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GENOME-WIDE IDENTIFICATION AND EXPRESSION PATTERN ANALYSIS OF THE TRIHELIX GENE FAMILY IN CUCUMBER

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Abstract

Trihelix family genes (TFGs) serve as a crucial transcription factor (TF) family influencing plant growth and development. Despite its significance, knowledge regarding TFGs in cucumber is scarce. This study uncovered the identification of 28 TFGs in cucumber, distributed across seven chromosomes and categorized into five subfamilies: SIP1, GTγ, SH4, GT-1, and GT-2. Synteny analysis revealed colinearity between 24 cucumber TFGs and 28 *Arabidopsis* TFGs, along with 21 cucumber TFGs and 33 rice TFGs. Tissue-specific expression analysis indicated varying expression profiles of TFGs across diverse tissues, with only the *CsaV3_7G033160* gene remaining unexpressed in all tissues. Expression pattern analysis of cucumber TFGs under different types of abiotic stress (AbS) and biotic stress (BS) such as high-temperature, chilling, salt, waterlogging, downy mildew, powdery mildew, *Phytophthora capsici*, *Fusarium* wilt, root-knot nematode and angular leaf spot treatments showed that the differential expression of *CsaV3_3G033700* gene under 6 types of AbS and BS, while *CsaV3_3G036680* and *CsaV3_6G004030* genes had differential expression under 5 types of AbS and BS, indicating their pivotal roles in cucumber growth and development. This comprehensive study on the identification, evolution, and expression patterns of the TFG provides valuable insights into potential candidates for breeding stress-resistant cucumber varieties, laying important foundation for future investigations into the molecular biological functions of cucumber TFGs.

Key words: Trihelix gene family, *Cucumis sativus* L., Evolutionary analysis, Expression pattern analysis.

Introduction

Transcription factors (TFs) serve as crucial regulators, influencing plant growth and responses to environmental stresses by binding to specific *cis*-regulatory elements in the promoter regions. This binding activates or represses the transcriptional activity of target genes (Riechmann *et al*., 2000; Zhang *et al*., 2011). At present, over 60 TF gene families have been identified in plants (Jin *et al*., 2016). Trihelix DNA-binding factors, as a plant-specific transcription factor gene family, have a unique DNA-binding domain with a special binding site for GT factors (Nagano, 2000). Previous reports have demonstrated that the sequence of the trihelix structure in GT factors closely resembles that of the Myb DNA-binding domains (Qin *et al*., 2014). However, the distinctive feature lies in the gaps between helix pairs, which contribute to the formation of a specific binding site for GT elements within the GT factors domain.

The initial trihelix family gene (TFG) *GT-1* was discovered in *Pisum sativum* (Green *et al*., 1987). and its homologous genes were then cloned in tobacco (Perisic & Lam, 1992) and *Arabidopsis thaliana* (Hiratsuka *et al*., 1994). TFGs have been identified in several major plants, such as rice (Ji *et al*., 2015), soybean (Osorio *et al*., 2012), tomato (Yu *et al*., 2015), and chrysanthemum (Song *et al*., 2016), playing essential roles in various stress responses and developmental processes. In *Arabidopsis thaliana*, the *PETAL LOSS* (*PTL*) gene from the GT-2 subfamily regulates the development of sepals, petals, and floral organs (Griffith *et al*., 1999; Brewer *et al*., 2004; Lampugnani *et al*., 2012). Another gene, *GT-2 Like 1* (*AtGTL1*), can act as a temporal regulator inhibiting the growth of root hair by binding to the *ROOT HAIR DEFECTIVE SIX-LIKE4* (*RSL4*) activator (Shibata *et al*., 2018). Loss-of-function mutations in the *AtGTL1* gene contribute to the plant's tolerance to water deficit (Yoo *et al*., 2010). In rice, *SHA1* is responsible for modulating the seed-scattering process (Lin *et al*., 2007). The GTγ evolution branch gene, *OsGTγ-1*, has also exhibited high expression levels under salt stress in rice (Fang *et al*., 2010). In soybean, the up-regulated expression of *GmGT-2A* and *GmGT-2B* results in high tolerance to salt, drought, and cold (Xie *et al*., 2009). In *Brassica napus*, *BnSIP1-1*, belonging to SIP1 subfamilies, improves seed germination by overexpressing under abscisic acid treatment, salt stress and osmotic pressure (Luo *et al*., 2017). The distinctiveness of TFGs in plants suggests their role in plant-specific gene regulation.

Cucumber (*Cucumis sativus* L.), the first vegetable crop to have its genome published in 2009 (Huang *et al*., 2009), holds a prominent position in national economic development. Despite extensive investigations into various gene families (e.g., WRKY (Ling *et al*., 2011), MADS-box (Hu & Liu, 2012), NBS (Wan *et al*., 2013), and bZIP (Baloglu *et al*., 2014)) in cucumber, the functional and evolutionary aspects of the cucumber TFG remain unexplored. In this study, we systematically and comprehensively identified the TFGs in cucumber through whole-genome analysis. We provided detailed information encompassing physicochemical characteristics, gene structure, chromosomal localization, phylogenetic tree, and collinearity relationships. Additionally, we assessed the expression profiles of cucumber TFGs using extensive data from cucumber transcriptome sequencing (TS). This analysis included tissue-specific expression patterns (EPs) and expression profiling under 10 types of AbS and BS, according to the latest cucumber genome data. Our study lays a crucial foundation for further exploration into the molecular functions of cucumber TFGs. Moreover, these findings provide a theoretical framework for molecular breeding strategies aimed at enhancing cucumber resistance.

Material and Methods

Identification and chromosomal distribution of TFGs in cucumber: The Hidden Markov Model (HMM) model file (PF13837) of TFGs was downloaded from the Pfam database. Subsequently, potential cucumber TFGs were identified through scanning with HMMER 3.0. Pfam and SMART website (Letunic *et al*., 2021) was employed for identifying the TFG members, and the genes containing Trihelix domains were designated as TFGs. The physicochemical characteristics of cucumber TFGs, including the number of amino acids, CDS size, isoelectric point, molecular weight, aliphatic index, instability index, and grand average of hydropathicity were analyzed using the TBtools software (Chen *et al*., 2020a). Subcellular localization of cucumber TFGs were estimated through CELLO (http://cello.life.nctu.edu.tw/) (Yu *et al*., 2004). Furthermore, the chromosomal location of cucumber TFGs was visualized using TBtools.

Structural and phylogenetic analyses of TFGs in cucumber: To depict the structural features of cucumber TFG members, the GFF3 file (general feature format 3) was utilized. The online website MEME was employed for analyzing conserved motifs within cucumber trihelix proteins (Bailey *et al*., 2006). The analysis involved a maximum of 10 motifs, with an optimal motif width ranging from 6 to 100 amino acid residues. For an in-depth understanding of the regulatory elements associated with cucumber TFGs, the PlantCare website was utilized. This analysis focused on *cis*-acting elements and was based on the examination of 1.5 kb upstream sequences of the TFGs (Lescot *et al*., 2002). To assess the evolutionary relationship between TFGs from *Arabidopsis* and cucumber, a phylogenetic tree was constructed. This involved the utilization of the neighbor-joining approach with specific parameters, including 1000 bootstrap replications and pairwise deletion, within the MEGA 11 software.

Synteny analysis of TFGs in rice, *Arabidopsis* and cucumber: To unravel the syntenic relationships within the TFGs from rice, *Arabidopsis*, and cucumber, collinearity analysis was executed using MCScanX software (Wang *et al*., 2012). The results were then visually presented through the Circos tool (Krzywinski *et al*., 2009).

RNA-seq re-analysis with cucumber TS big data: The cucumber TS big data was retrieved from the SRA database, and subsequently transformed into fastq format using the fasterq-dump.2.11.0. Quality assessment of the fastq data was carried out using FastQC (Brown *et al*., 2017). To enhance data integrity, low-quality sequences were eliminated utilizing the Trimmomatics plug-in (Bolger *et al*., 2014), resulting in a set of filtered and clean data. The filtered transcriptome data were aligned to the cucumber ChineseLong_V3 version genome using STAR (Li *et al*., 2009). The obtained SAM files were further converted into BAM files. Gene expression analysis was conducted with the StringTie Quantify plug-in (Pertea *et al*., 2015), followed by the identification of differentially expressed genes (DEGs) using the DESeq2 plug-in (Varet *et al*., 2016).

Tissue-specific expression analysis of cucumber TFGs: The cucumber TS project PRJNA80169 (Li *et al*., 2011) was obtained from the SRA database to investigate the EPs of TFGs across diverse cucumber tissues. Employing the RNA-seq analysis workflow, the transcriptome data underwent re-analysis using the ChineseLong_V3 version genome information of cucumber. Subsequently, a heatmap illustrating the expression profiles of cucumber TFGs in diverse tissues was generated using the TBtools.

Expression profiling of cucumber TFGs under AbS and BS: To analyze the EPs of cucumber TFGs under different stress conditions, the cucumber TS projects associated with AbS (high-temperature (PRJNA634519) (Chen *et al*., 2020b), chilling (PRJNA438923) (Li *et al*., 2020), salt (PRJNA511946) (Zhu *et al*., 2019), waterlogging (Kęska *et al*., 2021)) and BS (downy mildew (PRJNA285071) (Burkhardt & Day, 2016), powdery mildew (PRJNA321023) (Xu *et al*., 2017), *Fusarium* wilt (PRJNA472169) (Dong *et al*., 2020), *Phytophthora capsici* (PRJNA345040) (Mansfeld *et al*., 2017), angular leaf spot (PRJNA704621) (Słomnicka *et al*., 2021), root-knot nematode (PRJNA419665) (Wang *et al*., 2018)) were retrieved from SRA database. Following the aforementioned methods, RNA-seq re-analyses were conducted, and the resulting EPs were visualized through heatmaps using the TBtools software.

Results

Genome-wide identification of TFGs in cucumber: Overall 28 cucumber TFGs were screened out, and their detailed information is presented in Table 1. The results indicated that the CDS sizes of TFGs ranged from 369 bp to 2730 bp, encoding a varying number of amino acids between 122 and 909. Molecular weights showed diversity, ranging from 13.89 to 100.49 kD, while aliphatic indexes varied from 51.31 to 91.89. The theoretical isoelectric points of the 28 cucumber trihelix proteins ranged from 4.78 to 9.96. With the exception of CsaV3_1G033320 protein, which exhibited stability (instability coefficient less than 40), the instability indexes of the other cucumber trihelix proteins were greater than 40, classifying them as unstable proteins. The mean hydrophilicity of all cucumber trihelix proteins was less than zero, indicating their hydrophilic nature. Subcellular localization prediction demonstrated that only the *CsaV3_1G033320* gene was located in the extracellular matrix, while the remaining 27 TFGs were found in the nucleus (Table 1).

Chromosomal localization of cucumber TFGs: The 28 cucumber TFGs were unevenly anchored across the 7 chromosomes of cucumber, with chromosome 3 hosting the higher number (six TFGs) and chromosome 2 having the lowest number (two TFGs). Gene duplication event analysis revealed three pairs of tandem repeat gene pairs: *CsaV3_1G033310* and *CsaV3_1G033320*, *CsaV3_3G036680* and *CsaV3_3G036690*, *CsaV3_6G004020* and *CsaV3_6G004030*. Additionally, two pairs of segmental duplication gene pairs were identified: *CsaV3_1G033310* on chromosome 1 and *CsaV3_7G005690* on chromosome 7, as well as *CsaV3_3G033700* on chromosome 3 and *CsaV3_4G026300* on chromosome 4 (Fig. 1).

Table 1. The physicochemical features of the 28 TFGs in cucumber.

Gene ID	CDS size (bp)	Number of amino acids (aa)	Molecular weight (kD)	pI	Instability index	Aliphatic index	Grand average of hydropathicity	Prediction of subcellular location
CsaV3_1G000660	2730	909	100.49	8.37	45.23	87.51	-0.369	Nucleus
CsaV3_1G015790	1929	642	72.87	5.92	58.03	69.78	-0.721	Nucleus
CsaV3_1G033310	570	189	21.52	9.96	44.42	63.97	-0.676	Nucleus
CsaV3_1G033320	369	122	13.89	9.34	34.39	91.89	-0.39	Extracellular matrix
CsaV3_1G045640	573	190	21.68	9.36	54.74	60.58	-1.108	Nucleus
CsaV3_2G018070	1500	499	57.25	5.55	48.98	61.04	-1.097	Nucleus
CsaV3_2G025280	1194	397	45.45	6.09	56.44	64.89	-0.905	Nucleus
CsaV3_3G010680	1101	366	40.21	9.33	68.39	53.93	-0.829	Nucleus
CsaV3_3G033700	924	307	36.27	6.99	52.49	55.99	-1.142	Nucleus
CsaV3_3G036680	1320	439	50.51	5.92	60.95	51.96	-1.123	Nucleus
CsaV3_3G036690	1617	538	59.38	6.45	49.16	69.57	-0.548	Nucleus
CsaV3_3G036920	1554	517	59.15	6.61	49.77	51.95	-1.144	Nucleus
CsaV3_3G047250	936	311	34.92	4.88	52.47	79.36	-0.691	Nucleus
CsaV3_4G006900	825	274	32.71	7.71	49.68	55.66	-1.171	Nucleus
CsaV3_4G024170	1332	443	50.13	6.81	46.69	69.30	-0.849	Nucleus
CsaV3_4G026300	780	259	31.64	9.01	48.17	54.67	-1.238	Nucleus
CsaV3_5G001320	2217	738	81.56	5.16	66.31	55.91	-0.882	Nucleus
CsaV3_5G012890	1179	392	44.34	8.78	72.25	64.23	-0.949	Nucleus
CsaV3_5G035640	1218	405	46.24	6.66	58.18	51.31	-0.929	Nucleus
CsaV3_5G036820	1038	345	38.56	9.92	60.33	66.43	-1.022	Nucleus
CsaV3_6G004020	1692	563	63.41	5.90	60.57	55.93	-0.984	Nucleus
CsaV3_6G004030	1962	653	73.32	5.90	70.47	56.75	-1.051	Nucleus
CsaV3_6G005270	1350	449	51.52	6.22	42.71	65.79	-1.04	Nucleus
CsaV3_6G007760	1068	355	38.74	5.48	45.43	74.23	-0.679	Nucleus
CsaV3_7G005690	792	263	30.63	9.44	47.31	70.80	-0.801	Nucleus
CsaV3_7G026050	936	311	36.33	5.15	74.88	57.75	-1.153	Nucleus
CsaV3_7G033160	1158	385	44.60	4.78	49.32	51.66	-1.256	Nucleus
CsaV3_7G034170	1122	373	40.96	9.67	46.51	60.67	-0.901	Nucleus

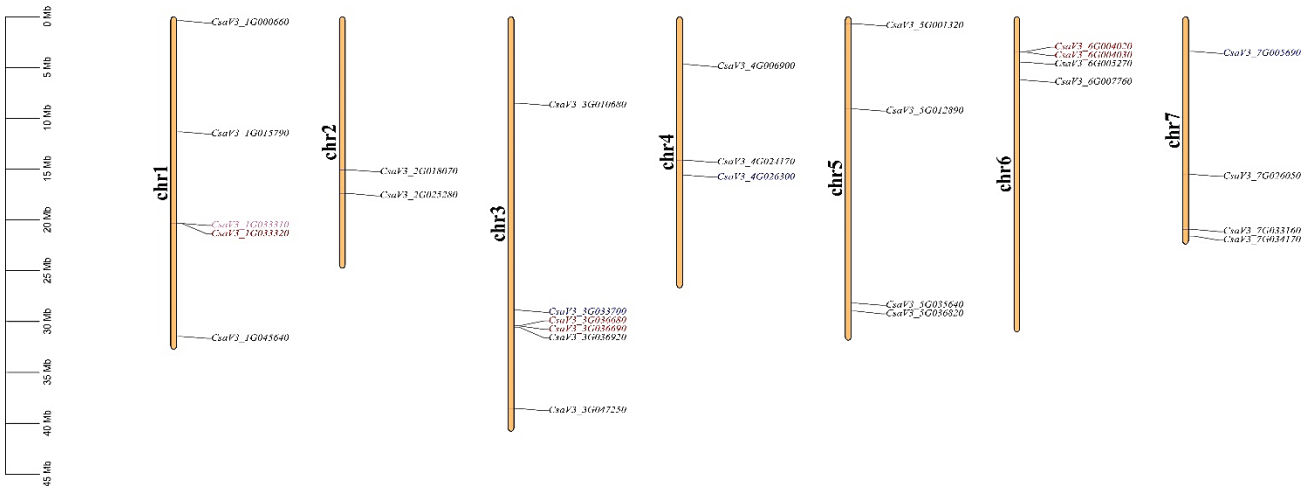

Fig. 1. The arrangement of TFGs across cucumber chromosomes. Note: Genes highlighted in red signify tandem duplication gene pairs, those in blue represent segmental duplication gene pairs, and those in pink are indicative of both tandem and segmental duplication.

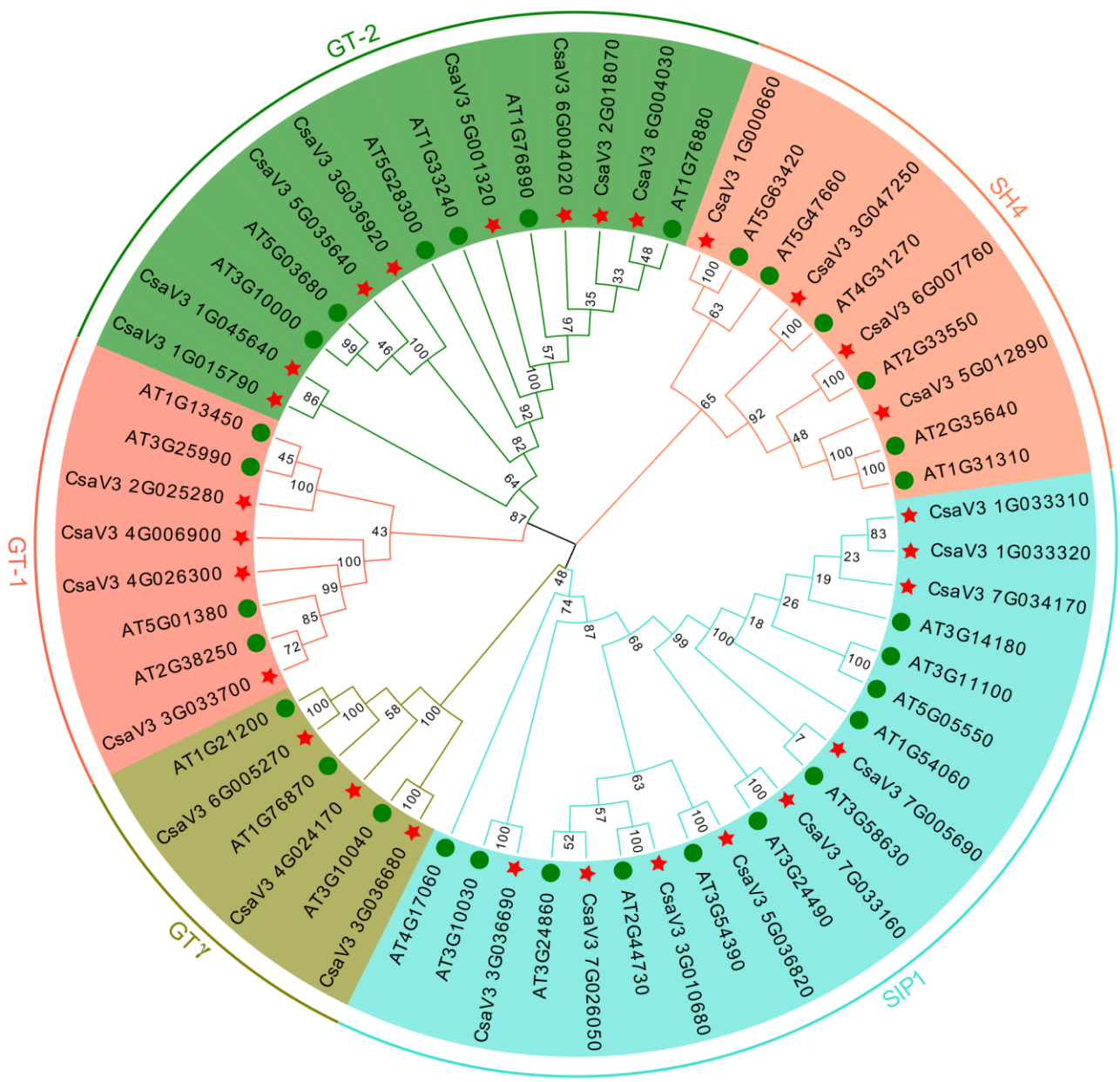

Fig. 2. Phylogenetic analysis of trihelix proteins from cucumber and *Arabidopsis*.

Phylogenetic analysis of TFGs in cucumber: To elucidate the categorization of trihelix proteins, phylogenetic trees were developed using trihelix proteins from both cucumber and *Arabidopsis*. Aligning with the classification outcomes of *Arabidopsis* TFGs, cucumber TFGs were grouped into five subfamilies: GT-1, GT-2, SH4, SIP1, and GTγ. Within these subfamilies, the SIP1 subfamily featured the highest count of TFGs, while the GTγ subfamily exhibited the smallest number of TFGs (Fig. 2). Furthermore, the phylogenetic analysis revealed 13 pairs of orthologous genes between cucumber TFGs and *Arabidopsis* TFGs, namely, *CsaV3_3G033700/ AT2G38250, CsaV3_6G004030/AT1G76880, CsaV3_ 1G000660/ AT5G63420, CsaV3_3G047250/ AT4G31270, CsaV3_ 6G007760/ AT2G33550, CsaV3_7G005690/ AT3G58630, CsaV3_ 7G033160/ AT3G24490, CsaV3_ 5G036820/ AT3G54390, CsaV3_3G010680/AT2G44730, CsaV3_7G026050/ AT3G24860, CsaV3_ 3G036690/ AT3G10030, CsaV3_3G036680/AT3G10040, CsaV3_ 6G005270/ AT1G21200*. Two pairs of paralogous genes existed among the cucumber TFGs, *CsaV3_1G015790/ CsaV3_1G045640* and *CsaV3_1G033310/ CsaV3_1G033320*, respectively.

The gene structure and conserved motifs of TFGs in cucumber: The structural analysis of all 28 cucumber TFGs revealed their classification into five subfamilies: GTγ, SIP1, SH4, GT-1, and GT-2 (Fig. 3). These classifications generally aligned with the clustering data observed in the comparison of TFGs between cucumber and *Arabidopsis* (Fig. 2). Overall, 10 conserved motifs (1–10) were identified in the 28 cucumber TFGs. The results demonstrated that trihelix proteins in different subfamilies exhibited distinct conserved sequences, while those within the same subfamily shared identical conserved sequences. For example, in the SIP1 subfamily, most genes contained motifs 4, 1, and 7, arranged in the same order. Conversely, in the GTγ subfamily, most genes contained motifs 10, 6, and 1, arranged in a consistent order. This observation suggests that the differential distribution of motifs among various subfamilies may contribute to the evolution of functional diversity. Meanwhile, the similar conserved motifs among TFGs within the same subfamilies may indicate similar functional roles.

Synteny analysis of TFGs among rice, *Arabidopsis* and cucumber: To explore the evolutionary relationships within the cucumber TFG, synteny analysis was conducted among TFGs from rice, *Arabidopsis*, and cucumber. The results indicated a total of 37 syntenic relationships involving 24 cucumber TFGs *(CsaV3_1G000660, CsaV3_3G036690, CsaV3_6G005270, CsaV3_2G025280, CsaV3_6G007760, CsaV3_3G036920, CsaV3_6G004020, CsaV3_5G035640, CsaV3_7G034170, CsaV3_5G001320, CsaV3_3G036680, CsaV3_3G010680, CsaV3_2G018070, CsaV3_3G033700, CsaV3_1G045640, CsaV3_4G026300, CsaV3_7G033160, CsaV3_5G036820, CsaV3_3G047250, CsaV3_1G033310, CsaV3_5G012890, CsaV3_7G026050, CsaV3_7G005690, CsaV3_1G015790)* and 28 *Arabidopsis* TFGs (*AT5G63420, AT3G10030, AT1G21200, AT3G25990, AT1G13450, AT2G33550, AT5G03680, AT1G76880, AT3G14180, AT1G33240, AT3G10000, AT3G10040, AT2G44730, AT2G38250, AT5G01380, AT1G54060, AT5G28300, AT3G24490, AT3G54390, AT4G31270, AT3G11100, AT5G05550, AT1G31310, AT3G58630, AT5G40340, AT2G35640, AT5G10140, AT5G65050*). There were 45 syntenic relationships between 21 cucumber TFGs (*CsaV3_1G000660, CsaV3_3G036690, CsaV3_6G005270, CsaV3_2G025280, CsaV3_4G024170, CsaV3_5G001320, CsaV3_2G018070, CsaV3_6G004020, CsaV3_5G012890, CsaV3_3G036680, CsaV3_4G006900, CsaV3_7G034170, CsaV3_1G033310, CsaV3_1G015790, CsaV3_7G005690, CsaV3_7G026050, CsaV3_5G036820, CsaV3_7G033160, CsaV3_6G007760, CsaV3_3G010680, CsaV3_3G033700*) and 33 rice TFGs (*Os02g33610, Os04g33300, Os11g06410, Os12g06640, Os04g40930, Os03g02240, Os02g43300, Os04g45750, Os04g57530, Os01g21590, Os04g51320, Os04g36790, Os02g35690, Os05g48690, Os02g06860, Os03g03100, Os01g48320, Os01g74440, Os06g30830, Os09g02830, Os02g47370, Os05g40250, Os08g37810, Os10g41460, Os09g38570, Os01g52090, Os04g32590, Os11g06030, Os11g09690, Os12g21880, Os07g05850, Os02g31160, Os05g06560*). Furthermore, two cucumber TFGs (*CsaV3_1G033320* and *CsaV3_6G004030*) were identified as conserved in cucumber but did not exhibit colinearity with any genes in *Arabidopsis* and rice (Fig. 4). As indicated by the earlier results (Fig. 1), the two pairs of TFGs in cucumber (*CsaV3_1G033310*/*CsaV3_7G005690* and *CsaV3_3G033700*/*CsaV3_4G026300*), which were segmental duplications, displayed syntenic relationships.

The *cis*-acting regulatory elements in the promoters of cucumber TFGs: In the promoter regions of 28 cucumber TFGs, 14 distinct *cis*-acting regulatory elements were identified. The majority, constituting 51%, were related to light-responsiveness, encompassing elements such as ACE, Box 4, G-box, I-box, and others. Additionally, various other *cis*-acting regulatory elements were detected, including those linked to hormone response (auxin, salicylic acid, abscisic acid, gibberellin, and MeJA), stress response (low temperature and drought), photoperiod regulation, endosperm expression, meristem expression, and others (Fig. 5). The presence of diverse *cis*-acting regulatory element members in the promoter regions suggests that cucumber TFGs play multiple roles during the growth and development of cucumber plants.

Tissue-specific expression analysis of TFGs in cucumber: To explore the tissue-specific EPs of TFGs in cucumber, the TS data from 10 diverse cucumber tissues were re-analyzed using the ChineseLong_V3 version genome. Among the 28 cucumber TFGs, the *CsaV3_7G033160* gene showed no expression across all 10 types of cucumber tissues. Additionally, four cucumber TFGs, namely, *CsaV3_1G015790, CsaV3_5G012890, CsaV3_3G033700* and *CsaV3_4G026300*, exhibited either no expression or low expression levels in any cucumber tissues. Two cucumber TFGs, *CsaV3_3G036680* and *CsaV3_3G036920*, were expressed at low levels or not expressed at all in the tendril, while they displayed expression in other tissues. The remaining cucumber TFGs were expressed in all 10 types of cucumber tissues and demonstrated tissue-specific EPs (Fig. 6).

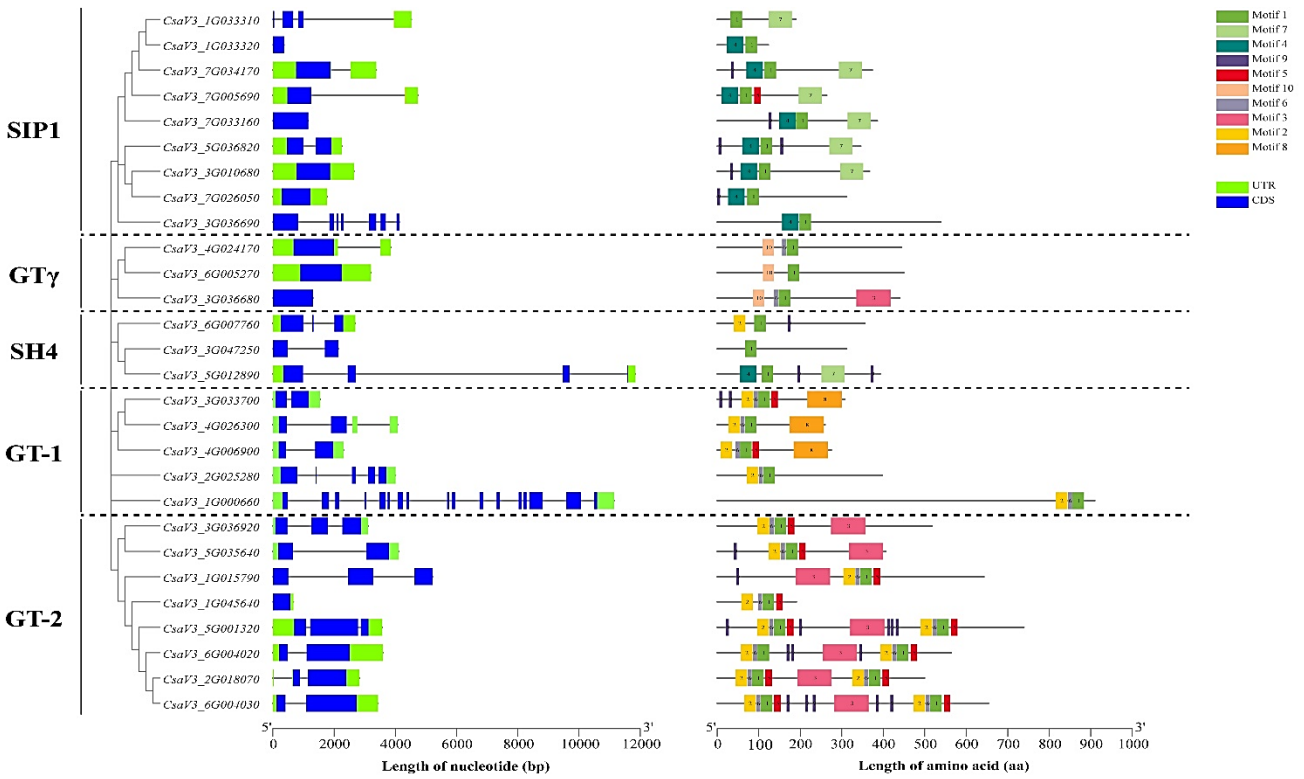

Fig. 3. The intron-exon structures of TFGs and a schematic representation of the amino acid motifs of trihelix proteins in cucumber.

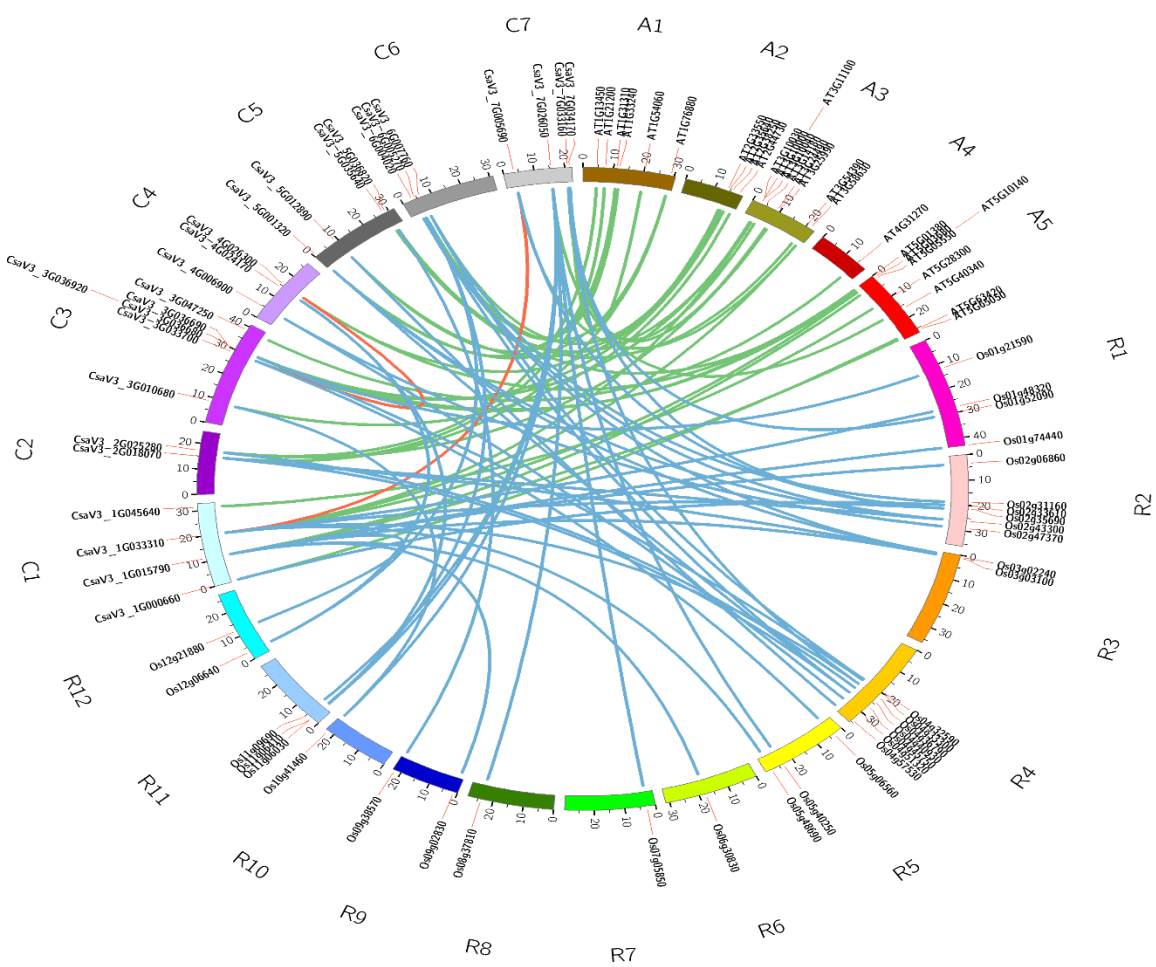

Fig. 4. The syntenic relationships of TFG in rice, *Arabidopsis* and cucumber.

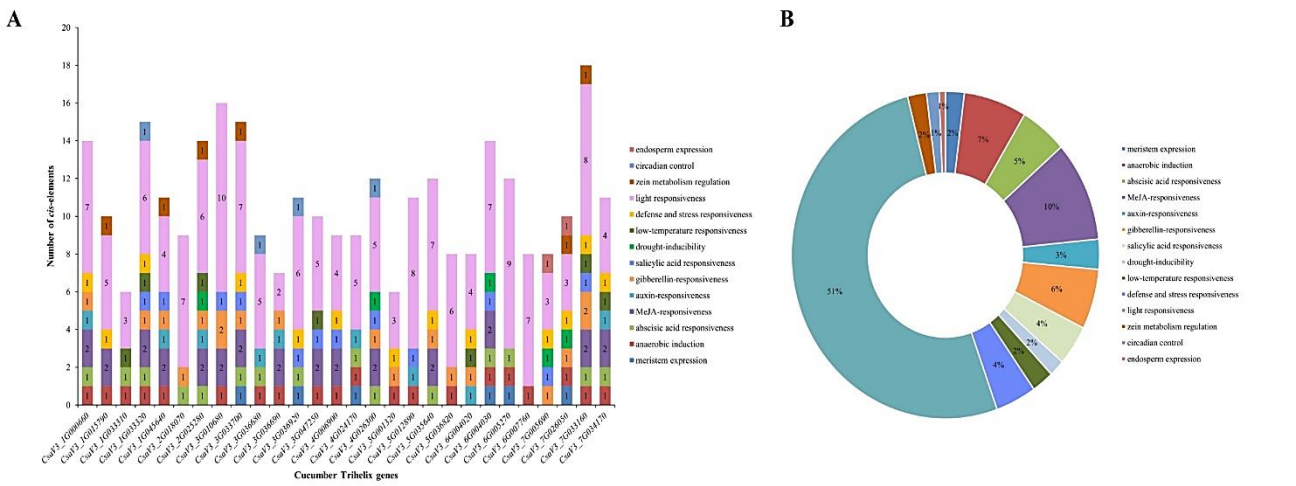

Fig. 5. The distribution of *cis*-acting regulatory elements in the promoter regions of cucumber TFGs. (A) The count of different *cis*-acting regulatory elements in the promoter regions of each cucumber TFG. (B) The proportions of various *cis*-acting regulatory elements in the promoter regions of cucumber TFGs, as shown in the pie chart. Note: the *cis*-acting regulatory elements with similar functions are indicated by a single color.

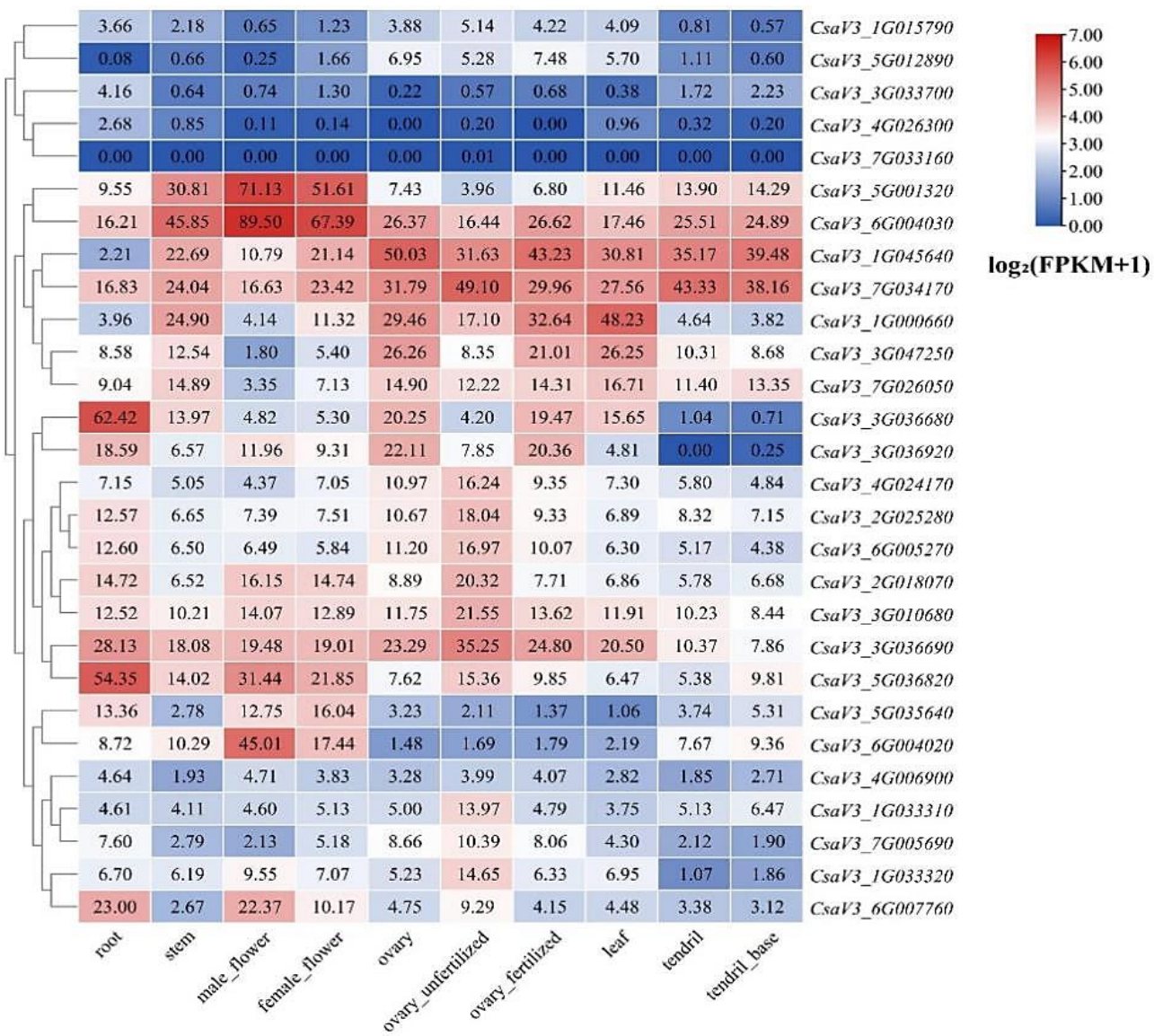

Fig. 6. The heatmap depicting the expression of the TFG in various cucumber tissues. Note: the data within the boxes represent the initial FPKM values.

Expression profiles of cucumber TFGs under AbS: Utilizing the available TS data of cucumber subjected to different AbS, including high-temperature, chilling, salt and waterlogging. RNA-seq re-analyses were performed with the ChineseLong_V3 version genome. The EPs of cucumber TFGs were subsequently analyzed (Fig. 7). Under high-temperature stress, *CsaV3_3G036680* gene exhibited significant up-regulation after 3 and 6 hours of heat treatment. *CsaV3_5G036820* gene showed significant up-regulation after 3 hours of heat treatment, while *CsaV3_3G047250* and *CsaV3_5G012890* genes were markedly down-regulated at 6 hours after heat treatment (Fig. 7A). During chilling stress, only *CsaV3_6G004030* gene displayed significant down-regulation (Fig. 7B). In response to salt stress, only *CsaV3_3G033700* gene exhibited significant up-regulation (Fig. 7C). Under waterlogging stress, *CsaV3_3G047250* and *CsaV3_5G012890* genes were remarkably up-regulated in the susceptible cucumber plant, while *CsaV3_3G036920* gene was markedly up-regulated in the resistant cucumber plant. In contrast, *CsaV3_5G036820* gene was obviously down-regulated in the resistant cucumber plant. Five cucumber TFGs, including *CsaV3_3G033700*, *CsaV3_3G036680*, *CsaV3_7G005690*, *CsaV3_3G036690* and *CsaV3_3G010680*, were markedly up-regulated in both resistant and susceptible cultivars. Three cucumber TFGs, including *CsaV3_6G004020*, *CsaV3_6G004030* and *CsaV3_5G001320*, were remarkably down-regulated in both resistant and susceptible cultivars (Fig. 7D).

Expression profiles of cucumber TFGs under BS: Utilizing the TS data of cucumber subjected to various BS, including powdery mildew, downy mildew, *Phytophthora capsici*, *Fusarium* wilt, root-knot nematode and angular leaf spot, RNA-seq re-analyses were performed with the ChineseLong_V3 version genome. Subsequently, the expression profiles of cucumber TFGs were evaluated (Fig. 8). In response to downy mildew stress, the *CsaV3_3G033700* gene was remarkably up-regulated in both susceptible and resistant cucumber cultivars, while the *CsaV3_7G026050* gene demonstrated significant upregulation only in the resistant cucumber cultivar. Several genes, including *CsaV3_6G005270*, *CsaV3_3G036690*, *CsaV3_1G033310*, *CsaV3_1G033320*, *CsaV3_6G004020*, *CsaV3_6G004030* and *CsaV3_1G000660*, were obviously down-regulated in both susceptible and resistant cucumber lines. Additionally, *CsaV3_2G018070*, *CsaV3_3G010680* and *CsaV3_6G007760* genes were markedly down-regulated in the resistant cucumber cultivar (Fig. 8A). Under powdery mildew stress, *CsaV3_3G033700* gene exhibited significant up-regulation in both susceptible and resistant cucumber materials. Both *CsaV3_6G004020* and *CsaV3_6G004030* genes were significantly down-regulated in the susceptible and resistant cultivars, while *CsaV3_5G001320* gene was remarkably down-regulated in the resistant cultivar (Fig. 8B). During *Fusarium* wilt stress, *CsaV3_4G026300* gene showed significant up-regulation from 24 hpi to 96 hpi, returning to normal expression levels at 192 hpi. *CsaV3_3G036680* gene was remarkably up-regulated at 96 hpi, whereas *CsaV3_3G036920* gene exhibited significant down-regulation at 96 hpi (Fig. 8C). Under *Phytophthora capsici* treatment, *CsaV3_6G007760* gene was markedly up-regulated in both susceptible and resistant cucumber lines. *CsaV3_3G036680* gene exhibited significant down-regulation in both resistant and susceptible cultivars. Both *CsaV3_6G004030* and *CsaV3_1G000660* genes were obviously down-regulated in the susceptible cultivar (Fig. 8D). Under angular leaf spot stress, *CsaV3_6G007760* and *CsaV3_3G033700* genes showed significant up-regulation in both susceptible and resistant cucumber lines. *CsaV3_1G045640* gene was obviously down-regulated in the resistant cultivar (Fig. 8E). Under root-knot nematode treatment, *CsaV3_3G033700* gene was remarkably down-regulated in both susceptible and resistant cultivars, while *CsaV3_3G036680* gene displayed significant up-regulation in both cultivars, with its expression levels gradually increasing along with the inoculation time. The *CsaV3_2G018070* gene was remarkably up-regulated in the susceptible cultivar, but not markedly changed in the resistant cultivar (Fig. 8F).

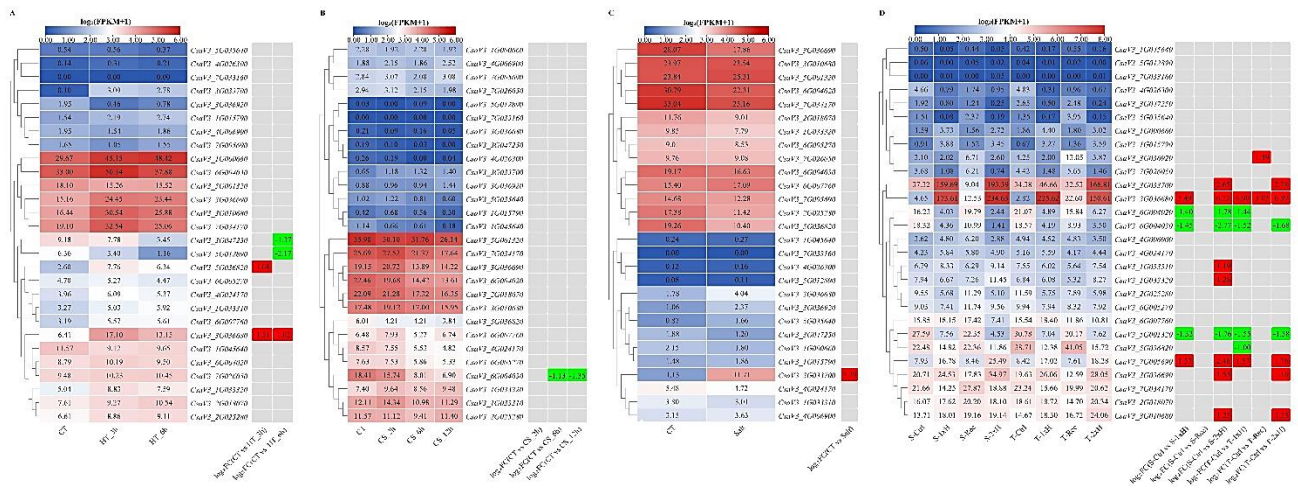

Fig. 7. The heatmap illustrating the expression of the cucumber TFG under various AbS. (A) EPs of cucumber TFGs under high-temperature stress; CT: control treatment; HT_3h: high-temperature treatment for 3 h. HT_6h: high-temperature treatment for 6 h. (B) Expression patterns of cucumber TFGs under chilling stress; CT: control treatment; CS_2h: chilling treatment for 2 h; CS_6h: chilling treatment for 6 h; CS_12h: chilling treatment for 12 h. (C) Expression patterns of cucumber TFGs under salt stress; CT: control treatment; Salt: salt treatment. (D) Expression patterns of cucumber TFGs under waterlogging stress; S: susceptible cultivar; R: resistant cultivar; Ctrl: untreated plants cultivated under optimal conditions; 1xH: non-primed plants waterlogged for 1 week only once; 2xH: primed plants waterlogged for 1 week and after 2 weeks of recovery, then waterlogged again; Rec: plants after 1 week of waterlogging and 2 weeks of recovery.

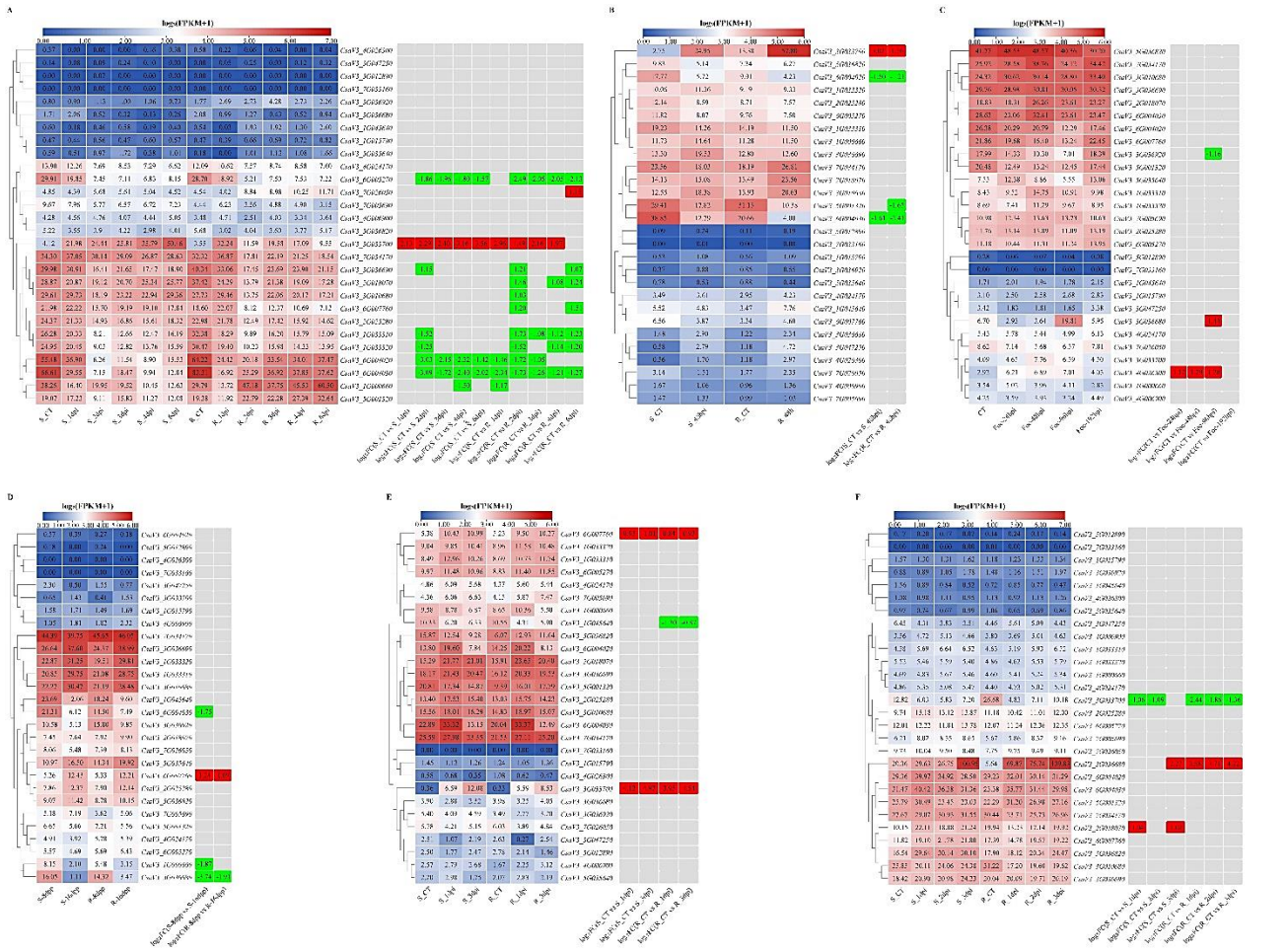

Fig. 8. The heatmaps depicting the EPs of cucumber TFG under different BS (A) EPs of TFGs under downy mildew stress. R: resistant cultivar; S: susceptible cultivar; 1, 2, 3, 4 and 6 dpi represent 1, 2, 3, 4 and 6 days after inoculation, respectively. (B) EPs of TFGs under powdery mildew stress. R: resistant cultivar; S: susceptible cultivar; CT: control; 48 hpi: 48 hours after inoculation. (C) EPs of TFGs under *Fusarium* wilt stress; CT, Foc-24hpi, Foc-48hpi, Foc-96hpi and Foc-192hpi were 0, 24, 48, 96 and 192 hours after inoculation with *Fusarium oxysporum* f. sp. *cucumerinum*, respectively. (D) EPs of TFGs under *Phytophthora capsici* stress; R: resistant cultivar; S: susceptible cultivar; 8dpp and 16dpp represent 8 and 16 days after pollination, respectively, indicating the fruit ages. (E) EPs of cucumber TFGs under angular leaf spot stress; R: resistant cultivar; S: susceptible cultivar; CT: before inoculation; 1dpi and 3dpi represent 1 and 3 days after inoculation with *Pseudomonas syringae* pv. *lachrymans*, respectively. (F) EPs of cucumber TFGs under root-knot nematode stress; R: resistant cultivar; S: susceptible cultivar; CT, 1, 2 and 3 dpi represent 0, 1, 2 and 3 days after inoculation with *Meloidogyne incognita*, respectively.

Regulation patterns of cucumber TFGs under AbS and BS: All the cucumber TFGs that exhibited differential expression under AbS and BS were selected and marked on the heatmap (Fig. 9). Out of the 28 cucumber TFGs, 21 TFGs showed differential expression in response to these diverse stresses. The highest number of differentially expressed cucumber TFGs was observed under waterlogging and downy mildew stresses, while the fewest were observed under chilling and salt stresses. Some cucumber TFGs exclusively responded to AbS, such as *CsaV3_3G047250*, *CsaV3_7G005690*, *CsaV3_5G012890* and *CsaV3_5G036820* genes. Conversely, specific cucumber TFGs were solely involved in response to BS, including *CsaV3_7G026050*, *CsaV3_ 4G026300*, *CsaV3_ 1G000660*, *CsaV3_ 1G045640*, *CsaV3_ 2G018070*, *CsaV3_6G005270* and *CsaV3_6G007760* genes. Additionally, 10 cucumber TFGs showed differential expression under both AbS and BS. Among them, the *CsaV3_3G033700* gene showed differential expression under six types of AbS and BS, suggesting its varying EPs in response to distinct stresses. The *CsaV3_3G036680* and *CsaV3_6G004030* genes were obviously regulated under five types of AbS and BS, respectively. These three genes actively participated in the response to AbS and BS, making them promising candidates for further investigation. The analysis of EPs of cucumber TFGs under AbS and BS serves as a valuable reference for future studies on the molecular biological functions of these genes.

Discussion

The transcriptional regulation of gene expression plays an essential role in the intricate processes of plant growth, development, and their adaptive responses to environmental changes. This regulatory complexity is often influenced by the vast genetic variations resulting

from genome duplication (Van de Peer *et al*., 2009). TFGs, serving as pivotal transcriptional regulators, have been demonstrated to participate in cellular development and stress responses (Breuer *et al*., 2009; Xi *et al*., 2012). Cucumber, a globally cultivated vegetable crop, had its genome sequenced back in 2009 (ChineseLong_V2 version). Although TFGs have been identified in many plants, such as wheat (Xiao *et al*., 2019), soybean (Liu *et al*., 2020) and sorghum (Li *et al*., 2021), the majority of these studies focused on field crops, leaving cucumbers relatively understudied. Therefore, our findings contribute more comprehensive insights into TFGs within the cucumber genome.

Herein, 28 TFGs were uncovered in cucumber, a number similar to that found in *Arabidopsis* but less than the counts observed in rice (41) (Li *et al*., 2019), soybean (63) (Osorio *et al*., 2012), and tomato (36) (Yu *et al*., 2015). This variation may be attributed to whole-genome duplication events occurring after the divergence of species from early land plants. Previous studies suggested classifying TFGs into 3 subfamilies: GTα, GTβ and GTγ (Fang *et al*., 2010). However, Kaplan-levy and co-workers (Kaplan-Levy *et al*., 2012), based on TFGs in *Arabidopsis* and rice, proposed a classification into 5 subfamilies: SIP1, GTγ, SH4, GT-1, and GT-2. Our phylogenetic analysis aligns with these findings, placing cucumber TFGs into five subfamilies. Members belonging to the identical subfamily exhibited analogous gene structures and motif compositions, indicating close evolutionary relationships. Further analysis revealed two tandem duplication and two segmental duplication gene pairs in cucumber, suggesting that the expansion of cucumber TFGs primarily resulted from tandem and segmental duplications, consistent with previous assertions (Kong *et al*., 2007). Comparative analysis of TFGs across rice, *Arabidopsis* and cucumber revealed two relatively conserved TFGs in cucumber, showing no collinearity with their counterparts in rice and *Arabidopsis*. On the other hand, the remaining 26 TFGs exhibited various collinearity patterns with *Arabidopsis* and rice TFGs, indicating species-specific gene expansion mechanisms. This phenomenon is a common occurrence in the exploration of various plant gene families (Zhang *et al*., 2005; Jain *et al*., 2006).

Previous research has implicated TFGs in the development of plant organs (Qin *et al*., 2014). Herein, we assessed the expression levels of cucumber genes in various tissues, including stem, leaf, male flower, female flower, expanded ovary (fertilized), expanded ovary (unfertilized), root, ovary, tendril, and base tendril. We conducted a re-analysis of RNA-seq data from cucumber tissues to determine gene expression. The results demonstrated that *CsaV3_7G033160* gene was not expressed in any of the tissues, while other TFGs exhibited variable expression profiles in different tissues. For instance, the *CsaV3_5G001320* and *CsaV3_6G004030* genes showed high expression levels in male and female flowers, suggesting their involvement in the development of plant reproductive organs. Similarly, the TFGs *CqTH27* and *CqTH42* were significantly up-regulated in the flowers of *C. quinoa* during flowering (Li *et al*., 2022).

It has been demonstrated that TFGs play crucial roles in various stress responses. For example, the trihelix TF members were either exclusively up-regulated in A17 (resistant material) or exclusively down-regulated in DZA (susceptible material) after infection with powdery mildew (*Erysiphe pisi*). Consistent with this finding, trihelix TF-binding motifs were strongly enriched only in the promoter of A17 (Gupta *et al*., 2020). In tomatoes, the TFG known as *ShCIGT* plays an essential role in improving drought and cold tolerance by interacting with SnRK1 (Yu *et al*., 2018). The trihelix TF AST1 in *Arabidopsis thaliana* conferred tolerance to osmotic and salt stresses through interaction with a novel AGAG-Box and various GT motifs (Xu *et al*., 2018). To further elucidate the molecular functions of cucumber TFGs in environmental adaptation, we conducted a comprehensive expression profiling analysis of these genes under 10 different types of AbS and BS. These stresses included high-temperature, chilling, salt, waterlogging, downy mildew, powdery mildew, *Fusarium* wilt, *Phytophthora capsici*, root-knot nematode and angular leaf spot treatments. The results revealed that, with the exception of 7 cucumber TFGs (*CsaV3_1G015790*, *CsaV3_2G025280*, *CsaV3_4G006900*, *CsaV3_4G024170*, *CsaV3_5G035640*, *CsaV3_7G033160* and *CsaV3_7G034170*), the remaining 21 cucumber TFGs were all differentially expressed in respond to these stresses. Notably, *CsaV3_3G033700* exhibited differential expression in most of the stresses, including two types of AbS (salt and waterlogging) and four types of BS (powdery mildew, downy mildew, root-knot nematode and angular leaf spot). Interestingly, the phylogenetic analysis of trihelix proteins in cucumber and *Arabidopsis* revealed that *CsaV3_3G033700/AT2G38250* formed one pair of orthologous genes. Previous studies have indicated that *AT2G38250* may participate in the induction of Calmodulin 4 (CAM4) in response to salt and pathogens (Li *et al*., 2017; Yu *et al*., 2019). Additionally, the cucumber TFGs *CsaV3_3G036680* and *CsaV3_6G004030* responded to two types of AbS and three types of BS. The orthologous gene of *CsaV3_3G036680*, *AT3G10040*, was up-regulated by oxygen deprivation (Giuntoli *et al*., 2014). In this study, we also found that *CsaV3_3G036680* gene was differentially up-regulated under waterlogging stress (oxygen deprivation). Furthermore, this gene was also markedly up-regulated under high-temperature, consistent with its up-regulation in anthers under heat stress in the previous study (Chen *et al*., 2021). The *CsaV3_6G004030* gene was differentially down-regulated in response to chilling, waterlogging, powdery mildew, downy mildew, and *Phytophthora capsica*. A prior study reported that *AT1G76880*, the orthologous gene of *CsaV3_6G004030*, was down-regulated at 48 h post-inoculation with *Agrobacterium tumefaciens* (Ditt *et al*., 2006).

The preceding discussions strongly indicate that similar molecular functions are observed in orthologous genes, affirming the reliability of our study's results. Expression profiling of cucumber TFGs under AbS and BS highlighted that *CsaV3_3G033700*, *CsaV3_3G036680* and *CsaV3_6G004030* genes as candidate genes for further investigation into their molecular functions. Additionally, these genes emerged as favorable candidates for molecular breeding efforts aimed at enhancing cucumber resistance.

Fig. 9. The heatmap depicting the regulation patterns of cucumber TFGs under AbS and BS. Note: the gray color indicates no alteration in expression level, red signifies an up-regulated EP, and green represents a down-regulated EP.

Conclusion

In summary, we identified 28 TFGs in cucumber. Through the integration of physicochemical features, chromosomal localization, gene structure, phylogenetic analysis, synteny, and EP analyses, we gained a comprehensive understanding of the evolution and EPs of cucumber TFGs. Notably, *CsaV3_3G033700* gene exhibited differential expression under six types of AbS and BS, while *CsaV3_3G036680* and *CsaV3_6G004030* genes were markedly regulated under five types of AbS and BS, respectively. This suggests that these three TFGs play pivotal roles in stress responses. Overall, these findings offer a scientific basis for further exploration into the molecular functions of cucumber TFGs and identify promising candidates for the development of stress-resistant cucumber varieties.

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