

The functional analysis of ABCG transporters in the adaptation of pigeon pea (*Cajanus cajan*) to abiotic stresses

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Abstract: ATP-binding cassette (ABC) transporters are a class of proteins that widely present in living organisms and mediate transmembrane transport of substances by hydrolyzing ATP, play a vital role in the physiological process of growth and development in plants. The ABCG is the largest subclass of ABC transporters family, which has the most complex functions in plant's response to abiotic stress. This study focused on the effect of ABCG transporters in pigeon pea adaptation to the adverse environments (such as drought, salt, temperature, etc.). Based on this, the functional analysis of ABCG transporters in pigeon pea to abiotic stresses was conducted. In this study, a total of 51 *ABCG* genes (*CcABCGs*) were identified, further, their phylogenetic analysis and physicochemical properties of the encoded proteins, subcellular localization prediction, and conserved domains were performed. Expression analysis showed that *ABCG* genes have different expression profiles under tissues and abiotic stresses. Results showed that *CcABCG28* was up-regulated at low temperature, however, *CcABCG7* was up-regulated by drought and aluminum stress. Initial results revealed that ABCG transporters is more effective in the abiotic stresses resistance of pigeon peas, which broaden its research direction in abiotic stress resistance.

Keywords: Pigeon pea (*Cajanus Cajan*); ABCG transporters; Abiotic stresses; Gene expression

Introduction

ABC transporters, also known as ATP-binding cassette transporters, are a part of the largest and oldest known protein families and are widely found in the eukaryotic and prokaryotic organisms (Martinoia et al., 2002; Mosser et al., 1993). ABC transporters play an extremely important role in the growth and development of plants by participating in the detoxification of exogenous toxins for the response to abiotic stress, transporting the molecular of metabolites such as intercellular peptides, sugars, lipids, alkaloids, and inorganic ions and other metabolic substances (Mendez & Salas, 2001; Morris & Zhang, 2006; Mourez et al., 1997). *AtABCB1* (also known as *AtMDR1*) identified in *Arabidopsis* was the first reported in plants in 1992 (Dudler & Hertig, 1992). In the plant genomes, ABC transporters are divided into 8 subfamilies (ABCA-ABCG and ABCI) according to the HUGO (Human Genome Organization) scheme. Then, 129, 128, 261 ABC transporters have been identified in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*) and soybean (*Glycine max*) genomes, respectively (Mishra et al., 2019; Schulz & Kolukisaoglu, 2006; Sanchez-Fernandez et al., 2001).

The ATP-binding cassette subfamily G is the largest subfamily of ABC transporters family. It is found that ABCG protein is widely distributed in plants and plays an important role in many fundamental physiological processes (Kretschmar et al., 2011). A typical structural feature of ABCG transporters has a highly conservative amino acid of 1-2 nucleotide-binding domains (NBDs) and a highly hydrophobic transmembrane domain of 1-2 trans-membrane domains (TMDs) (Verrier et al., 2008). ABCG subfamily is divided into WBCs (white-brown complex) and PDRs (pleiotropic drug resistance). WBCs are semi-molecular ABCG transporter, contain one NBD domain, and one TMD domain, while PDRs belong to the full-molecule ABCG transporter, include two NBD

domains and two TMD domains (Jasinski et al., 2009). The NBD domain consists of three highly conserved regions of approximately 200 amino acids and include Walker A box [GX₄GK (ST)], Walker B box [(RK) X₃GX₃L (hydrophobic)₃], and Walker C between them with approximately 120 amino acids in length (J. E. Walker, 1982). The TMD domain contains 4~6 α -helices, which constitute the channel for the transport of substrate molecules for transmembrane transport (Hyde et al., 1990; Schneider & Hunke, 1998). Therefore, it is generally believed that the mechanism of ABCG transporters is that the substrate recognition site on the TMD domain recognize and bind the transport substrates located near the cell membrane, while NBD domain on the cell membrane hydrolyzes ATP to provide energy for substrate transport and cause conformational changes in the membrane structure (Davidson & Maloney, 2007). *SpTUR2*, the first full-molecule ABCG transporter gene in plants, have been identified in the perennial aquatic plant *Spirodella polyrhiza* in 2002 (van den Brule et al., 2002). So far, more than 40 ABCG transporters have been identified in the *Arabidopsis*, rice and soybean genomes, respectively. ABCG transporters are involved with many physiological activities of the plant. For example, *OsABCG31* identified in rice could decrease the evaporation of plant leaves, which is predicted to relate to the drought stress response of rice (Chen et al., 2011). *Arabidopsis AtPDR8* is involved with Na⁺ excretion, increasing the plant's tolerance to salt and drought stress (Kim et al., 2007). It was also found that *AtPDR36* in *Arabidopsis thaliana* responded to the toxic effects of heavy metal lead and participated in the stomatal self-regulation of leaves (Kim et al., 2010).

Cajanus cajan (L.) Millsp., as known as pigeon pea, is a diploid plant (2n=22) with a genome size of approximately 858 Mbp and grows in tropical and subtropical regions, which has formed a stable regulatory system to adapt to the adverse environment of high temperature, high salt and drought (Singh et al., 2013; Varshney et al., 2011; Wu et al., 2011; Yadu et al., 2018). Meanwhile, chilling is an important environmental factor in the practice of pigeon pea migration to the north. Besides, pigeon pea has widely grown in acidic soil (pH5-7), and its good aluminum resistance has brought it much attention. Pigeon pea produces numerous secondary metabolites that play an important role in the adaptation of the plant to the adverse environment (H Shepherd, 1986). Pigeon pea possesses medicinal properties and widely used in chemical industry (Ogoda et al., 2002; Pandey & Pandey, 1991).

However, the current research work focused on the trans-membrane transport of secondary metabolite, antibiotics, heavy metal ions by ABCG transport protein hydrolysis of ATP (Yazaki, 2006; Badri et al., 2008; Le Hir et al., 2013; Fourcroy et al., 2014). The inhibition of ABCG transporters decreases plant flavonoid content (Morris & Zhang, 2006; Imai et al., 2004). It was found that plants are transporting some hormones (such as ABA) which increases the survival of plants in a drought and other adversity environments (Kuromori et al., 2010; Kang et al., 2010). It reminds us that ABCG transporters can positively affect plant adversity by transporting certain substances. To reveal the important role of ABCG transporters in resisting environmental stress in pigeon peas, identification, phylogenetic analysis, and expression analysis, etc. of the pigeon pea ABCG gene family were performed in our study. A total of 51 ABCG transporters were identified and further analyzed. We have found that some cis-acting elements related to stress-responsive existed in several genes identified above, suggesting that ABCG transporter had a related regulatory effect in pigeon pea. To explore the expression profile of ABCG gene in pigeon pea, we analyzed the expression of ABCG gene under different organs and different stress treatments and observed the tissue specificity of ABCG gene and stress expression response. Our findings will lay the foundation for exploring the mechanism of ABCG transporters in resistance in several abiotic stresses of pigeon pea.

Materials and Methods

Identification of ABCG transporters in pigeon pea genome

The whole 129 *Arabidopsis* ABC protein sequences were downloaded in the Phytozome v12.1 database (<https://phytozome.jgi.doe.gov/pz/portal.html>). All of these *Arabidopsis* ABCs as queries were used to identify the

ABC transporters in *Cajanus cajan* (L.) Millsp (taxid: 3821) database using BLASTP search in NCBI (National Center for Biotechnology Information) with an initial cut-off e-value of 1.0×10^{-10} and max target sequence of 500. Also, the HMM (Hidden Markov Model) profiles of ABC transporters (such as PF00005, PF00664, and PF10614) were downloaded from the Pfam database (<http://pfam.xfam.org/search#tabview=tab1>) and used HMMER search server against the pigeon pea proteome with an E-value setting of 1.0×10^{-5} (<https://www.ebi.ac.uk/Tools/hmmer/>) (Potter et al., 2018). Then, the resulting protein sequences were further identified whether it contained a conserved domain of ABC based on CD-search (Conserved Domain search) on the NCBI website with a threshold e-value of 1.0×10^{-5} . Through the above methods, members of the ABC transporters were identified.

Then, multiple-sequence alignments of ABC transporters identified above and several ABC proteins of *Arabidopsis thaliana*, rice, and soybean were performed using clustalW with default settings. The phylogenetic tree of the ABC transporters was constructed using the neighbor-joining (NJ) method with the 1000 replications of bootstrap and p-distance of a model in MEGA6.0 software (<https://www.megasoftware.net/>) (Tamura et al., 2013). The phylogenetic tree was visualized with iTOL website (<http://itol.embl.de/help.cgi>) (Sugiyama et al., 2007). Finally, ABCG transporters were identified through the phylogenetic analysis of the ABC family of pigeon pea.

Phylogenetic tree construction and chromosome localization

The chromosomal information of *ABCG* genes was obtained through the NCBI website (<https://www.ncbi.nlm.nih.gov/>) and was systematically named based on the number and relative position of the *ABCG* genes in the pigeon pea chromosome. The phylogenetic tree of pigeon pea ABCG transporters was constructed in MEGA6.0 as described above.

Based on the chromosomal location information of the ABCG transporters, the chromosomal location of the *CcABCGs* was mapped using the MapChart software (<https://www.wur.nl/en/show/Mapchart.html>) (Voorrips, 2002).

Protein property prediction, subcellular localization

The exon information of *CcABCGs* was collected on the NCBI website. The number of amino acid sequences, relative molecular weight, and the isoelectric point (pI) of *CcABCGs* was predicted in the ExPASy ProtParam server database (<http://expasy.org/>). Subcellular localization prediction of ABCG proteins was performed by the Cell-PLoc 2.0 database (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>) (Chou & Shen, 2008).

To verify the above predictions of subcellular localization, two-week-old seedlings of pigeon pea were used as cDNA template to amplify the full-length coding region of *CcABCG7* gene. The primers were: F: 5'-ATGGTGATGATATGGGAAATGTTAC-3' and R: 5'-TTATATTGGAAGTTTGGGGACA-3'. The recovered PCR production was ligated to the T vector pMD19-T (TaKaRa, Japan). Subsequently, it was cloned into the eGFP-pROK II vector by double digestion with Kpn I /Xba I to construct the *CcABCG7*-eGFP-pROK II expression vector and transformed into agrobacterium strain GV3101 (Shanghai Weidi Biotechnology, China). One-month-old tobacco seedlings were injected into the Agrobacterium liquid on the back of the tobacco leaves with a disposable syringe and placed in a dark place for 3 days for observation. Approximately 0.5 cm^2 of the material was taken around the injection site, observed, and photographed under a Leica SP8 laser confocal microscope (Leica, Germany).

Analysis of motifs and conserved domain

To examine the characteristics and properties of the pigeon pea ABCG subfamily protein domain, the conserved motif of *CcABCGs* was analyzed on the MEME software (<http://meme-suite.org/tools/meme>) with following parameters: the number of motifs was set 10 and the optimum motif width was set between 20 and 200, the other

parameters were the defaults (Bailey & Elkan, 1994). Then InterPro program (<http://www.ebi.ac.uk/interpro/scan.html>) was performed to annotate all 10 motifs.

The conserved domain of CcABCGs was performed in HMMER servers (<https://www.ebi.ac.uk/Tools/hmmer/>) and visualized using TBtools software (Chen, 2018). At the same time, representative WBC and PDR transporter amino acid sequences were selected as target sequences used to select the template with higher homology and better coverage Swiss-model server with automatic mode (<https://swissmodel.expasy.org/interactive>). The model results were evaluated using SAVES server (<https://servicesn.mbi.ucla.edu/SAVES/>), and the homology model of CcABCG proteins was visualized by the PyMol software.

Gene structure and cis-elements analysis of CcABCGs

Based on the evolution analysis of *CcABCGs*, the intron/exon structures of *ABCG* genes were analyzed by the gene annotation file (GFF) of *C. cajan*_V1.0, and visualized using TBtools software (Chen, 2018). Then the 2000bp upstream region of all *CcABCGs* identified was extracted from pigeon pea genome to identify the stress-related or other functions cis-acting regulatory elements of promoter sequences using TBtools software. All promoter sequences were analyzed on the PlantCARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Rombauts et al., 1999).

GO annotation and function prediction

GO (Gene Ontology) integrates and unifies the description and standards of gene products in the current database, and provides the most comprehensive description of gene functions and gene products. In this study, the ABCG protein sequences were used for BLAST alignment used *Arabidopsis thaliana* sequences as queries in the SWISS-PROT database (<https://www.uniprot.org/blast/>), and then the alignment results were imported to the go annotation website (<http://geneontology.org/>) to complete the annotation and classification of the *ABCG* gene (Ashburner et al., 2000; Consortium, 2019; Ehlert et al., 2006; Yang et al., 2016).

Plant materials and treatments

On the one hand, the seeds of pigeon pea (ICPL87119) were sown in mixed soil of nutrient soil, vermiculite and perlite (1:1:1) in the greenhouse with 10-14h/day of average annual lighting conditions, 18~28°C in temperature and 45-70% of the relative humidity at Beijing Forestry University in China. After three months, the roots, stems, leaves, and flowers from pigeon pea trees were selected and stored at -80°C to extract RNA and further analyze tissue-specific expression.

On the other hand, the pigeon pea seeds were surface-sterilized with 75% Ethanol for 30 seconds, then soaked in sodium hypochlorite solution for 6 minutes, and finally washed 5 times with sterile water for 30 seconds each time. These sterilized seeds were grown in Murashige and Skoog (MS) medium at pH=5.8 in growth chambers at 24±2°C with a photoperiod of 16h light/ 8h dark, 400 $\mu\text{M m}^{-2} \text{s}^{-1}$ light intensity. To analyze the expression of *CcABCG* genes in various abiotic stresses, the 14-days old pigeon pea seedlings were chosen to different treatments: for heat and cold stress, the seedlings were cultured at 4°C and 42°C in incubators, respectively; for salt stress and metal stress, the seedlings were grown in solid MS medium with 200 nmol/L NaCl and 100 $\mu\text{mol/L AlCl}_3$, respectively; for drought stress, seedlings of pigeon pea were treated with 250 nmol/L mannitol in solid MS medium. Roots and leaves of these seedlings were sampled at 0h, 6h, 12h after various treatments. Three biological replicates of each sample were immediately frozen in liquid nitrogen and stored at -80°C until expression analysis of variously abiotic stresses.

RNA isolation and quantitative real-time PCR analysis

Based on the above analysis of cis-elements in the promoter region, 10 genes containing elements that responded to adversity were selected for tissue specificity analysis and abiotic stress analysis. The total RNA was isolated using CTAB method. And then, the first-strand cDNA was synthesized from 1 µg of total RNA using a PrimeScript RT kit (Takara) according to the manufacturer's manual. The quality and concentration of cDNA were assessed using a Nano Photometer N50 (Implen GmbH, Munich, Germany). Expression analysis of ABCG transporter under different tissues and abiotic stress was performed using qRT-PCR. Real-time RT-PCR analysis was performed on a CFX connect (Bio-Rad, California, USA) using the SYBR Green PCR Master Mix (TaKaRa, Tokyo, Japan) with *CcActin* as a reference gene. The primers of genes selected were synthesized on Sangon Biotech website (<http://www.sangon.com/newPrimerDesign>) and were listed on Table S1. All the analysis were performed with three biological replicates in this study, and qRT-PCR data was analyzed using IBM SPSS 22 software (IBM Corporation, USA).

Results

Identification of ABCG transporter genes in pigeon pea genome

To identify all members of the pigeon pea ABCG transporters, 129 Arabidopsis ABC proteins were downloaded and used as queries to search in pigeon pea genome database using BLASTP server. A total of 222 pigeon pea ABC proteins were identified after removing the redundancy. Then, the HMMER search was performed with Hidden Markov Model (HMM) of ABC transporters (such as PF00005, PF00664, and PF10614) to screen for ABC transporters identified by BLASTP. Further, 155 pigeon pea ABC proteins were eventually confirmed that have the NBD/TMD domains (Table S2). To explore the phylogenetic evolutionary relationship between pigeon pea and other species ABC proteins such as Arabidopsis, rice, and soybean, a Neighbor-Joining (NJ) tree was constructed (Fig. S1). Phylogenetic relationship indicated that the pigeon pea *ABC* gene family was classified into 8 different groups (ABCA-ABCI), and ABCG subfamily, contained 51 members, was the largest group of pigeon pea ABC transporters, which was participated in many fundamental biological processes in a recent report.

Phylogenetic tree construction and chromosome localization

All of the ABCG transporters identified above were named *CcABCG1-CcABCG51* based on chromosomal location. A Neighbor-Joining (NJ) tree was constructed to analyze the phylogenetic relationship of ABCG transporters (Fig. 1). Phylogenetic analysis indicated that the ABCG transporters were further divided into WBC and PDR, of which WBC contained 33 ABCG transporters and PDR contained 18 transporters.

Chromosome localization of ABCG transporters in pigeon pea genome was performed, which indicated all identified *CcABCGs* were diversely distributed on pigeon pea chromosomes except chromosome 7. Chromosome 1 and 8 both contained one ABCG transporter, which had the least ABCG transporter in all the chromosomes of pigeon. Chromosome 2, 9, 11 included 5, 5, 8 transporters, respectively. Besides, 17 genes, distributed on unplaced scaffolds, were mapped onto a single chromosome called the ChrUn (Fig. 2). Two pairs of genes (*CcABCG8/9*, *CcABCG32/33*) closely linked on the chromosome and belonged to the paralogous genes. Then, the *ABCG* gene localization was annotated using NCBI website and added the information in Table 1.

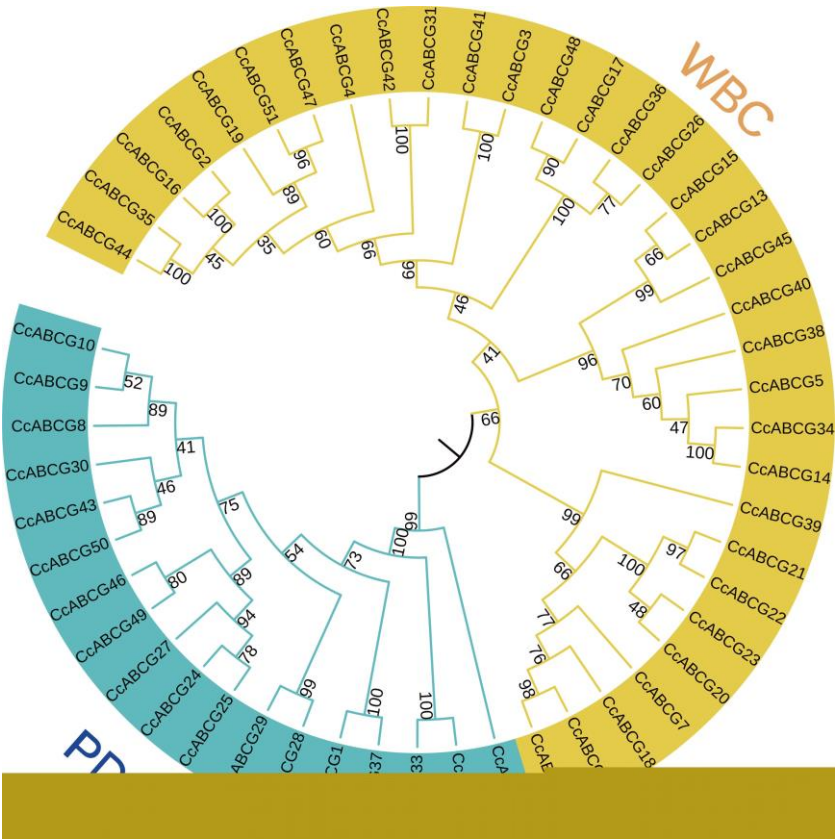


Figure 1. Phylogenetic analysis of the ABCG transporters among pigeon pea. Using the MEGA6.0 program, the NJ (Neighbor-Joining, NJ) tree was constructed using the amino acid sequence of the pigeon pea ABCG transporters. The numbers beside the branches represent bootstrap values based on 1,000 replications. The outer side of the phylogenetic tree is a branch labeled with two subgroups of the pigeon pea ABCG transporters, and shown in different colors. WBC, white-brown complex; PDR, pleiotropic drug resistance.

Protein property prediction, subcellular localization of CcABCGs

All of 51 CcABCGs identified were predicted their protein property, such as amino acid lengths, relative molecular weight, and isoelectric points (pIs) (Table 1). Results indicated that the lengths of all identified pigeon ABCG proteins ranged from 609 aa (CcABCG34) to 1500 aa (CcABCG29) amino acid; similarly, relative molecular mass varied from 68159.95 Kd (CcABCG34) to 170370.83 Kd (CcABCG29), and the theoretical isoelectric points ranged from 6.75 (CcABCG29) to 9.47(CcABCG4). The exons number of *ABCG* genes changed from 1 to 26.

To determine the active site of CcABCGs, subcellular localization of CcABCGs was performed. The prediction of subcellular localization indicated that all CcABCGs were localized in the cell membrane. Also, 3 ABCG transporters (CcABCG26, 28, 29) were found to be localized in chloroplast, not just on cell membranes (Table 1). To verify the above prediction, the subcellular localization of ABCG transporter was verified by transiently expressing *CcABCG7-eGFP* in tobacco leaves. The results showed that the green fluorescent signal of CcABCG7-eGFP-pROK II was mainly detected in the cell membrane (Fig. S2).



Figure 2. The chromosomal location of the pigeon pea *ABCG* genes. The pigeon pea *ABCG* genes were named *CcABCG1*-*CcABCG51* based on chromosomal location information. All of *ABCG* genes were located in all pigeon pea chromosomal except of chromosomal 7. ChrUn contained 17 members, which were mapped based on the length of those unplaced scaffold sequences with 17 genes, respectively.

Table 1. Prediction of physicochemical properties and subcellular localization of ABCG transporters.

Gene	Accession	Location	Positio	Length	Length (aa)	Mw	pI	Chr	Exon	Sub-loc
						(aa)				
<i>CcABCG1</i>	KYP76113	LOC109804364		6,006,382–6,022,606	1,421	159155.33	7.76	1	23	Cell membrane
<i>CcABCG2</i>	KYP72481	LOC109789679		5,026,814–5,030,449	645	72010.85	9.08	2	5	Cell membrane
<i>CcABCG3</i>	KYP73490	LOC109810734		15,320,209–15,323,794	684	77318.74	8.11	2	10	Cell membrane
<i>CcABCG4</i>	KYP74721	LOC109795335		28,723,419–28,730,237	668	73512.6	9.47	2	3	Cell membrane
<i>CcABCG5</i>	KYP74838	LOC109806747		29,968,224–29,970,344	619	69012.83	9.4	2	1	Cell membrane
<i>CcABCG6</i>	KYP75362	LOC109815813		35,437,420–35,445,042	726	79570.36	8.27	2	12	Cell membrane
<i>CcABCG7</i>	KYP70503	LOC109797228		14,492,247–14,496,242	660	73883.51	8.81	3	9	Cell membrane
<i>CcABCG8</i>	KYP71088	LOC109796083		20,447,829–20,456,456	1,448	164560.21	8.28	3	20	Cell membrane
<i>CcABCG9</i>	KYP71089	LOC109796084		20,469,958–20,479,590	1,444	163478.51	8.27	3	20	Cell membrane
<i>CcABCG10</i>	KYP71090	LOC109796805		20,488,744–20,497,576	1,454	164868.63	7.95	3	20	Cell membrane
<i>CcABCG11</i>	KYP68041	LOC109798752		1,617,897–1,630,220	718	80600.64	9.1	4	9	Cell membrane
<i>CcABCG12</i>	KYP68044	LOC109798893		1,641,751–1,647,610	686	76872.23	8.88	4	10	Cell membrane
<i>CcABCG13</i>	KYP67437	LOC109799601		355,237–358,794	814	91841.52	8.95	5	6	Cell membrane
<i>CcABCG14</i>	KYP67863	LOC109799748		5,000,555–5,003,051	643	72113.02	7.88	5	1	Cell membrane
<i>CcABCG15</i>	KYP66379	LOC109800436		13,422,611–13,426,469	721	81114.25	8.97	6	3	Cell membrane
<i>CcABCG16</i>	KYP66524	LOC109801031		15,158,990–15,162,297	663	74678.78	8.6	6	5	Cell membrane
<i>CcABCG17</i>	KYP66999	LOC109800523		19,843,419–19,853,680	1,092	121456.99	8.87	6	14	Cell membrane
<i>CcABCG18</i>	KYP61725	LOC109804023		4,405,565–4,412,603	704	78466.57	8.83	8	10	Cell membrane
<i>CcABCG19</i>	KYP61105	LOC109804765		8,122,601–8,126,214	632	70593.09	9.02	9	4	Cell membrane
<i>CcABCG20</i>	KYP61155	LOC109805263		8,663,643–8,668,819	658	73428.07	7.99	9	8	Cell membrane
<i>CcABCG21</i>	KYP61157	LOC109805167		8,722,014–8,728,825	679	75362.12	8.36	9	8	Cell membrane
<i>CcABCG22</i>	KYP61159	LOC109805166		8,746,304–8,752,313	681	75328.34	8.65	9	8	Cell membrane
<i>CcABCG23</i>	KYP61160	LOC109804802		8,756,197–8,761,747	648	71982.39	8.81	9	9	Cell membrane
<i>CcABCG24</i>	KYP58337	LOC109805773		11,158–17,707	1,427	161183.4	7.36	10	21	Cell membrane
<i>CcABCG25</i>	KYP59575	LOC109805875		14,201,296–14,246,400	1,431	161968.54	7.97	10	21	Cell membrane
<i>CcABCG26</i>	KYP60108	LOC109805696		19,992,101–20,040,472	1,104	123273.83	8.89	10	16	Cell membrane. Chloroplast.
<i>CcABCG27</i>	KYP53996	LOC109808825		1,626,589–1,635,183	1,429	162016.24	7.32	11	22	Cell membrane
<i>CcABCG28</i>	KYP54148	LOC109808608		3,046,631–3,056,753	1,482	167729.07	8.19	11	23	ll membrane. Chloroplast.
<i>CcABCG29</i>	KYP54262	LOC109807460		4,014,294–4,034,321	1,500	170370.83	6.75	11	26	Cell membrane. Chloroplast.
<i>CcABCG30</i>	KYP55666	LOC109807699		19,989,558–20,004,511	1,418	161820.65	8.9	11	26	Cell membrane
<i>CcABCG31</i>	KYP56982	LOC109809098		34,818,776–34,826,902	741	81702.72	9.05	11	12	Cell membrane
<i>CcABCG32</i>	KYP57733	LOC109807641		43,015,398–43,024,752	1,449	164070.17	7.67	11	24	Cell membrane
<i>CcABCG33</i>	KYP57734	LOC109809552		43,027,240–43,038,856	1,445	163218.52	8.03	11	24	Cell membrane
<i>CcABCG34</i>	KYP57934	LOC109807560		45,075,476–45,077,364	609	68159.95	8.65	11	1	Cell membrane

<i>CcABCG35</i>	KYP53034	LOC109810595	391,122–395,936	681	75466.37	8.91	Un	5	Cell membrane
<i>CcABCG36</i>	KYP53068	LOC109810569	840,758–848,796	1,115	123587.99	9.16	Un	14	Cell membrane
<i>CcABCG37</i>	KYP52287	LOC109811196	2,121–13,637	1,229	138171.93	8.81	Un	20	Cell membrane
<i>CcABCG38</i>	KYP52347	LOC109811227	785,413–787,392	651	73181.09	8.45	Un	1	Cell membrane

Table 1 (continued)

Gene	Accession	Location	Position	Length (aa)		Mw pI	Chr	Exon	Sub-loc
<i>CcABCG39</i>	KYP51780	LOC109811628	147,015–153,318	723	80384.61	8.89	Un	12	Cell membrane
<i>CcABCG40</i>	KYP51380	LOC109811918	541,176–543,014	612	68365.89	9.39	Un	1	Cell membrane
<i>CcABCG41</i>	KYP49769	LOC109813109	127,353–133,820	683	77708.58	9.05	Un	10	Cell membrane
<i>CcABCG42</i>	KYP49445	LOC109813363	17,796–28,760	744	82372.18	8.91	Un	12	Cell membrane
<i>CcABCG43</i>	KYP48955	LOC109813737	400,317–424,748	1,358	154173.34	8.61	Un	21	Cell membrane
<i>CcABCG44</i>	KYP48157	LOC109814303	92,212–96,948	678	75455.58	9.06	Un	5	Cell membrane
<i>CcABCG45</i>	KYP47060	LOC109815112	59,170–61,921	755	83546.16	9.22	Un	1	Cell membrane
<i>CcABCG46</i>	KYP41536	LOC109818995	187,197–197,961	1,445	163678.86	8.75	Un	24	Cell membrane
<i>CcABCG47</i>	KYP39219	LOC109788852	72,316–76,120	624	69345.94	8.87	Un	4	Cell membrane
<i>CcABCG48</i>	KYP37548	LOC109790064	6,674–12,941	1,087	120433.13	8.96	Un	14	Cell membrane
<i>CcABCG49</i>	KYP36564	LOC109790672	14,766–22,348	1,457	164896.42	8.25	Un	24	Cell membrane
<i>CcABCG50</i>	KYP35274	LOC109791517	44,302–58,642	1,473	167555.55	7.74	Un	20	Cell membrane
<i>CcABCG51</i>	KYP32835	LOC109793033	5,717–9,684	628	70573.41	8.74	Un	4	Cell membrane

were localized in the cell membrane. Three ABCG transporters (*CcABCG26*, *28*, *29*) were found to be localized in the chloroplast and not just on the cell membranes (Table 1). To confirm our prediction, we verified the subcellular localization of the ABCG transporter by transiently expressing *CcABCG7*-eGFP in tobacco leaves. The results showed that

the green fluorescent signal of *CcABCG7*-eGFP-pROK II was mainly detected in the cell membrane (Fig. S2).

Analysis of motifs and conserved domain of *CcABCGs*

To identify the structure and function of the ABCGs in the pigeon pea, motif analysis was performed based on the phylogenetic analysis (Fig. 3A). A total of 10 conserved motifs were predicted and the width of those motifs ranged from 32 to 123 amino acids.

The number of motifs in each amino acid varied from 2 to 10. Results showed that the number of motifs in the WBC was significantly less than the number of motifs detected in the PDR. All 10 motifs were annotated using the InterPro program to identify the motif structure. Annotation analysis demonstrated that Motifs 3, 7, 9, and 10 did not annotate at all; Motif 6 and Motif 8 annotated in the ABC_2_trans structure (IPR013525); Motif 1,2,4,5 annotated in the P_loop_NTPase structure (IPR027417); Motifs 1, 4, and 5 were annotated in the ABC_transporter_like structure (IPR003439). Our results indicated that the motifs of the same annotation were relatively conservative in structure, such as Motifs 1, 4, and 5 (Table S3).

We examined the conserved domain of *CcABCGs* to explore the ABCG transporter's domain function using

HMMER servers (Fig. 3B). The HMME model, “ABC2_membrane” indicated the TMD domain, and “ABC_tran” indicated the NBD domain. The results showed that the ABCG transporters were different from other members of the ABC transporter family with an inverted “TMD-NBD” domain arrangement pattern. All ABCG transporters contained the NBD domain, but several ABCG transporters did not contain the TMD domain. The WBC subgroup contained one NBD domain with a length that increased from 97 to 153 and one or no TMD domains with a length of approximately 200, while the PDR subgroup contained two NBD domain with an amino acid length of 138 to 200 and a domain approximately 200 TMD long.

CcABCG35 was selected for homology modeling to explore the molecular functions of CcABCGs. The best template of CcABCG35 was 6hij.1A (Seq Identity:33.05%, GMQE:0.58, Coverage:0.85) using the Swiss-model program. According to the PROCHECK evaluation results, Ramachandran Plot results in the model evaluation revealed that in each ABCG model >90% of the amino acid residues were distributed in the allowed region, indicating that the quality of the CcABCG protein model obtained by homology modeling was reliable (Fig. S3A). A model of CcABCG35 revealed that Walker A and Walker B were located in the NBD domain, while the TMD domain contained six alpha helices (Fig. S3B).

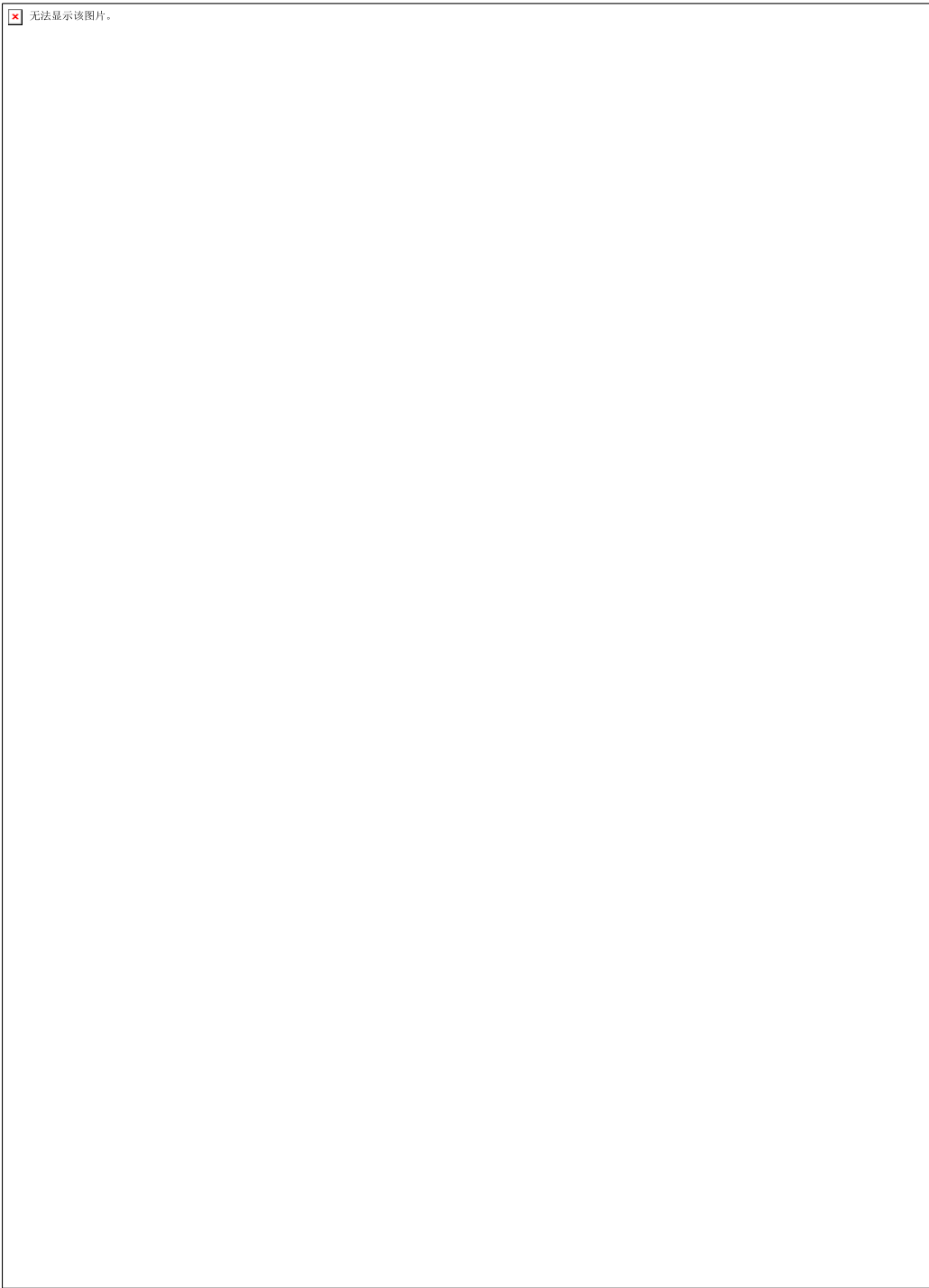


Figure 3. Motifs and conserved domain of CcABCGs. (A) The 10 conserved motif of CcABCGs on the MEME software. Motif annotation is shown as color legends. Annotates of motif were listed on Table S3. (B) Conserved domain of CcABCGs. Blue indicates the NBD (nucleotide binding domain), and orange indicates the another conserved domain TMD (trans-membrane domains).

Gene structure and cis-elements analysis of CcABCGs

Gene structure was analyzed based on the phylogenetic relationship of the ABCG gene family to better understand their structural evolution (Fig. 4A). The highly conserved exon sequence is an essential sequence for the ABCG transporter to perform gene functions, and the differences in introns may be based on dissimilar regulatory mechanisms for the existence of genes. The results indicated that all ABCG transporters in the pigeon pea contained different numbers of exons and introns. The number of exons in CcPDRs was significantly higher than that in

CcWBCs. The number of exons of CcPDRs ranged from 5 to 25, while the number of exons of CcWBCs varied from 1 to 14 and there were large differences in the number of introns. There were approximately 20 introns in CcPDRs and about 5 in CcWBCs.

A 2,000 bp region upstream of the promoter was selected for analysis of the cis-acting element of the pigeon pea ABCG gene family in order to explore the expression elements of the promoter region (Fig. 4B). Three cis-acting elements were screened, and focused on hormone-responsive elements, light-responsive elements, and stress-responsive elements. The number of light-responsive elements in the promoter region of the pigeon pea ABCG transporter was found to be relatively large. Hormonal response elements had a large number of copies in the promoter region of the ABCG transporter, including auxin response elements (AuxRR-core, TGA-element, etc.) and gibberellin response elements. There were fewer stress response elements than hormone response elements and some genes did not contain stress response elements (CcABCG14, CcABCG10, etc.). Drought-related cis-acting elements were identified simultaneously in CcABCG7 and CcABCG24. Low-temperature stress-related cis-acting elements (LTR) were identified in CcABCG28 and their elements were highly conserved with short sequences composed of CCGAAA.

Go annotation and function prediction

The ABCG gene in pigeon pea was annotated and divided into three categories: molecular function, biological processes, and cell components. The annotation results showed that all 50 genes were annotated into three major categories, but CcABCG48 did not show results from the blast alignment method and UniProtKB ID and all other usable data are provided in Data S5. The transmembrane transport (GO:0055085) had the highest prominence and 45 genes were annotated in the transmembrane transport. CcABCG28 and CcABCG29 were blasted in AB36G_ARATH (UniProtKB ID), and annotated in 42 GO annotations, which included “response to salt stress” (GO:0009651) and “root development” (GO:0048364). Fifty ABCG genes were annotated to the broad category of molecular functions related to ATPase activity and ATP binding. The major category of cellular components found most of the ABCG genes to be annotated on the membrane (Fig. 5).

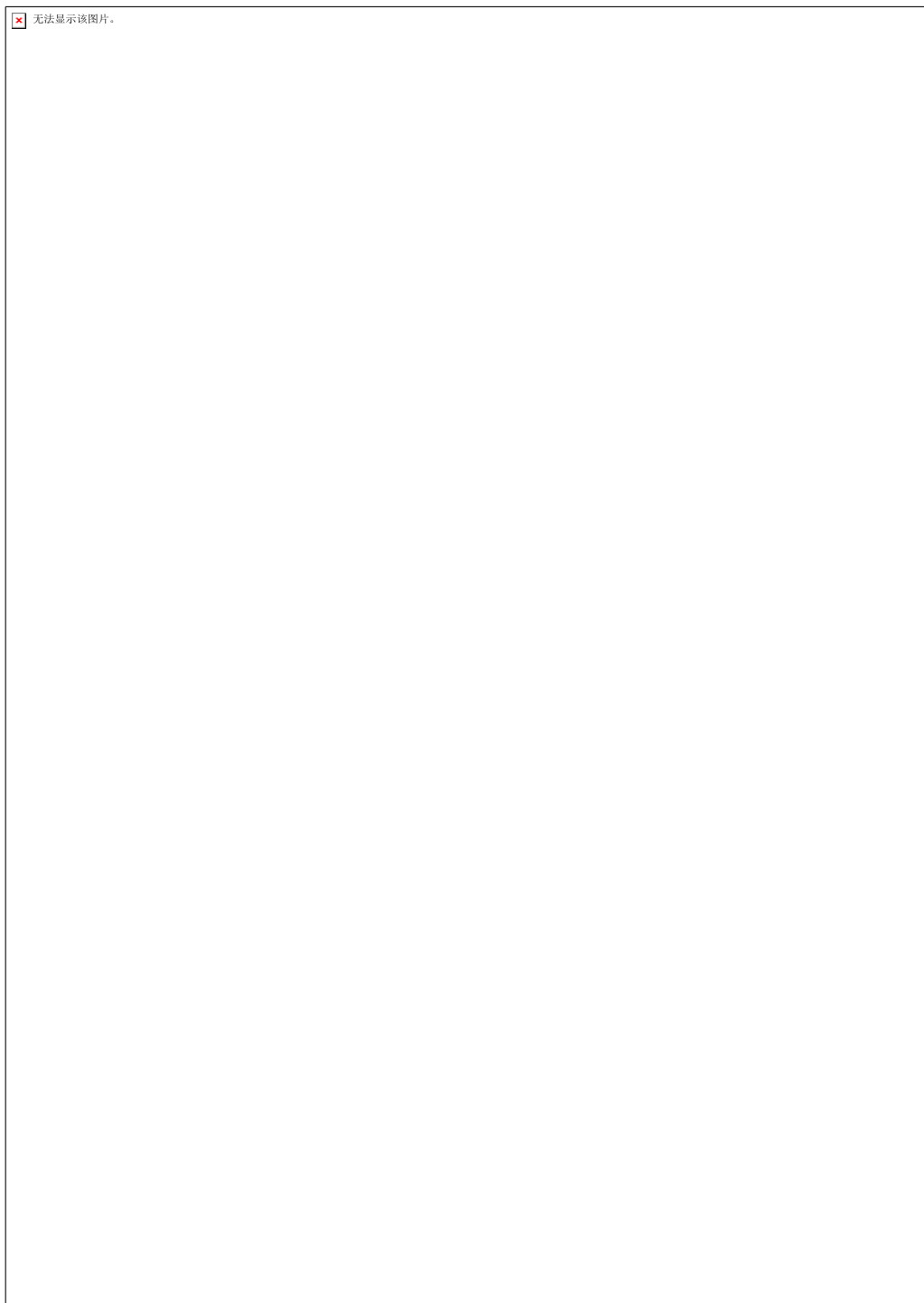


Figure 4. Gene structure and cis-elements analysis of ABCG genes. (A) Intron-exon structures of CcABCG genes. Yellow rectangles: coding sequences (CDSs); thin lines: introns; blue rectangles: untranslated regions (UTRs). (B) Putative regulatory cis-elements in the ABCG gene promoters. The relative positions of elements are labeled with capital letters in the figure.

Expression analysis of the pigeon pea ABCG gene family in different organs

To explore the expression pattern of the ABCG gene in different organs during the growth and development of pigeon peas, the organs (roots, stems, leaves, and flowers) of one-year-old pigeon peas were collected to extract RNA for qRT-PCR analysis for the expression of the ABCG genes. Tissue-specific expression analysis found that the expression of ABCG transporters in different tissues was not significantly different. However, there was an obvious difference in the expression level of the ABCG transporter gene in flowers, which was significantly lower than that in the roots, stems, and leaves. The expression of CcABCG24 was significantly higher than that of any other gene (Fig. 6).

Expression analysis of the pigeon pea ABCG transporters under different abiotic stresses

To explore the expression level of the pigeon pea ABCG gene family under different abiotic stresses, two-week-old pigeon pea seedlings were selected and transplanted into the culture environments at 4 °C, 42 °C, 200 nmol/L NaCl, 100 μmol/L AlCl₃ and 250 nmol/L mannitol in solid MS medium, respectively. CcABCG5,7,14,19,21 belonged to the WBC subgroup, and CcABCG10,24,28,29,32 belonged to the PDR subfamily. We found that the expression difference of 10 genes in the roots was significantly higher than in the leaves (Fig. 7). The two major subfamilies of ABCG transporters had significant differences in expression in the roots (Figs. 7A,7C,7E,7Gand7I) under 4 °C stress and NaCl stress. The PDR family genes were significantly up-regulated under both stressors. CcABCG24 was up-regulated 40-fold after 12 h at 4 °C and CcABCG28 increased 65 times. Under the initial NaCl treatment, CcABCG24 increased 10 times. However, it is worth noting that with an increase of the treatment time, the expression of CcABCG24 decreased to below the initial level at 12 h. We found that the relative expression changes of the WBC subgroups under drought and aluminum stress treatments were opposite, as shown in Figs. 7Eand7G. The expression level of CcABCG5 increased under drought stress at 6 h but did not change significantly under aluminum stress. CcABCG19 also exhibited the same pattern of opposite expression in the treatments of mannitol stress and AlCl₃ stress. Under high-temperature stress, we observed that the expression of CcABCG7 was down-regulated but the expression of CcABCG7 in leaves was up-regulated.

There was a relatively mild difference in the expression levels in leaves (Figs. 7B,7D, 7F,7Hand7J).

CcABCG28 experienced up-regulation under 4 °C treatment. However, CcABCG7 was significantly up-regulated after 6 h of drought stress. In the same situation, CcABCG7 was up-regulated 11.3 times after 12 h of aluminum stress treatment. However, under sodium stress, CcABCG7, 19, 21 were down-regulated. The ABCG transporter was found to have different expressions in response to different environmental stresses.

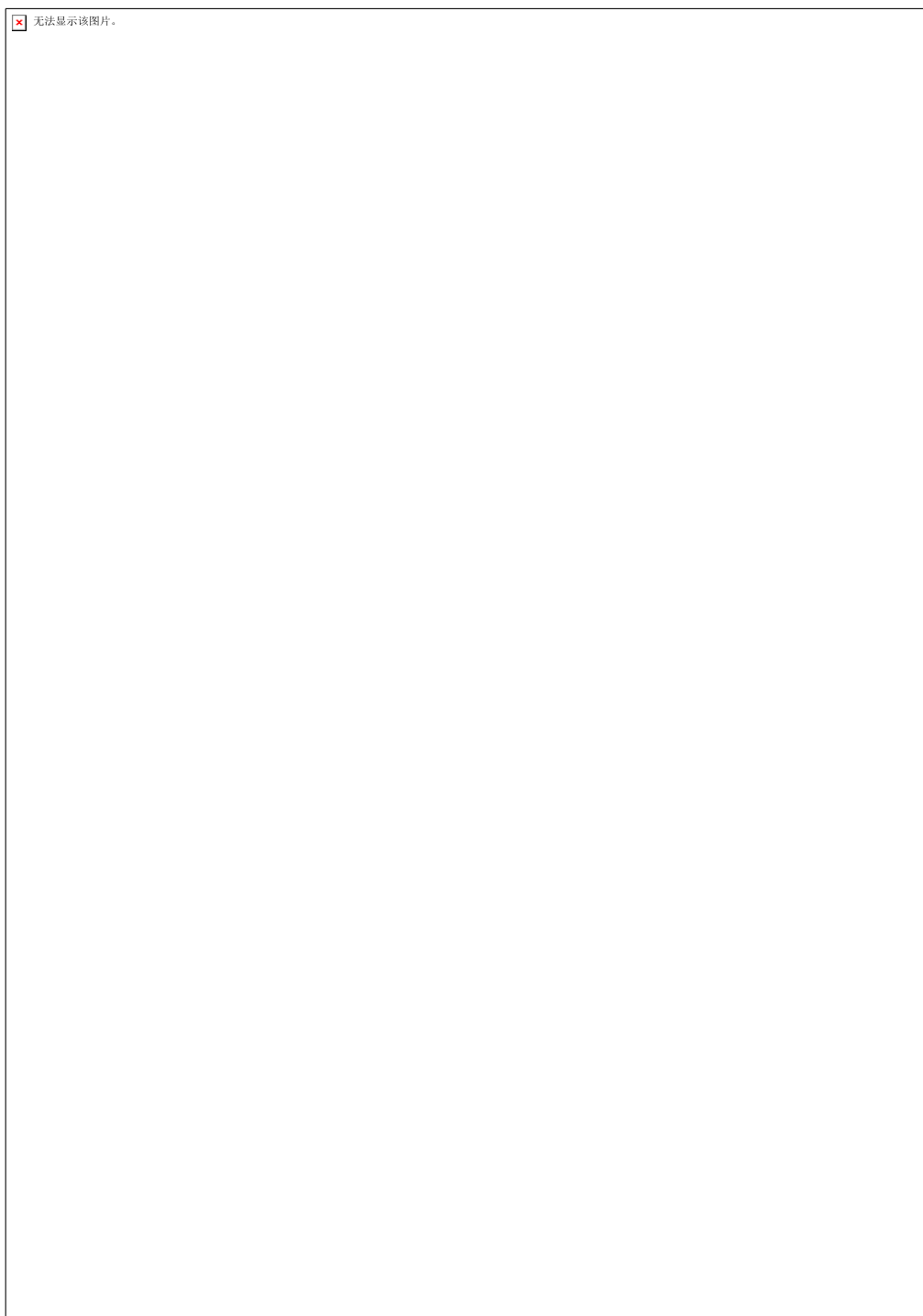


Figure 5. Go annotation of ABCG transporters in pigeon pea. GO annotations of ABCG transporters in pigeon pea were predicted using Swiss-prot database serve. Red means molecular function, yellow means cell component and blue means biological process. Displaying only results for FDR $P < 0.05$.

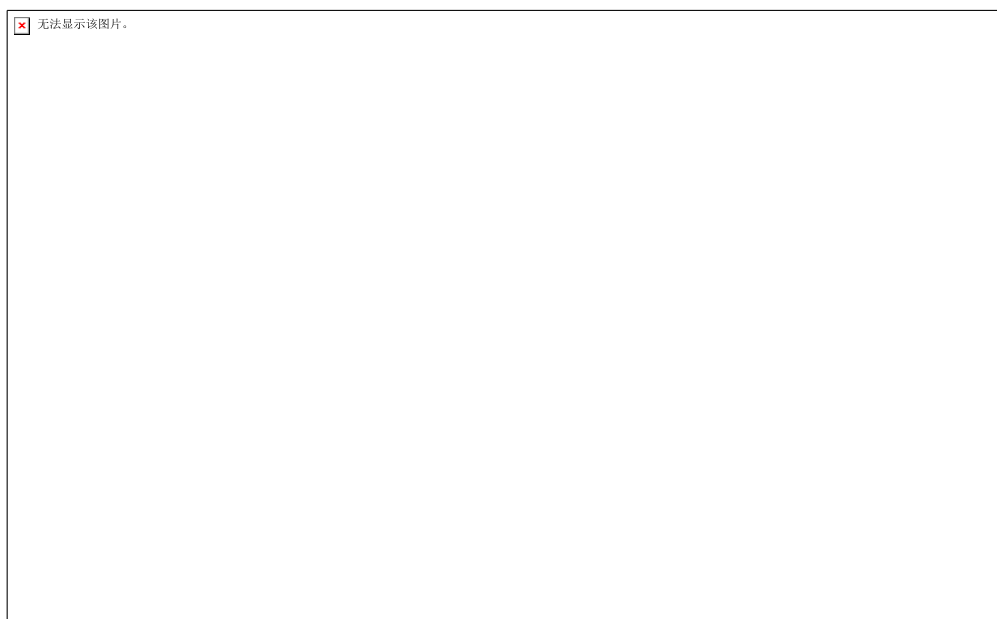


Figure 6. Expression analysis of pigeon pea ABCG gene in different organs. The expression levels of 10 genes in different tissues are shown in the heat map. Three biological replicates per sample. Values represent means $\pm$ SEM.

Discussion

ABC transporters are found in animals and plants and have a large number of complex biological functions. A total of 155 ABC transporters were identified in the pigeon pea genome, which were divided into 8 subgroups of ABCA-ABCI (Fig. S1). Among them, the ABCE and ABCF subfamily had no transport function because their proteins were localized to the endoplasmic reticulum without a transporter region. The ABCB subfamily may be involved in the transport of auxins, secondary metabolites, and heavy metal salts (Kang et al., 2011; Verrier et al., 2008). The ABCC subfamily may be involved in plant chlorophyll transport and cell detoxification among other functions (Hashimoto & Yamada, 2003). The ABCG transporters contained 51 members and was the largest group of ABC transporters in the pigeon pea, which was larger than reported for *Arabidopsis* (44) and rice (50), but smaller than the 116 members of ABCG reported in soybean (Mishra et al., 2019; Jasinski et al., 2003).

As shown in Fig. 1, the ABCG subfamily could be further divided into a full-molecular transporter PDRs and a semi-molecular transporter WBCs, in which PDRs contained 18 ABCG transporters and WBCs included 33 ABCG transporters. The conserved domain of the ABCG transporter is composed of NBD and TMD, and the ABCG transporter is different from the conserved domain arrangement of other ABC transporter subfamilies. The conserved domain composition of the ABCG transporter is a trans-TMD-NBD structure (Fig. 3) (Van den Brule & Smart, 2002). The arrangement of ABC domains and their transmembrane domains are highly conserved, while the number of transmembrane helices and their arrangements is not necessarily conserved, and determines their functional differences (Andolfo et al., 2015; Goodman, Casati & Walbot, 2004; Locher, 2004).

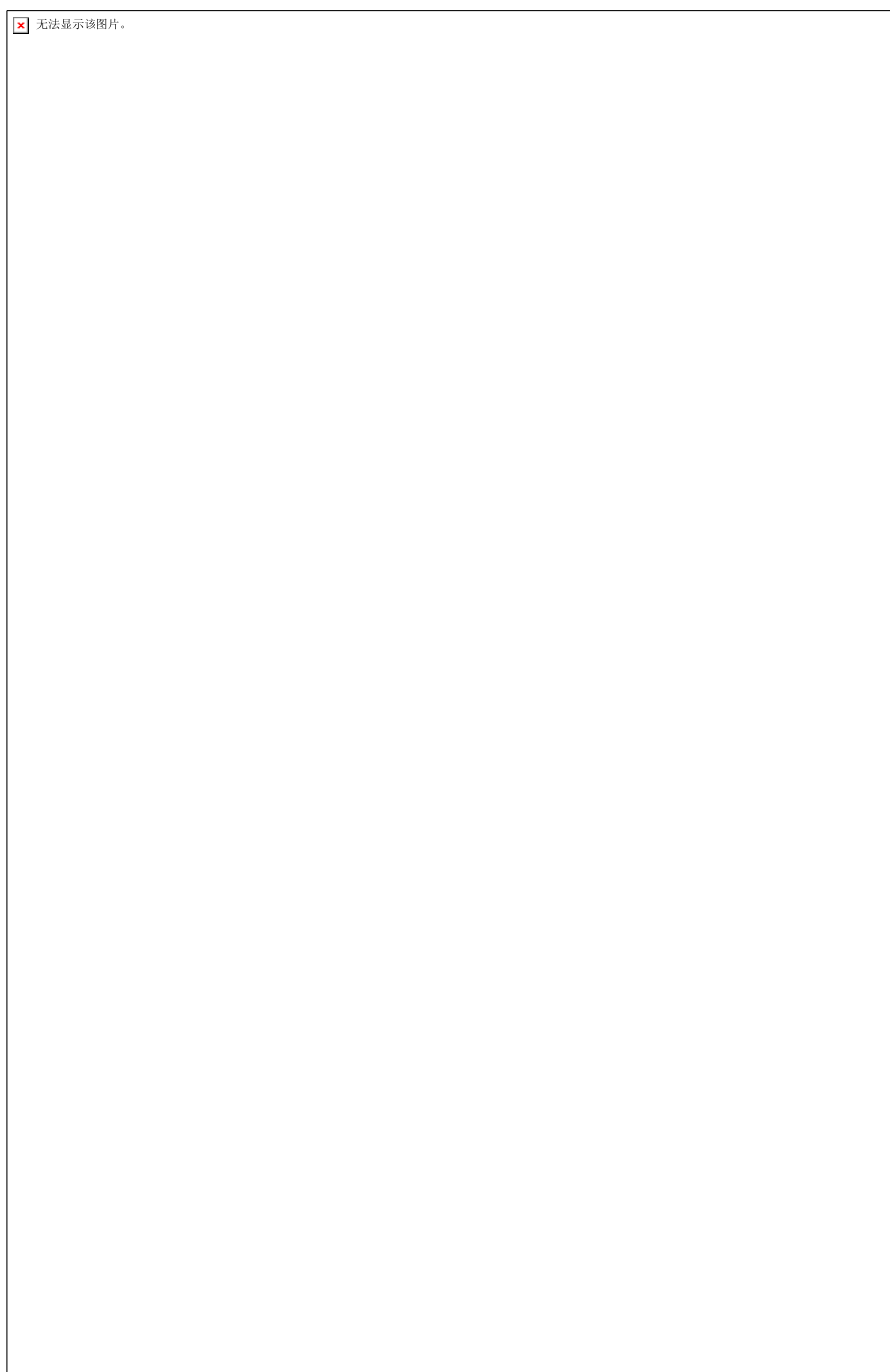


Figure 7. Relative expression analysis of the pigeon pea ABCG genes under different abiotic stresses. Relative expression level of the ABCG genes in pigeon pea under different abiotic stresses of 4°C (A, B), 42°C (C, D), 250 nmol/L mannitol (E, F), 100 μ mol/L AlCl₃ (G, H), 200 nmol/L NaCl (I, J). Expression analysis was performed using a relative quantitative method 2^{-OOCq}, and CcActin as internal reference gene. Relative expression level of roots: A, C, E, G, I; relative expression level of leaves: B, D, F, H, J. Three biological replicates of each sample. Values represent means $\pm$ SEM. Asterisks indicate significant difference.

ABCG transporters are involved in many physiological activities of plants, including the transport of small molecular compounds, secondary metabolites, and are active in disease resistance, hormonal regulation, and adaptation to changes in the external

environment (Alejandro et al., 2012; Bird et al., 2007; Lee et al., 2010). Arabidopsis cutin and wax secretion require the participation of AtWBC11 (Panikashvili et al., 2007a).

Rice OsPDR9 could be induced by methyl jasmonate, and AtPDR8 could be induced by salicylic acid (Kim et al., 2007; Kuromori et al., 2010; Moons, 2008). External stressors on plants cause the signal receptors on plant cells to first sense the external stress signal and produce a second messenger transmitted inside the cell, such as Ca^{2+} , reactive oxygen and ABA. The stress response also mediates proteins, causing phosphorylation to activate downstream transcription factors and stress-related target genes, thereby resisting the destruction of plant cells (Mittler, Finka & Goloubinoff, 2012; Farooq et al., 2009). Many ABCG genes annotated hormone transport and regulation-related functions (Fig. 4B and 5). AtABCG22 was induced by drought stress, possibly by affecting the stomata

and increasing transpiration (Kuromori, Sugimoto & Shinozaki, 2011). In our study, CcABCG28 (PDR) may be involved in the response to abiotic stresses including colder temperatures and salinity, while CcABCG7 (WBC) in the pigeon pea tended to respond to drought and aluminum stress, as shown in Fig. 7.

The analysis of the location of the ABCG family in the pigeon pea chromosome found that most of the ABCG gene was located on chromosome 11, as shown in Fig. 2. The major part of most plant genomes consisted of different repeating DNA elements. These sequence elements are essential for the large-scale organization and evolution of the plant genome (Kubis, Schmidt & Heslop-Harrison, 1998). Our results showed that there were two pairs of paralogous genes (CcABCG8/9, CcABCG32/33) closely linked to chromosome 3 and chromosome 11, and the tandem replication led to the expansion of these two genes. Introns are important components in the genome of eukaryotes. The typical 5r-

GT...AG- 3r of intron is an important marker of gene splicing and an important feature

of introns in eukaryotic mRNA sequences (Rose et al., 2016; Mukherjee et al., 2018). We performed exon/intron analysis of the ABCG transporter to determine the stress regulation of the splicing process in Fig. 4A. Our results indicated that PDR contained

more intron structures than WBC. It is thought that PDR had more variable splices and functions and may respond more frequently to stress. Furthermore, introns may also affect gene expression, and more introns may have a stronger regulatory effect (Rose et al., 2016; Mukherjee et al., 2018). There is no evidence supporting the theory that more exons make them prone to more regulation. PDR/WBC have different regulatory effects, which depends on deeper molecular mechanisms. The cis-acting elements are involved in the binding of transcription factors (TF) and regulate the expression of the gene (Toledo et al., 2011). Our study found that there were many low temperature-related elements in the promoter region of the pigeon pea, which might be induced by chilling

in the pigeon pea (Fig. 4B). The promoter region of the pigeon pea ABCG transporter had a large number of hormone-regulated expression elements (abscisic acid responsiveness, MeJA responsiveness), which also demonstrated the important role of ABCG transporters in the regulation of plant hormones (Kuromori et al., 2010; Wu, Lewis & Spalding, 2007). Kuromori, Sugimoto & Shinozaki (2011) found that AtABCG25 is mainly expressed in vascular tissues and can transport ABA out of cells.

ABC transporters are essential for plant development and play a role in the processes of gametogenesis, seed development, seed germination, organ formation, and secondary growth (Do, Martinoia & Lee, 2018). We performed expression analysis of the pigeon pea in different organs and under different abiotic stresses (Figs. 6 and 7). The expression levels of all genes in flowers were not overtly different than in other tissues, while CcABCG24 was more highly expressed in other tissues. CcABCG24 was expressed in

the stems of pigeon peas in significantly higher amounts than in other tissues, showing that ABCG transporters are found in active regions such as trans-membrane proteins. AtABCG25 is mainly expressed in vascular tissues, can transport ABA to outside of cells (Kuromori, Sugimoto & Shinozaki, 2011), and is closely related to the formation of plant vascular bundles. We showed that the expression of most genes in the roots was significantly higher than that in the leaves by analyzing the expression levels of the

pigeon seedlings under different stresses. Interestingly, the expression of CcABCG28 is up-regulated, regardless of any tissue (root or leaf), especially in the leaves. The up-regulation of this expression is significantly higher than any other gene under low-temperature stress. At present, the regulation mechanism of the CcABCG28 homologous gene in

other species is still unclear, but it was shown to play an important role in the growth and development of the pigeon pea. We also found that CcABCG24 was highly expressed under NaCl treatment, and its homologous gene AtPDR12 was of great significance for Pb(II) resistance in *Arabidopsis* (Lee et al., 2005). CcABCG24 played an important role in response to salt stress in this experiment. In response to drought and salt stress in plants, the cuticle lipid coding ABCG transporter gene was significantly up-regulated,

indicating that the ABCG transporter has an important role in adapting plants to drought and saline-alkali environments (Luo et al., 2007; Panikashvili et al., 2007a). The expression level of CcABCG7 was up-regulated in pigeon pea roots under drought and aluminum stress, which proved its important role in stress tolerance.

CONCLUSIONS

A total of 51 ABCG transporters were identified and divided into two subgroups: WBC and PDR. We analyzed the protein structure and gene structure cis-elements on the pigeon pea ABCG transporters. The highly conserved NBD domain determines the important function of the ABCG transporter. CcABCG28 was significantly up-regulated under low temperature stress, while CcABCG7 responded to drought stress. In conclusion, our results reveal the role of ABCG transporters in abiotic stress resistance and broaden the research direction in abiotic stress resistance of pigeon peas.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Lili Ni analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, software, and approved the final draft.
- Hanghang Li conceived and designed the experiments, performed the experiments, analyzed the data,

prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Zhihua Song performed the experiments, prepared figures and/or tables, and approved the final draft.
- Biying Dong conceived and designed the experiments, prepared figures and/or tables, and approved the final draft.
- Hongyan Cao conceived and designed the experiments, performed the experiments, prepared figures and/or tables, and approved the final draft.

Data Availability

The following information was supplied regarding data availability: Raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.10688#supplemental-information>.

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Analysis of motifs and conserved domain of CcABCGs

To identify the structural and functional of ABCGs in pigeon pea, motif analysis was performed based on the phylogenetic analysis (Fig. 3A). In our study, a total of 10 conserved motifs were predicted, and the width of those motifs ranged from 32 to 123 amino acids, the number of motifs in each amino acid varied from 2 to 10. Results showed that the number of motifs in the WBC was significantly less than the number of motifs detected in the PDR. Further, all of 10 motifs were annotated using the InterPro program to identify information of motif structure. Annotation analysis demonstrated that Motif 3, 7, 9, 10 were annotated nothing; Motif 6 and Motif 8 were annotated ABC_2_trans structure (IPR013525); Motif 1, 2, 4, 5 were annotated P_loop_NTPase structure (IPR027417); Motif 1, 4, 5 were annotated ABC_transporter_like structure (IPR003439). Results indicated that motifs of the same annotation were relatively conservative in structure, such as Motif 1, 4, 5 (Table S3).

The conserved domain of CcABCGs was performed to explore ABCG transporter's domain function using HMMER servers (Fig. 3B). In the HMMER model, "ABC2_membrane" indicates the TMD domain, and "ABC_tran" indicates the NBD domain. The results showed that the ABCG transporters were different from other members of the ABC transporters family with an inverted "TMD-NBD" domain arrangement pattern. All ABCG transporters contained the NBD domain, but several ABCG transporters didn't contain the TMD domain. WBC subgroup contained one NBD domain with a length changed from 97 to 153 and one or no TMD domain with a length of about 200, while PDR subgroup contained two NBD domain with amino acid length of 138 to 200 and an

approximately 200-length TMD domain.

CcABCG35 was selected for homology modeling to explore the molecular functions of CcABCGs. The best template of CcABCG35 was 6hij.1.A (Seq Identity:33.05%, GMQE:0.58, Coverage:0.85) using the Swiss-model program. According to the PROCHECK evaluation results, Ramachandran Plot results in the model evaluation shown that in each ABCG model, > 90% of the amino acid residues were distributed in the allowed region, indicating that the quality of the CcABCG protein model obtained by homology modeling is reliable (Fig. S3A). Model of CcABCG35 shown that Walker A and Walker B were located in the NBD domain, while the TMD domain contained 6 alpha helices (Fig. S3B).

Gene structure and cis-elements analysis of CcABCGs

Gene structure was analyzed based on the phylogenetic relationship of the ABCG gene family to better understand their structural evolution (Fig. 4A). The highly conserved exon sequence is an essential sequence for the ABCG transporter to perform gene functions, and the differences in introns may be different regulatory mechanisms for the existence of genes. The results indicated that all ABCG transporters in pigeon pea contained different numbers of exons and introns, but the significant difference was that the number of exons in *CcPDRs* was significantly higher than that in *CcWBCs*. The exons number of *CcPDRs* ranged from 5 to 25, while the exons number of *CcWBCs* varied from 1 to 14. Moreover, there were large differences in the number of introns. The average number of introns in *CcPDRs* was about 20, while those in *CcWBCs* was about 5.

To explore the expression elements of the promoter region, a region of 2000 bp upstream of the promoter was selected for cis-acting element analysis of the pigeon pea ABCG gene family (Fig. 4B). Three cis-acting elements were screened in our study, which focused on hormone-responsive elements, light-responsive elements, and stress-responsive elements. It was found that the number of light-responsive elements in the promoter region of the pigeon pea ABCG transporter was relatively large. Hormonal response elements had a large number of copies in the promoter region of the ABCG transporter, including auxin response elements (AuxRR-core, TGA-element, etc.), gibberellin response elements, etc. Compared with hormone response elements, there were fewer stress response elements, and some genes didn't contain stress response elements (*CcABCG14*, *CcABCG10*, etc.). At the same time, drought-related cis-acting elements were identified in *CcABCG7*, *CcABCG24*. Low-temperature stress-related cis-acting elements (LTR) were also identified in *CcABCG28*, in which their elements were highly conserved with short sequences composed of CCGAAA.

Go annotation and function prediction

Since the gene term of pigeon pea was not be annotated, the blast alignment method was used to perform go annotation in the swiss-prot database used *Arabidopsis thaliana* sequences as queries. The ABCG gene in pigeon pea was annotated and divided into three categories: molecular function, cell component, and biological process. The annotation results showed that all 43 genes were annotated into three major categories. Among the major categories of biological processes, the gene frequency with higher prominent is the transmembrane transport, furthermore, the suberin biosynthetic process, abscisic acid transport also was annotated. In the broad category of molecular functions, 43 ABCG genes are annotated to the molecular functions related to ATPase activity; while in the major category of cellular components, most of the ABCG genes are annotated on the plasma membrane (Fig. 5).

Expression analysis of the pigeon pea ABCG gene family in different organs

To explore the expression pattern of the ABCG gene in different organs during the growth and development of pigeon, organs (roots, stems, leaves, and flowers) of the one-year-old pigeon pea were collected to extract RNA and

used it for qRT-PCR for analyzing the expression of the ABCG genes. Tissue-specific expression analysis found that the expression of ABCG transporters in different tissues was not significantly different. However, the relatively obvious difference was that the expression level of the ABCG transporter gene in flowers was significantly lower than that in roots, stems, and leaves. The expression of the gene *CcABCG24* was significantly higher than that of any other gene (Fig. 6).

Expression analysis of the pigeon pea ABCG transporters under different abiotic stresses

To explore the expression level of the pigeon pea ABCG gene family under different abiotic stresses, two-week-old pigeon pea seedlings were selected and transplanted to the culture environment of 4°C, 42°C, 200 nmol/L NaCl, 100 μmol/L AlCl₃ and 250 nmol/L mannitol in solid MS medium, respectively. Among them, *CcABCG5,7,14,19,21* belong to the WBC subgroup, and *CcABCG10,24,28,29,32* belong to the PDR subfamily. It was found that the expression difference of 10 genes in roots was significantly higher than that in leaves (Fig. 7).

In the roots (Fig. 7A, C, E, G, I), the two major subfamilies of ABCG transporters had significant differences in expression under 4°C stress and NaCl stress. The PDR family genes were significantly up-regulated under both stresses, and *CcABCG24* was up-regulated 40-fold after 12 h under 4°C treatment. The *CcABCG28* increased by 65 times. Under the initial NaCl treatment, *CcABCG24* increased by 10 times, but it was worth noting that with the further increase of the treatment time, the expression of *CcABCG24* decreased to below the initial level at 12h. Further, we found that the relative expression changes of WBC subgroups under drought and aluminum stress treatments were opposite differences, as shown in Fig. 7E, G. Expression level of *CcABCG5* increased under drought stress at 6h, while wasn't change significantly under aluminum stress. Similarly, *CcABCG19* also exhibited the same pattern of opposite expression in the treatments of mannitol stress and AlCl₃ stress. Under high-temperature stress, we observed that the expression of *CcABCG7* was down-regulated to a certain extent, but the expression of *CcABCG7* in leaves was up-regulated.

The expression differences in leaves were relatively gentle (Fig. 7B, D, F, H, J). It was worth noting that *CcABCG28* also was a certain up-regulation under 4°C treatment. However, *CcABCG7* was significantly up-regulated after 6h of drought stress. In the same situation, *CcABCG7* was up-regulated 11.3 times after 12h of aluminum stress treatment. However, under sodium stress, *CcABCG7, 19, 21* were down-regulated. In conclusion, under different stress conditions, the ABCG transporter has different expressions in response to different environmental stresses.

Discussion

ABC transporters are distributed in animals and plants, but ABC transporters in plants are characterized by a large number and complex function, such as 129,127 ABC transporters were identified in *Arabidopsis* and rice. As shown in Fig. S1, a total of 155 ABC transporters were identified in the pigeon pea genome, which was divided into 8 subgroups of ABCA-ABCI. Among them, ABCE and ABCF subfamily had no transport function because of their proteins were localized to the endoplasmic reticulum without a transporter region. ABCB subfamily might be involved in the transport of auxins, secondary metabolites, heavy metal salts (Kang et al., 2011; Verrier et al., 2008). ABCC subfamily might be involved in plant chlorophyll transport, cell detoxification, and other functions (Hashimoto & Yamada, 2003). ABCG transporters contained 51 members, was the largest group of ABC transporters in the pigeon pea, which was larger than reported for *Arabidopsis* (44) and rice (50), but smaller than the 116 members of ABCG reported for soybean (Mishra et al., 2019; Jasinski et al., 2003).

As shown in Fig. 1, the ABCG subfamily could be further divided into a full-molecular transporter PDRs and a semi-molecular transporter WBCs, in which PDRs contained 18 ABCG transporters and WBCs included 33 ABCG transporters. The conserved domain of the ABCG transporter is composed of NBD and TMD, and the ABCG transporter is different from the conserved domain arrangement of other ABC transporter subfamilies. The

conserved domain composition of the ABCG transporter is a trans- "TMD-NBD" structure (Fig. 3)(van den Brule & Smart, 2002). The arrangement of ABC domains and their transmembrane domains are highly conserved, while the number of transmembrane helices and their arrangements is not necessarily conserved, which determines their functional differences (Andolfo et al., 2015; Goodman et al., 2004; Locher, 2004). ABCG transporters were involved in many physiological activities of plants, transport small molecular compounds, secondary metabolites, and played an important role in disease resistance, hormonal regulation, and adaptation to changes in the external environment (Alejandro et al., 2012; Bird et al., 2007; Lee et al., 2010). Arabidopsis cutin and wax secretion required *AtWBC11* participation (Panikashvili et al., 2007). Rice *OsPDR9* could be induced by methyl jasmonate, and *AtPDR8* could be induced by salicylic acid (Kim et al., 2007; Kuromori et al., 2010; Moons, 2008). When plants are stressed by external environmental factors, the signal receptors on the plant cell first sense the external stress signal and generate a second messenger transmitted inside the cell, such as Ca^{2+} ions, reactive oxygen molecules, ABA, etc., and then mediates the phosphorylation of downstream proteins to activates downstream transcription factors, which can specifically activate target genes related to stress response, and thus resist cell damage from factors such as drought and temperature changes (Mittler et al., 2012; Farooq et al., 2009) As shown in Fig. 4B and Fig. 5, there were found that many *ABCG* genes were annotated to hormone transport and regulation related functions. *AtABCG22* was induced by drought stress, possibly by affecting stomata and increasing transpiration (Kuromori et al., 2011). In our study, *CcABCG28*(PDR) might be involved in the response of pigeon pea to abiotic stresses such as chilling, salt; while *CcABCG7*(WBC) tended to respond to drought and aluminum stress as shown in Fig. 7.

The analysis of the location of the ABCG family in the pigeon pea chromosome found that most of the *ABCG* gene was located on chromosome 11 as shown in Fig. 2. The major part of most plant genomes consisted of different repeating DNA elements. These sequence elements are essential for the large-scale organization and evolution of the plant genome (Kubis et al., 1998). Our results showed that there were two pairs of paralogous genes (*CcABCG8/9*, *CcABCG32/33*) closely linked to chromosome 3 and chromosome 11, and the tandem replication led to the expansion of these two genes. Introns are important components in the genome of eukaryotes. The typical 5'-GT ... AG-3'of intron is an important marker of gene splicing and an important feature of introns in eukaryotic mRNA sequences(Rose et al., 2016; Mukherjee et al., 2018). Further, the exon/intron analysis of ABCG transporter was performed to learn the stress regulation of the splicing process in Fig. 4A. Our results indicated that PDR contained more intron structures than WBC, speculating PDR had more variable splices and functions, and might be more changes in response to stress. Furthermore, introns may also affect gene expression, and more introns may have a stronger regulatory effect (Rose et al., 2016; Mukherjee et al., 2018). But the argument that having more exon make them prone to more regulation is theoretical and no evidence was provided in support of that statement. PDR/WBC indeed have a different regulatory effect more depends on deeper molecular mechanisms. The cis-acting elements are involved in the binding of transcription factors (TF) and regulating the expression of the gene (Toledo et al., 2011). Our study found that there were many low temperature-related elements in the promoter region of the pigeon pea, which might be induced by chilling induction in pigeon pea (Fig. 4B). It was worth noting that the promoter region of the pigeon pea ABCG transporter had a large number of hormone-regulated expression elements (abscisic acid responsiveness, MeJA responsiveness), which also demonstrated the important role of ABCG transporters in the regulation of plant hormones(Kuromori et al., 2010b; Wu et al., 2007). Kuromori et al. (Kuromori et al., 2011) found that *AtABCG25* is mainly expressed in vascular tissues and can transport ABA out of cells.

ABC transporters are essential for plant development and play a role in the processes of gametogenesis, seed development, seed germination, organ formation, and secondary growth (Thanh, etc, 2018). Our study performed the expression analysis of pigeon pea in different organs and under different abiotic stresses (Fig. 6 and Fig. 7). The expression levels of all genes in flowers were not obvious than in other tissues, while *CcABCG24* was highly

expressed in other tissues. *CcABCG24* was expressed in the stems of pigeon peas significantly higher than other tissues, showing that ABCG transporters active regions as trans-membrane proteins. *AtABCG25* is mainly expressed in vascular tissues and can transport ABA to the outside of cells (Kuromori et al., 2011). It is demonstrated that it is closely related to the formation of plant vascular bundles. Analysis of the expression levels of the pigeon seedlings under different stresses shown that the expression of most genes in the roots was significantly higher than that in the leaves. Interestingly, *CcABCG28*, regardless of any tissue (root or leaf), the expression level is up-regulated, especially in the leaves, the up-regulation of this expression is significantly higher than any other gene under low-temperature stress. At present, the regulation mechanism of *CcABCG28* homologous gene in other species is still unclear, but this gene certainly plays an important role in the growth and development stage of pigeon pea. In our study, we also found that *CcABCG24* was highly expressed under NaCl treatment, and its homologous gene *AtPDR12* was of great significance for Pb(II) resistance in Arabidopsis (Lee et al., 2005). However, it was proved that *CcABCG24* played an important role in response to salt stress in this experiment. In response to drought and salt stress in plants, the cuticle lipid coding gene ABCG transporter gene will be significantly up-regulated, indicating that the ABCG transporter has an important role in adapting plants to drought and saline-alkali environments (Luo et al., 2007; Panikashvili et al., 2007b). The expression level of *CcABCG7* was up-regulated in roots under drought and aluminum stress, which proved its important role in stress tolerance of pigeon pea.

Conclusions

In the present study, a total of 51 ABCG transporters were identified and divided into two subgroups, WBC and PDR. Further, analysis of protein structure, gene structure cis-elements on the pigeon pea ABCG transporters were performed. The highly conserved NBD domain determines the important function of the ABCG transporter. *CcABCG28* was significantly up-regulated under low temperature stress, while *CcABCG7* responded to drought stress. In conclusion, the results will initially reveal the role of ABCG transporters in abiotic stress resistance and broaden research direction in abiotic stress resistance of pigeon peas.

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