Article

Preliminary Classification of the ABC Transporter Family in *Betula halophila* and Expression Patterns in Response to Exogenous Phytohormones and Abiotic Stresses

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Abstract: ATP-binding cassette (ABC) transporters comprise a transport system superfamily which is ubiquitous in eukaryotic and prokaryotic cells. In plants, ABC transporters play important roles in hormone transport and stress tolerance. In this study, 15 BhABC transporters encoded by genes identified from the transcriptome of *Betula halophila* were categorized into four subfamilies (ABCB, ABCF, ABCG, and ABCI) using structural domain and phylogenetic analyses. Upon *B. halophila* exposure to exogenous phytohormones and abiotic stressors, gene expression patterns and transcriptional responses for each subfamily of genes were obtained using semi-quantitative RT-PCR analysis. The results demonstrated that expression of most genes belonging to ABCB and ABCG subfamilies changed in response to exogenous phytohormone exposures and abiotic stress. These results suggest that *BhABC* genes may participate in hormone transport and that their expression may be influenced by ABA-dependent signaling pathways involved in abiotic stress responses to various stressors.

Keywords: *Betula halophila*; ATP-binding cassette transporter; hormone response; abiotic stress response; gene expression

1. Introduction

ATP-binding cassette (ABC) transporters are complex proteins that are responsible for the translocation of substrates across membranes. They constitute one of the largest protein families yet discovered, with widespread distribution among eukaryotes and prokaryotes [1,2]. ABC transporters contain a common core structure of nucleotide-binding domains (NBDs) and transmembrane domains (TMDs) [3]. In plants, ABC transporters can be classified into full transporter, half transporter, and soluble ABC protein subgroups based on arrangements of these domains [4]. Generally, full transporters contain two NBDs and two TMDs, half transporters contain one NBD and one TMD, and soluble ABC proteins only contain one NBD [4]. According to phylogenetic analyses, ABC transporters can also be classified into eight subfamilies based on evolutionary relatedness (A–G and ABCI; ABCH is not found in plants) [5,6].

In 1992, the first plant ABC transporter gene (*AtPGP1*) was cloned from *Arabidopsis* [7]. Since then, the volume of research focusing on the functions of plant ABC transporters has gradually increased and has revealed diverse roles of these proteins. Indeed, the current body of accumulated evidence indicates that these proteins transport plant hormones, metal ions, lipids, secondary metabolites, and exogenous substances, in addition to their roles in regulating plant ion channels and plant–pathogen interactions. Members of the soluble ABC protein subgroup, such as ABCE (Ribonuclease L inhibitor), participate in ribosome recycling and translation termination functions that influence leaf development [8,9].

To date, research on plant hormone and abiotic stress responses has been predominantly focused on the investigation of these processes in herbs, such as *Arabidopsis thaliana*.

Using a salt stress model, ABCA subfamily transporters have been shown to be downregulated after NaCl treatment in *Arabidopsis* [10], whereas ABCB transporters are mainly involved in the transport of plant hormones, although they can also enhance tolerance of plants to heavy metals [11–15]. ABCC transporters participate in cellular detoxification mainly by pumping toxins and heavy metals out of cells or vacuoles. [16–19]. The ABCG subfamily PDR subgroup of full transporter proteins is primarily responsible for managing abiotic stress and is also involved in hormone regulation [20–22], whereas the ABCG subfamily half transporter (WBC) subgroup of proteins is mainly involved in the transport of plant hormones [23–25].

Betula halophila, a member of the Betulaceae family, is found in the Altay region of northern Xinjiang, China, where it grows in salt marshes to an altitude of 1500 m. Due to its strong salt and drought resistance, *B. halophila* is a useful model species for studying plant resistance to abiotic stress. In a previous study, after *B. halophila* had been exposed to 200 mM NaCl for 0 h or 24 h to simulate salt stress, cDNA libraries were prepared from its leaves. Subsequently, high-throughput sequencing was conducted to identify differentially expressed genes (SRA: SRP146369) [26]. Gene expression profile analysis detected 15 differentially expressed *BhABC* genes in response to salt stress. Based on this result, we studied the expression profiles of these 15 genes in leaves, xylem, roots, and apical buds to better understand *BhABC* gene transcriptional responses to exogenous plant hormones and abiotic stressors under various conditions.

2. Materials and Methods

2.1. Plant Materials and Treatment Methods

B. halophila seeds were collected in Altay, Xinjiang, China and all seeds were half-siblings. After the seeds germinated, they were transplanted into soil within 10 × 10 cm culture pots and then cultivated at the Birch Breeding Base of the Northeast Forestry University of Harbin, China. Seedlings were grown for one year and only those with the same growth rate over this period were used in this study.

Seedlings were treated with exogenous hormones and abiotic stress reagents. For hormone treatments, seedlings were sprayed with 25 mg/L gibberellin (GA₃), 1 µmol/L methyl jasmonate (MeJA), 350 µmol/L salicylic acid (SA), 0.2 mg/L brassinolide (BR), 50 mg/L cytokinin (6-BA), 100 µmol/L abscisic acid (ABA), or 50 mg/L auxin (IAA), respectively. For samples undergoing abiotic stress, 20% Polyethylene Glycol (PEG), 0.4 mol/L sodium chloride (NaCl), 150 µmol/L cadmium chloride (CdCl₂), and 0.3 mol/L sodium bicarbonate (NaHCO₃) were used for watering, with no additional watering performed over the study period.

Every day at 8:00 a.m., the spraying of hormones and the irrigation of abiotic stress reagents were carried out. Plants were sampled at various time points during abiotic stress and ABA treatments, namely 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h. For all other hormone treatments, plants were sampled at 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, and 72 h. At each time point, xylem, roots, leaves, and apical buds of at least five seedlings were collected and pooled, immediately frozen in liquid nitrogen, then stored at −80 °C for RNA analysis.

2.2. Experimental Methods

2.2.1. Bioinformatics Analysis

We used the online National Center of Biotechnology Information (NCBI) Conserved Domains tool (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to predict protein open reading frame (ORF) length and conserved structural domains. The ExPASy tool (<https://web.expasy.org/protparam/>) was used to reveal amino acid sequences of proteins encoded by these genes and to calculate the theoretical isoelectric point of each protein. Subcellular localization was performed using ProtComp v9.0 (

softberry.com/berry.phtml?group=programs&subgroup=proloc&topic=protcompan). Signal peptides were predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). To carry out transmembrane structural region prediction for BhABC transporter proteins, we used the transmembrane structure analysis program TMHMM v2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). The phylogenetic tree was drawn using MEGA 5.0 (developer: Pennsylvania State University, State College, PA, USA) based on the following parameters: neighbor-joining tree method, complete deletion, and 500 bootstrap replicates.

2.2.2. Semi-Quantitative RT-PCR

For RNA extraction, we used an RNA extraction kit produced by Universal Plant Total RNA Extraction Kit (BioTeKe, Beijing, China). To perform reverse transcription of RNA to produce cDNA, we used a kit (Rever Tra Ace[®]qPCR RT Master Mix with gDNA Remover, TOYOBO, Shanghai, China). Dilutions of cDNA were used as templates for semi-quantitative RT-PCR analysis. Upstream and downstream primers were designed based on the specific sequence of each gene, with 18s RNA serving as internal reference gene (Table 1). The reaction system for performing semi-quantitative RT-PCR was as follows: TaKaRa rTaqTM (5 U/ μ L) (TaKaRa, Beijing, China) 0.2 μ L, cDNA 1.0 μ L, 1 μ L of each upstream and downstream primer (10 μ mol/L), dNTP mixture (2.5 mmol each) 1.6 μ L, and 10 $\times$ PCR buffer (Mg²⁺ free) 3.2 μ L. Sufficient ddH₂O was then added to each sample to adjust the total volume to 20 μ L. Semi-quantitative RT-PCR was conducted using thermal cycling at 94 $^{\circ}$ C for 30 s, 58 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s. Amplification was repeated for a total of 35 cycles. The volume of each reaction product solution was adjusted to achieve a consistent concentration of internal reference gene PCR product across samples (to represent a constant number of original plant cells per final adjusted unit volume). In this way, variations in fluorescence intensity of *BhABC* PCR product bands, as detected during gel electrophoresis, would reflect differences in *BhABC* mRNA quantity in the original cells instead of differences due to variations in sample loading among gel lanes. Each standardized PCR product solution was then electrophoresed on 1% agarose gels and the intensity of each amplified fragment on the electropherogram was analyzed using Tanon Gis software (developer: Tanon, Shanghai, China) to generate a number representing the relative expression level of a given gene. Finally, heat maps were generated using HemI v1.0 software (developer: The CUCKOO Workgroup, China) based on the following parameter selections: linear normalized intensity data, average linkage clustering method, and similarity metric of Euclidean distance.

Table 1. Primer sequences of *BhABC1–BhABC15* genes and internal reference gene 18s RNA.

Gene	Upstream Primer Sequence (5'→3')	Downstream Primer Sequence (5'→3')	Length of Product
<i>BhABC1</i>	CTTACCGGTACATACCTGTAGG	GTATCATGGTGCATTGTTGTCC	295 bp
<i>BhABC2</i>	GGTGGTGGACGTAGAAGATCC	GGAAAGCTGGAAGAGCATGCC	297 bp
<i>BhABC3</i>	TCTCATTCGCTTACGTGTCC	GACCTTACTGAGCAGCTTCC	289 bp
<i>BhABC4</i>	CCTCTACCTCCAGGATTAATGC	GCATCACTACCTGATGGTCTGG	282 bp
<i>BhABC5</i>	GGATGTTAGCTATCGACCTCC	TGCCACAAAGAACCTCTCAGG	274 bp
<i>BhABC6</i>	GAATGGATGAGCAGCAAGTTCC	AACCATCCACAATGAGCTCACG	279 bp
<i>BhABC7</i>	AAAGGCAATGCAGAAAGCAGG	CTGGTGTCAATTTGGCTACTGC	251 bp
<i>BhABC8</i>	CGAACAAAGAAGCTTCGAAGC	CCACATCTCTTCTCTCTACC	273 bp
<i>BhABC9</i>	GGATGTTGGAAGTACTACAGC	GAATCTATCACGAGCTGTCC	272 bp
<i>BhABC10</i>	CATAAGCAGTTCCAACACCTGG	CGGTCCTTGAGCTTTAACAGG	276 bp
<i>BhABC11</i>	GCATGTTGGATGGTCACTGG	TAGCATGACGAGAGCAAGAAGC	261 bp
<i>BhABC12</i>	TCCGTATGGAGGAACAATGC	GCAATTCACACTGTCTGAGG	290 bp
<i>BhABC13</i>	GAGAACCGTGCATCATTTCTGG	TTCCTCTTCTCGTCTTGTCTGC	254 bp
<i>BhABC14</i>	GAATCTCGATTGGAATGGTGC	GGTCAACCTTGTGGTCTTCG	260 bp
<i>BhABC15</i>	GGCTGCTTTAGAGAGACTGC	GTAGGCAATGCTCTGCTTCC	285 bp
18s rRNA	ATCTTGGGTGGGCAGATCG	CATTACTCCGATCCCAAGG	265 bp

3. Results

3.1. Sequence Analysis of *BhABC* Genes in *B. halophila*

3.1.1. Analysis of Physicochemical Properties of *BhABC* Genes

We analyzed basic characteristics including open reading frame (ORF) length, amino acid number, isoelectric point, subcellular localization, signal peptide, and transmembrane structure of proteins

predicted from 15 *BhABC* genes predicted from the *B. halophila* transcriptome (Table 2). ORF lengths within genes encoding BhABC proteins varied greatly, from 786 bp (BhABC2) to 4296 bp (BhABC12). Isoelectric points of BhABC proteins ranged between 5.41 and 9.68. It was predicted that only BhABC14 contained a signal peptide. Transporter proteins predicted to be located on the cell membrane were BhABC1, BhABC2, BhABC3, BhABC7, BhABC9, BhABC10, BhABC11, BhABC12, and BhABC14. BhABC15, BhABC4, BhABC6, and BhABC8 were predicted to be localized to chloroplast, whereas BhABC5 and BhABC13 were predicted to be located within mitochondria and nucleus, respectively. The number of transmembrane helices in BhABC transporters varied greatly. BhABC2, BhABC4, BhABC5, BhABC6, BhABC8, and BhABC13 had no transmembrane helix. By contrast, BhABC1, BhABC10, and BhABC12 proteins each had 13 transmembrane helices, whereas BhABC9 and BhABC11 had 11 and 10 transmembrane helices, respectively. BhABC15 had seven transmembrane helices, BhABC14 had six, and BhABC3 and BhABC7 each had four.

Table 2. Analysis of physical and chemical properties of *BhABC* genes in *Betula halophila*.

Name	Length of ORF (bp)	Number of Amino Acids	Isoelectric Point	Subcellular Localization ¹	Signal Peptide	Number of Transmembrane Helix
<i>BhABC1</i>	4266	1421	8.54	PM	NO	13
<i>BhABC2</i>	786	261	5.41	PM	NO	0
<i>BhABC3</i>	2265	754	9.34	PM	NO	4
<i>BhABC4</i>	789	262	6.44	CM	NO	0
<i>BhABC5</i>	837	278	9.59	M	NO	0
<i>BhABC6</i>	2148	715	5.55	CM	NO	0
<i>BhABC7</i>	3742	1205	9.12	PM	NO	4
<i>BhABC8</i>	900	299	5.81	CM	NO	0
<i>BhABC9</i>	3528	1175	6.97	PM	NO	11
<i>BhABC10</i>	4281	1426	6.65	PM	NO	13
<i>BhABC11</i>	3564	1187	8.27	PM	NO	10
<i>BhABC12</i>	4296	1431	8.57	PM	NO	13
<i>BhABC13</i>	1176	391	9.68	N	NO	0
<i>BhABC14</i>	3231	1076	9.17	PM	YES	6
<i>BhABC15</i>	3447	1148	5.47	PM	NO	7

¹ Mitochondrial membrane (M); chloroplast membrane (CM); plasma membrane (PM); nuclear (N).

3.1.2. Analysis of BhABC Protein Domains

The ABC transporter domain has various histological forms. In this paper, BlastP provided by the National Center of Biotechnology Information (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) was used to predict conserved NBDs of 15 BhABC proteins (see Figure 1). Using numbers of NBDs, lengths of protein molecules, and results of phylogenetic tree analysis, the 15 BhABC proteins were divided into subfamilies (ABCB, ABCG, ABCF, and ABCI). Within these subfamilies, ABCB (MDR) and ABCG (PDR) proteins are full transporters and each contains two NBDs, whereas ABCB (TAP) and ABCG (WBC) proteins are half transporters with one NBD each. ABCF and ABCI subfamily proteins are soluble ABC proteins containing one NBD domain each.

3.1.3. Phylogenetic Analysis of BhABC Proteins

For phylogenetic analysis, a total of 68 ABC transporters were selected, comprising 24 *Oryza sativa* transporters, 29 *Arabidopsis* transporters [27], and 15 BhABC transporters. Phylogenetic tree analysis, carried out using MEGA software, sorted the 68 protein sequences into eight subfamilies (ABCA-ABCG and ABCI) unique to plants (see Figure 2). The ABCB subfamily was further divided into MDR, TAP, and ATM subgroups and the ABCG subfamily into PDR and WBC subgroups. Most BhABC transporters were found within a limited set of subfamilies (with or without subgroup in parentheses) as follows: ABCB (MDR), ABCB (TAP), ABCF, ABCG (PDR), ABCG (WBC), and ABCI. More specifically, BhABC7 and BhABC11 belonged to ABCB (MDR); BhABC3 belonged to ABCB (TAP); BhABC1, BhABC9, BhABC10, BhABC12, and BhABC15 belonged to ABCG (PDR); BhABC14 belonged

to ABCG (WBC); and BhABC5 and BhABC6 belonged to subfamily ABCF. BhABC2, BhABC4, BhABC8, and BhABC13 belonged to subfamily ABCI.

Figure 1. Prediction of conserved domains of *BhABC* genes. Gray areas represent exons, blue areas represent introns, and orange areas represent locations of nucleotide-binding domains (NBDs).

Figure 2. Phylogenetic tree of ABC transporter proteins (*BhABC* transporters noted with red/orange diamonds). Phylogenetic subclasses are marked with different colors and corresponding subfamily names. The phylogenetic tree was drawn using MEGA 5.0 and was based on the following parameters: neighbor-joining tree method, complete deletion, and 500 bootstrap replicates. OsABC denotes *Oryza sativa* ABC proteins (including 24 members), AtABC denotes *Arabidopsis* ABC proteins (including 29 members), and BhABC denotes *B. halophila* ABC proteins (including 15 members). Numeric values indicate estimated evolutionary distances.

3.2. Responses of *BhABC* Genes to Hormone Treatments

3.2.1. Responses of *BhABC* Genes to 6-Benzylaminopurine (6-BA)

Under 6-BA treatment, altered mRNA expression of most *BhABC* genes encoding full transporters and half transporters was observed in xylem, roots, and leaves, whereas in apical buds only the expression of *BhABC* genes encoding full transporters changed in response to treatment (see Figure 3). In xylem, mRNA levels reflecting expression of *BhABC1*, *BhABC2*, *BhABC4*, *BhABC7*, and *BhABC11* genes were mainly upregulated at all treatment time points except at 72 h. Meanwhile, mRNA expression of *BhABC1* and *BhABC2* peaked at 6 h and of *BhABC4* peaked at 24 h. *BhABC8*, *BhABC12*, and *BhABC13* genes showed upregulated transcriptional responses after 6 h of treatment, whereas *BhABC15* exhibited a transcriptional response that was first upregulated then downregulated. *BhABC3*, *BhABC5*, *BhABC6*, *BhABC9*, *BhABC10*, and *BhABC14* expression levels did not change with treatment. In roots, mRNA expression levels of *BhABC4* and *BhABC14* were transiently upregulated at 6 h and 48 h, respectively. *BhABC1*, *BhABC2*, *BhABC7*, *BhABC8*, *BhABC11*, and *BhABC15* genes were mainly upregulated at time points during the middle stage of treatment. Both *BhABC1* and *BhABC2* exhibited peak expression at 6 h. Expression levels of *BhABC12* and *BhABC13* genes increased during middle and late treatment stages, whereas expression of *BhABC3*, *BhABC5*, *BhABC6*, *BhABC9*, and *BhABC10* genes did not change. In leaves, *BhABC1*, *BhABC4*, *BhABC7*, *BhABC11*, *BhABC12*, *BhABC13*, and *BhABC15* were mainly upregulated after 12 h, but *BhABC1* and *BhABC4* reached peak expression levels only at 72 h and expression of other genes in leaves remained unchanged. In apical buds, only *BhABC1*, *BhABC7*, and *BhABC15* responded to 6-BA treatment, with expression of *BhABC15* in bud similar to expression in xylem, such that both were upregulated then downregulated, whereas *BhABC1* and *BhABC7* exhibited upregulation at 12 h and 4 h of treatment, respectively.

Figure 3. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after 6-BA treatment. Heat map showing responses of *BhABC* genes to 6-BA (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.2.2. Responses of *BhABC* Genes to Brassinolide (BR)

During BR treatment, *BhABC* full transporter genes were the main genes with altered expression in xylem and apical buds, whereas most *BhABC* genes among all three subgroups (full transporter, half transporter, soluble ABC proteins) responded to BR in roots and leaves (see Figure 4). In xylem, only *BhABC1*, *BhABC11*, *BhABC12*, *BhABC14*, and *BhABC15* responded to BR, with upregulated expression of each after 12 h, whereas expression of *BhABC1* peaked at 48 h. In roots, *BhABC15* was first upregulated then downregulated, *BhABC12* was upregulated early, and *BhABC3* and *BhABC10* genes were upregulated at 72 h. *BhABC1*, *BhABC2*, *BhABC4*, *BhABC5*, *BhABC8*, *BhABC11*, and *BhABC14* genes all showed different degrees of upregulation in middle and late treatment stages, with *BhABC1* mRNA expression peaking at 72 h. *BhABC6*, *BhABC7*, *BhABC9*, and *BhABC13* were unresponsive to BR treatment, with mRNA expression levels remaining unchanged. In leaves, only *BhABC3*, *BhABC13*, and *BhABC14* mRNA levels remained unchanged, whereas the remaining genes responded to BR hormone treatment; the expression level of *BhABC15* was initially downregulated then upregulated, whereas other genes were mainly upregulated in the middle and late stages of treatment and *BhABC4* expression peaked at 72 h. In apical buds, only *BhABC1*, *BhABC5*, *BhABC10*, *BhABC11*, *BhABC12*, and *BhABC15* responded to BR, with *BhABC15* mainly showing a downregulated transcriptional response and the other genes exhibiting upregulation during middle and late treatment stages.

Figure 4. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after BR treatment. Heat map showing responses of *BhABC* genes to BR (right); semi-quantitative RT-PCR analysis with 18s rRNA internal reference (left).

3.2.3. Responses of *BhABC* Genes to Gibberellin (GA₃)

During GA₃ treatment (see Figure 5), only *BhABC11*, *BhABC14*, and *BhABC15* mRNA expression levels changed in xylem, with downregulation of expression of *BhABC11* and upregulation of *BhABC14* and *BhABC15* observed after 12 h. In roots, mRNA levels of *BhABC1*, *BhABC2*, *BhABC3*, *BhABC4*, *BhABC5*, *BhABC7*, *BhABC9*, *BhABC10*, *BhABC12*, and *BhABC14* were upregulated after 6 h, with expression of *BhABC1* and *BhABC3* peaking at 48 h. *BhABC6*, *BhABC8*, *BhABC11*, and *BhABC13* expression did not change, whereas *BhABC15* was downregulated after 12 h. In leaves, *BhABC1*, *BhABC2*, *BhABC5*, and *BhABC11* showed upregulated transcriptional responses during the middle stage of treatment, with *BhABC1* and *BhABC2* reaching highest expression levels at 48 h. *BhABC10*, *BhABC12*, *BhABC13*, *BhABC14*, and *BhABC15* were upregulated during middle and late stages of treatment, whereas *BhABC3*, *BhABC4*, *BhABC6*, *BhABC7*, *BhABC8*, and *BhABC9* genes were not expressed. In apical buds, *BhABC15* showed alternate upregulation and downregulation, whereas mRNA levels of *BhABC1*, *BhABC5*, *BhABC10*, *BhABC11*, *BhABC12*, *BhABC13*, and *BhABC14* genes were upregulated at most time points throughout the experiment, with expression of *BhABC1* reaching a peak at 48 h. No changes in the expression of remaining genes were observed.

Figure 5. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after GA₃ treatment. Heat map showing responses of *BhABC* genes to GA₃ (right); semi-quantitative RT-PCR analysis with 18s rRNA internal reference (left).

3.2.4. Responses of *BhABC* Genes to Salicylic Acid (SA)

During SA treatment, genes encoding three subgroups of two subfamilies of *BhABC* transporters, namely *ABCG* (*PDR*), *ABCG* (*WBC*), and *ABCB* (*MDR*), exhibited differing degrees of responses among all four tissue sites (see Figure 6). In xylem, mRNA-level expression of *BhABC11* and *BhABC15* was downregulated during early treatment stages, whereas *BhABC13* and *BhABC14* showed upregulated expression after 12 h. No changes in the expression of remaining genes were observed. In roots, only seven genes responded to SA, with expression of *BhABC15* downregulated, whereas *BhABC12* was upregulated at 72 h. Expression levels of *BhABC2*, *BhABC3*, *BhABC7*, *BhABC11*, and *BhABC14* peaked in middle and later treatment stages, with mRNA transcription levels of *BhABC2* and *BhABC3* highest at 48 h. In leaves, *BhABC14* and *BhABC15* were downregulated in later treatment stages, *BhABC1*, *BhABC2*, *BhABC3*, *BhABC5*, *BhABC6*, *BhABC7*, *BhABC10*, *BhABC11*, and *BhABC12* genes were upregulated in middle and later treatment stages, and no changes in expression of *BhABC4*, *BhABC8*, *BhABC9*, and *BhABC13* were observed. In apical buds, *BhABC14* exhibited upregulation then downregulation of transcriptional response. Meanwhile, *BhABC11* and *BhABC12* were briefly upregulated between 2 h and 4 h, whereas *BhABC1*, *BhABC6*, *BhABC10*, and *BhABC15* genes were upregulated during middle and later treatment stages, and *BhABC15* expression peaked at 72 h. No changes in the expression of remaining genes were observed in response to SA treatment.

Figure 6. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after SA treatment. Heat map showing responses of *BhABC* genes to SA (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.2.5. Responses of *BhABC* Genes to Jasmonic Acid (JA)

During JA treatment, generally most *BhABC* full transporter and half transporter gene subgroups responded to JA in all four tissues (see Figure 7). In xylem, only *BhABC11*, *BhABC14*, and *BhABC15* responded to JA, with mRNA expression of *BhABC11* and *BhABC14* upregulated then downregulated during middle and later treatment stages. In roots, five genes responded to JA, including *BhABC14*, which was upregulated then downregulated, whereas *BhABC9*, *BhABC11*, *BhABC12*, and *BhABC15* were all upregulated and *BhABC15* peaked at 2 h. In leaves, *BhABC2*, *BhABC7*, *BhABC9*, *BhABC11*, and *BhABC12* were all upregulated after 24 h. By contrast, expression levels of *BhABC14* and *BhABC15* decreased during early treatment stages and the remaining genes experienced no changes in expression. In apical buds, *BhABC15* exhibited upregulation followed by downregulation, whereas *BhABC1*, *BhABC2*, *BhABC3*, *BhABC4*, *BhABC6*, *BhABC7*, *BhABC9*, *BhABC11*, *BhABC13*, and *BhABC14* were upregulated during middle and late treatment stages. No changes were observed in *BhABC5*, *BhABC8*, *BhABC10*, *BhABC12*, and *BhABC13* expression.

Figure 7. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after JA treatment. Heat map showing responses of *BhABC* genes to JA (right); semi-quantitative RT-PCR analysis with 18S rRNA internal reference (left).

3.2.6. Responses of *BhABC* Genes to Indole-3-Acetic Acid (IAA)

After IAA treatment (see Figure 8), in xylem and roots *BhABC* genes responding to IAA were mainly full and half transporters, whereas in leaf and terminal buds all three types of *BhABC* proteins responded to IAA. In xylem, only *BhABC14* responded to IAA and exhibited upregulated expression in middle and late treatment stages. In roots, *BhABC2*, *BhABC11*, *BhABC14*, and *BhABC15* were upregulated during middle and later stages of IAA treatment, whereas remaining genes were not induced. In leaves, *BhABC2*, *BhABC3*, *BhABC4*, *BhABC5*, *BhABC6*, *BhABC7*, *BhABC9*, *BhABC11*, *BhABC14*, and *BhABC15* exhibited different degrees of upregulation during middle and late treatment stages, with *BhABC11* peaking at 48 h and expression of *BhABC1*, *BhABC8*, *BhABC10*, *BhABC12*, and *BhABC13* unchanged. Expression patterns of *BhABC* genes in apical buds were similar to patterns in leaves, with *BhABC11* expression peaking at 48 h as well.

Figure 8. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after IAA treatment. Heat map showing responses of *BhABC* genes to IAA (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.2.7. Responses of *BhABC* Genes to Absciscic Acid (ABA)

In response to ABA treatment, expression of the three subgroups of *BhABC* genes in all four studied tissues responded to ABA (see Figure 9). In xylem, only *BhABC3*, *BhABC11*, *BhABC14*, and

BhABC15 showed upregulated expression, which occurred during middle and late treatment stages, with remaining genes not induced. In roots, *BhABC1*, *BhABC6*, *BhABC8*, *BhABC11*, *BhABC12*, *BhABC14*, and *BhABC15* were upregulated during middle and late stages of treatment, whereas no other genes were induced. In leaves, *BhABC1*, *BhABC2*, *BhABC3*, *BhABC6*, *BhABC8*, *BhABC11*, *BhABC12*, *BhABC14*, and *BhABC15* all exhibited upregulated expression after 48 h, with expression levels of *BhABC1* and *BhABC15* peaking at 72 h and remaining genes not induced. In apical buds, *BhABC4*, *BhABC5*, and *BhABC13* RNA levels were unchanged, but the other genes exhibited upregulated expression to varying degrees during middle and late stages of treatment, with *BhABC1*, *BhABC2*, *BhABC3*, and *BhABC15* upregulated to the greatest extent between 48 h and 72 h.

Figure 9. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after ABA treatment. Heat map showing responses of *BhABC* genes to ABA (right); semi-quantitative RT-PCR analysis with 18s rRNA internal reference (left).

3.3. Expression Characteristics of *BhABC* Genes under Abiotic Stress

3.3.1. Expression of *BhABC* Genes under NaCl Stress

Under conditions of NaCl stress, the three subgroups of *BhABC* proteins showed variable degrees of responsiveness among all four tissue sites (see Figure 10). In xylem, *BhABC15* exhibited

downregulated expression after 6 h, whereas *BhABC2*, *BhABC3*, *BhABC5*, *BhABC6*, *BhABC10*, *BhABC11*, *BhABC12*, *BhABC13*, and *BhABC14* all exhibited upregulated expression to varying degrees during middle and later stages of treatment. No changes in expression of *BhABC1*, *BhABC4*, *BhABC7*, *BhABC8*, and *BhABC9* were observed. In roots, *BhABC15* exhibited downregulated expression, whereas *BhABC2* and *BhABC6* exhibited upregulated expression at 72 h and *BhABC7* exhibited a transient increase in expression at 24 h. Meanwhile, *BhABC3*, *BhABC5*, *BhABC11*, *BhABC13*, and *BhABC14* showed higher expression levels during middle or later treatment stages, whereas expression of *BhABC3* and *BhABC11* peaked at 24 h. No changes in the expression of remaining genes were observed. In leaves, only *BhABC8* did not respond to NaCl treatment, whereas *BhABC15* showed upregulation followed by downregulation. All remaining genes were upregulated during middle and late treatment stages, with expression of *BhABC1* and *BhABC9* peaking at 24 h. In apical buds, *BhABC3*, *BhABC8*, and *BhABC15* were not induced, whereas all other genes exhibited upregulation during the middle treatment stage.

Figure 10. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after NaCl treatment. Heat map showing responses of *BhABC* genes to NaCl (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.3.2. Expression of *BhABC* Genes under CdCl_2 Stress

As for NaCl stress, three subgroups of *BhABC* proteins responded to CdCl_2 stress in all four tissues (see Figure 11). In xylem, *BhABC11* and *BhABC15* were upregulated then downregulated, whereas *BhABC1*, *BhABC2*, *BhABC5*, *BhABC6*, *BhABC8*, *BhABC12*, and *BhABC14* were upregulated during middle and later stages of treatment, with expression of *BhABC1*, *BhABC2*, and *BhABC6* genes significantly upregulated at 48 h. In roots, only *BhABC7*, *BhABC10*, and *BhABC13* did not respond to CdCl_2 , whereas *BhABC1*, *BhABC3*, *BhABC6*, and *BhABC8* exhibited downregulation. *BhABC11*, *BhABC12*, and *BhABC15* showed upregulation then downregulation, whereas all remaining genes were upregulated to varying degrees. In leaves, all genes except *BhABC7* responded to CdCl_2 treatment, with *BhABC14* and *BhABC15* exhibiting upregulation then downregulation and remaining genes exhibiting upregulation to varying degrees during middle and later treatment stages. In apical buds, *BhABC10* was downregulated in the middle treatment stage, whereas *BhABC1*, *BhABC9*, *BhABC14*, and *BhABC15* exhibited upregulation then downregulation. Meanwhile, *BhABC3*, *BhABC4*, *BhABC5*, and *BhABC6* were upregulated dramatically at 6 h, with upregulation and high expression of *BhABC11* observed at 72 h. No changes in the expression of remaining genes were observed.

Figure 11. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after CdCl_2 treatment. Heat map showing responses of *BhABC* genes to CdCl_2 (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.3.3. Expression of *BhABC* Genes under NaHCO_3 Stress

During NaHCO_3 stress, *BhABC* genes generally exhibited low levels of expression in xylem, leaves, and apical buds (see Figure 12). In xylem, only *BhABC1*, *BhABC3*, *BhABC14*, and *BhABC15* responded to NaHCO_3 stress, with *BhABC15* showing downregulated expression during the middle treatment stage, *BhABC1* and *BhABC3* showing upregulation at 6 h, and upregulation observed for *BhABC14* at 72 h. In leaves, regulation of *BhABC15* expression resembled its regulation pattern in xylem, showing a downregulated expression pattern during later treatment stages. *BhABC2* expression was upregulated to a relatively high level at 24 h, whereas *BhABC11* was upregulated during middle and late treatment stages. In apical buds, *BhABC2*, *BhABC11*, *BhABC12*, *BhABC14*, and *BhABC15* expression levels were mainly upregulated during middle and later treatment stages. In roots, *BhABC1*, *BhABC3*, *BhABC6*, *BhABC7*, *BhABC8*, *BhABC9*, *BhABC10*, *BhABC11*, *BhABC14*, and *BhABC15* exhibited upregulation during middle and later stages of treatment, with *BhABC3*, *BhABC7*, *BhABC9*, *BhABC11*, and *BhABC14* exhibiting significantly increased expression levels at 72 h. No changes in the expression of remaining genes were observed.

Figure 12. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after NaHCO_3 treatment. Heat map showing responses of *BhABC* genes to NaHCO_3 stress (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

3.3.4. Expression of *BhABC* Genes under Polyethylene Glycol (PEG) stress

Genes exhibiting altered mRNA expression after plant exposure to PEG stress were mainly *BhABC* full and half transporters, with altered expression observed in the four tissues studied (see Figure 13). In xylem, *BhABC11* and *BhABC15* showed opposing transcriptional responses of upregulation and

downregulation, respectively. *BhABC1* and *BhABC2* were downregulated at 6 h and 48 h, respectively, whereas *BhABC6* and *BhABC14* were upregulated at 72 h. In roots, *BhABC11* was downregulated during the middle treatment stage, whereas *BhABC12* and *BhABC15* were upregulated then downregulated. Meanwhile, *BhABC1*, *BhABC2*, *BhABC3*, *BhABC5*, and *BhABC14* genes were upregulated at some time points, whereas no changes in the expression of remaining genes were observed. In leaves, expression levels of *BhABC6* and *BhABC15* were first downregulated then upregulated and expression levels of *BhABC1*, *BhABC2*, *BhABC3*, *BhABC5*, *BhABC7*, *BhABC8*, *BhABC11*, *BhABC12*, and *BhABC14* were all upregulated to varying degrees over the course of treatment. No changes in the expression of remaining genes were observed. In apical buds, *BhABC11*, *BhABC14*, and *BhABC15* were all upregulated then downregulated, whereas *BhABC1*, *BhABC5*, *BhABC6*, *BhABC7*, *BhABC8*, and *BhABC12* were all upregulated, with expression of *BhABC12* peaking during the first PEG treatment stage. No changes in the expression of remaining genes were observed.

Figure 13. Expression patterns of *BhABC* genes in xylem, roots, leaves, and apical buds after PEG treatment. Heat map showing responses of *BhABC* genes to PEG (right); semi-quantitative RT-PCR analysis with 18s RNA internal reference (left).

4. Discussion

After analyzing physical and chemical properties of 15 *BhABC* family proteins deduced from transcriptome gene sequences, six *BhABC* proteins were found to lack transmembrane domains. Subsequent prediction of structural domains and phylogenetic tree analysis revealed six genes coding for soluble ABC proteins belonging to ABCF and ABCI subfamilies. We constructed a preliminary

phylogenetic tree of BhABC transporter proteins showing that they were distributed among six subfamilies of which several contained subgroups (in parentheses): ABCB (MDR), ABCB (TAP), ABCF, ABCG (PDR), ABCG (WBC), and ABCI. Except for the ABCI subfamily, all other subfamilies were associated with single phylogenetic tree branches (see Figure 2), a result that aligns with previously reported phylogenetic tree results for *Arabidopsis* and *O. sativa* ABC transporter proteins [27]. As ABCI proteins contain only a single domain (NBD or auxiliary domain), clustering of members of this subfamily was observed within three distinct phylogenetic tree branches, each closely associated with different subfamilies. The phylogenetic tree shows that protein pairs with greatest homology, namely BhABC7 paired with BhABC11 and BhABC10 paired with BhABC15, exhibited 72.33% and 92.87% amino acid sequence similarity, respectively. Comparisons among ABC transporters of *B. halophila*, *A. thaliana*, and *O. sativa* demonstrated variability in relative evolutionary distances between proteins. For example, three pairs of close homologs, BhABC8 and OsABCI15, BhABC14 and AtABCG24, and BhABC2 and AtABCI17, exhibited protein sequence similarities of 77.03%, 60.59%, and 70.79%, respectively. Greater homology of transporters across subfamilies than among members within a given subfamily may be explained by the functional conservation of structure between protein homologs of herbs and woody plants. However, although the similarity between BhABC3 and OsABCB25 was 65%, the similarity between BhABC5 homologs of *A. thaliana* and *O. sativa* was only about 50%, suggesting that substantial genetic and functional differences exist between woody and herbaceous plants.

We studied expression characteristics of BhABC genes in response to exogenous plant hormones and abiotic stressors using semi-quantitative RT-PCR analysis. Of the genes responding to these stimuli, a high proportion belonged to ABCB and ABCG subfamilies, which happen to be the same plant subfamilies currently under study for their suspected roles in exogenous hormone and abiotic stressor responses [4]. As BhABCs are known to exhibit variable expression patterns in response to exogenous hormones, under IAA treatment BhABC11 (ABCB subfamily, subgroup MDR) was upregulated in roots and BhABCB (MDR) genes BhABC7 and BhABC11 were upregulated in leaves and apical buds. At the same time, IAA led to increased gene expression in *B. halophila* roots, leaves, and apical buds of ABCG (PDR) genes BhABC9 and BhABC15. In *A. thaliana*, the AtABCB19, AtABCB4, AtABCB11/MDR8, AtABCB14/MDR12, and AtABCB15/MDR13 genes are involved in auxin transport and regulation [11,12,28], with Indole-3-Butyric acid (IBA) serving as a storage form of IAA. AtABCG36/PDR8 mediates IBA output and regulates its accumulation in cells, contributing to the regulation of intracellular auxin dynamic balance [29]. We found that treatment with IAA led to increased gene expression in *B. halophila* roots, leaves, and apical buds of ABCG (PDR) genes BhABC9 and BhABC15. Meanwhile, cytokinins are substances mainly synthesized in root tips which regulate plant growth during water transport from xylem to aboveground plant tissues. The AtABCG14 gene, a member of subgroup ABCG (WBC), is involved in transport of cytokinins in roots [24]. Notably, in *B. halophila*, 6-BA treatment led to upregulated expression of the ABCG (WBC) gene BhABC14 only in roots, raising speculation that BhABC14 may share functionality with AtABCG14. Likewise, after ABA treatment, some BhABCG subfamilies showed upregulated expression patterns in the four tissue types studied here; some of these results align with results of an earlier study demonstrating that stomata of loss-of-function AtABCG40 mutants closed more slowly in response to ABA, resulting in reduced drought tolerance [21]. In addition, another transport protein, AtABCG22, may play a role in ABA signal transduction and ABA biosynthesis [30,31]. Meanwhile, GmPDR12 in soybean (*Glycine max*) can be expressed rapidly and in large quantities in response to treatment with salicylic acid and its functional derivatives [32]; here, after salicylic acid treatment BhABC15 (PDR) was downregulated in xylem, roots, and leaves. Taken together, the collective results demonstrate that ABC transporters may have different functions in woody versus herbaceous plants.

When under abiotic stress from cadmium treatment, *Arabidopsis* seedlings upregulate AtPDR8 (ABCG subfamily) in roots, stems, and other plant tissues [33], whereas ABCB HMT1 protein can improve cadmium tolerance in yeast cells [34,35]. The relationship between ABC transporter proteins and drought resistance has also been studied, with expression of proteins encoded by the ABCG gene subfamily in millet found to vary to differing degrees in response to PEG stress [36]. Moreover, in

A. thaliana, *AtABCG40* (PDR) gene expression relies on the ABA signaling pathway to regulate the rate of stomatal closure, thereby increasing the ability of plants to resist drought [19]. Meanwhile, *AtABCG36* helps plants resist drought and salt by reducing the amount of sodium in the plant [37]. Here, CdCl₂ treatment resulted in variable changes in expression levels of most *BhABCG* (PDR) genes in the four plant tissues studied, with *BhABCB* genes mainly downregulated in xylem and roots. During PEG treatment, *ABCG* gene expression was altered in all four tissues, whereas during NaCl stress, expression of *ABCG* (PDR) genes in xylem, leaves, and apical buds were all upregulated.

Under stress, the balance between various hormone levels is disrupted, leading to disturbances in plant growth rhythms and disordered metabolic functions. Therefore, below a certain limit, plants can apparently adjust their physiological functions to compensate for changing hormone levels in order to adapt to adverse environmental conditions. In the abovementioned experiment, all genes that were upregulated in response to ABA hormone treatment were upregulated in all four tissues in this study. The expression patterns of *BhABC* transporters in response to NaCl, PEG, NaHCO₃, CdCl₂, and ABA treatments suggest that *BhABC* genes, which are involved in different abiotic stress responses, may be regulated by ABA-dependent stress signaling pathways.

5. Conclusions

The 15 identified *BhABC* genes were assigned to four major subfamilies. In the *ABCB* subfamily were *BhABC7* and *BhABC11* (MDR) and *BhABC3* (TAP); in the *ABCG* subfamily were *BhABC1*, *BhABC9*, *BhABC10*, *BhABC12*, *BhABC15* (PDR), and *BhABC14* (WBC); the *ABCF* subfamily contained *BhABC5* and *BhABC6*; all remaining genes (*BhABC2*, *BhABC4*, *BhABC8*, and *BhABC13*) were assigned to subfamily ABCI. After treatment with seven exogenous phytohormones and four abiotic stress treatments, it was found that most *ABCB* and *ABCG* genes were involved in plant responses to hormones and abiotic stress. We note that molecular mechanisms underlying these responses require further study.

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Abbreviations

NBD	Nucleotide binding domains
TMD	Transmembrane domains
ORF	Open reading frame
6-BA	6-Benzylaminopurine
BR	Brassinolide
GA ₃	Gibberellin
SA	Salicylic acid
JA	Jasmonic acid
IAA	Indole-3-acetic acid
ABA	Absciscic acid
PDR	Pleiotropic drug resistance
WBC	White-brown complex
ATH	ABC2 homologue
MDR	Multidrug resistance protein
TAP	Transporter associated with antigen processing
ATM	ABC transporter of the mitochondria
MRP	Multidrug resistance-associated protein

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