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Characterization of Germin-like Proteins (GLPs) and Their Expression in Response to Abiotic and Biotic Stresses in Cucumber

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Abstract: Germins and germin-like proteins (GLPs) are glycoproteins closely associated with plant development and stress response in the plant kingdom. Here, we carried out genome-wide identification and expression analysis of the *GLP* gene family in cucumber to study their possible functions. A total of 38 *GLP* genes were identified in cucumber, which could be mapped to six out of the seven cucumber chromosomes. A phylogenetic analysis of the GLP members from cucumber, *Arabidopsis* and rice showed that these GLPs could be divided into six groups, and cucumber GLPs in the same group had highly similar conserved motif distribution and gene structure. Gene duplication analysis revealed that six cucumber *GLP* genes were located in the segmental duplication regions of cucumber chromosomes, while 14 genes were associated with tandem duplications. Tissue expression profiles of cucumber *GLP* genes showed that many genes were preferentially expressed in specific tissues. In addition, some cucumber *GLP* genes were differentially expressed under salt, drought and ABA treatments, as well as under DM inoculation. Our results provide important information for the functional identification of *GLP* genes in the growth, development and stress response of cucumber.

Keywords: cucumber; germin-like protein (GLP); abiotic stresses; downy mildew (DM); expression analysis

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1. Introduction

Germin was first identified in wheat's embryos and labeled as a germination marker; it was subsequently characterized as a homohexamer glycoprotein with oxalate oxidase (OXO, EC 1.2.3.4) activity [1,2]. Proteins with similar amino acid sequences and structures to wheat germin are defined as germin-like proteins (GLPs) [3–5]. GLPs are a type of soluble glycoproteins with a "cupin" domain (PF00190) belonging to the "cupin superfamily" ubiquitously present in plants [6,7]. GLPs harbor a conserved β-sheet barrel domain in metal binding and play enzymatic and non-enzymatic roles by altering the region of the active site [3,8,9]. Most GLPs are stable oligomers, but they have enzyme activities most often in typically hexameric structures, being trimers of dimers form [4,10]. Besides OXO activity, various other enzymatic activities were found in GLPs, such as superoxide dismutase (SOD, EC 1.15.1.1) [10,11], ADP glucose pyrophosphatase/phosphodiesterase (AGPPase, EC 3.1.4.1) [12], and polyphenol oxidases (PPO) [13,14].

To date, many *GLP* genes have been isolated and functionally characterized in a variety of plant species, indicating their key roles in plant growth and development. For example, rice *OsGLP1* (Os08g35760, also named as *OsGLP8-14*) is mainly found in green vegetative tissues and plays a determinant role in plant height [15]. *OsGLP2-1* (Os02g29000)

acts as an important regulator of seed dormancy in rice in the abscisic acid (ABA) and gibberellic acid (GA) signaling pathways [16]. Overexpression of rice *OsRGLP1* (Os08g09080, also named as *OsGLP8-11*) in tomato resulted in morpho-physiological traits, including increased chlorophyll and relative water content, dwarfism, high density and diversity of trichomes [17]. In addition, GLPs are also related to the responses of plants to biotic and abiotic stress. For instance, among the eight tea plant *GLPs*, six and two genes were induced and repressed by insect herbivory, respectively [18]. The expression levels of many rice *GLP* genes were obviously altered under various biotic (brown plant hopper, *Agrobacterium tumefaciens* and fungal infestation) and abiotic (cold, salt, drought and anoxia) stress conditions [19]. Specifically, *OsGLP8-14* was found to be involved in pathogen resistance [15], salt stress response [20] and acclimation to UV-B radiation [21]. In recent years, there has been increasing evidence demonstrating the crucial regulatory roles of *GLP* genes in plant disease resistance. For example, transgenic tobacco plants overexpressing *Lilium regale LrGLP1* exhibited considerably enhanced resistance to *Fusarium oxysporum* infection [22]. Transgenic *Solanum tuberosum* and *Medicago truncatula* plants overexpressing *OsRGLP1* also displayed higher resistance to *Fusarium oxysporum* [23,24]. Another rice *GLP* gene, *OsGLP2-4* (Os02g32980), was found to confer resistance to fungal blast and bacterial blight in the jasmonic acid (JA)-dependent pathway [25]. Overexpression of sunflower *HaGLP1* in *Arabidopsis* improved the resistance to fungal pathogens by promoting the accumulation of reactive oxygen species (ROS) [11]. Transgenic tobacco plants overexpressing soybean *GmGLP10* gene displayed enhanced resistance to *Sclerotinia sclerotiorum* infection [26]. Cotton *GhABP19* is also associated with the JA-mediated defense response to *Verticillium dahliae* and *Fusarium oxysporum* infection [27].

In recent years, genome-wide analysis of the *GLP* gene family has identified a large number of *GLP* genes in various plant species, such as *Physcomitrella patens* [28], soybean [29,30], tea plant [18], wheat [31], rice and *Arabidopsis* [6]. However, there is very limited information about the *GLP* genes in cucumber, an important vegetable crop frequently affected by diverse stresses. This study systematically analyzed the *GLP* family genes in cucumber. The findings may provide a theoretical basis for the functional analysis of *GLP* genes in cucumber and other plant species.

2. Materials and Methods

2.1. Identification and Property Characterization of GLPs in Cucumber

To identify all members of the *GLP* gene family in cucumber, the hidden Markov model (HMM) profile of the GLP feature domain (PF00190) was downloaded from the Pfam database (http://pfam.xfam.org/, Pfam 34.0, 15 March 2021), and then the HMMER3 software was employed to identify the *GLP* genes in the cucumber (Chinese Long 9930) genome database (http://cucurbitgenomics.org/organism/2, 15 March 2021). To ensure accuracy, all putative GLP protein sequences were subjected to Pfam (http://Pfam.sanger.ac.uk/, 15 March 2021) and SMART (http://smart.embl-heidelberg.de/, 15 March 2021) to verify the presence of the "cupin" domain (PF00190). The length of amino acids (aa), theoretical molecular weight (MW) and isoelectric point (pI) of the deduced CsGLP proteins were determined by the ProtParam program (http://web.expasy.org/protparam, 15 March 2021). Subcellular localization prediction of the CsGLP proteins was carried out with the online tools including Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi, 15 March 2021) and CELLO v.2.5 (http://cello.life.nctu.edu.tw, 15 March 2021).

2.2. Phylogenetic, Conserved Motif and Gene Structure Analyses

To investigate the phylogenetic relationship between GLP proteins of cucumber and other plant species, the full-length GLP protein sequences of cucumber, *Arabidopsis* and rice were aligned using MAFFT with the default options, and a phylogenetic tree was constructed using the MEGA 7.0 software by the neighbor-joining (NJ) method with a bootstrap analysis of 1000 replications. To investigate the conserved motifs of GLP members from cucumber, the complete amino acid sequences of GLP proteins were analyzed

using the online MEME tool (http://meme-suite.org/tools/meme, 15 March 2021). The parameters for the analysis were as follows: maximum number of motifs, 10; minimum motif width, 6; and maximum motif width, 50. The genomic library and coding sequence (CDS) information of cucumber were downloaded from the cucumber (Chinese Long) genome database (http://cucurbitgenomics.org/organism/2, 15 March 2021). For gene structure analysis, the CDS and corresponding genomic sequences of cucumber *GLP* genes were retrieved, and the gene structure was analyzed by the Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/, 15 March 2021).

2.3. Chromosomal Distribution and Gene Duplication Analysis

The chromosomal locations of the *CsGLP* genes were analyzed by MapGene2Chrom (http://mg2c.iask.in/mg2c_v2.0/, 15 March 2021), a tool for the online reconstruction of the gene chromosome position map. Tandem and segmental duplication events were identified using the MCScanX software based on previously published criteria [32].

2.4. RNA-Seq Expression Analysis of the CsGLP Genes

The raw RNA-seq data from various cucumber tissues (roots, stems, leaves, flowers, ovaries and tendrils) and cucumber inoculated with downy mildew (DM, *Pseudoperonospora cubensis*) were download from the NCBI database (PRJNA80169 and SRP009350) and analyzed with StringTie according to our previous study [33]. For tempo-spatial expression analysis, a total of 10 tissues from *Cucumis sativus* var. *sativus* line 9930 were sampled, including the root, stem, leaf, male and female flowers, base part of tendril, tendril, unexpanded ovary, expanded ovary under fertilization (7 days after flowering) and expanded ovary not fertilized (7 days after flowering) [34]. For DM inoculation expression analysis, leaf samples were collected from *C. sativus* cv. 'Vlaspik' at different time points after inoculation with *P. cubensis* [35]. The transcripts per kilobase million (TPM) values were $\log_2$ transformed using the TBtools software for generating heat map and cluster analysis [36].

2.5. qRT-PCR Analysis of CsGLP Genes in Response to Abiotic Stress

Seeds of the cucumber line 9930 were surface sterilized and germinated on wet filter paper in a growth chamber at 28 °C for 1 day. Then, the germinated seeds were transplanted onto poly trays containing peat, sand and pumice at a 1:1:1 ratio. The seedlings were subsequently transferred into hydroponic boxes filled with 1/2 Hoagland nutrient solution in a greenhouse under 16-h light/8-h dark conditions once the cotyledon was fully unfolded. Seedlings at the three-leaf stage were treated with the salt, drought and ABA treatments by using 200 mM NaCl, 10% PEG-6000 (*w*/*v*) and 100 µM ABA. Seedlings were subjected to salt and drought stress treatments by the addition of 10% (*w*/*v*) PEG-6000 (Sigma-Aldrich, Shanghai, China) and 200 mM NaCl in 1/2 Hoagland nutrient solution, respectively. For ABA treatment, seedlings were treated with 100 µM abscisic acid (ABA) as described previously [37]. Leaf samples were collected at 0, 6, 12 and 24 h after treatments and used for qRT-PCR analysis.

Total RNA was isolated using the Eastep Super Total RNA Extraction Kit (Promega, Madison, WI, USA), and cDNA synthesis was performed using the M-MLV reverse transcriptase (Invitrogen, USA) according to the protocols. qRT-PCR was performed with the Roche Lightcyler 480II PCR System using the TB Green Premix Ex TaqII Kit (TaKaRa, Dalian, China) under the guidelines, as previously described [32]. Three biological replicates were performed for each experiment. The *CsAct3* gene was used as a control for qRT-PCR analysis with the $2^{-\Delta\Delta Ct}$ relative quantitative method [32].

3. Results

3.1. Identification of the CsGLP Genes in Cucumber

In total, 38 candidate *GLP* genes were identified in the cucumber genome, which were assigned according to their chromosomal locations (Table 1). Then, the 38 deduced protein sequences were verified through scanning of Pfam and SMART. The results revealed that

all the sequences had the cupin domain (PF00190), which is the typical configuration of GLP proteins, suggesting that they belong to the *GLP* gene family. The detailed information of each GLP member, including the locus ID, chromosomal location, length of CDS, gDNA and protein, is presented in Table 1. The gDNA of *CsGLPs* ranged from 564 bp (*CsGLP1-2*) to 3868 bp (*CsGLP7-4*) and encoded the polypeptides with lengths ranging from 124 aa (CsGLP5-3) to 927 aa (CsGLP3-1). The MW ranged from 13.54 kDa (CsGLP5-3) to 112.93 kDa (CsGLP3-1), and the pI values from 5.16 (CsGLP1-3) to 9.43 (CsGLP7-3) (Table 1). Prediction of the subcellular localization of CsGLP proteins revealed that two CsGLPs were located in the nucleus, 12 CsGLPs were located in the vacuole, and 23 CsGLPs were located in the cell wall, while CsGLP3-2 was predicted to be located in both the cell wall and vacuole (Table 1).

3.2. Phylogenetic Analysis of GLP Proteins from Cucumber and Other Plant Species

To assess the evolutionary relationships of the 38 CsGLP proteins, a phylogenetic tree was constructed with MEGA 7.0 using the full-length GLP amino acid sequences from cucumber, *Arabidopsis* and rice. The phylogenetic tree demonstrated that these GLPs could be divided into six groups (Groups a–f), with Group a being the largest group in all three species (Figure 1). Group f (15 CsGLPs) and Group a (11 CsGLPs) comprised a larger number of cucumber members, while there were only five, three, two and two members from cucumber in Group e, Group b, Group c and Group d, respectively (Figure 1).

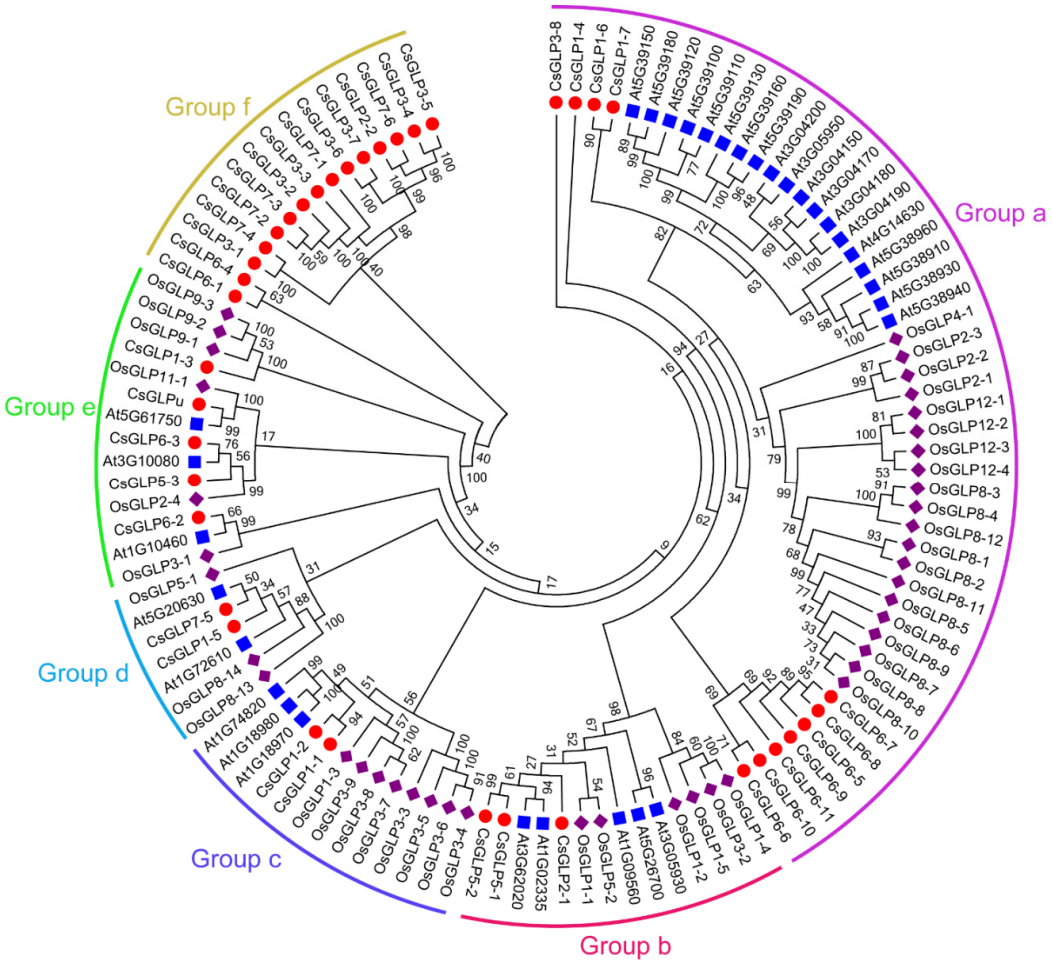

Figure 1. Phylogenetic relationships and subfamily designations of GLP proteins from cucumber, rice and *Arabidopsis*. The analysis involved 113 amino acid sequences—cucumber (38), rice (43) and *Arabidopsis* (32)—and their IDs are listed in Table S2. The phylogenetic tree of GLP proteins from cucumber, rice and *Arabidopsis* was constructed using the NJ method with 1000 bootstrap replicates, and GLP proteins in the tree were divided into six groups (Groups a–f).

Table 1. Identification and characterization of *GLP* family genes in cucumber.

Gene	Locus	Chromosome	Chromosomal Position	gDNA (bp)	CDS (bp)	Protein			
						Length (aa)	MW (Da)	pI	Subcellular Prediction
CsGLP1-1	Csa1G007890.1	1	1255048-1256300	1253	666	221	23,666.44	7.73	Cell wall
CsGLP1-2	Csa1G007900.1	1	1258859-1259500	642	564	213	22,984.84	7.81	Cell wall
CsGLP1-3	Csa1G166250.1	1	10344063-10344626	564	675	187	19,869.54	5.16	Cell wall
CsGLP1-4	Csa1G537570.1	1	19610111-19612225	2115	672	217	23,108.48	5.79	Cell wall
CsGLP1-5	Csa1G596420.1	1	22537064-22539018	1955	642	215	22,364.19	6.81	Cell wall
CsGLP1-6	Csa1G662790.1	1	26758087-26759600	1514	648	224	23,991.83	7.82	Cell wall
CsGLP1-7	Csa1G662810.1	1	26786634-26788514	1881	654	223	24,103.81	6.82	Cell wall
CsGLP2-1	Csa2G035370.1	2	3544666-3546134	1469	654	217	22,892.41	9.30	Cell wall
CsGLP2-2	Csa2G174130.1	2	9968726-9970593	1868	1464	487	55,464.56	8.83	Vacuole
CsGLP3-1	Csa3G146460.1	3	9816841-9820025	3185	1440	927	112,934.56	8.43	Nucleus
CsGLP3-2	Csa3G218160.1	3	14499431-14500893	1463	1023	340	37,226.71	5.83	Cell wall, Vacuole
CsGLP3-3	Csa3G218170.1	3	14512631-14514977	2347	1071	356	38,684.19	5.19	Vacuole
CsGLP3-4	Csa3G384800.1	3	18869164-18870960	1797	2784	501	57,611.85	7.20	Vacuole
CsGLP3-5	Csa3G386310.1	3	18896546-18898442	1897	1485	494	56,770.06	8.60	Vacuole
CsGLP3-6	Csa3G386810.1	3	18901334-18903344	2011	447	479	54,416.18	5.71	Vacuole
CsGLP3-7	Csa3G386820.1	3	18920239-18921873	1635	1047	348	40,180.34	5.51	Vacuole
CsGLP3-8	Csa3G644800.1	3	25260870-25261483	614	1506	148	16,322.28	6.63	Cell wall
CsGLP5-1	Csa5G128780.1	5	3162233-3164925	2693	675	233	25,061.75	6.06	Cell wall
CsGLP5-2	Csa5G129280.1	5	3171528-3174320	2793	702	224	23,714.30	6.96	Cell wall
CsGLP5-3	Csa5G614670.1	5	24218204-24218890	687	375	124	13,540.72	6.51	Cell wall
CsGLP6-1	Csa6G290870.1	6	14050717-14053164	2448	1527	508	58,057.85	5.74	Vacuole
CsGLP6-2	Csa6G404270.1	6	18237396-18238128	733	663	224	24,344.08	8.37	Cell wall
CsGLP6-3	Csa6G452110.1	6	21556627-21557475	849	657	207	22,359.95	8.77	Cell wall
CsGLP6-4	Csa6G502040.1	6	25250904-25253534	2631	648	661	78,191.37	6.23	Nucleus
CsGLP6-5	Csa6G525540.1	6	28560994-28561809	816	648	219	23,425.11	7.80	Cell wall
CsGLP6-6	Csa6G525550.1	6	28563130-28563904	775	651	216	22,819.23	6.69	Cell wall

Table 1. *Cont.*

Gene	Locus	Chromosome	Chromosomal Position	gDNA (bp)	CDS (bp)	Protein			
						Length (aa)	MW (Da)	pI	Subcellular Prediction
CsGLP6-7	Csa6G525580.1	6	28567264-28568054	791	660	215	22,597.97	5.47	Cell wall
CsGLP6-8	Csa6G525590.1	6	28570081-28570968	888	651	215	22,664.07	5.84	Cell wall
CsGLP6-9	Csa6G525600.1	6	28572339-28573248	910	624	216	23,153.73	7.85	Cell wall
CsGLP6-10	Csa6G525610.1	6	28574435-28575220	786	1986	218	22,902.41	6.26	Cell wall
CsGLP6-11	Csa6G525620.1	6	28576583-28577614	1032	675	220	23,148.55	6.95	Cell wall
CsGLP7-1	Csa7G281380.1	7	9939437-9941121	1685	1071	356	38,300.97	5.82	Vacuole
CsGLP7-2	Csa7G337100.1	7	12086664-12088637	1974	708	339	37,153.37	5.37	Vacuole
CsGLP7-3	Csa7G368140.1	7	12991068-12992188	1121	1431	235	25,649.72	9.43	Vacuole
CsGLP7-4	Csa7G380130.1	7	14118117-14121984	3868	1458	485	55,928.57	6.94	Vacuole
CsGLP7-5	Csa7G450510.1	7	18555157-18556979	1823	627	208	21,395.90	6.39	Cell wall
CsGLP7-6	Csa7G452090.1	7	18921704-18923747	2044	1020	476	54,181.04	7.69	Vacuole
CsGLPu	CsaUNG024810	Scaffold000221	4018-4951	934	690	229	25,058.56	7.00	Cell wall

3.3. Conserved Motif Analysis of Cucumber GLP Proteins

The conserved motifs of the 38 cucumber GLP proteins were analyzed using the MEME online software, resulting in the prediction of 10 conserved motifs (Figure 2). Among them, motif 1 was present in all CsGLPs except for CsGLP3-8, and motif 2 and motif 3 were also present in most CsGLP proteins. Most CsGLPs in the same group harbored common motifs. For example, motif 4 and motif 5 were only observed in CsGLPs from Groups a–e, while motif 7, motif 8 and motif 9 were exclusively present in the CsGLPs of Group f (Figure 2B). In addition, motif 6 was widely present in CsGLPs from Groups a–e, as well as in *CsGLP7-1* from Group f. It is worth noting that CsGLP3-1, CsGLP6-1, CsGLP6-4, CsGLP7-3 and CsGLP7-4 from Group f harbored two motif 1, while CsGLP3-7 and CsGLP6-10 contained an additional motif 9 and motif 10, respectively (Figure 2B).

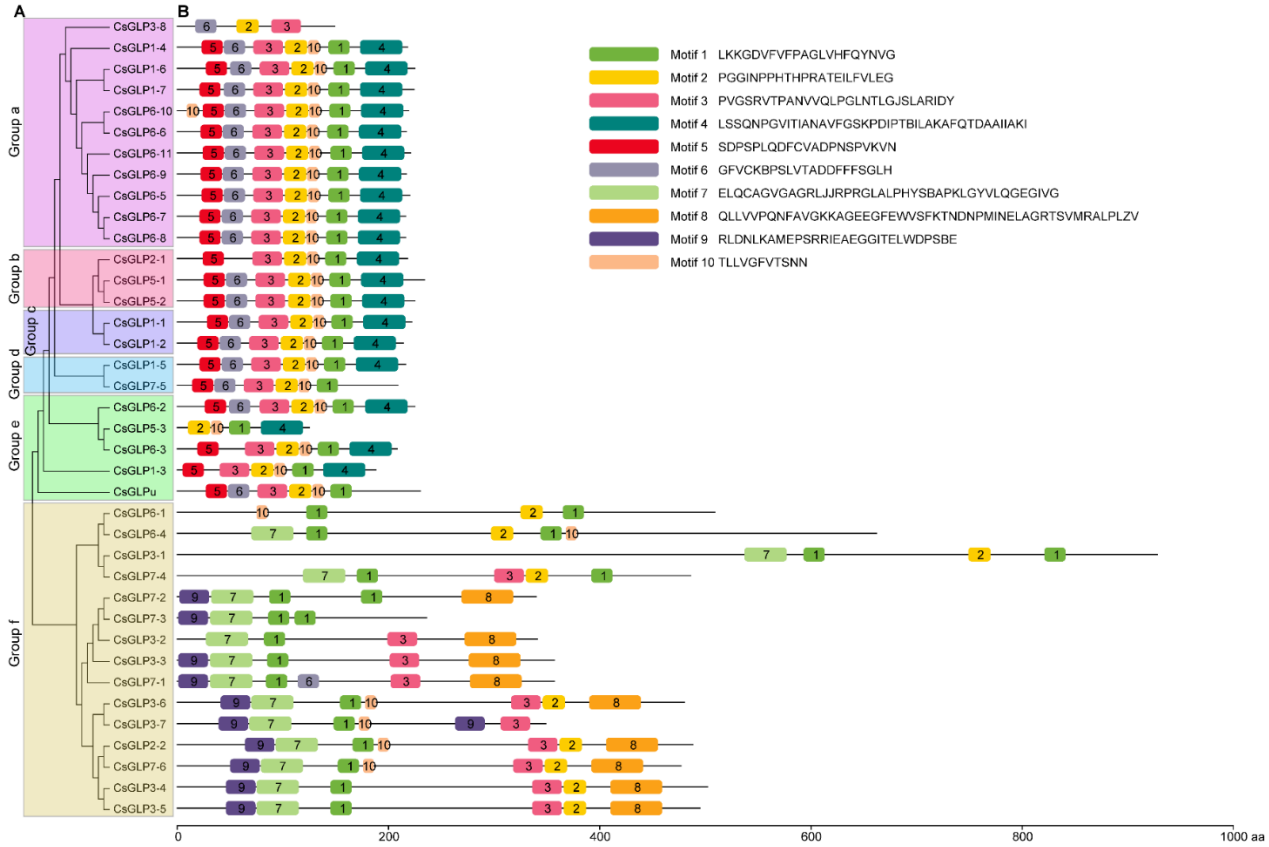

Figure 2. Phylogenetic relationship (**A**) and conserved motif arrangement (**B**) of cucumber GLP family members. (**A**) The phylogenetic tree of CsGLP proteins was created using the NJ method with 1000 bootstrap replicates, and the CsGLP proteins can be divided into six groups (Groups a–f). (**B**) Distribution of 10 conserved motifs among CsGLP proteins identified by MEME, and their amino acid sequences are shown at the right edge.

3.4. Structure Analysis of CsGLP Genes

Gene structure diversity is a possible mechanism for the evolution of multiple gene families [38]. We then analyzed the structural diversity of *CsGLP* genes with the GSDS tool. As shown in Figure 3, the intron number of the *CsGLP* genes ranged from 0 to 5, and nearly half of the *CsGLP* genes (18/38) were transcriptionally encoded by two exons and one intron. Specifically, seven *CsGLP* genes (*CsGLP1-1*, *CsGLP1-2*, *CsGLP1-3*, *CsGLP5-3*, *CsGLP6-2*, *CsGLP6-3* and *CsGLP7-5*), which were clustered in Groups c–e, were found to be intronless (Figure 3). In addition, nearly all *CsGLP* genes in Group a and Group b harbored only one intron, except for *CsGLP3-8*, which possessed three introns.

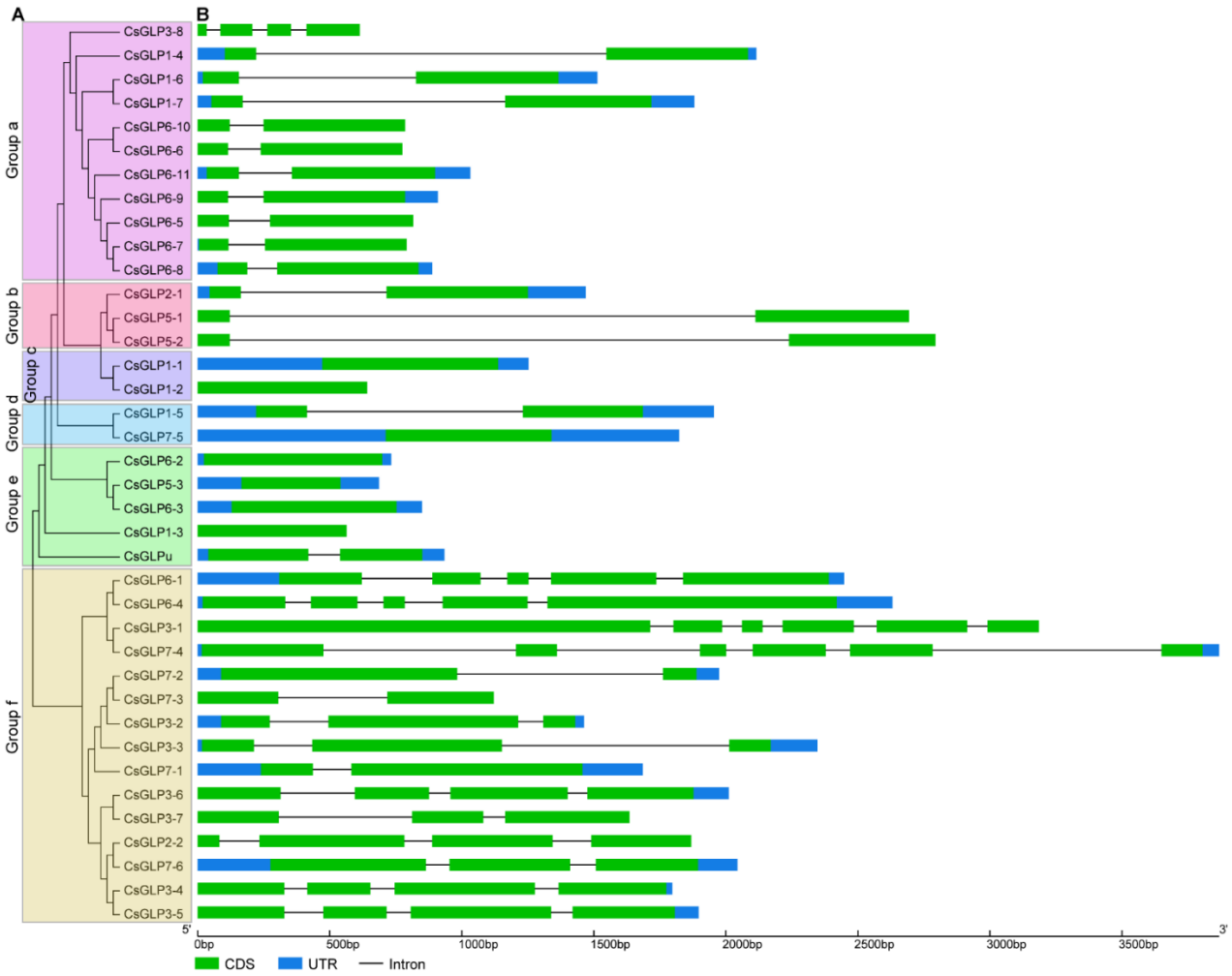

Figure 3. Phylogenetic relationship (**A**) and gene structure (**B**) of cucumber GLP family members. (**A**) The phylogenetic tree of CsGLP proteins was created using the NJ method with 1000 bootstrap replicates, and CsGLP proteins were divided into six groups (Groups a–f). (**B**) Gene structures of *CsGLP* genes analyzed by GSDS. Green box: CDS; Blue box: UTR; Black line: intron.

3.5. Chromosomal Location and Gene Duplication of CsGLP genes

To determine the chromosomal locations of *CsGLP* genes, a chromosomal map was constructed (Figure 4). A total of 37 *CsGLP* genes were distributed on six of seven cucumber chromosomes (except for chromosome 4), and one *CsGLP* gene was assigned to the scaffold000221. Amongst them, 11 *CsGLP* genes were found on chromosome 6, eight genes on chromosome 3, seven genes on chromosome 1, six genes on chromosome 7, three genes on chromosome 5 and only two genes on chromosome 2 (Figure 4). We also determined the duplication events of the *CsGLP* genes. The results showed that three pairs of *CsGLP* genes underwent segmental duplication, including *CsGLP1-4*/*CsGLP6-6*, *CsGLP1-5*/*CsGLP7-5* and *CsGLP2-2*/*CsGLP3-4*. In addition, five tandem duplication events involving 14 *CsGLP* genes were also observed (Figure 4).

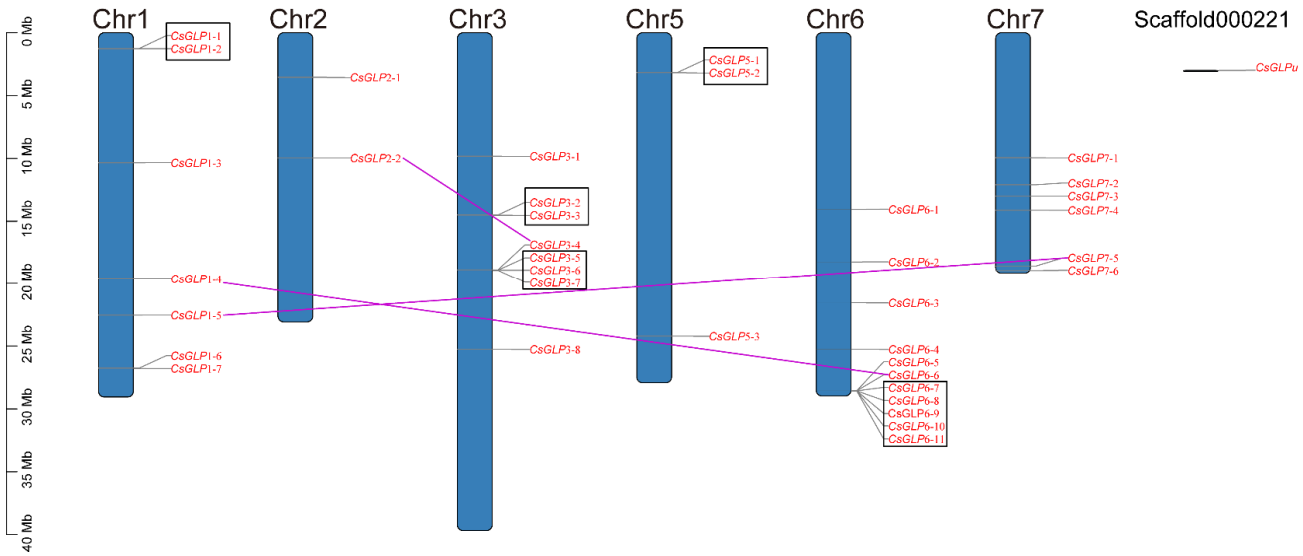

Figure 4. Chromosomal distributions and gene duplications of *GLP* genes in cucumber genome. The chromosome numbers are shown at the top of each chromosome.

3.6. Expression Profiles of CsGLP Genes in Various Tissues

To gain insights into the possible functions of the *CsGLP* genes, their temporal and spatial transcription patterns in various tissues were analyzed based on the published transcriptome data (Figure 5). A total of 24 *CsGLP* genes were found to be expressed in at least one of the tested tissues. Amongst them, some *CsGLP* genes were exclusively expressed in certain tissues, while other genes showed constitutive expression in different tissues. For example, *CsGLP3-5* and *CsGLP3-6* were specifically expressed in stems and tendrils, respectively (Figure 5). *CsGLP6-1* and *CsGLP6-4* showed preferential expression in the male flower, while *CsGLP1-5* exhibited the highest transcriptional level in leaves. Several *CsGLP* genes, such as *CsGLP1-6*, *CsGLP1-7*, *CsGLP5-2*, *CsGLP5-3*, *CsGLP6-3*, *CsGLP6-7*, *CsGLP6-8*, *CsGLP6-11* and *CsGLPu*, showed higher expression levels in roots than in other tissues (Figure 5), suggesting their specific roles in root development. In addition, *CsGLP3-2* and *CsGLP3-3* exhibited remarkable accumulation of transcripts in unfertilized and fertilized ovaries but not in the unexpanded ovary, and some other *CsGLP* genes including *CsGLP1-1*, *CsGLP1-4*, *CsGLP1-5*, *CsGLP1-6*, *CsGLP2-1*, *CsGLP5-3*, *CsGLP7-1*, *CsGLP7-5* and *CsGLP7-6* were also differentially transcribed in ovaries (Figure 5).

3.7. Expression Patterns of CsGLP Genes in Response to DM Treatment

To study the possible roles of *CsGLP* genes in response to biotic stress, the expression levels of *CsGLP* genes under downy mildew (DM) inoculation were determined based on the available RNA-seq data [35]. Compared with those in the mock control, a total of nine *CsGLP* genes (*CsGLP1-1*, *CsGLP1-6*, *CsGLP2-1*, *CsGLP5-3*, *CsGLP6-7*, *CsGLP6-8*, *CsGLP6-9*, *CsGLP6-11* and *CsGLP7-5*) displayed up-regulated expression under DM inoculation (Figure 6). In particular, *CsGLP2-1* and *CsGLP5-3* were significantly up-regulated at the earlier time point of infection (1 dpi) and also showed increases in transcription at the later time points. However, the other seven *CsGLP* genes were significantly up-regulated at the later time points, especially at 6 dpi and 8 dpi (Figure 6). In addition, the expression of five *CsGLP* genes (*CsGLP1-5*, *CsGLP5-2*, *CsGLP6-2*, *CsGLP7-1* and *CsGLP7-2*) was significantly decreased by DM treatment compared with the control (Figure 6). The results suggested that the *CsGLP* genes might play key roles in the response of cucumber to DM infection.

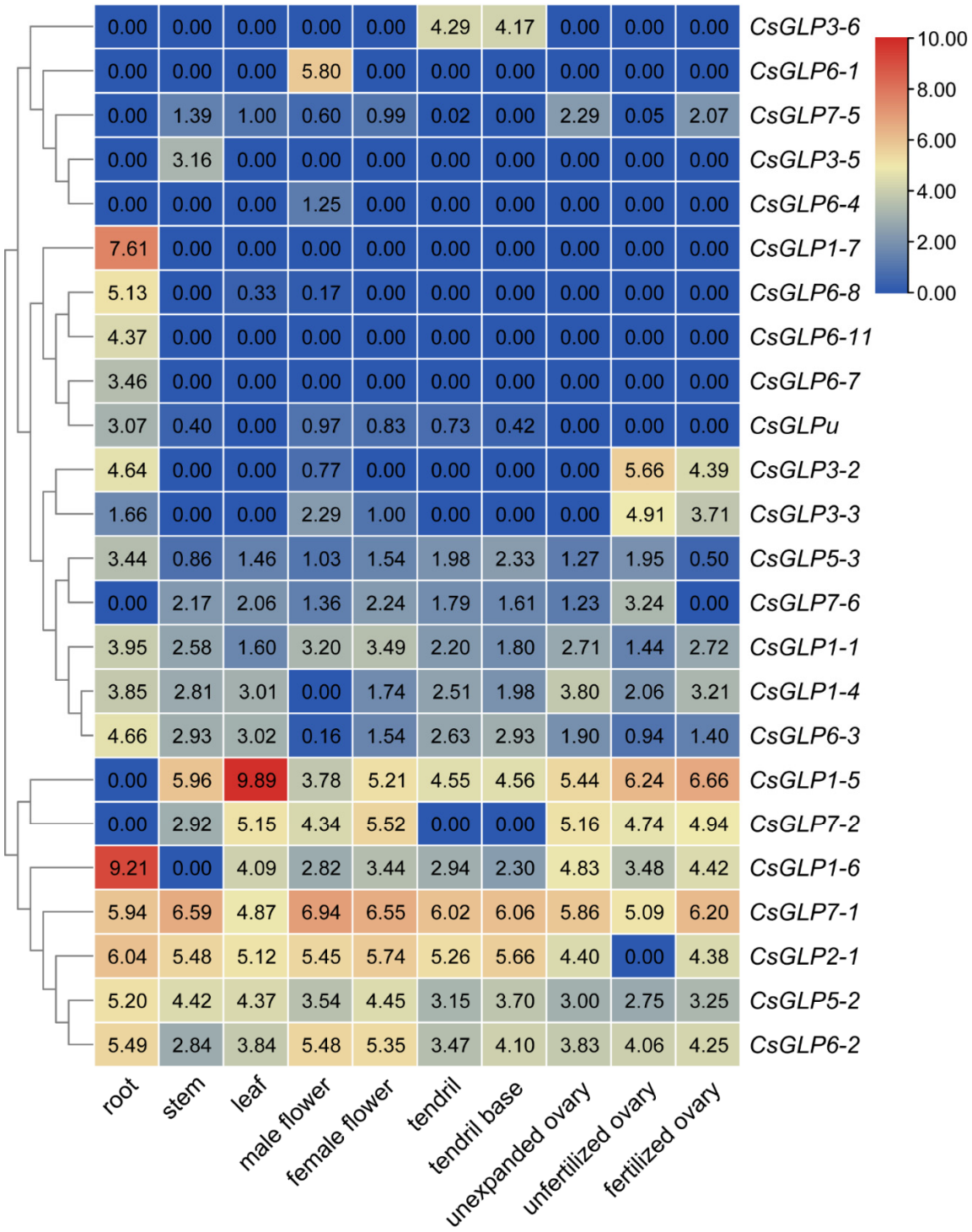

Figure 5. Expression levels of *CsGLP* genes in different cucumber tissues.

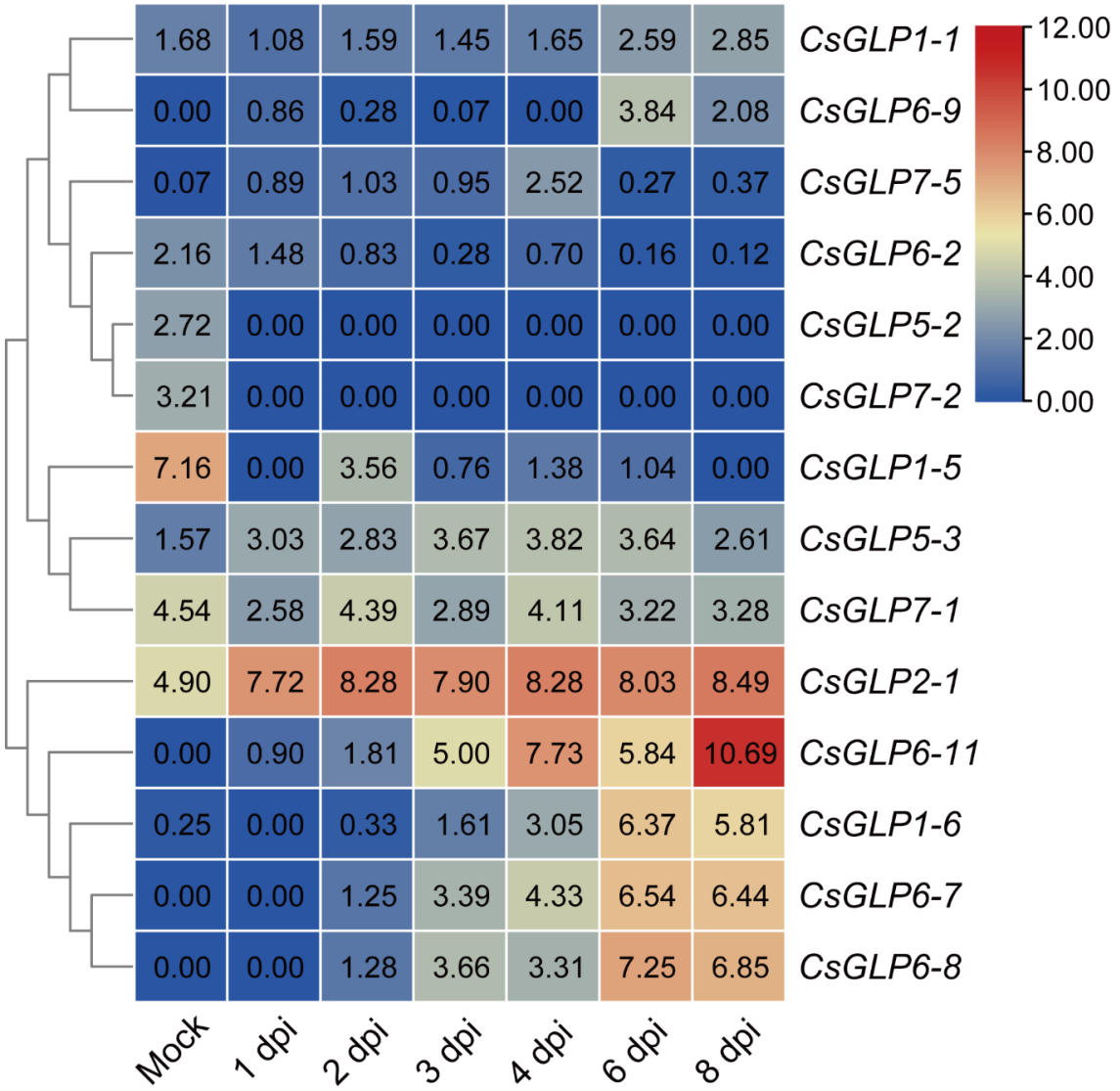

Figure 6. Expression patterns of *CsGLP* genes in response to inoculation with downy mildew for 1 to 8 days post inoculation (dpi). Mock, mock-inoculated sample collected at 1 dpi.

3.8. Expression Patterns of Selected CsGLP Genes in Response to Salt, Drought and ABA Treatments

The expression patterns of five selected *CsGLP* genes in response to salt, drought and ABA treatments were determined by qRT-PCR. Under salt treatment, the transcription levels of *CsGLP1-6*, *CsGLP5-2* and *CsGLP7-1* were obviously increased at 12 h, followed by a decrease at 24 h. And the transcription of *CsGLP1-5* was observably down-regulated, while the transcription of *CsGLP2-1* was dramatically up-regulated after salt treatment at all time points (Figure 7A). Under drought treatment, *CsGLP1-5* and *CsGLP1-6* exhibited a decrease in expression at 6 h, followed by increases at the subsequent time points. The transcription of *CsGLP1-5*, *CsGLP1-6*, *CsGLP2-1* and *CsGLP7-1* was induced and reached the peak at 12 h or 24 h, while that of *CsGLP5-2* was significantly down-regulated after drought treatment at all time points (Figure 7B). Under ABA treatment, the expression of *CsGLP1-5* and *CsGLP2-1* was significantly induced and reached the highest level at 12 h, while that of *CsGLP1-6*, *CsGLP5-2* and *CsGLP7-1* displayed significant decreases at all time points (Figure 7C). The results indicated that these *CsGLP* genes are differentially regulated in response to salt, drought and ABA treatments.

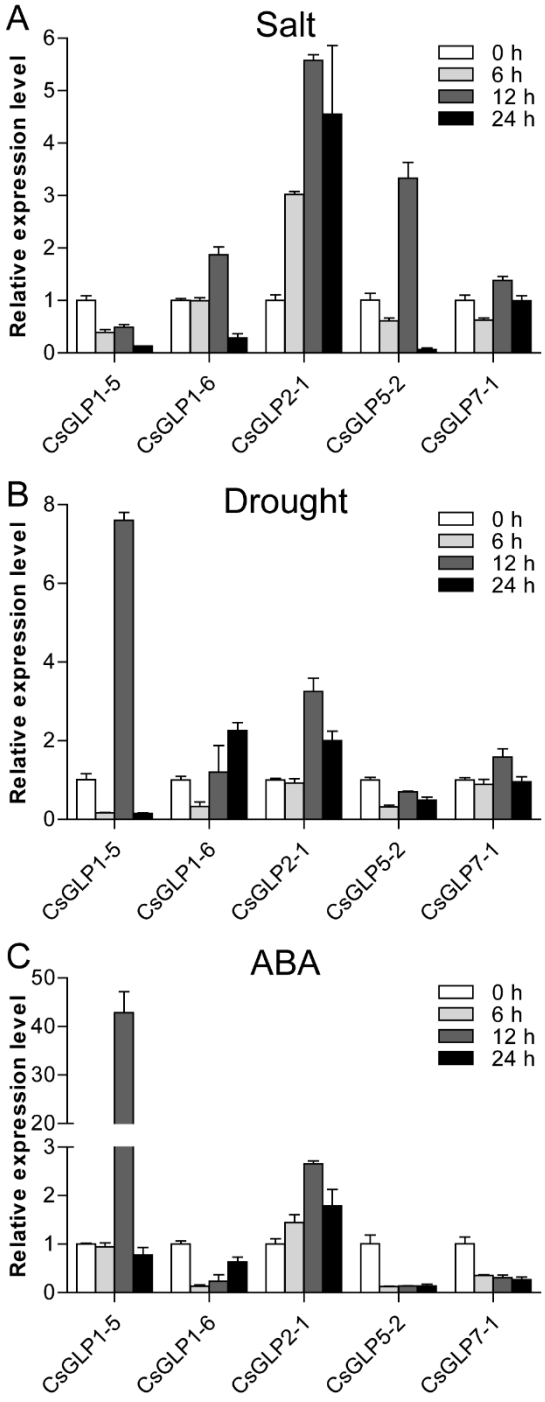

Figure 7. qRT-PCR analysis of expression patterns of five selected *CsGLP* genes in responses to salt (**A**), drought (**B**) and ABA (**C**) treatments.

4. Discussion

GLPs are a class of glycoproteins encoded in a multigene family and widely present in many plants. For example, it has been reported that there are 32 *GLP* genes in *Arabidopsis* [6], 43 in rice [6,39], 69 in soybean [30], 77 in *P. patens* [28] and 258 in wheat [31]. In the present study, a total of 38 *GLP* family members were identified and characterized in cucumber (Table 1). The number of cucumber *GLP* genes is comparable to that of rice and *Arabidopsis*, but much larger than that of the tea plant, which only has eight *GLP* genes [18]. The number of *GLP* genes shows no proportional variations along with the genome size of the above-mentioned plants, suggesting that gene duplication plays an

important role in the expansion of *GLP* genes for diversification. In this study, three pairs of *CsGLP* genes were involved in segmental duplication, and 14 *CsGLP* genes constituted tandem duplication events (Figure 4).

Consistent with the results in rice and *Arabidopsis* [6], the 38 CsGLPs were clustered into six groups (Groups a–f), among which the largest number of members from cucumber belonged to Group f (Figure 1). We further examined the conserved motif distribution of cucumber GLP proteins according to evolutionary relationships. In total, 10 conserved motifs were identified, whose distributions exhibited strong evolutionary conservation (Figure 2), indicating that GLP proteins in the same group might have similar functions. Gene structure can also provide important insights into the evolutionary relationships among gene families [38]. In our results, most *CsGLP* members were found to contain only one intron (Figure 3), which is in accordance with the results in previous studies [6,11]. Moreover, the genes in the same group tended to exhibit similar intron numbers and CDS lengths (Figure 3), indicating that their functions may be similar. In addition, about 18% *CsGLP* genes (7 out of 38) were found to contain no intron at all, which was also observed in soybean [30], rice and *Arabidopsis* [6].

Previous studies have shown that GLPs play various roles in many physiological processes, such as plant height [15], fiber development [40] and seed dormancy [16]. We then investigated the tissue expression patterns of *CsGLP* genes based on the transcriptome data from different tissues of cucumber [34]. As shown in Figure 5, many *CsGLP* genes showed preferential expression in specific tissues. For example, predominant expression levels of some *CsGLP* genes were found in roots (*CsGLP1-6*, *CsGLP1-7*, *CsGLP6-7*, *CsGLP6-8*, *CsGLP6-11* and *CsGLPu*), stems (*CsGLP3-5*), leaves (*CsGLP1-5*), male flowers (*CsGLP6-1*), and tendrils (*CsGLP3-6*), indicating that they play essential roles in these tissues. In addition, some *CsGLP* genes exhibited differential expression in different developmental stages of ovaries (Figure 5), suggesting their possible regulatory roles in the ovary development of cucumber.

In cucumber, DM caused by *P. cubensis* is a serious disease that results in severe damage to the production of cucumber around the world [41,42]. We then analyzed the expression of *CsGLP* genes to explore their possible roles in response to DM infection. As a result, nine and five *CsGLP* genes were up-regulated and down-regulated in response to the inoculation of DM (Figure 6), implying their possible roles in response to DM infection. It should be noted that *CsGLP6-7*, *CsGLP6-8*, *CsGLP6-9* and *CsGLP6-11* were significantly induced under DM inoculation, particularly at the later stage of infection (Figure 6), and they were found to be tandemly duplicated (Figure 4). Similar results were also reported in other plants. For example, a total of 12 rice *OsGLP* genes clustered in chromosome 8 confer resistance against two fungal pathogens: *Magnaporthe oryzae* and *Rhizoctonia solani* [43]. In wheat, most of the *Blumeria graminis* f. sp. *tritici* (Bgt) resistance-related *TaGLP* genes are also repeated in large tandem on the 4A, 4B and 4D chromosomes [31].

Plant GLPs also play key roles in the regulation of the abiotic stress response. Some GLPs were found to have SOD activity and could protect plants from antioxidant stress by converting harmful ROS into H_2O_2. For example, overexpression of sunflower *HaGLP1* in *Arabidopsis* resulted in higher resistance to fungal pathogens by promoting ROS accumulation [11]. *Craterostigma plantagineum* CpGLP1 is induced by dehydration and ABA, shows SOD activity and is involved in ROS metabolism and dehydration-related cell wall folding during desiccation [44]. Cotton *GhGLP2* can also confer tolerance to oxidative stress in transgenic *Arabidopsis* plants by eliminating excess ROS due to its SOD activity [10]. In the present study, we observed that the five selected *CsGLP* genes showed differential expression in response to salt, drought and ABA treatments (Figure 7). Notably, *CsGLP2-1* was significantly induced by the three treatments, and *CsGLP1-6* and *CsGLP7-1* were up-regulated under salt and drought stress but down-regulated under ABA treatment, suggesting their positive roles in regulating the salt and drought stress response in the ABA-dependent signal pathways. In addition, *CsGLP1-5* and *CsGLP5-2* displayed an oppo-

site expression pattern under salt and drought stress (Figure 7A,B), suggesting that they might play divergent regulatory roles in response to the two stresses.

5. Conclusions

In this study, we performed a comprehensive characterization of the *GLP* genes in cucumber through systematic analysis of their phylogenetic relationships, conserved motifs, gene structures and expression profiles in response to salt, drought and ABA treatments, as well as under DM inoculation. A total of 38 *GLP* family genes were identified in the cucumber genome, and both segmental and tandem duplications are main mechanisms for *GLP* gene expansion in cucumber. Based on their phylogenetic relationship to the corresponding members in *Arabidopsis* and rice, CsGLPs could be classified into six groups (Group a–f), with highly similar conserved motif distribution and exon-intron structure within the same groups. Expression analysis based on the transcriptome data demonstrated that some *CsGLP* genes are preferentially expressed in specific tissues and may participate in specific tissue and organ development. In addition, the expression of some *CsGLP* genes was significantly changed under DM inoculation, as well as in response to salt, drought and ABA treatments, suggesting that *CsGLP* genes may play key roles in the response of cucumber to DM infection and various abiotic stresses. Our findings may lay a solid foundation for studying the functions of *CsGLP* genes in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7100412/s1—Table S1: The gene-specific primers used for qRT-PCR, Table S2: The accession numbers of GLPs for phylogenetic analysis.

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