *B. chinense* and *B. scorzonerifolium*, and assess their role in the root development in *Bupleurum*.

Methods: The gene sequences of HO family were selected from *iso*-seq full-length transcriptome data of *B. chinense* and *B. scorzonerifolium*, and were analyzed in physicochemical properties, conserved domains, motifs and phylogenetic relationship. In addition, the expression patterns of HO gene in different parts of roots were compared via transcriptome sequencing and qRT-PCR in the two species.

Results: Five *Bupleurum* HO genes (*BcHO1-BcHO5*) belonging to the HO1 subfamily were identified from the transcriptome data, whereas the HO2 subfamily member was not identified. The expression levels of *BcHO1* and *BcHO2* were significantly higher than those of other three HO members in the transcriptome analysis. In addition, the expression profile of *BcHO1* showed consistency with lateral root development in *B. chinense* and *B. scorzonerifolium*.

Conclusion: Hos might participate in the auxin-induced morphogenesis of lateral roots. The yield of saikosaponin may be improved by manipulating expression of these genes.

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1. Introduction

Bupleuri Radix (dried roots of *Bupleurum chinense* DC. or *B. scorzonerifolium* Willd) is a famous traditional Chinese medicine, in which saikosaponins are the major bioactive compounds (Huang et al., 2009; Tian, Xie, & Liu, 2009). It was reported that saikosaponins are primarily distributed in the vascular cambium and secondary phloem of the mature roots, indicating that higher quality plants have slender taproots and more lateral roots than mediocre plants (Tan, Cai, Hu, & Ni, 2008). Therefore, the root architecture directly affects the content and total yield of saikosaponins in *Bupleurum* L. It was found that *B. scorzonerifolium* showed shorter roots and less lateral roots than *B. chinense*, but why they are different is elusive at the molecular level (Pan, Shun, Bo, & Bao, 2002).

Heme oxygenase (HO), a key enzyme in heme degradation, occurs widely in animals, plants, and microorganisms (Zhao, Ozen, Wong, & Stevenson, 2015). Genes of HO involved the regulation of diverse aspects of plant biology, including lateral root formation (Guo, Xia, & Yang, 2008; Han et al., 2012; Terry, Linley, & Kohchi, 2002), phytochrome chromophore synthesis (Emborg, Walker, Noh, & Vierstra, 2006), and protection against oxidative cell damage (Gisk, Yasui, Kohchi, & Frankenberg-Dinkel, 2010; Shekhawat & Verma, 2010). Previous studies have demonstrated that auxin, methyl jasmonate, apocynin, and cobalt chloride can induce HO expression and promote the lateral root formation (Chen, Chao, Hsu, Hong, & Kao, 2012; Chen, Chao, Hsu, & Kao, 2013; Hsu, Chao, & Kao, 2013a,b).

To determine the mechanism of root development in the genus *Bupleurum* L., we compared transcript profiles at different stages of lateral root development in *B. chinense* and *B. scorzonerifolium* (Yu et al., 2021). In the present study, we identified gene sequences of HO family from *iso*-seq and transcriptome data in *B. chinense* and *B. scorzonerifolium*, predicted their physicochemical properties,

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https://doi.org/10.1016/j.chmed.2022.03.006
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conserved domains and motifs, and analyzed the phylogenetic relationship. In addition, the association between HO gene expression and lateral root development was investigated to determine whether HO is involved in the root development process of *Bupleurum* genus.

2. Materials and methods

2.1. Plant materials

The *B. chinense* cultivar ChuanbeiChai No.1 (CBC1) and *B. scorzonerifolium* cultivar ChuanHongChai No.1 (CHC1) were used as experimental materials in this study. CBC1 and CHC1 plants, were bred by Dr. Jianhe Wei, Institute of Medicinal Plant Development (Beijing, China), Chinese Academy of Medical Sciences & Peking Union Medical College, and Dr. Dabin Hou, Southwest University of Science and Technology (Mianyang, China). In the RNA-Seq analysis of both species, we sampled the full roots on the fifth day after germination as Stage 1 (S1), root tips on the fifteenth day (S2), and the differentiated region of roots on the fifteenth day (S3). The samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction (Yu et al., 2021).

2.2. RNA isolation and transcriptome sequencing on Illumina platform

Total RNA was extracted from CBC1 and CHC1 using TRIzol (Invitrogen™, Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. The quality and quantity of the extracted RNA were assessed using an Agilent Bioanalyzer 2000 (Agilent Technologies, Palo Alto, USA). An RNA-Seq library with a 150-bp insertion size was then constructed and sequenced on the Illumina sequencing platform (Novogene, Beijing, China) (Yu et al., 2021).

The sequence data from this study have been deposited in the Short Read Archive of NCBI under accession numbers PRJNA645610 for *B. chinense* (SRR12223465-SRR12223473) and PRJNA662700 for *B. scorzonerifolium* (SRR12647620-SRR12647628). The genes associated with triterpenoid saponin biosynthesis and lateral root development have been identified (Yu et al., 2021); we focus on the HO genes in this study.

2.3. HO bioinformatics analysis

The protein coding sequences (CDSs) were blasted against the NCBI non-redundant protein (Nr), non-redundant nucleotide sequence (Nt), protein family (Pfam), eukaryotic orthologous groups (KOG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes and Gene Ontology databases, using *E* value $\leq 10^{-5}$ as a threshold value. With HO protein sequence of *Daucus carota* L. and *Arabidopsis thaliana* (L.) Heynh. as templates, the local BLAST was used to search the transcript database and compare against other data bases (Yu et al., 2021). The NCBI Conserved Domain Database (CDD) as well as the protein family search were used to integrate and analyze the data results and remove redundant sequences.ProtParam (https://web.expasy.org/protparam/), MEME (https://alternate.meme-suite.org/tools/meme), and the SMART (https://smart.embl-heidelberg.de/) online software were used to analyze the corresponding basic physical and chemical properties, subcellular locations, and conserved motifs of the HO family. MEME parameter setting: The maximum motif was set to 15, the motif length ranged from 6 to 100 amino acids, and other parameters were set at their default values. The HO amino acid sequences of CBC1, CHC1, *A. thaliana*, *D. carota* and *Oryza sativa* Linn. were compared using MEGA-X (https://www.megasoftware.net/), and the Neighbor-Joining (NJ) method was used to construct the phylogenetic tree with the following parameters: P-distance model, pairwise deletion of gap options, bootstrap method value of 1000.

2.4. Expression of Bupleurum HO genes during root development

The expression data of the HO gene family were based on the RPKM value of expression data in the in-house RNA-Seq database constructed by our group, and the heat map was created after homogenization of expression data of HO gene family using the OmicStudio tools (https://www.omicstudio.cn/tool).

2.5. Effect of auxin on expression of Bupleurum HO genes

The hydroponic seedlings were treated along different time gradients with optimum auxin concentrations for the root development of CBC1 and CHC1. After treatment, the roots were collected and the total RNA (RNAprep Pure Plant Kit, Tiangen) was extracted and reverse-transcribed into cDNA (TransScript II First-Strand cDNA Synthesis Super Mix, TransGene). The HO gene expression was quantified using real-time qPCR.

3. Results

3.1. Identification of Bupleurum HO family and analysis of protein characteristics

Based on the transcriptome data, a sequence homology search was performed with local BlastP against the HO sequences of *A. thaliana*; 14 *Bupleurum* HO (BcHO) genes were identified, then the conserved domains were predicted via CCD and Pfam searches. The incomplete domain sequences and repetitive sequences were removed, and five BcHO protein sequences were obtained from each of two species respectively. In the multiple sequence alignment of HO proteins using ClustalX software, the conserved HO signature sequence (QAFICHFYNI) was identified in almost all amino acid sequences of HO proteins, which is important for heme binding (Fig. 1); these proteins were similar in the length of amino acids and molecular weight, with isoelectric point ranging from 8.26 (BcHO4) to 8.9 (BcHO1) (Table 1). The instability index of all HO proteins was larger than 41, and all grand averages of hydropathicity were negative, indicating that the five HO proteins were unstable hydrophilic proteins. It was predicted that all five HO proteins were located in chloroplasts.

3.2. Evolutionary analysis of Bupleurum HO family proteins

The phylogenetic relationships between *Bupleurum* HOs were compared with other well-characterized HO members of the model plants *A. thaliana*, *D. carota*, and *O. sativa*. The phylogenetic tree was constructed using the NJ method, based on the sequence alignment of 13 full-length HO amino acid sequences of four species (Fig. 2A). The 13 sequences were separated into two clusters. BcHOs were all located in Class I and had the highest homology with *D. carota* of the family Umbelliferae.

3.3. Motif analysis of Bupleurum HO family proteins

To better understand the function of HO proteins in *Bupleurum*, the HO protein sequences were further analyzed. The multiple sequence alignment of HO full-length amino acid sequences, isolated from a diverse set of species using ClustalX, showed that most of the proteins retained the HO signature sequence, which is important for heme binding (Fig. 1). The MEME software was used to analyze the composition and number of conserved motifs in the HO gene family of four plants, and 10 relatively conserved

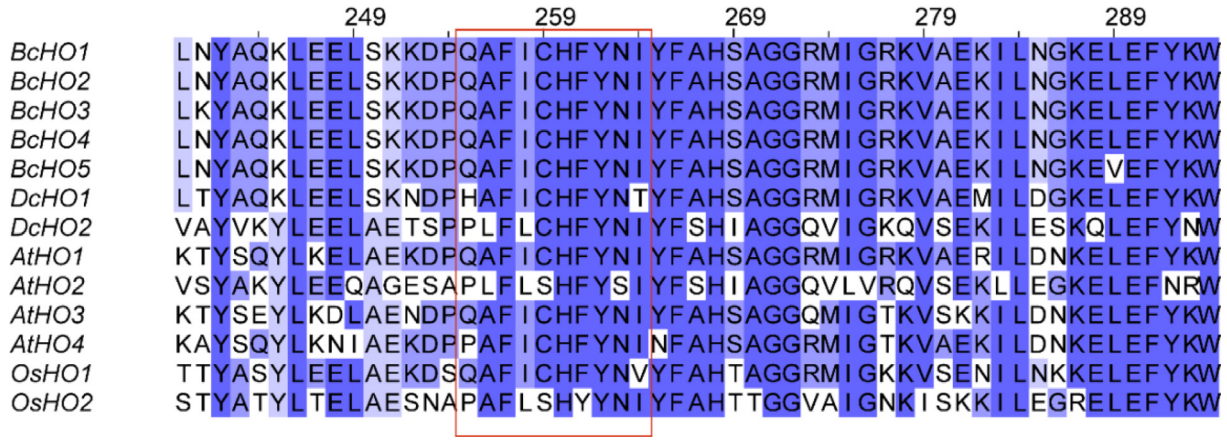

Fig. 1. Multiple sequence alignment of BcHO genes and other HO in plant species. Conserved HO amino acids in the red block.

Table 1
Heme oxygenase homologs from four plant species and their physiochemical parameters.

Gene ID	Structure domain	Length (AA)	Exon NO.	Molecular weight (kDa)	pI	Instability index	Aliphatic index	Grand average of hydropathicity	Subcellular localization
BcHO1	72–279	281	5	32.09	8.90	41.67	77.08	−0.570	Chloroplast
BcHO2	72–279	281	5	32.09	8.90	41.67	77.08	−0.570	Chloroplast
BcHO3	74–278	280	5	31.95	8.89	43.92	74.93	−0.580	Chloroplast
BcHO4	74–278	280	5	31.99	8.26	44.41	76.29	−0.564	Chloroplast
BcHO5	74–278	280	5	31.98	8.88	44.94	74.93	−0.595	Chloroplast
AtOH1	73–280	280	5	32.49	6.50	44.70	75.96	−0.604	Chloroplast
AtOH2	127–297	299	4	34.90	5.80	50.50	79.20	−0.795	Chloroplast
AtOH3	76–283	285	5	32.43	7.02	42.19	70.21	−0.604	Chloroplast
AtOH4	28–281	283	2	32.95	6.98	45.25	80.64	−0.530	Chloroplast
OsOH1	83–287	289	5	31.91	6.28	55.34	72.35	−0.426	Chloroplast
OsOH2	159–335	337	2	37.27	4.96	56.98	79.97	−0.407	Chloroplast
DcOH1	73–277	279	5	31.81	8.22	46.12	73.44	−0.624	Chloroplast
DcOH2	130–300	302	5	34.59	6.29	49.81	77.75	−0.501	Chloroplast

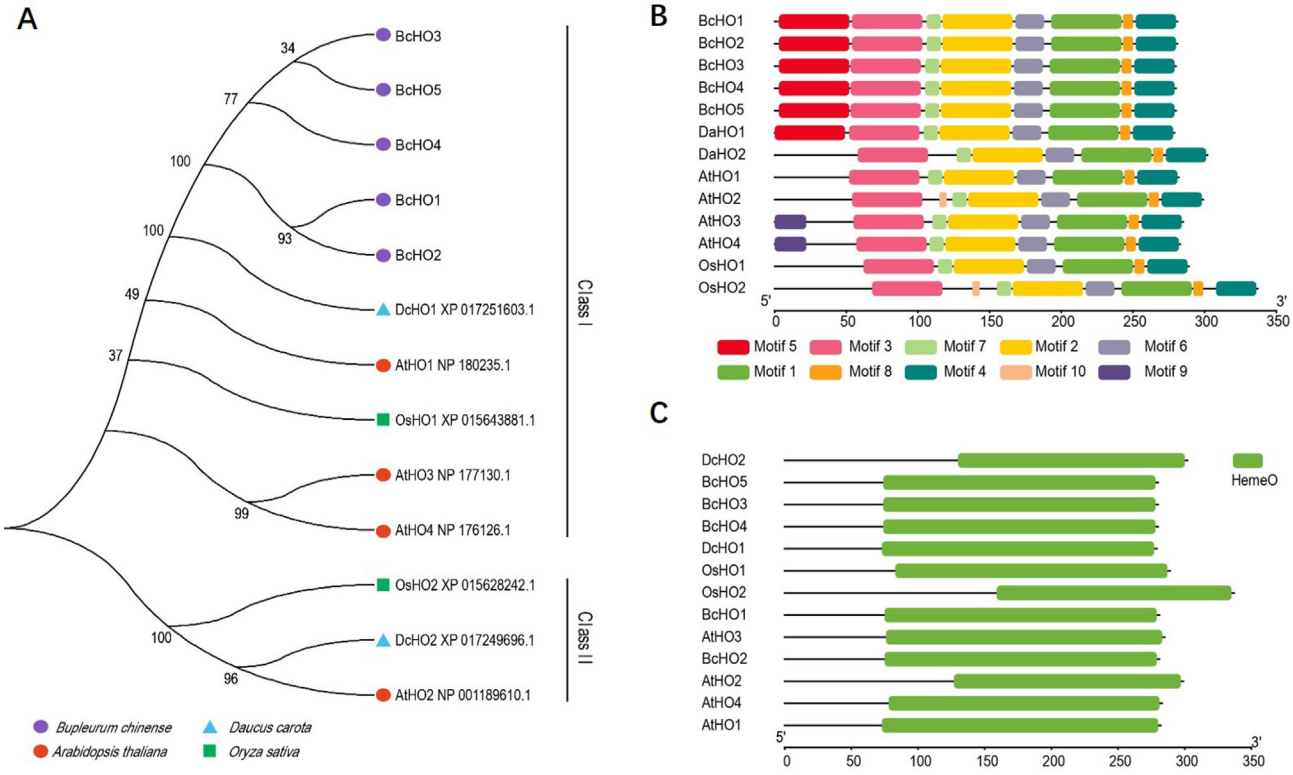

Fig. 2. A: Phylogenetic tree showing evolutionary relationships between BcHO protein sequences and HOs from *Arabidopsis*, *Daucus* and *Oryza*. B: Conserved motifs of heme oxygenase proteins. C: Exon of heme oxygenase genes from different plant species.

motifs were identified (Fig. 2B); Motif 5 was identified in all five HO genes of *Bupleurum* and *DcHO1* of *D. carota*. In addition, all 13 investigated HO genes contained motifs 3, 7, 2, 6, 1, 8, and 4, indicating the highly conserved HO structural region. Thirteen HO protein domains were analyzed online using the CDD, and all HOs contained HO domains (Fig. 2C).

3.4. Expression of Bupleurum HO genes during root development

The expression levels of five HO members in three different tissues were extracted from the transcriptome data of CBC1 and CHC1, and a heat map was created after homogenization of the expression data using OmicStudio tools (Fig. 3A). Most HOs were expressed in the full roots on the 5th day (Fig. 3B), the root tip and maturation zone of roots on the 15th day in two *Bupleurum* species (Fig. 3C), and the expression of *BcHO5* in all tissues was very low or not expressed at all. As roots grew and developed, the expression of HO decreased. The expression level of five HOs was the highest in S1, and BcHO1 had the highest expression level. The expression levels of *BcHO1* and *BcHO2* were significantly higher than those of *BcHO3*, *BcHO4*, and *BcHO5*.

The *BcHO1* exon coding sequence was used to design primers for the qPCR analysis of auxin-treated roots of CBC1 and CHC1. The relative expression of *BcHO1* in CBC1 reached the highest level at 12 h after treatment (Fig. 3D), and the relative expression of *BcHO1* in CHC1 reached the highest level at 8 h after treatment (Fig. 3E).

4. Discussion

4.1. It might be an effective way to improve saikosaponin production by increasing lateral roots, the saikosaponin biosynthetic factories

There are two ways to improve the yield of saikosaponin in *B. chinense* and *B. scorzonerifolium*, i.e. biosynthetic pathway enhancement and expansion of saikosaponin biosynthetic facto-

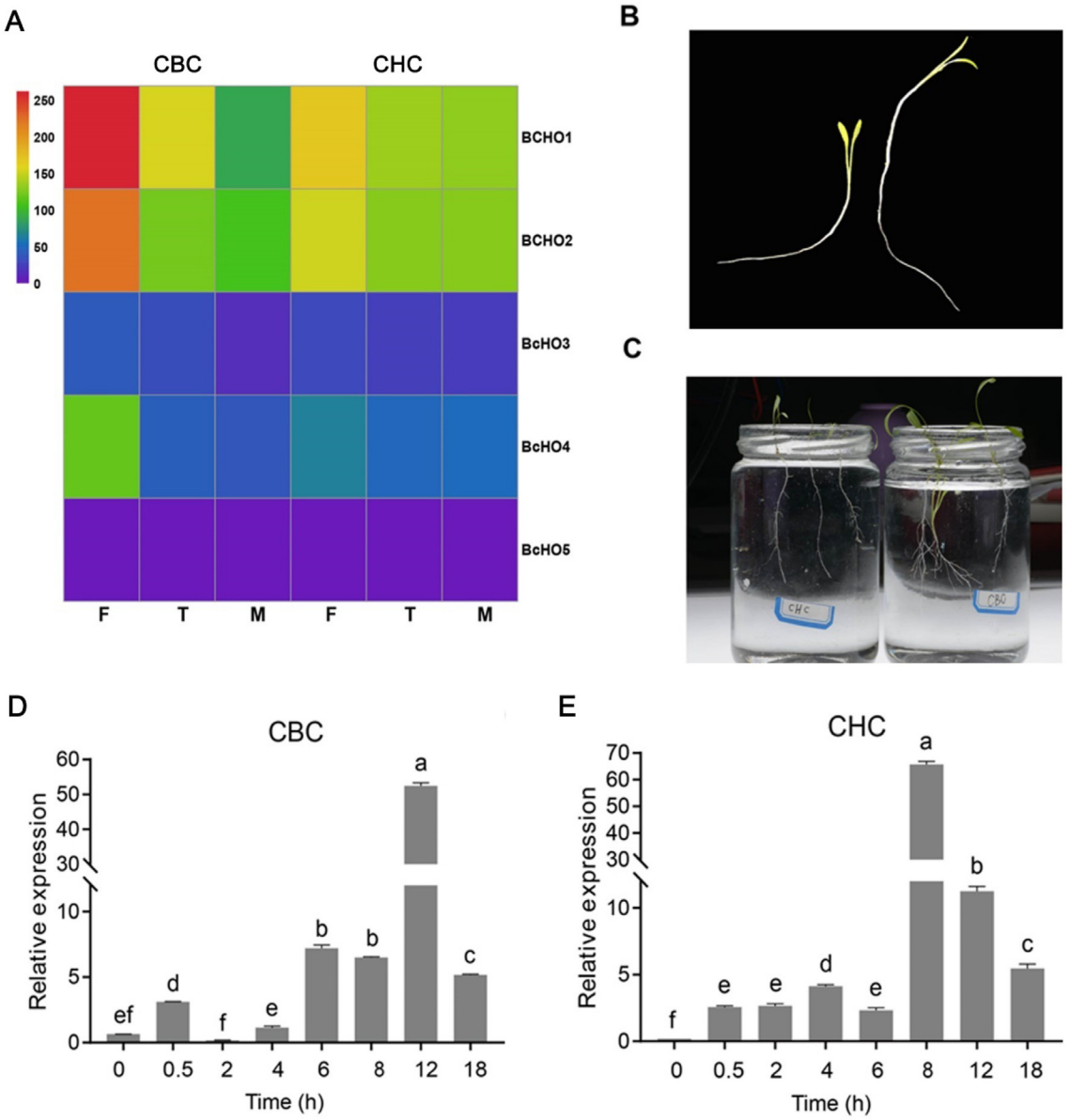

Fig. 3. A: Heatmap representation of BcHO genes of CBC and CHC during root development (F: full roots at 5th day, T: root tip at 15th day, M: maturation zone of root at 15th day). B: Seedling on the fifth day after germination, CHC1 on the left, CBC1 on the right. C: Seedling on the fifteenth day after germination, CHC1 on the left, CBC1 on the right. D: Effect of different auxin treatment duration on BcHO1 gene expression in CBC. E: Effect of different auxin treatment duration on BcHO1 gene expression in CHC; the error bars indicate standard deviation, the letters above error bars stand for different significant levels, $P < 0.05$.

ries.However, it is hard to improve the saikosaponin production by enhancing the expression of single gene. We have identified 77 unigenes of eight families probably involved in the biosynthetic pathway of saikosaponins in *B. chinense* and *B. scorzonerifolium* (Yu et al., 2020; He et al., 2021). Saikosaponins were reported to be distributed in tissues of cork and cortex (Liang et al., 2014; Tan, Cai, Hu, & Ni, 2008), and it might be an easier way to induce more cork and cortex by increasing lateral roots. During lateral root development, the expression of HO promoted the lateral root formation and root hair proliferation in *Solanum lycopersicum* L., *Nicotiana tabacum* Linn., and *Glycine* max (Linn.) Merr. (Cheng et al., 2018; Guo, Xia, & Yang, 2008; Santa-Cruz et al., 2010). In addition, the root formation and HO activity were simultaneously reduced by the HO specific inhibitor zinc protoporphyrin in *O. sativa* (Chen, Chao, Hsu, Hong, & Kao, 2012). Therefore, the promotion of HO on root growth might be beneficial to increase the total yield of saikosaponins (Fig. 4A).

4.2. BcHO1 might be involved in auxin regulation in promoting the development of lateral roots

To understand whether HO is involved in the lateral root formation in *Bupleurum*, we analyzed the expression of HO in the full roots on the 5th day, the root tip and maturation zone of roots on the 15th day in two *Bupleurum* species (Fig. 4B). We found that the HO expression decreased with the development of lateral roots. During exogenous auxin induction, the expression of *BcHO1* increased with the extension of auxin treatment time, reaching a

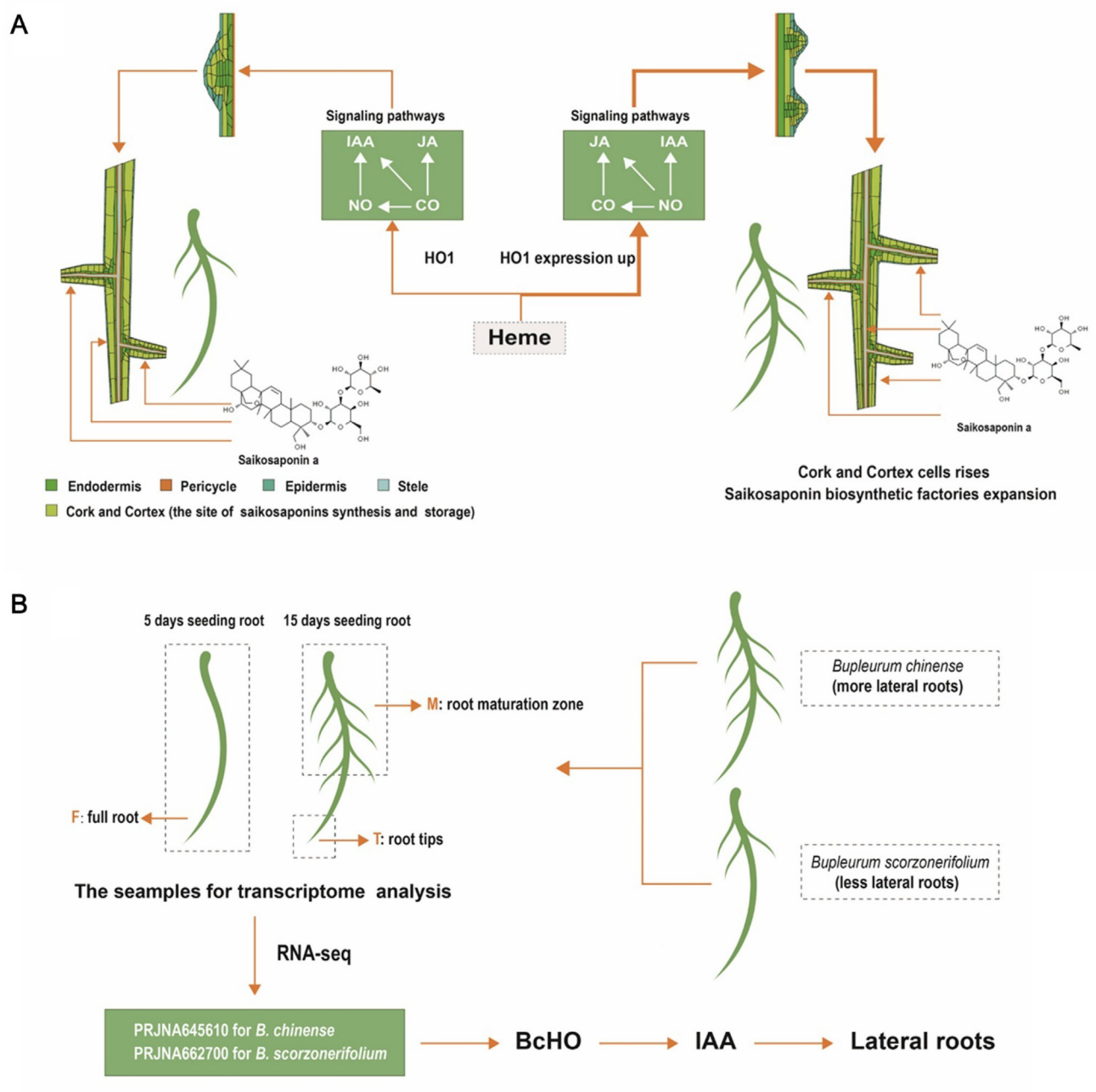

Fig. 4. A: HO expression upregulation may induce the root development of *Bupleurum* and expand the biosynthetic factories of saikosaponins. B: HO is involved in the lateral root formation in *Bupleurum*.

maximum at 12 h (CBC1) and 8 h (CHC1) respectively, before ultimate decline. This is consistent with our prior studies, which showed that the exogenous IAA treatment increased the number of lateral root primordia within 12 h in CBC1 and within 8 h in CHC1, respectively (Yu et al., 2021). These suggest that the HO gene is involved in the auxin induced development of lateral roots in CBC1 and CHC1, and it is necessary to identify the interaction of BcHO1 with auxin on promoting the lateral root formation at the transgenic level.

5. Conclusion

This is the first study to investigate the involvement of HO in lateral root formation in *Bupleurum*. Five HO genes were identified in the transcriptome data. We found that *BcHO1* is involved in the auxin induced lateral roots development in both species of *B. chinense* and *B. scorzonerifolium*. Further study is being pursued on the functional verification through genetic transformation of *BcHO1* to clarify how it interacts with auxin and promotes lateral root formation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financially supported by China Agriculture Research System of MOF and MARA, the CAMS Innovation Fund for Medical Sciences (CIFMS) (No. 2016-I2M-2-003), Opening Project Fund of Key Laboratory of Rubber Biology and Genetic Resource Utilization, Ministry of Agriculture/State Key Laboratory Breeding Base of Cultivation & Physiology for Tropical Crops/Danzhou Investigation & Experiment Station of Tropical Crops, Ministry of Agriculture" (No. RRL-KLOF202201).

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