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Genome-Wide identification and expression analysis of *CsABF/AREB* gene family in cucumber (*Cucumis sativus* L.) and in response to phytohormonal and abiotic stresses

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Abscisic acid (ABA)-responsive element binding factors (ABF)/ABA-responsive element binding proteins (AREB)/ABA insensitive protein 5 (ABI5) all belong to the basic leucine zipper (bZIP) transcription factor A subfamily. The bZIP transcription factor family contains 13 subfamilies, namely groups A, B, C, D, E, F, G, H, I, J, K, M and S, and the *ABF/AREB/ABI5* gene belongs to A subfamily of the bZIP transcription factor. However, genomic analysis of *CsABF/AREB* in cucumber (*Cucumis sativus* L.) has not been systematically studied. In this study, we analyzed the characterization of *CsABF/AREB* family members and their response to phytohormonal and abiotic stresses. The results showed that a total of 8 genes family members were identified in cucumber. Structural domain analysis showed that the proteins of these family members are highly similar, and all of them belong to the bZIP structural domain. qRT-PCR analysis showed that *CsABF/AREB* members are expressed in root, stem, and leaf, with the highest expression in root, followed by stem and leaf. In addition, all 8 *CsABF/AREB* genes respond to ABA and methyl jasmonate (Me-JA). Among them, *CsABF7* has the highest expression under both ABA and Me-JA treatments. Drought and salt stress significantly induce *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8* expression. Drought and NaC1 stresses significantly induce the expression of *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8*. This study provides a basis for a further understanding of the role of *CsABF/AREB* homologous genes in response to abiotic stress and lays the foundation for further research on the function of *CsABF/AREB*.

Keywords Cucumber, ***CsABF/AREB***, Bioinformatics analysis, Abiotic stress, Hormones, Expression pattern

Abiotic stresses such as drought, salinity, low and high temperatures are important factors that jeopardize crop development, quality and yield. Plants have evolved a variety of complex and fine-grained regulatory networks to cope with adversity stress, such as the abscisic acid (ABA) signaling pathway, the Ca^{2+}signaling pathway, and the mitogen-activated protein kinase (MAPK) cascade pathway[1–3]. Various stress-induced signaling pathways intersect with each other and constitute a complex regulatory network that jointly regulates plant responses to adversity stress. The plant hormone ABA plays a very important and critical role in plant seed dormancy, stomatal opening and closing, organ abscission and response to abiotic stresses[4]

The promoter region of ABA-responsive genes usually contains an ABRE, and ABF transcription factors are a class of bZIP proteins that specifically recognize the ABRE, belong to subfamily A of bZIP transcription factors[5]. The bZIP transcription factors have a conserved bZIP structural domain consisting of 2 structural features: a basic region that specifically binds DNA and a basic region of the leucine zipper dimerization motif[6]. In addition to the bZIP structural domain, the bZIP transcription factor has several other structural domains, such as glutamine-rich motifs and phosphorylation sites (R/KxxS/T)[7]. To bind to DNA, the N-terminal half of

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the basic region binds to the major groove of double-stranded DNA, while the C-terminal half of the Leu zipper forms an overlapping coiled structure by dimerization. The bZIP transcription factors have been reported to be involved in developmental and physiological processes under stressful growth conditions and are considered to be important regulators in response to a variety of abiotic stresses, such as drought, high salt, and low-temperature stresses in Arabidopsis[8], rice[9], wheat[10], tomato[11], and chili pepper[12]. Thus, they are important for various plants to withstand unfavorable environmental conditions[6].

Cucumber (*Cucumis sativus* L.) is an annual herbaceous plant of the genus *Cucumis* in the family Cucurbitaceae, which is the third largest vegetable crop in the world and has important economic and nutritional value[13]. Because of its unique flavor with high nutritional quality improving the quality of cucumber gradually becomes one of the important means to enhance market competitiveness[14]. In recent years, there has been a rapid increase in genomic and biotechnological tools for cucumber and other crops, including genome assembly and annotation, extensive transcriptomics analysis, multifunctional genome database construction, and rapid development of whole-genome sequencing technologies[15]. As the entire cucumber genome has been sequenced and the partial contigs have been already deposited in the NCBI database (http://www.ncbi.nlm.nih.gov), significant progress in research into the molecular biology of this species should be expected[16]. Cucumber is a temperature-loving vegetable crop, and abiotic stresses such as low temperature and low light, high temperature and drought are important constraints to efficient cucumber production and quality[17]. In this study, the *CsABF/AREB* gene family was identified using bioinformatics methods and systematically analyzed for chromosomal localization and physicochemical properties, protein secondary and tertiary structure prediction, gene structure, conserved motifs, conserved structural domains, promoter *cis*-acting elements, phylogenetic tree, sequence alignment, GO functional and KEGG pathway enrichment, protein phosphorylation, and analysis of interacting proteins. The expression pattern of *CsABF/AREB* genes in different tissues under hormonal as well as abiotic stresses was also analyzed by qPCR. Therefore, the study provides theoretical basis for further exploring the function of *CsABF/AREB* genes and improving plant abiotic stress tolerance.

Results

Identification and chromosomal localization of *CsABF/AREB* genes family members

To identify all ABF genes in the cucumber genome, the Blastp program was initially employed to search for the cucumber proteins database based on 9 Arabidopsis *ABF/AREB/ABI5* proteins sequences. After using the SMART, NCBI-CDD, and Pfam databases to confirm the structural domain integrity, a total of 8 *ABF/AREB/ABI5* genes were identified in named *CsABF1-CsABF8* based on their locations on homologous cluster chromosomes and gene homology (Table 1). The 8 *CsABF/AREB* genes are unevenly distributed on chromosomes (Chr 1 ~ Chr 7), and the number of genes on each chromosome is independent of chromosome size (Fig. 1). Among them, *CsABF1* is located on Chr1, *CsABF2* on Chr2, *CsABF3* on Chr3, *CsABF4* on Chr4, *CsABF5*, *CsABF6*, *CsABF7* on Chr6, and *CsABF8* on Chr7. Proteins physicochemical property analysis using ExPaSy online tool showed that the amino acid length of CsABF/AREB proteins rangs from 204 to 446 aa, and the relative molecular weights range from 23014.97 to 47673.45 kDa, with the smallest number of amino acids and relative molecular mass of *CsABF4*, and the largest number of amino acids and relative molecular mass of *CsABF3* (Table 1). The isoelectric points are between 7.60 ~ 9.55, so all are basic proteins. A higher aliphatic index value represents higher thermal stability of CsABF/AREB proteins. The instability indexes range from 44.55 (*CsABF2*) to 64.84 (*CsABF4*), and 90% of the members have indices > 40, indicating that they are relatively stable. The aliphatic indexes range from 64.61(*CsABF4*) to 86.13(*CsABF7*). From the subcellular location analysis, *CsABF1-CsABF8* are all expressed in the nucleus, and it was hypothesized that this gene is related to the storage and replication of genetic material. Among them, replication events occur for *CsABF5*, *CsABF6*, and *CsABF7*, located on chromosome Chr6, indicating that only one pair of segments replicated.

Gene structure, conserved motifs, and conserved structural domains of the CsABF/AREB proteins

To better understand the relationship between the 8 CsABF/AREB proteins identified, we used TBtools software to visualize the structure, conserved motifs, and conserved structural domains of the *CsABF/AREB* gene. The gene structure showed that the number and distribution of introns and exons of *CsABF/AREB* family members

Gene name	Gene ID	Chr. No.	Protein Length (aa)	Molecular weight (kDa)	PI	Instability index	Aliphatic index	Grand average of Hydropathicity	Subcellular Localization
CsABF1	Csa_ 101,222,720	1	270	30510.40	9.35	55.73	71.22	−0.868	Nucleus
CsABF2	Csa_ 101,223,043	2	411	44351.78	9.55	44.55	67.71	−0.642	Nucleus
CsABF3	Csa_ 101,209,887	3	446	47673.45	8.46	57.43	72.42	−0.488	Nucleus
CsABF4	Csa_ 101,212,875	4	204	23014.97	9.44	64.84	64.61	−0.765	Nucleus
CsABF5	Csa_ 101,213,915	6	321	35912.51	8.56	56.40	70.50	−0.846	Nucleus
CsABF6	Csa_ 101,203,423	6	339	38817.81	7.60	55.38	68.44	−0.796	Nucleus
CsABF7	Csa_ 101,204,702	6	266	29511.14	7.85	64.80	86.13	−0.685	Nucleus
CsABF8	Csa_ 101,203,950	7	409	44875.65	9.53	50.53	69.14	−0.603	Nucleus

Table 1. Characterization of *CsABF/AREB* transcription factors. Note: pI, isoelectric point Mol. Wt., molecular weight.

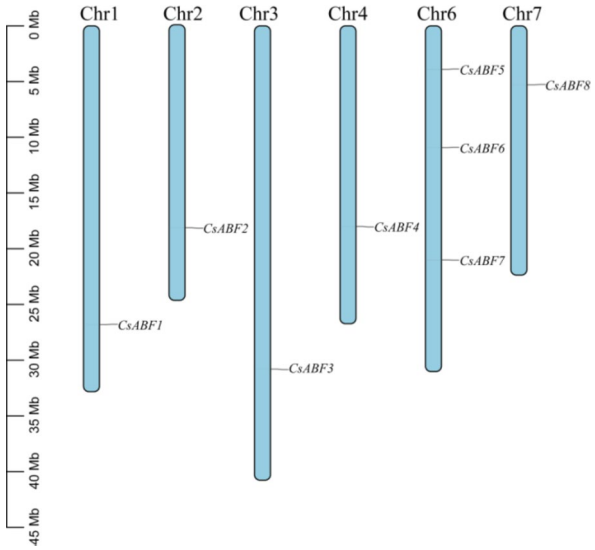

Fig. 1. Chromosomal localization of *CsABF/AREB* gene family members. Chromosome numbers are shown at the top of each bar graph. Gene names, chromosome numbers are in black, and scale bars are on the left.

are similar, with the number of introns ranging from 2 to 4 and the number of exons ranging from 3 to 4 (Fig. 2A). The types and numbers of conserved motifs were different for different subfamilies or subgroups, and the conserved motifs were similar for the same subfamily or subgroup. C*sABF5* and *CsABF7* have similar and longer introns, *CsABF2* and *CsABF8* have similar and longer introns, and *CsABF4* has a shorter intron. Conserved motifs were showed that a total of 10 CsABF/AREB proteins conserved motifs (motif 1 ~ motif 10) were identified (Fig. 2B; Table 2), and the specific sequence information is shown in Fig. 2C; Table 2. Notably, Motif 1 is the basic core of the bZIP structural domain and is present in all CsABF/AREB proteins. All proteins except CsABF4 contain Motif2, Motif3, and Motif4; all proteins except CsABF4 and CsABF7 contain Motif5; all proteins except CsABF3 and CsABF7 contain Motif6. In addition, Motif7 is only present in CsABF4 and CsABF7 proteins, Motif8 is only present in CsABF2, CsABF6, and CsABF8 proteins, and Motif9 and Motif10 are present in all proteins except CsABF2 and CsABF8. It can be inferred that *CsABF/AREB* members are highly conserved and may have similar functions. A search for conserved structural domains of CsABF/AREB proteins using NCBI Bacth CD-search showed that all members of the *CsABF/AREB* family contain the bZIP conserved structural domain (Fig. 2D; Table 3). It further suggests that there are some conservations of gene structure in the same evolutionary branch.

Width (aa): number of amino acids included in the motif. The results were obtained by MEME.

Phylogenetic and sequence comparison analysis of *CsABF/AREB* family genes

The gene structure analysis showed that all members of the *CsABF/AREB* family contain the bZIP conserved structural domain. To further validate the result, we also downloaded the amino acid sequence of the *CsABF/AREB* gene from the NCBI database and performed a multiple sequence comparison between cucumber and Arabidopsis ABF/AREB (Fig. 3). The results showed that the CsABF/AREB proteins has four conserved phosphorylation sites, which is similar with Arabidopsis. The N-terminus consists of C1, C2, and C3, whereas the C-terminus consists of C4 and the bZIP region (basic region and leucine zipper) with the unique BRLZ structural domain of the bZIP transcription factor. To further understand the developmental affinities of *CsABF/AREB* family members, we compared the full-length amino acid sequences of the proteins in Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), cottonwood (*Populus deltoides*), potato (*Solanum tuberosum*), and cucumber in the full-length amino acid sequences of the proteins and jointly constructed a phylogenetic tree (Fig. 4). Based on the homology, the CsABF/AREB proteins of these plants were categorized into three subgroups (Group A, Group B, and Group C), Group A contains two *CsABF2*, *CsABF8*, which are closer related to cottonwood *PdABF1* and *PdABF7*. Group B contains four *CsABF5*, *CsABF1*, *CsABF4*, *CsABF7*, of which *CsABF5* is highly homologous to *PdABF3* with 99% affinity, *CsABF1* is closer to *SIABF1*, *CsABF4* is highly homologous to *PdABF2* with 81% affinity, and *CsABF7* is highly homologous to *SIABF2* with 99% affinity. Group C contains two *CsABF3* and *CsABF6*, which are closer to *PdABF4* and *PdABF6*.

Functional prediction analysis of promoter *cis*-acting elements of the *CsABF/AREB* genes

The promoter *cis*-elements can regulate the gene expression level[18]. Here, in order to understand the expression regulation of *CsABF/AREB* genes, we analyzed the *cis*-elements composition of 8 *CsABF/AREB* promoters with the length of 2 kb upstream of the gene sequence using Plant CARE (Figure S1). Three main types of *cis*-acting elements were identified, including light, hormone, and stress response elements (Fig. 5). Among them, 7 elements (GATA-motif, AE-box, G-box, MRE, SPI, BOX4, TCT-motif) are related to the light response, 5 elements (MBS, LTR, TC-rich repeats, ARE, Circadian) are related to the stress response, and 6 elements (ABRE, P-box, TATC-box, CGTCA-motif, TGACG-motif, TCA-element) are associated with hormonal responses (Table

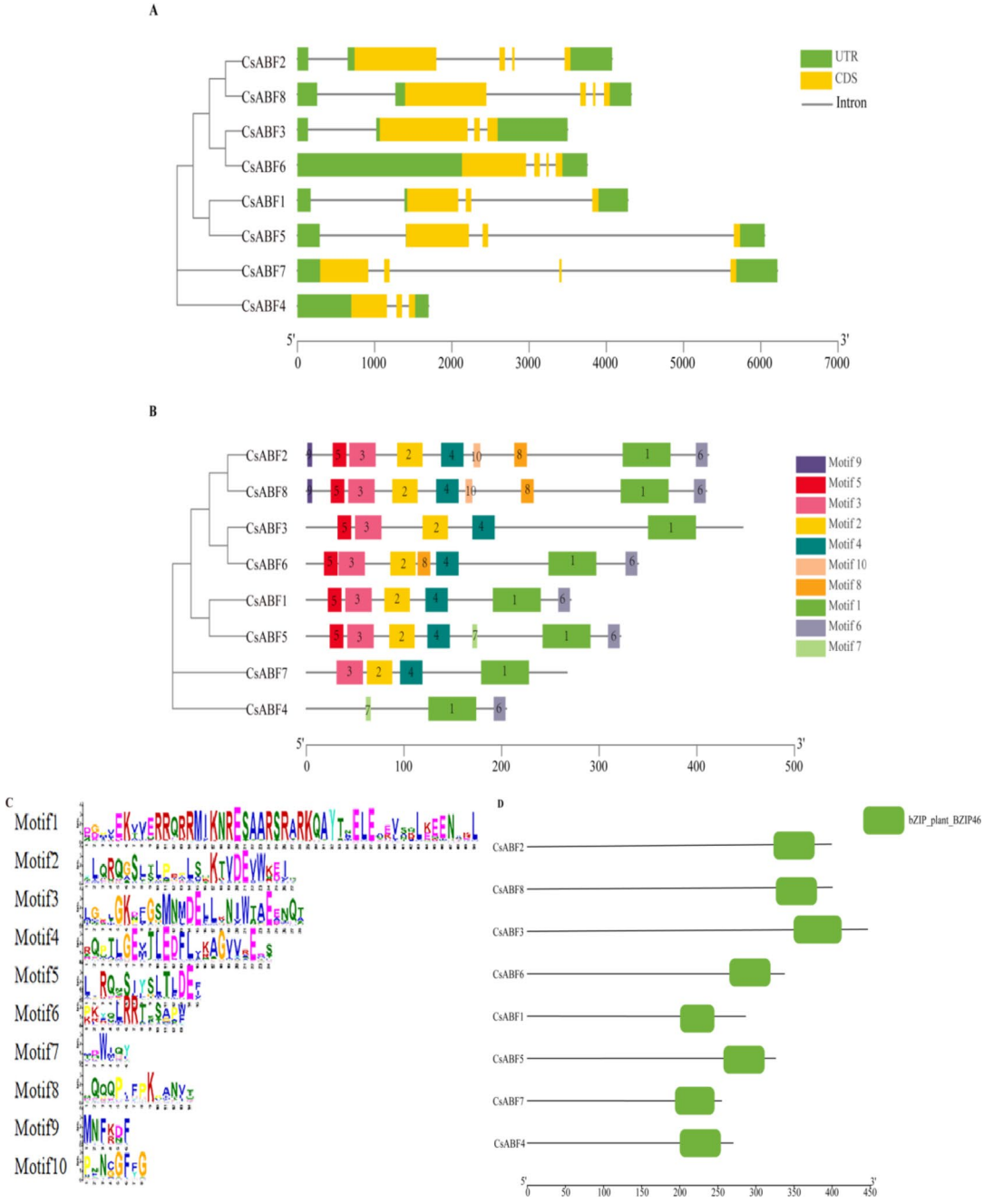

Fig. 2. *CsABF/AREB* gene structure and motif. (**A**) The gene structure of *CsABF/AREB* was mapped using TBools software (v1.09876), where the green thick box represents the uncompiled region (UTR), the yellow thick box represents the exon (CDS), and the black line represents the intron (Intron). The scale bar represents1000bp. The scale bar represents the length of the DNA sequence. (**B**) Conserved motifs of CsABF/AREB proteins were analyzed using the online tool MEME. Different colors represent different conserved motifs. (**C**) Amino acid sequences of different conserved motifs displayed by stacks of letters at each position. The total height of the stack represents the information content of the relative amino acid in the position of each letter in the motif in bits. The height of the individual letter in a stack was calculated by the probability of the letter at that position times the total information content of the stack. The X- and Y-axes represent the width and the bits of each letter, respectively. (**D**) The green box indicates the conserved structural domain of CsABF/AREB.

S1). The G-box element is not distributed in any of the other *CsABF/AREB* genes except *CsABF5*, and the G-box element is the most abundant in the light response. The Box4 element is distributed in all *CsABF/AREBs* except *CsABF4* and is highest in *CsABF3* and *CsABF8*. TCT-motif elements are distributed in *CsABF/AREB* except for *CsABF2* and *CsABF5*. MBS is not distributed in any of the other *CsABF/AREB* except *CsABF8*, and the MBS

Motif	Width (aa)	Motif sequence
Motif 1	50	DGPVEKVVERRQRRMIKNRESAARSRARKQAYTNELEAEVSQLKEENAKL
Motif 2	27	YLQRQGSLTLPRPLSQKTVDEVWKEIQ
Motif 3	28	LGGLGKDFGSMNMDELLKNIWTAEENQT
Motif 4	24	RQPTLGEVTLEDFLVKAGVVREDS
Motif 5	15	LARQNSIYSLTLDEF
Motif 6	13	PKYQLRRTSSAPW
Motif 7	6	MDWIQY
Motif 8	14	HQQQPJFPKPANVT
Motif 9	6	MNFKDF
Motif 10	8	PNNCGFFG

Table 2. Detailed information of 10 conserved motifs of CsABF/AREB proteins.

Protein	E-Value	Bitscore	Accession	Short name	Incomplete	Superfamily
CsABF1	254	3.05176e-15	68.1084	cd14707	bZIP_plant_BZIP46	cl21462
CsABF2	385	3.50638e-25	96.9984	cd14707	bZIP_plant_BZIP46	cl21462
CsABF3	413	5.55286e-24	94.302	cd14707	bZIP_plant_BZIP46	cl21462
CsABF4	179	1.92514e-20	80.4348	cd14707	bZIP_plant_BZIP46	cl21462
CsABF5	305	1.61963e-22	88.524	cd14707	bZIP_plant_BZIP46	cl21462
CsABF6	320	2.16246e-20	83.1312	cd14707	bZIP_plant_BZIP46	cl21462
CsABF7	235	6.21971e-17	72.7308	cd14707	bZIP_plant_BZIP46	cl21462
CsABF8	385	3.54035e-25	96.9984	cd14707	bZIP_plant_BZIP46	cl21462

Table 3. Conserved structural domains of CsABF/AREB proteins.

component is the least abundant in the stress response. The ABRE and ARE components are all distributed in the *CsABF/AREB*, indicating that this component is the most responsive and obvious response, with *CsABF7* being the highest in ABRE and *CsABF1* and *CsABF6* being the highest in ARE. Thus, these results suggest that the *CsABF/AREB* gene may play a potentially critical role in regulating plant growth and stress responses.

Analysis of the proteins secondary structure of *CsABF/AREB* family genes

Based on the primary structure prediction of the CsABF/AREB proteins, the secondary structure prediction of the CsABF/AREB protein family was performed. The results showed that 4 protein secondary structures existed in the protein family, including alpha helix (30.66–52.94%), extended chain (0.75–10.29%), a beta of turns (0.88–2.22%), and random coil (35.29–59.64%) (Table S2). The tertiary structureprediction showed that CsABF/AREB proteins are predominantly α-helical and irregularly coiled, consistent with the secondary structure prediction (Fig. 6).

CsABF/AREB proteins phosphorylation, proteins interactions analysis and annotation

To better understand the potential function and regulatory mechanisms of CsABF/AREBs proteins, the phosphorylation prediction analysis of CsABF/AREB proteins members was performed using the online website CBS NetPhos (www.cbs.dtu.dk/services/NetPhos). The CsABF1 protein contains 35 phosphosites, of which 24 sites are phosphorylatable at serine (Ser), 8 sites at threonine (Thr), and 3 sites at tyrosine (Tyr) (Fig. 7A). The CsABF2 protein contains 35 phosphosites, of which 21 sites can be phosphorylated and modified on serine (Ser), 11 sites can be phosphorylated and modified on threonine (Thr), and 3 sites can be phosphorylated and modified on tyrosine (Tyr) (Fig. 7B). The CsABF3 protein contains 40 phospho-sites, of which 27 sites can be phosphorylated on serine (Ser), 12 sites can be phosphorylated on threonine (Thr), and 1 site can be phosphorylated on tyrosine (Tyr) (Fig. 7C). The CsABF4 protein contains 32 phospho-sites, of which 16 sites can be phosphorylated and modified on serine (Ser), 5 sites on threonine (Thr), and 1 site on tyrosine (Tyr) (Fig. 7D). The CsABF5 protein contains 34 phosphosites, of which 20 sites can be phosphorylated at serine (Ser), 10 Thr sites can be phosphorylated at threonine (Thr), and 4 sites can be phosphorylated at tyrosine (Tyr) (Fig. 7E). The CsABF6 protein contains 36 phospho-sites, of which 26 sites can be phosphorylated at serine (Ser), 9 sites can be phosphorylated at threonine (Thr), and 1 site can be phosphorylated at tyrosine (Tyr) (Fig. 7F). The CsABF7 protein contains 28 phosphorylation sites, of which 19 sites can be phosphorylated on serine (Ser), 8 sites can be phosphorylated on threonine (Thr), and 1 site can be phosphorylated on tyrosine (Tyr) (Fig. 7G). The CsABF8 protein contains 45 phospho-sites, of which 25 sites can be phosphorylated and modified on serine (Ser), 18 sites on threonine (Thr), and 2 sites on tyrosine (Tyr) (Fig. 7H). The STRING 11.5 database (https://cn.string-db.org/) was utilized to predict the proteins interactions properties of CsABF/AREB (Fig. 8). There are

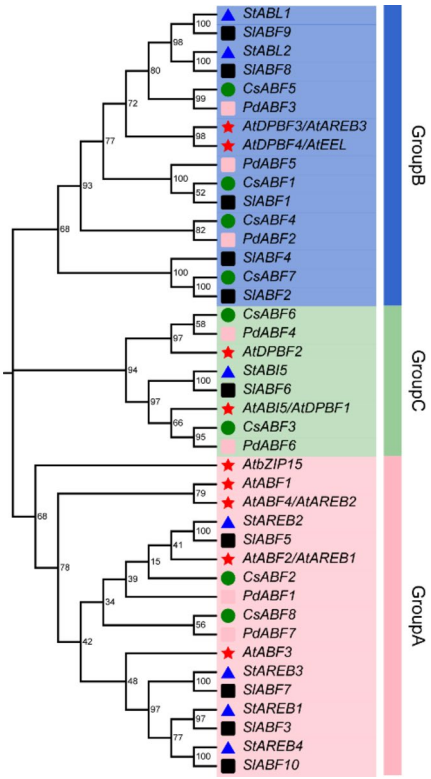

Fig. 3. Multiple sequence alignment of *CsABF/AREB* members. The positions of C1 to C4 are conserved motifs and basic regions are represented by lines above the proteins sequence. Potential phosphorylation residues (R-S-S-X/T) of the characteristic phosphorylation sites are shown in blue. The positions of the conserved Leu residues in the Leu zipper domain are indicated by a red stars. Domains are indicated by red stars.

13 nodes in the whole proteins interactions network, and a total of 40 sets of proteins interactions exist between the nodes. Among them, *CsABF1* ~ *CsABF8* have a strong correlation with 5 proteins, including Csa-2G286490, Csa-4G456680, Csa-4G457180, Csa-4G454150, and Csa-4G455680 (Fig. 8). In addition, although there are interactions correlations among some members, the specific mechanism between each member is not clear, and further in-depth study is still needed.

CsABF/AREB genes GO function and KEGG pathway enrichment

Gene Ontology (GO) analysis was performed to better understand the functional relevance of the *CsABF/AREB* gene family. We used the STRING 11.5 database was utilized to predict GO functional enrichment (biological process molecular function,) of CsABF/AREB proteins, KEGG pathway enrichment, with similarity >= 0.8. Biological processes mainly include cellular response to alcohol, ABA-activated signaling pathways, cellular response to lipids, positive regulation of transcription, DNA templating, and signal transduction (Fig. 9A). The cellular components are mainly localized in the nucleus. The results show that the molecular functions of CsABF/AREB protein mainly include proteins serine/threonine kinase activity, DNA-binding transcription factor activity, DNA binding, and sequence-specific DNA binding (Fig. 9B). In addition, KEGG pathway enrichment indicated that the *CsABF/AREB* genes were significantly linked to plant hormone signal transduction pathway (Fig. 9C).

Analysis of the expression pattern of *CsABF/AREB* genes in different tissues

To determine the expression specificity of *CsABF/AREB* genes in different growth stages and tissues, qRT-PCR was used to detect the expression levels of *CsABF/AREB* genes in different tissues. The results showed that *CsABF1-CsABF8* have the highest expression in roots, which is significantly higher than that in stems and leaves (Fig. 10). Compared with that in roots, the expression of *CsABF1*, *CsABF3*, *CsABF4*, and *CsABF8* in stems is decreased by 38%, 65%, 22%, and 55%, respectively; the expression of *CsABF1*, *CsABF2*, *CsABF6*, and *CsABF8* in leaves is decreased by 64%, 53%, 55%, and 81%, respectively. Our results suggest that these genes are expressed at different levels in different tissues or organs and may play a specific role in cucumber.

Expression pattern analysis of *CsABF/AREB* genes under different hormonal and abiotic stresses

Based on the fact that the promoters of the *CsABF/AREB* genes contain many elements respons to hormonal and abiotic stresses (Figure S1), it was predicted that the proteins may also respond to both hormonal and

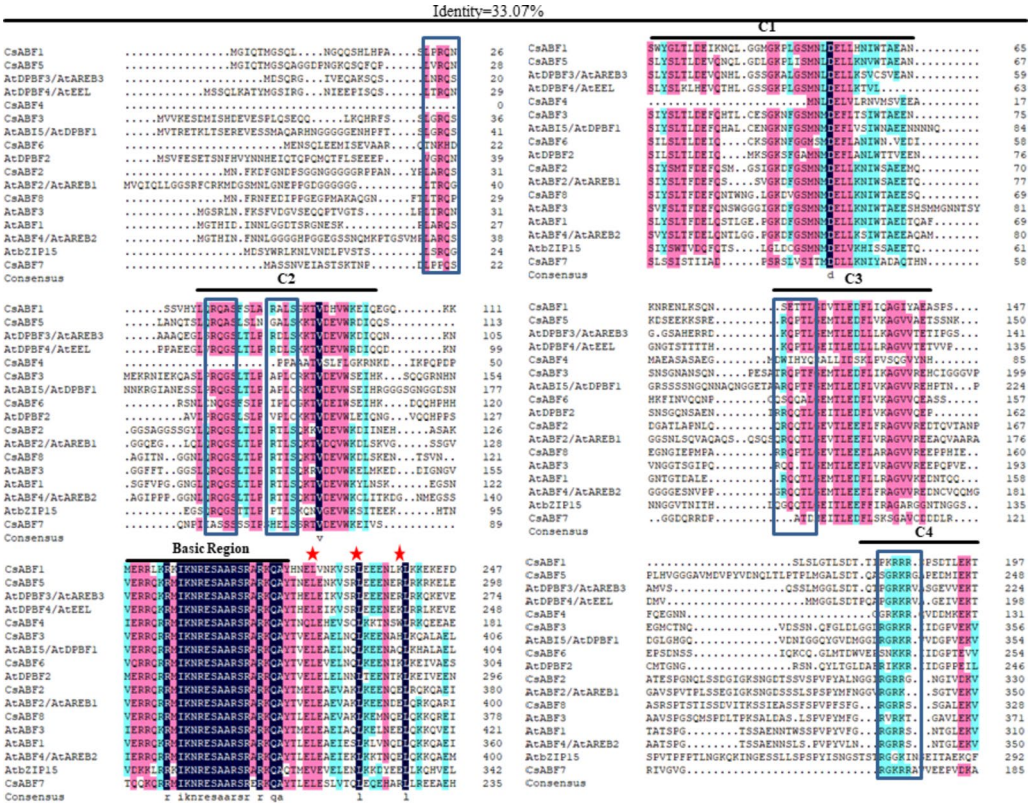

Fig. 4. Phylogenetic tree of *CsABF/AREB* in cucumber (Cs), Aabidopsis (At), potato (St), tomato (Sl), and cottonwood (Pd). In total, 8 *CsABF/AREBs*, 9 *AtABF/AREBs*, 7 *StABF/AREBs*, 10 *SlABF/AREBs*, and 7 *PdABF/AREBs* were included. The phylogenetic trees of CsABF/AREB proteins sequences (green circles), Arabidopsis (red pentagrams), potato (blue triangles), tomato (black squares), and cottonwood (pink squares) were constructed using maximum likelihood method in MEGA 11(v11.0.13) Bootstrap values from 1000 replicates are displayed at each node. The proteins on the tree can be divided into three groups, Group A, Group B, and Group C. Different groups are represented by different colors.

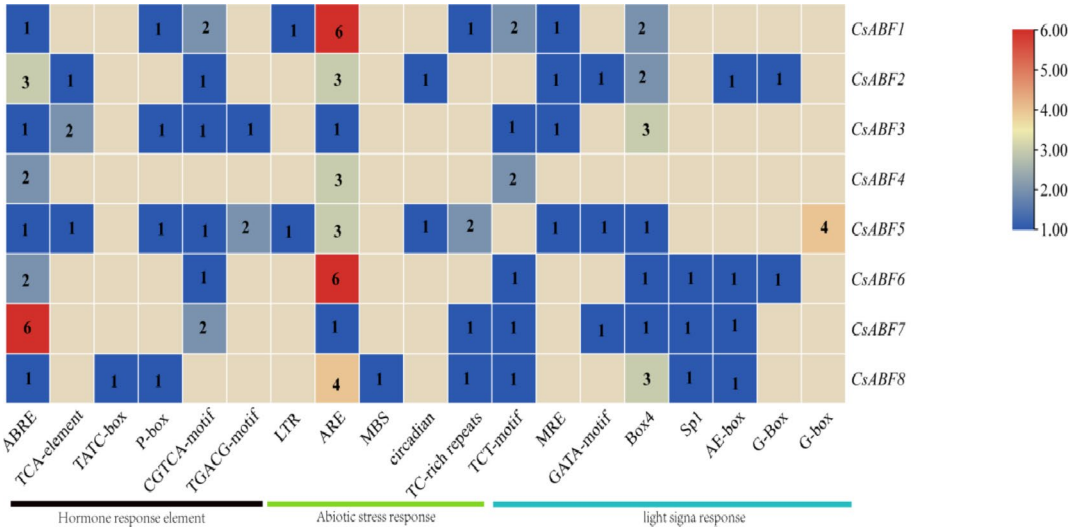

Fig. 5. The number of *cis*-acting elements *CsABF/AREB* genes. The different colors and numbers of the grid indicate the number of different *cis*-acting regulatory elements in these *CsABF/AREB* genes.

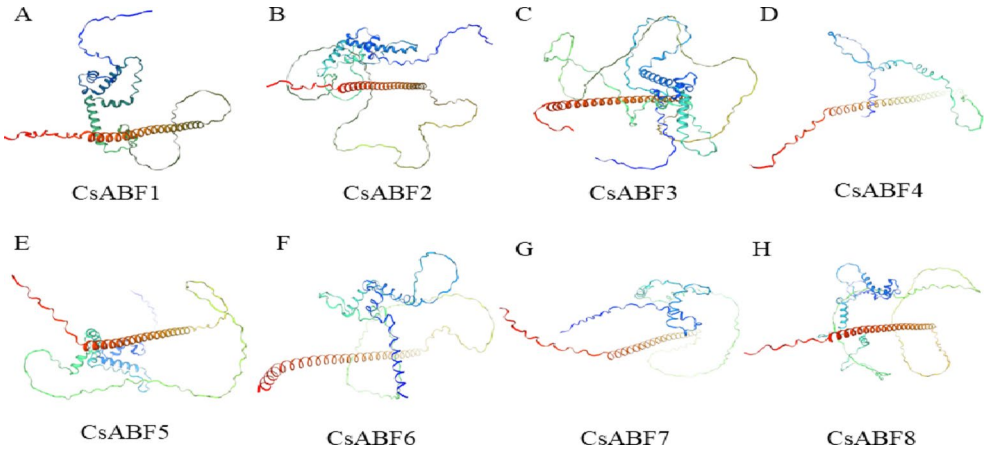

Fig. 6. Tertiary structure prediction of the CsABF/AREB protein family. Red indicates alpha helix, yellow indicates beta turn, blue indicates extended chain, and green indicates random coil.

abiotic stresses. In order to further validate this prediction, we studied the expression levels of 8 *CsABF/AREB* genes under NaCl (50 mM), PEG 6000 (8%), SA (100 µM), Me-JA (100 µM) and ABA (50 mM) treatments (Fig. 11). The results showed that 8 *CsABF/AREB* genes are significantly induced by ABA in root (Fig. 11A), stem (Fig. 11B) and leaf (Fig. 11C), among which, *CsABF7* shows the highest expression. After 12 h of treatment with NaC1, the expression of *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8* is significantly higher than that of the control in root, stem, and leaf. Among them, *CsABF2* shows the highest expression. At 24 h, compared with the control, PEG treatment up-regulates the expression of *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8* in root, stem, and leaf. The highest expression of *CsABF8* is obtained. The expression of *CsABF2*, *CsABF3*, and *CsABF8* are significantly up-regulated in root, stem, and leaf at 24 h of SA treatment. The expression of *CsABF3*, *CsABF6*, and *CsABF7* is significantly up-regulated in root, stem, and leaf after treatment with Me-JA for 24 h compared with the control.

Discussions

Transcription factors are structurally specialized proteins molecules that function to regulate gene expression and generally play an important role in the cell nucleus[19]. The polypeptide chain of *ABF/AREB*transcription factor was found to be 254–485 amino acids in length[20]. *ABF/AREB*transcription factor is one of the important transcription factors responsible for abiotic stress responses. It participates in ABA signal transduction and stress response in plants[21]. So far, the *ABF/AREB* family has been found in a wide range of crops. For example, 9 *ABF/AREB*genes were identified in Arabidopsis[22]. 7 *ABF/AREB*genes were identified in potato[23]. 14 *ABF/AREB*genes were identified in poplar[24]. 10 *ABF/AREB*genes were identified in tomato[25]. These findings reveal the prevalence and diversity of *ABF/AREB* genes across species.

In this study, a total of 8 *CsABF/AREB* genes in were identified based on the latest genomic data (Table 1), which were named *CsABF1-CsABF8* based on chromosome location, randomly distributed on 6 chromosomes (Fig. 1). This is close to the number of ABF family members in jute (*Corchorus olitorius*)[26]. The ABF transcription factors in this study are 321–474 amino acids in length, and the *CsABF/AREB* genes were both located in the nucleus (Table 1), which is similar to *BvABF*[27]. The study of exons and introns has helped to understand the differences in gene structure and function[28]. The gene structure analysis in the present study showed that the number of introns in *CsABF2* and *CsABF8* both were 4 in number (Fig. 2). It was proposed that the rates of intron creation are higher during earlier periods of plant evolution[29]. The types and numbers of conserved motifs were different for different subfamilies or subgroups, and the conserved motifs were similar for the same subfamily or subgroup.

A total of 10 conserved motifs (motif1-motif10) were identified in this study, with motif1 being the core of the bZIP structural domain (Fig. 2). In addition to the bZIP structural domain located at the C-terminal end, the Arabidopsis ABF/AREB/ABI5 proteins also contains four conserved phosphorylation motifs C1 ~C4 (R-XX-S/T), of which C1 ~C3 are located at the N-terminal end and C4 is located at the C-terminal end, which are capable of being phosphorylated by serine/threonine proteins kinases[19]. These corresponding phosphorylation sites in Arabidopsis *AREB1*are essential for the regulation of proteins activity[30]. *AREB1/ABF2*, *AREB2/ABF4*, and *ABF3*can be phosphorylated by SnRK2 kinase[31]. SnRK2 s kinase OST1 phosphorylates threonine residues in C4 of ABF3 protein to produce a binding site for 14-3−3protein, and the two form a stable complex to regulate downstream ABA-responsive gene expression[32]. In this study, the CsABF/AREB proteins contain a highly conserved bZIP structural domain, and the C-terminal end of this region has a unique BRLZ structural domain (Fig. 3), which serves to recognize and bind specific DNA sequences[33]. Moreover, CsABF/AREB proteins also have four conserved potential phosphorylation sites C1 ~C4 (R-XX-S/T), where serine, threonine, and tyrosine undergo phosphorylation modification and affect proteins stability. We used maximum likelihood method to construct phylogenetic tree (Fig. 4). According to the sequence similarity, the 8 CsABF/AREB proteins were divided into 3 subfamilies. Among them, *CsABF7* and *SlABF2* proteins in Group B are highly similar and have a similar genetic relationship, and it can be inferred that there is functional similarity. Among them, CsABF1-

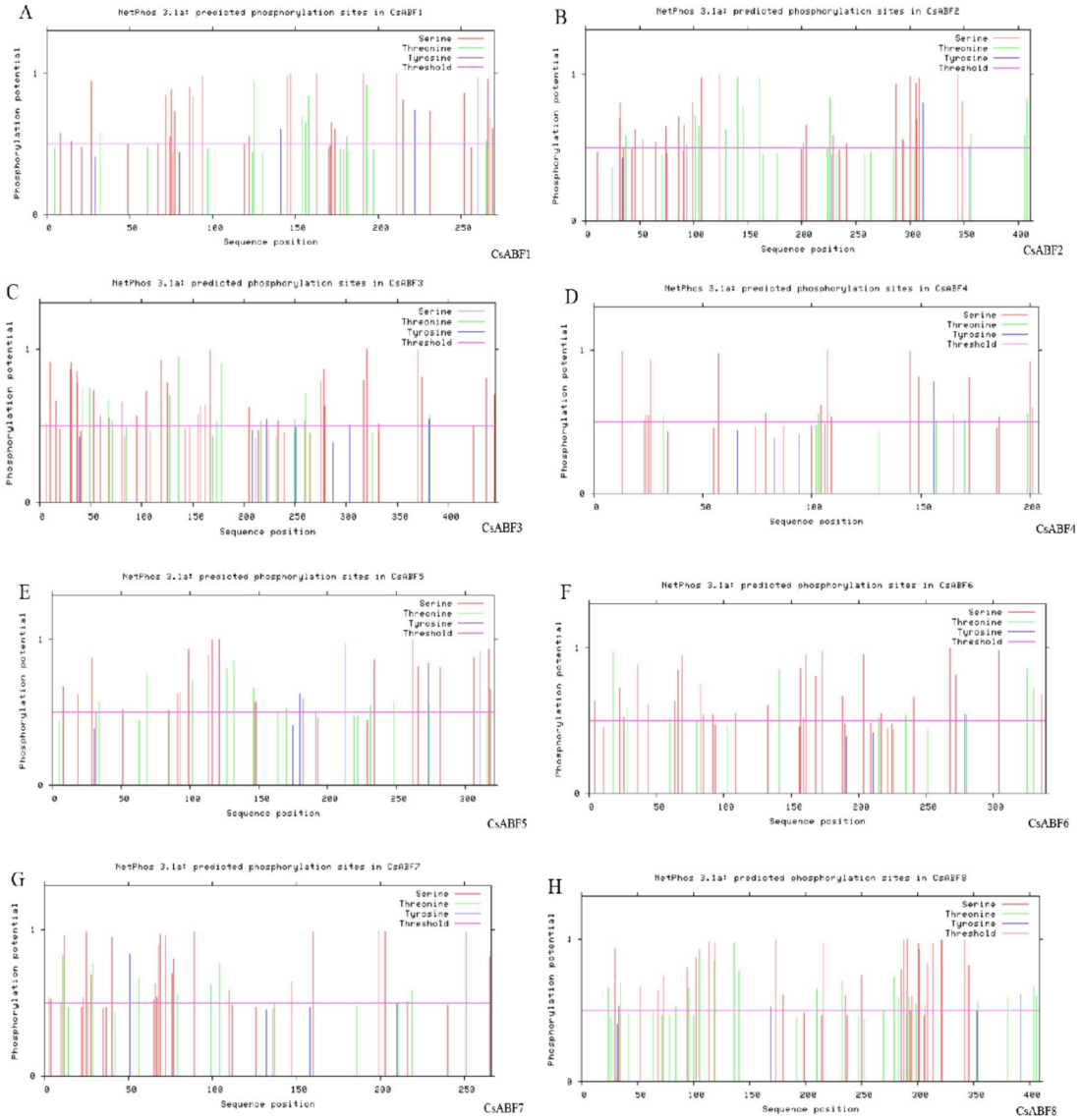

Fig. 7. Predicted phosphorylation of CsABF/AREB proteins. CsABF1 (**A**), CsABF2 (**B**), CsABF3 (**C**), CsABF4 (**D**), CsABF5 (**E**), CsABF6 (**F**), CsABF7 (**G**), and CsABF8 (**H**) Serine stands for serine and is represented by the red line; Threonine stands for threonine and is represented by the green line; Tyrosine represents tyrosine, indicated by blue line; and Threshold represents threshold, indicated by purple line.

CsABF8 have a strong correlation with 5 proteins, including Csa-2G286490, Csa-4G456680, Csa-4G457180, Csa-4G454150, and Csa-4G455680. Csa-2G286490 functions as the core of the protein interactions network (Fig. 8), which contains protein kinase structural domains and belong to the protein kinase superfamily[34]. GO functional and KEEG pathway enrichment showed that *CsABF/AREB* family genes have transcription factor molecular functions such as DNA-binding transcription factor activity, DNA binding, and sequence-specific DNA binding, which are widely involved in biological processes such as growth, development, response to adversity and stress, biosynthesis, etc. (Fig. 9).

Promoter *cis*-acting elements are able to bind to transcription factors to influence gene expression and participate in signal transduction in response to environmental signals[35]. Expression of Arabidopsis *AtABFs*is induced by light, ABA, and abiotic stresses (drought, salt, and low-temperature stress)[22]. Expression of potato *StABF1*can be induced by light, ABA and abiotic stresses (drought, salt and low temperature stress)[19]. The Expression of *PmABFs*in mei could be induced by light, hormones and abiotic stress induction (drought, low temperature)[36]. Tobacco *ABF*gene expression was induced by abscisic acid, methyl jasmonate, salinity, low temperature and drought[37]. Similarly, the results of this study on the *cis*-acting elements of the *CsABF/AREB* gene promoter showed that the *CsABF/AREB* promoter region also contains light, hormone (SA, ABA, Me-JA, and GA), and abiotic stress (hypoxia, drought, and low temperature) signaling response elements (Figure S1). Among them, most of the *CsABF/AREBs* contain ABA-responsive primordial (ABRE) and Me-JA (CGTCA-Motif and TGACG-Motif) response elements (Table S1). In addition, *CsABF1* and *CsABF5* contain low-

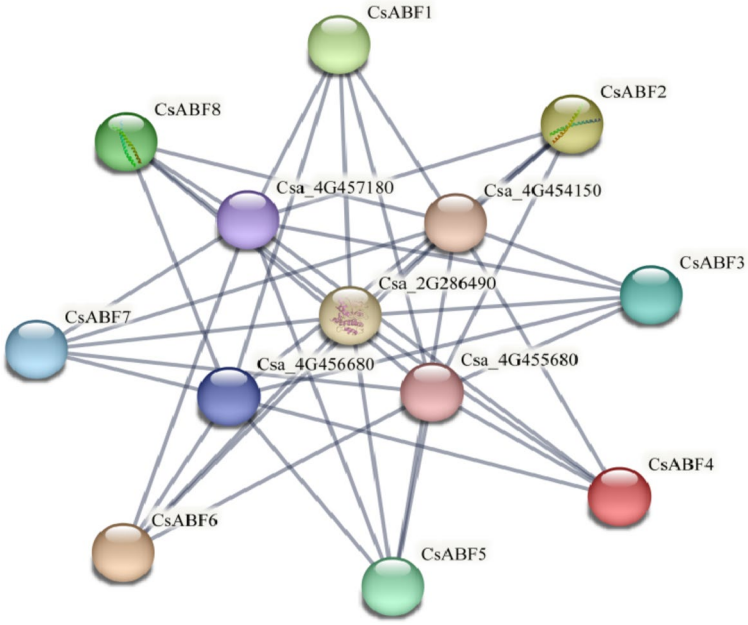

Fig. 8. Proteins interaction network model of CsABF/AREB. Network nodes represent proteins; empty nodes represent proteins with unknown 3D structures; while filled nodes represent proteins with known or predicted 3D structures, and edges represent protein-to-protein associations.

temperature response elements (LTR), and *CsABF8*contains MBS elements (drought), which is similar to the results of tomato studies[25]. Thus, our results also show that *CsABF/AREBs* also play important functions in growth and development and stress response. In the present study, it was shown by further quantitative analysis that all *CsABF/AREB* genesare highly expressed in root (Fig. 10), suggesting that the genes may act mainly in root. Similarly, *ABF/AREB*genes are also highly expressed in root in tomato[25], maize[38]and soybean[39]. Moreover, all *CsABF/AREBs* responded to both ABA and Me-JA treatments to varying degrees (Fig. 11). Among them, *CsABF7* had the highest expression under both ABA and Me-JA treatments, followed by *CsABF2*, which further supports the prediction of promoter analysis. In addition, we found that *CsABF/AREB* respond differently to drought and salt stress, in which, *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8* are significantly up-regulated under PEG and NaCl treatments (Fig. 11). However, *CsABF3* is significantly up-regulated under SA treatment, and *CsABF2* and *CsABF5* are expressed at lower levels under SA treatment. Therefore, we hypothesized that *CsABF/AREB* members may be involved in hormone and abiotic stress response. Of course, this needs to be further verified.

Conclusions

In this study, a total of eight *CsABF/AREB* genes were identified in the genome, and this gene family contains a common conserved structural domain bZIP, and all of them contain the ABA-responsive element ABRE in their promoters. In addition Me-JA, SA, light, and stress-responsive elements are shown in the promoters of most *CsABF/AREB* members. The expression level of all *CsABF/AREB* genes is highly expressed in root, suggesting that all the genes may act mainly in root. *CsABF/AREB* gene members mainly responde to ABA and Me-JA, among which, *CsABF7* has the highest expression under both ABA and Me-JA treatments. Drought and NaCl stress significantly induce *CsABF1*, *CsABF2*, *CsABF7*, and *CsABF8* expression. Therefore, *CsABF/AREB* may play an important role in hormone signaling and abiotic stress response. The results lay a foundation for further study on the potential functions of *CsABF/AREB* family members.

Materials and methods

Identification of members of the *CsABF/AREB* gene family

In Arabidopsis, there are 9 *ABF/AREB* subfamily genes: *AtABF1*, *AtABF2/AREB1*, *AtABF3*, *AtABF4/AREB2*, *AtABI5/DPBF1*, *AtDPBF2*, *AtDPBF3/AREB3*, *AtDPBF4*, *AtbZIP15*. The 9 Arabidopsis ABF/AREB proteins sequences, downloaded from TAIR (https://www.arabidopsis.org/) were used as queries to search the corresponding subject sequences in the genomic data of the cucumber by Blastp with an E-value cut-off of 1e − 22 to reduce false positives. The cucumber genome database and annotation information was downloaded form (https://www.NCBI.nlm.nih.gov/datasets/genome/GCF_000004075.3/)[40]. The conserved structural domains of ABF/AREB proteins in cucumber were further validated using the PFAM database (http://pfam.xfam.org/) and the SMART (http://smart.embl-heidelberg.de/) database.

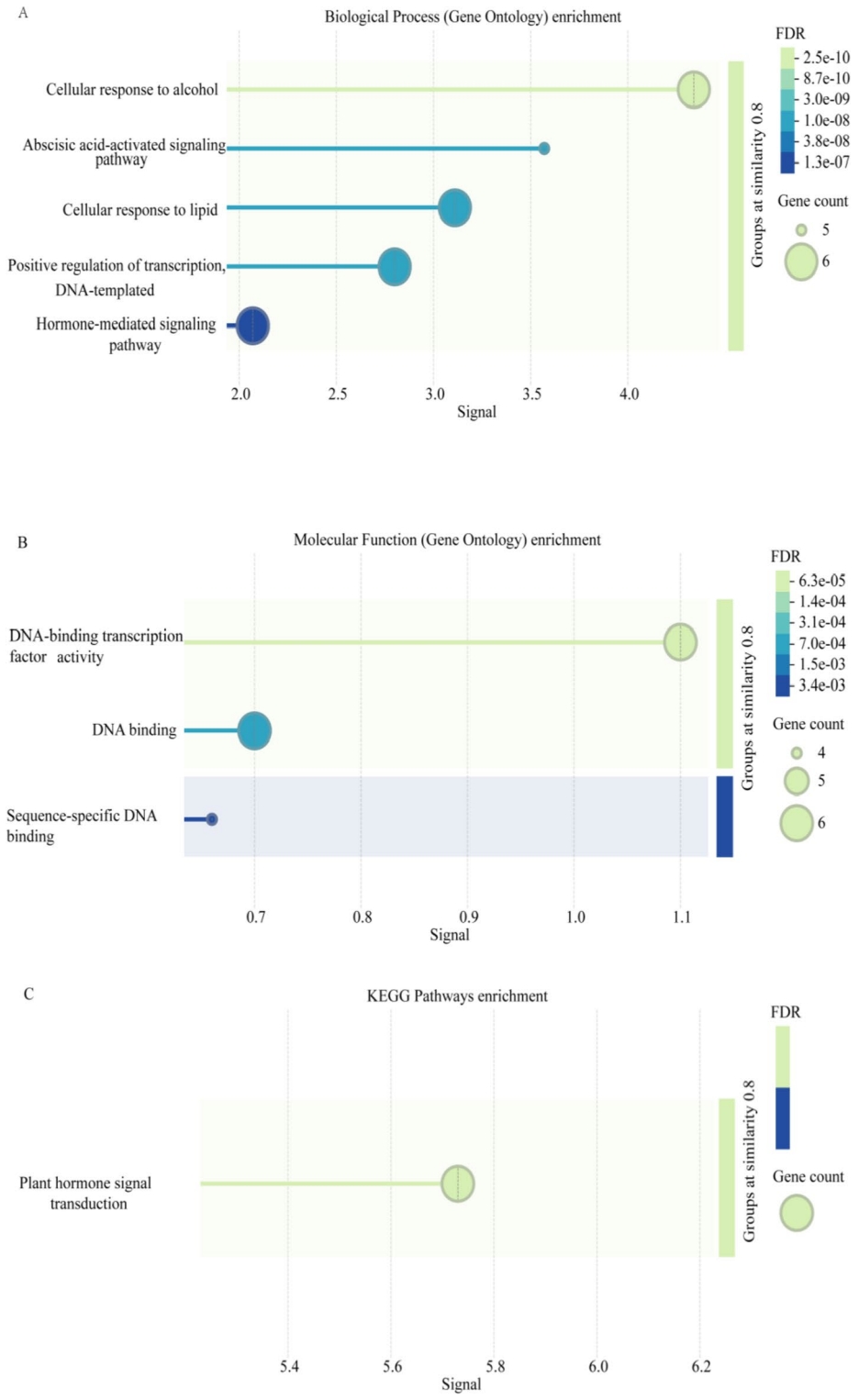

Fig. 9. *CsABF/AREB* GO and KEGG pathway enrichment analysis. (**A**) Biological process; (**B**) Molecular function; (**C**) KEGG pathway enrichment.

Chromosomal localization of the *CsABF/AREB* gene family

The whole genome information (GFF3, FASTA, PEP, CDS) of tomato was analyzed using TBtools (toolbox for ecological battle) v1.0985 software[41], and finally the cucumber genome file was screened and mapped according to the gene ID of the identified *CsABF/AREB* family members.

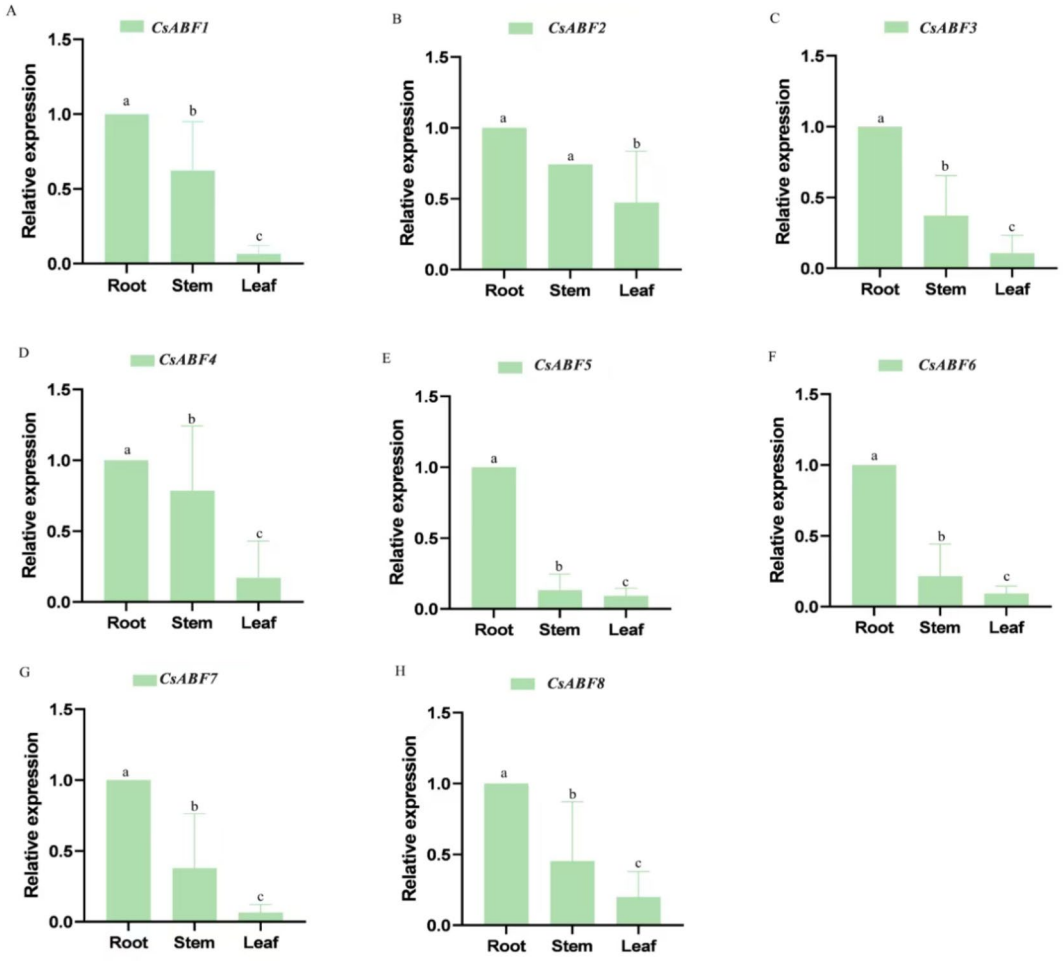

Fig. 10. Expression levels of *CsABF/AREB* genes in root, stem and leaf. Data are expressed as means ± standard deviation ($n = 3$). Different letters over the bars indicate significant differences between mean values according to Tukey's test ($p < 0.05$).

Analysis of *CsABF/AREB* genes structure, conserved motifs, and conserved structural domains

The gene structure of each member of *CsABF/AREB* was analyzed using the 'Visualize Gene Structure (from GTF/GFF3 File)' function in TBtools software. The cucumber GFF3 file was downloaded from the online database. The distribution of the conserved motifs based on amino acid sequence was conducted with the online MEME (http://meme-suite.org/tools/meme) website[42]. The maximum number of Motif discoveries was set to 10, whereas other parameters were the default values. Then, the corresponding motif information and the evolutionary tree information of *CsABF/AREB* family derived from MEGA 11 (v11.0.13) were combined to be analyzed via the gene structure view (Advances) function of TBtools software to visualize the conserved motifs of *CsABF/AREB* members.

Phylogeny of *CsABF/AREB* genes, analysis of multiple sequence comparisons

The ABF/AREB proteins sequences of *Arabidopsis thaliana*, potato, Oriental poplar, and tomato were downloaded from TAIR (https://www.arabidopsis.org/). The proteins sequences of Arabidopsis, potato, Oriental poplar, and tomato were combined in the same file, and the evolutionary tree was constructed using the MEGA 11 software (v11.0.13), in which the neighbor-joining method was used; the number of replicates was set to 1000, and the rest of the options were set to the default values. The website Evolview (https://evolgenius.info//evolview-v2/#login) was then used for the further modification of the evolutionary tree. The multiple sequence alignment of cucumber family was done by through ClustalX and Jalview software.

Cis-Acting element analysis of *CsABF/AREB* genes

The 'Gtf/Gff3 Sequences Extract' and 'Fasta Extract (Recommended)' functions of TBtools were used to extract the 2000 upstream *CsABF/AREB* genes of from the cucumber gene databases. Here, 2000 bp of data upstream of the *CsABF/AREB* genes were extracted from the cucumber gene databases; these were submitted to the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) database for gene homeotic element (promoter) analysis and were visualized using TBtools.

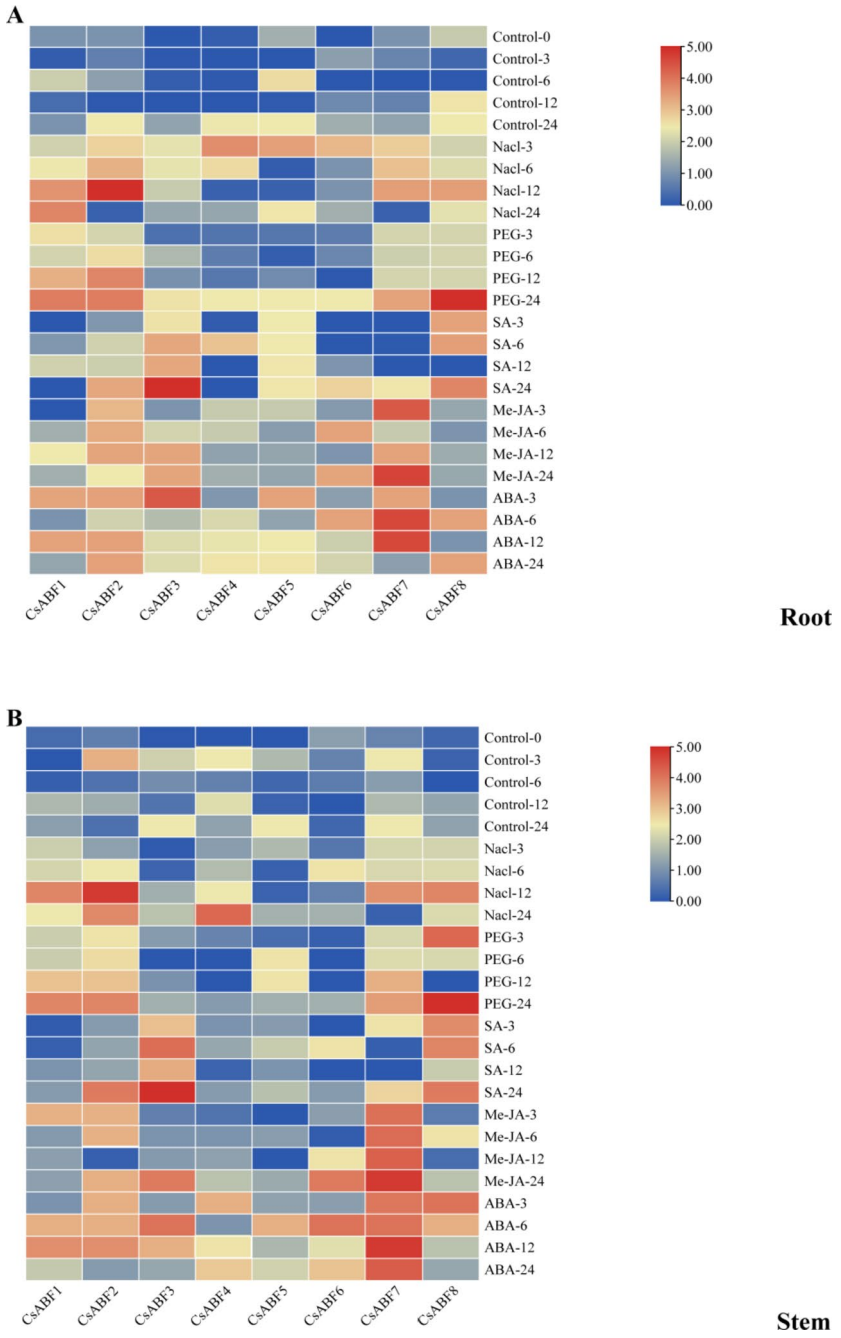

Fig. 11. Heatmap of *CsABF/AREB* gene expression in different treatments. Each color represents the corresponding expression value: the larger the value, the higher the expression; the smaller the value, the lower the expression. (**A**) The gene expression in root. (**B**) The expressionin stem. (**C**) The expression in leaf.

Analyzing proteins structure and expression patterns

The subcellular localization of proteins was predicted using the online software PlantmPLoc (v2.0) (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/)[40]. To analyze the physicochemical properties of the *CsABF/AREB* genes family, the molecular weights, instability coefficients, isoelectric points, and hydrophilicity of each member of the horse *CsABF/AREB* genes were analyzed using the Expasy online website (https://web.expasy.org/protparam/). The secondary structure of the proteins encoded by the *CsABF/AREB* genes was predicted by the online software SOPMA (https://npsa-prabi.ibcp.fr/), which includes four different forms of composition: α-helical, β-turned, irregularly coiled, and strand-extended structures. The tertiary structure of the proteins encoded by the *CsABF/AREB* genes was predicted by the online software SWISS-MODEL (https://swissmodel.expasy.org/). Tissue expression information for the *CsABF/AREB* genes was obtained from the eFP (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) database. Then, the data were sorted out and the expression patterns of *CsABF/AREB* in different tissues were drawn by TBtools.

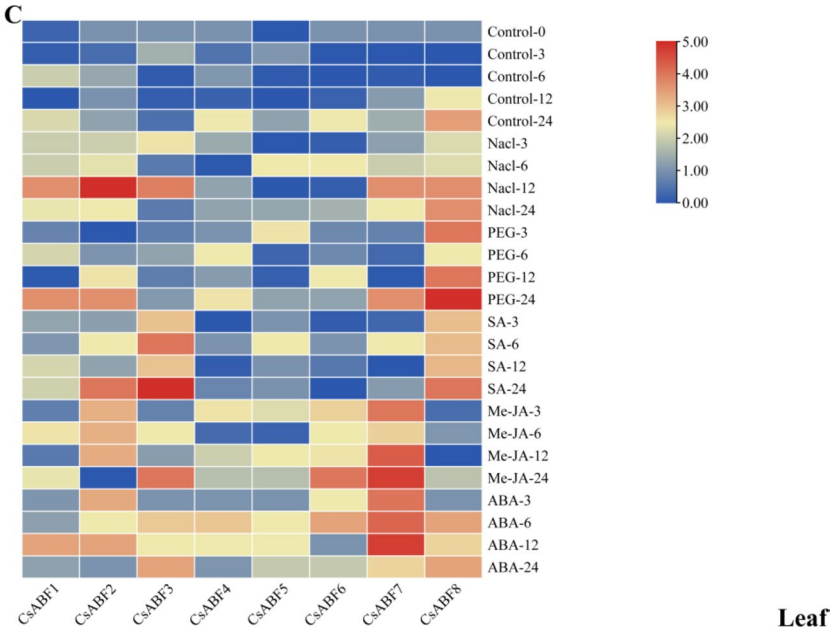

Figure 11. (continued)

CsABF/AREB proteins phosphorylation, proteins interactions analysis and annotation

Phosphorylation prediction analysis of CsABF/AREB proteins was performed via the online website CBS NetPhos (www.cbs.dtu.dk/services/NetPhos)[43]. The interaction network relationships for CsABF/AREB proteins were constructed using the STRING 11.5 Database (https://cn.string-db.org/). The selected value was > 0.400, and the interaction prediction analysis was carried out to investigate the role of CsABF/AREB proteins members.

CsABF/AREB genes GO function and KEEG pathway enrichment

Visualization of GO functional enrichment of *CsABF/AREB* was predicted using the STRING 11.5 database (https://cn.string-db.org/) with KEEG pathway, similarity > = 0.8.

Plant materials, cultivation conditions and treatments

Cucumber (*Cucumis sativus* L. 'New Spring IV') seed material was provided by our laboratory. All the experimental research on plants was conducted according to the proper guidelines and legislation of national and international regulations. After soaking in water at 30 °C for 3–4 h, 5% sodium hypochlorite (NaClO) was selected for surface disinfection for 10–15 min, and then rinsed with water for 4–5 times, and then placed flatly in a glass petri dish with moistened filter paper. Next, they were placed in an incubator (Accurate Biotechnology, Hunan, China at a constant temperature (28 ± 0.5 °C) for 1–2 d of germination, and when the two true leaves were fully expanded (20 d after seed germination), they were planted in a nutrient solution hydroponic tank with Yamazakis Special Cucumber Nutrient Solution (SCNS), which was changed every 3–4 days, and cultivated hydroponically in an artificial climatic chamber with an environment controlled to have a photoperiod of 14/10 h (light/dark), a temperature of 25 °C/18 °C (day/night), and a light intensity of 250 μmol m^{-2} $s^{-1}$and 70% relative humidity in an artificial climate chamber hydroponically[44]. The treatments were initiated when the seedlings grew to two leaves and one heart, and the concentrations were: NaCl (50 mM), SA (100 μM), Me-JA (100 μM), ABA (50 mM) and PEG 6000 (8%). These concentrations were selected on the basis of our previous studies[45]. Samples were taken at 0, 3, 6, 12, and 24 h after treatment, respectively; and cucumber roots, stems, and leaves were selected as sample materials. 0.5 g of samples were weighed and then dispensed with tin foil, and the collected samples were immediately frozen with liquid nitrogen and stored in a vertical ultra-low-temperature refrigerator at −80 °C (Qingdao Haier Specialty Appliances Co. Ltd., Qingdao, China). Each treatment contain 3 biological replicates. Root, stem, and leaf samples were collected at 0, 3, 6, 12 and 24 h after treatment for qRT-PCR experiments.

RNA extraction and qRT-PCR fluorescence quantification

Total RNA was extracted from different tissues and treated with MiniBEST plant RNA Extraction Kit (Takara, Dalian, China). The FastQuant (Tiangen, Beijing, China) Synthesis Kit was used to synthesize First Strand cDNA fragments according to the manufacturer's method. The CDS sequences of *CsABF/AREB* genes were input into the homepage of Shanghai Biology Company (Shanghai, China) for online primer design, and then the primer sequences were synthesized. *CsActin 1* and *CsActin 2*were used as an internal reference gene[46,47]. The primers were designed by Primer 5.0 software and listed in Supplementary Table S3. The amplification system contained

2 µL cDNA, 0.6 µL 10 µM upstream primers, 0.6 µL 10 µM downstream primers, 10 µL 2 × SuperReal PreMix Plus and 6.8 µL RNase-free ddH_2O. qRT-PCR cycle conditions included: 95 °C for 15 min, 90 °C for 10 s and 60 °C for 20 s, 40 cycles. There were three biological replicates per treatment. The *CsABF/AREB* gene was used to normalize relative expression levels. The $2^{-\Delta\Delta Ct}$method was used to calculate the relative expression[48].

Data availability

All data generated or analyzed during this study are included in this article.

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Author contributions

W.L. conceived the project and original research plans; S.L., Y.Q., X.P. and X.C. performed experiments and analyzed and interpreted the data; W.S., A.L. and X.L. participated in data interpretation and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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