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Article

Pan-Genome Analysis of TRM Gene Family and Their Expression Pattern under Abiotic and Biotic Stresses in Cucumber

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Abstract: Cucumber (*Cucumis sativus* L.) is a vital economic vegetable crop, and TRM (TONNEAU1 Recruiting Motif) gene plays a key role in cucumber organ growth. However, the pan-genomic characteristics of the TRM gene family and their expression patterns under different stresses have not been reported in cucumber. In this study, we identified 29 CsTRMs from the pan-genomes of 13 cucumber accessions, with *CsTRM29* existing only in PI183967. Most CsTRM proteins exhibited differences in sequence length, except five CsTRMs having consistent protein sequence lengths among the 13 accessions. All CsTRM proteins showed amino acid variations. Analysis of *CsTRM* gene expression patterns revealed that six *CsTRM* genes strongly changed in short-fruited lines compared with long-fruited lines. And four *CsTRM* genes strongly responded to salt and heat stress, while *CsTRM14* showed responses to salt stress, powdery mildew, gray mold and downy mildew. Some *CsTRM* genes were induced or suppressed at different treatment timepoint, suggesting that cucumber TRM genes may play different roles in responses to different stresses, with expression patterns varying with stress changes. Remarkably, the expression of *CsTRM21* showed considerable change between long and short fruit and in responses to abiotic stresses (salt stress, heat stress) as well as biotic stresses (powdery mildew, gray mold), suggesting a dual role of *CsTRM21* in both fruit shape determination and stress resistance. Collectively, this study provided a base for further functional identification of *CsTRM* genes in cucumber plant growth and stress resistance.

Keywords: pan-genome; cucumber; TRM; fruit shape; abiotic stress response; biotic stress response

1. Introduction

TRM (TONNEAU1 Recruiting Motif) family genes play important roles in the growth and development of plants, exerting significant functions in various plant species. In Arabidopsis, 34 TRM proteins were identified, and half of them are putative microtubule-associated proteins [1]. *AtTRM1* and *AtTRM2* regulate leaf morphology by positively promoting longitudinal polar cell elongation [2]. The *Attrm5* mutant causes slow leaf growth, delayed flowering, and shortened root length [3]. *AtTRM61*, has a conserved functional structure and possesses conserved binding motifs for cofactor S-adenosyl-L-methionine (AdoMet), affects embryo arrest and seed abortion [4]. Additionally, TRMs can interact with TON1(TONNEAU1) and PP2A (Protein Phosphatase 2A) through their M2 and M3 domains, respectively, forming the TTP (TON1-TRM-PP2A) protein complex. This complex is targeted to microtubules (MT) [5], regulating microtubule organization and preprophase band (PPB) formation, thus influencing cell division and/or growth. This regulation ultimately affects the size and shape of plant organs [5–10]. In tomatoes, TRMs can interact with OFPs through their M8 domain. The OFP-TRM protein complex undergoes relocalization between the cytoplasm and microtubules, maintaining dynamic balance to regulate cell division and organ growth, ultimately affecting fruit shape [11,12]. *SlTRM5* positively regulates fruit elongation by influencing cell division [13]. In the LA1589 background, although *SlTRM3/4* minimally influenced fruit shape, the absence of

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SlTRM5 led to a slight flattening of the fruit [14]. The fruit shape of the double mutant lacking both *SlTRM3/4* and *SlTRM5* closely resembles that of the single mutant lacking only *SlTRM5* [14]. Introducing the non-functional versions of either *SlTRM3/4* or *SlTRM5* into *ovate*/*sov1* near-isogenic lines (NILs) partially restored the pear shape of the fruit. Moreover, when both non-functional alleles of *SlTRM3/4* and *SlTRM5* were combined in *ovate*/*sov1* NILs, the fruit shape index (FSI) was similar to that of wild-type (WT) fruits [14,15], indicating the additive effects of *SlTRM3/4* and *SlTRM5* in regulating fruit elongation. Fruit shape analyses of the null mutants of *SlTRM17/20a*, *SlTRM19*, or *SlTRM26a* in the LA1589 background, generated using CRISPR/Cas9, revealed an interesting finding. It suggested that *SlTRM17/20a* and *SlTRM19* work together synergistically to regulate fruit elongation, while SlTRM26a has a minor effect on fruit shape. The null alleles of *SlTRM5* and *SlTRM19*, whether in the LA1589 or *ovate*/*sov1* backgrounds, were observed to counterbalance each other in the regulation of fruit elongation. This suggests that *SlTRM5* and *SlTRM19* have opposing effects on fruit elongation [14]. In rice, the TRM homologous genes OsGW7/GL7/SLG7 interact with TON1 and PP2A through their M2 and M3 domains, respectively, and target them to the cortical microtubules. By influencing cell length and width, they regulate grain size and quality [16–18]. In cucumber, *CsTRM5* affects fruit shape by influencing the direction of cell division and cell expansion. Additionally, ABA participates in regulating cucumber fruit elongation through *CsTRM5*-mediated cell expansion [19].

TRM gene family members are often localized to microtubules [2,5,13]. Microtubules are crucial components of the plant cell skeleton, and they play vital roles in maintaining cell shape, adapting to growth, development, and environmental changes, as well as in processes such as cell division, intracellular transport, immune responses, and stress tolerance [20–29]. MICROTUBULE-DESTABILIZING PROTEIN 25 (MDP25) is a hydrophilic cation-binding protein of the plant-specific developmentally regulated plasma membrane polypeptide(DREPP) family [30]. It is postulated that *AtMDP25* similarly modulates stomatal closure, root hydrotropic response, and immune responses by influencing microtubule dynamics [31–33]. *OsDREPP2* exhibits an affinity for microtubules and, in vitro, it inhibits microtubule polymerization [34], and *MtDREPP* induces the fragmentation of microtubules within membrane nanodomains during rhizobial infections [35]. Ethylene signaling regulates microtubule reassembly by up-regulating microtubule-stabilizing protein WAVE-DAMPENED2-LIKE5 (WDL5) expression in response to salt stress [36]. Katanin1 (KTN1) acts as a microtubule-severing protein, aiding in the maintenance of the organized microtubule structure. Under hypersalinity, the microtubule-associated protein KTN1 regulates hypersalinity-induced microtubule disassembly/assembly, thereby enhancing salinity tolerance [37]. Microtubules under high temperature stress undergo depolymerization [38]. High temperature stress (35°C-37°C) primarily disrupts the formation of excessive microtubule-organizing centers, which bind to the minus end of microtubules, consequently regulating their elongation and the shortening of microtubule arrays [39]. The changes in microtubule dynamics impact vesicular transport, protein trafficking, and cell wall deposition [40–43]. Currently, there are no reports on the involvement of cucumber TRM family genes in biotic or abiotic stress.

Pangenomics seeks to capture the full spectrum of genetic variation within a species through the assembly and comparative analysis of genome sequences from multiple individuals and displayed powerful potential in discovering novel genes or gene novel function [44]. In cucumber, a graph-based pan-genome was constructed based on 12 accessions [45], which provided a resource for characterizing variations of TRM proteins. In this study, we identified a total of 29 CsTRM genes in the pan-genome of cucumber and found that most of them vary in protein length between the 13 accessions, and all CsTRM proteins showed amino acid variations. In addition, we analyzed the expression patterns of the *CsTRM* genes using transcriptomic data in fruit and under different stresses that may play roles in different stresses. Therefore, our study provides a reference for investigating the potential role of TRMs for fruit shape and stress resistance in cucumber.

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2. Materials and Method

2.1. Identification of TRM Genes in Cucumber

To identify TRM genes in cucumber, download the cucumber pan-genome assembly and annotation files from https://www.ncbi.nlm.nih.gov/, and the 'PI183967' genome assembly from http://www.cucurbitgenomics.org/. Use TBtools to extract CDS sequences and translate them into protein sequences. Retrieve *AtTRM* family members from https://www.arabidopsis.org/, and employ these sequences as queries in TBtools to predict TRM family members in cucumber. Perform a conserved motif analysis using the online MEME tool (https://www.omicsclass.com/article/432). Visualize the results with TBtools and screen for the final *CsTRM* family members based on the conserved M2 motif.

2.2. Protein Length, Motif Composition and Gene Structure Analysis

The protein sequences of CsTRMs in different cucumber accessions were extracted and the proteins' lengths were counted using TBtools. The variation of amino acids was analyzed using DNAMAN program. The conserved motifs were identified using TBtools. The location information of CDSs and UTRs was extracted from the genomic annotation database and graphed using TBtools [46].

2.3. Gene Duplication and Synteny Analysis

The genomic databases of cucumber, Arabidopsis, rice, tomato, and maize were downloaded from http://cucurbitgenomics.org/organism/20 and http://plants.ensembl.org/index.html, and then, the gene duplication events and the syntenic relationships were obtained using the Multiple Collinearity Scan toolkit (MCScanX) [47] with the default parameters. The results were constructed using TBtools [46].

2.4. Transcriptome Analysis of CsTRM Genes in Friut

The publicly available transcriptomic data of cucumber fruit carpel numbers (SPR182933) [48], long fruit 408 and short fruit 409(SPR045470) [49], WT and *CsFUL1*^{A}-OX-29(SPR117025) [50] were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/geo/browse) to analyze the expression patterns of *CsTRMs* in fruit. The genome-wide expression of the *CsTRMs* gene was shown on a heatmap using TBtools [46]. For the transcriptome analysis of the *CsTRMs*, a threshold of FDR (or p-value) ≤ 0.05 and an absolute value of log2 (fold-change) ≥ 1 or log2 (fold-change) ≤ -1 were used to define DEGs.

2.5. Transcriptome Analysis of CsTRMs in Response to Abiotic and Biotic Stresses

The publicly available transcriptomic data of cucumber treated with salt (GSE116265) [51], heat (GSE151055) [52], PM (GSE81234) [53], GM (SRP062592) [54] and DM (SRP009350) [55] were downloaded from https://www.ncbi.nlm.nih.gov/ to analyze the expression patterns of *CsTRMs* under different stresses. After aligning the gene IDs to the cucumber genome, the genome-wide expression of the *CsTRMs* gene was shown on a heatmap using TBtools [46]. For the transcriptome analysis of the *CsTRMs*, a threshold of FDR (or p-value) ≤ 0.05 and an absolute value of log2 (fold-change) ≥ 1 or log2 (fold-change) ≤ -1 were used to define DEGs.

3. Result

3.1. Identification of CsTRM Genes Based on the Cucumber Pan-Genome

To investigate the variation of the TRM genes across cucumber accessions, we identified CsTRM genes from the pan-genome including 13 cucumber accessions [45]. A total of 29 putative TRM genes were identified among the genomes of the 13 cucumber accessions (Table 1, Table S1). We renamed them *CsTRM01-CsTRM29* based on their order on the chromosomes to avoid confusion in this study (Table 1). Additionally, *CsTRM04* exhibits multiple copies in W4. There were 28 *CsTRM* genes identified from '9930', being consistent with the previous study [56], and from PI183967, lacking

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CsTRM03 and possessing a unique *CsTRM29* (Table 1); 27 from 'Cu2', 'Cuc64', 'W4', 'Hx14', 'Hx17' 'Cuc37', 'Gy14' and '9110gt'; 26 from 'XTMC'; 25 from 'Cuc80' and 'W8' (Table 1). *CsTRM01*, *02*, *06*, *07*, *09*, *11*, *12*, *13*, *14*, *15*, *17*, *18*, *19*, *21*, *23*, *24*, *25*, *26*, *27* and *28*, all are present in the 13 cucumber accessions. *CsTRM3* is absent in Cuc80 and PI; *CsTRM04* is absent in Cuc80 and W8; *CsTRM05* is absent in *XTMC* and W8; *CsTRM08* is absent in *XTMC*; *CsTRM10* is absent in Cu2 and Cuc80; *CsTRM16* is absent in Cuc64, W4, W8, Hx14, Hx17 and Cuc37; *CsTRM20* is absent in Gy14; *CsTRM22* is absent in 9110gt. *CsTRM29* only existing in PI183967 is identified as a new member of the *CsTRM* gene family in the 13 cucumber accessions

Table 1. Identification of TRM genes in the 13 cucumber accessions.

Gene name	9930	XTMC	Cu2	Cuc80	PI	Cuc64	W4	W8	Hx14	Hx117	Cuc37	Gy14	9110gt
CsTRM01	*1G003000*	*1G002960*	*1G003190*	*1G003020*	*1G02950*	*1G003020*	*1G003020*	*1G003050*	*1G008150*	*1G003070*	*1G003050*	*1G002970*	1G003150
CsTRM02	*1G006080*	*1G006040*	*1G006230*	*1G006090*	*1G06220*	*1G006110*	*1G006160*	*1G006130*	*1G012280*	*1G009330*	*1G006130*	*1G005930*	1G006390
CsTRM03	*1G024390*	*1G023100*	*1G019380*			*1G034690*	*1G022250*	*1G022640*	*1G033380*	*1G035450*	*1G025870*	*1G016710*	1G021460
CsTRM04	*1G036240*	*1G038300*	*1G031670*		*1G23930*	*1G045200*	*1G039460* *1G050980*		*1G048780*	*1G052690*	*1G036240*	*1G023410*	1G034790
CsTRM05	*2G002210*		*2G001130*	*2G002170*	*2G01120*	*2G001150*	*2G001150*		*2G002120*	*2G002150*	*2G001120*	*2G001120*	2G002200
CsTRM06	*2G006910*	*2G004780*	*2G004780*	*2G005680*	*2G04550*	*2G004690*	*2G004760*	*2G005690*	*2G006680*	*2G005730*	*2G004660*	*2G004680*	2G005790
CsTRM07	*2G013800*	*2G013420*	*2G014430*	*2G016150*	*2G11310*	*2G012220*	*2G015170*	*2G022290*	*2G022190*	*2G018160*	*2G012230*	*2G011350*	2G015370
CsTRM08	*3G000320*		*3G000290*	*3G000270*	*3G00310*	*3G000310*	*3G000300*	*3G000290*	*3G000310*	*3G000300*	*3G000310*	*3G000260*	3G000300
CsTRM09	*3G008900*	*3G014120*	*3G011330*	*3G009320*	*3G08770*	*3G009130*	*3G011320*	*3G009390*	*3G013030*	*3G018440*	*3G009280*	*3G008870*	3G011230
CsTRM10	*3G009320*	*3G014570*			*3G09200*	*3G009570*	*3G011790*	*3G009840*	*3G013470*	*3G018890*	*3G009740*	*3G009280*	3G011660
CsTRM11	*3G016640*	*3G023990*	*3G019120*	*3G016980*	*3G16440*	*3G027380*	*3G019160*	*3G017460*	*3G023810*	*3G029290*	*3G017050*	*3G016550*	3G018960
CsTRM12	*3G020250*	*3G028160*	*3G024300*	*3G021230*	*3G20290*	*3G031530*	*3G023320*	*3G021650*	*3G030910*	*3G038450*	*3G021080*	*3G020040*	3G025120
CsTRM13	*3G028590*	*3G044970*	*3G034490*	*3G039640*	*3G27110*	*3G050790*	*3G034230*	*3G032590*	*3G049730*	*3G053340*	*3G043310*	*3G025270*	3G034790
CsTRM14	*3G033690*	*3G052230*	*3G039760*	*3G045880*	*3G31210*	*3G055180*	*3G039400*	*3G038880*	*3G057040*	*3G059810*	*3G049600*	*3G029050*	3G041170
CsTRM15	*3G035160*	*3G053700*	*3G041160*	*3G047320*	*3G32570*	*3G056620*	*3G040870*	*3G040290*	*3G058490*	*3G061290*	*3G050990*	*3G030380*	3G042680
CsTRM16	*3G036950*	*3G056500*	*3G043950*	*3G049050*	*3G34290*							*3G032070*	3G044470
CsTRM17	*3G045060*	*3G067760*	*3G055880*	*3G057270*	*3G42630*	*3G066680*	*3G051990*	*3G050610*	*3G070610*	*3G071560*	*3G061110*	*3G040150*	3G052920
CsTRM18	*4G024630*	*4G030170*	*4G024030*	*4G078840*	*4G14290*	*4G027840*	*4G018900*	*4G021930*	*4G026900*	*4G030090*	*4G084710*	*4G013800*	4G026010
CsTRM19	*4G031780*	*4G042910*	*4G034540*	*4G090510*	*4G21450*	*4G044030*	*4G027440*	*4G033570*	*4G040430*	*4G039790*	*4G095540*	*4G020000*	4G035410
CsTRM20	*5G002760*	*5G003630*	*5G003640*	*5G002610*	*5G05360*	*5G003650*	*5G002590*	*5G005490*	*5G006540*	*5G003570*	*5G002660*		5G003770
CsTRM21	*5G003260*	*5G004130*	*5G004140*	*5G003110*	*5G05880*	*5G003020*	*5G003100*	*5G004990*	*5G007040*	*5G004090*	*5G003170*	*5G003160*	5G004310

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CsTRM22	*5G005590*	*5G007580*	*5G007530*	*5G005560*	*5G08200*	*5G000650*	*5G006510*	*5G001620*	*5G009400*	*5G009680*	*5G005630*	*5G005580*	
CsTRM23	*5G026190*	*5G042130*	*5G041190*	*5G050680*	*5G17200*	*5G021470*	*5G028290*	*5G024190*	*5G042910*	*5G054980*	*5G043620*	*5G016900*	5G034200
CsTRM24	*5G038540*	*5G060730*	*5G054610*	*5G063070*	*5G29400*	*5G043920*	*5G040890*	*5G049340*	*5G063180*	*5G067580*	*5G056820*	*5G028860*	5G046740
CsTRM25	*6G016870*	*6G024320*	*6G018060*	*6G025320*	*6G14470*	*6G015450*	*6G015250*	*6G019390*	*6G025200*	*6G018200*	*6G015300*	*6G014340*	6G017280
CsTRM26	*6G022550*	*6G035270*	*6G023850*	*6G053560*	*6G17180*	*6G022100*	*6G019950*	*6G023190*	*6G032860*	*6G030040*	*6G019540*	*6G016650*	6G023980
CsTRM27	*6G040450*	*6G052040*	*6G035430*	*6G079450*	*6G25260*	*6G035100*	*6G032700*	*6G033070*	*6G052400*	*6G045570*	*6G036810*	*6G024690*	6G035970
CsTRM28	*7G025430*	*7G031600*	*7G024250*	*7G035470*	*7G13640*	*7G025890*	*7G021920*	*7G034950*	*7G031600*	*7G031470*	*7G037050*	*7G012470*	7G023340
CsTRM29					*UnG00530*								

3.2. Analysis of Protein Length and Amino Acid Variations in the CsTRM Proteins

To further understand protein length variation of CsTRMs among the cucumber accessions, we showed the length of the identified CsTRM proteins in Table 2. There were 5 CsTRMs with the same protein length among 13 cucumber accessions, namely, CsTRM04, 11, 14, 15 and 21. The length of CsTRM01, 02, 05, 06, 13, 18, 22, 24 and 26 differed in only one of the accessions. And others showed differences in protein length among multiple accessions. Length difference data are marked in red in Table 2.

Table 2. The predicted lengths of TRM proteins (amino acid residues) in the 13 cucumber accessions.

Protein number	**9930**	**XTMC**	**Cu2**	**Cuc80**	**PI**	**Cuc64**	**W4**	**W8**	**Hx14**	**Hx117**	**Cuc37**	**Gy14**	**9110gt**
CsTRM01	1048	1048	1048	1048	1048	1048	1043	1048	1048	1048	1048	1063	1048
CsTRM02	1040	1067	1067	1067	1067	1067	1067	1067	1067	1067	1067	1067	1067
CsTRM03	780	788	788			788	781	781	781	781	781	788	781
CsTRM04	402	402	402		402	402	402/402		402	402	402	402	402
CsTRM05	776	803	803	803	803	803	803	803	803	803	803	803	803
CsTRM06	722		722	722	722	722	722		722	750	722	722	722
CsTRM07	893	478	893	891	891	893	893	899	893	891	891	922	891
CsTRM08	893		879	879	893	879	879	879	893	879	879	904	879
CsTRM09	930	933	933	933	932	933	933	933	933	933	933	932	933
CsTRM10	346	346			344	344	344	344	346	344	346	305	346
CsTRM11	616	616	616	616	616	616	616	616	616	616	616	616	616
CsTRM12	953	953	954	954	953	953	953	953	953	952	953	954	953
CsTRM13	963	963	963	963	963	963	963	963	963	963	963	927	963
CsTRM14	353	353	353	353	353	353	353	353	353	353	353	353	353
CsTRM15	888	888	888	888	888	888	888	888	888	888	888	888	888
CsTRM16	472	472	550	550	472							472	550
CsTRM17	1091	1038	1091	1091	1091	1091	210	353	440	600	1091	1058	357
CsTRM18	961	961	961	961	961	922	961	961	961	961	961	961	961
CsTRM19	903	903	987	987	906	987	906	906	906	906	906	906	906
CsTRM20	785	785	785	785	781	781	781	781	785	785	745		785
CsTRM21	476	476	476	476	476	476	476	476	476	476	476	476	476
CsTRM22	495	495	495	495	495	495	495	495	495	495	495	449	
CsTRM23	794	794	794	848	795	795	794	795	794	794	795	794	736
CsTRM24	1049	1049	1049	1049	1049	1049	1049	1049	1049	1049	1049	1049	958
CsTRM25	1011	1011	1011	1011	902	959	1009	1011	1022	1009	1011	940	1011

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CsTRM26	936	936	936	936	936	936	938	936	936	936	936	936	936
CsTRM27	505	505	505	505	505	505	505	736	505	473	505	505	505
CsTRM28	960	960	960	959	959	994	995	1047	895	995	995	976	978
CsTRM29					788								

Among the proteins with different lengths, CsTRM01 in 'W4'; CsTRM02 in'9930'; CsTRM03 in '9930'; CsTRM05 in '9930'; CsTRM07 in 'XTMC'; CsTRM09 in '9930'; CsTRM13 in 'Gy14'; CsTRM18 in 'Cuc64'; CsTRM19 in '9930' and 'XTMC' ; CsTRM20 in 'Cuc37' ; CsTRM22 in 'Gy14'; CsTRM23 and CsTRM24 in '9110gt'; CsTRM25 in 'PI183967'; CsTRM27 in 'Hx117' and CsTRM28 in 'Hx14' had shorter lengths compared to those in other accessions, while CsTRM16 in 'Cu2', 'Cuc80' and 'Gy14'; CsTRM19 in 'Cu2', 'Cuc80' and 'Cuc64' and CsTRM26 in 'W4' were longer than those in other accessions. In addition, the lengths of some proteins showed multiple polymorphism. For example, the protein length of CsTRM17 was the same in '9930', 'Cu2', 'Cuc80', 'PI', 'Cuc64' and 'Cuc37', but totally different in other accessions, furthermore, dramatically shortened in 'W4', 'W8', 'Hx14', 'Hx117' and '9110gt' (Table 2).

Besides protein length, amino acid substitution also can change a protein's function [57]. The amino acid variations of CsTRMs in different cucumber accessions was analyzed (Table S1). Amino acid variations were annotated using CsTRMs protein sequence of 9930 as reference, and all CsTRM proteins exhibit amino acid variations. CsTRM04, 11, 14, 15 and 21 have 6, 5, 3, 7 and 2 amino acid variations, respectively, but these do not lead to changes in protein length (Table 2). Some CsTRMs exhibit amino acid insertions leading to an increase in protein length. For example, CsTRM02 has 27 amino acid insertions in accessions other than 9930. In CsTRM06, 17, 26, 27 and 28, there are frame shift leading to amino acid variations. Some amino acid variations are quite significant, such as CsTRM07, 17, and 24.

Some amino acid variations are quite significant, such as CsTRM07, 17, and 24 (Table 2). Further comparisons of CsTRM07, 17, and 24 gene structures and gene conservative motifs (Figure 1). CsTRM07 in XTMC has only 478 amino acids, which is significantly shorter than that in the other 12 accessions (Table 2), and its gene structure underwent changes along with alterations in some conserved motifs, experiencing an increase in gene length, but not leading to the loss of conserved motifs in Gy14 and PI183967 (Figure 1A). For CsTRM17, the protein length varied from 210 amino acids to 1091 amino acids across the 13 accessions (Table 2), with corresponding changes in gene structure and some conserved motifs, especially in W4, there are only two conserved motifs (Figure 1B). In CsTRM24 of 9110gt, alterations in gene structure caused the decreased protein length, but without a reduction in conserved motifs.

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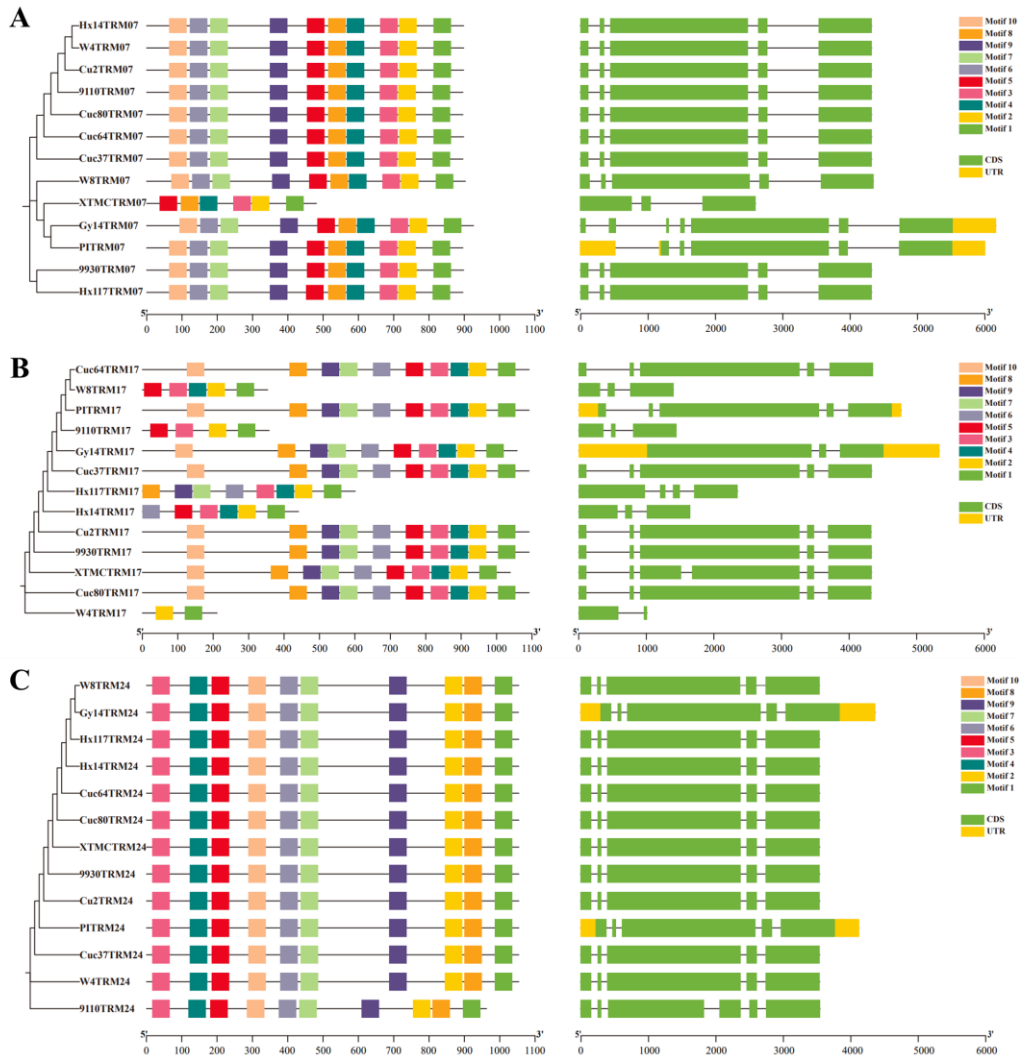

Figure 1. Comparison of the conserved motifs and gene structures of *CsTRM07* (A), *CsTRM17* (B) and *CsTRM24* (C) in the 13 cucumber accessions.

3.3. Synteny Analysis of CsTRM Genes

The phylogenetic relationship of the cucumber TRM family were further explored by constructing comparative syntenic maps of cucumber associated with four representative species, including two monocots (rice and maize) and two dicots (Arabidopsis and tomato) (Figure 2). 1, 3, 8, and 19 *CsTRM* genes showed syntenic relationships with those in the other four species: maize, rice, Arabidopsis, and tomato, respectively (Figure 2). Only 1 TRM collinear gene pairs between cucumber and maize were identified, followed by cucumber and rice (4), cucumber and Arabidopsis (9), and cucumber and tomato (20) (Table S2). It is evident that dicotyledonous plants exhibit a notably higher number of homologous genes compared to those shared between dicotyledonous and monocotyledonous plants. This observation aligns with the patterns expected in biological evolution. *CsTRM18* and its collinear gene pairs with maize are observed in rice and tomato, but not in Arabidopsis, indicating differences in the evolutionary process of *CsTRM18*. Additionally, collinear gene pairs between cucumber and rice, maize, and Arabidopsis are observed in cucumber and tomato, suggesting that cucumber and tomato may have undergone a common evolutionary history.

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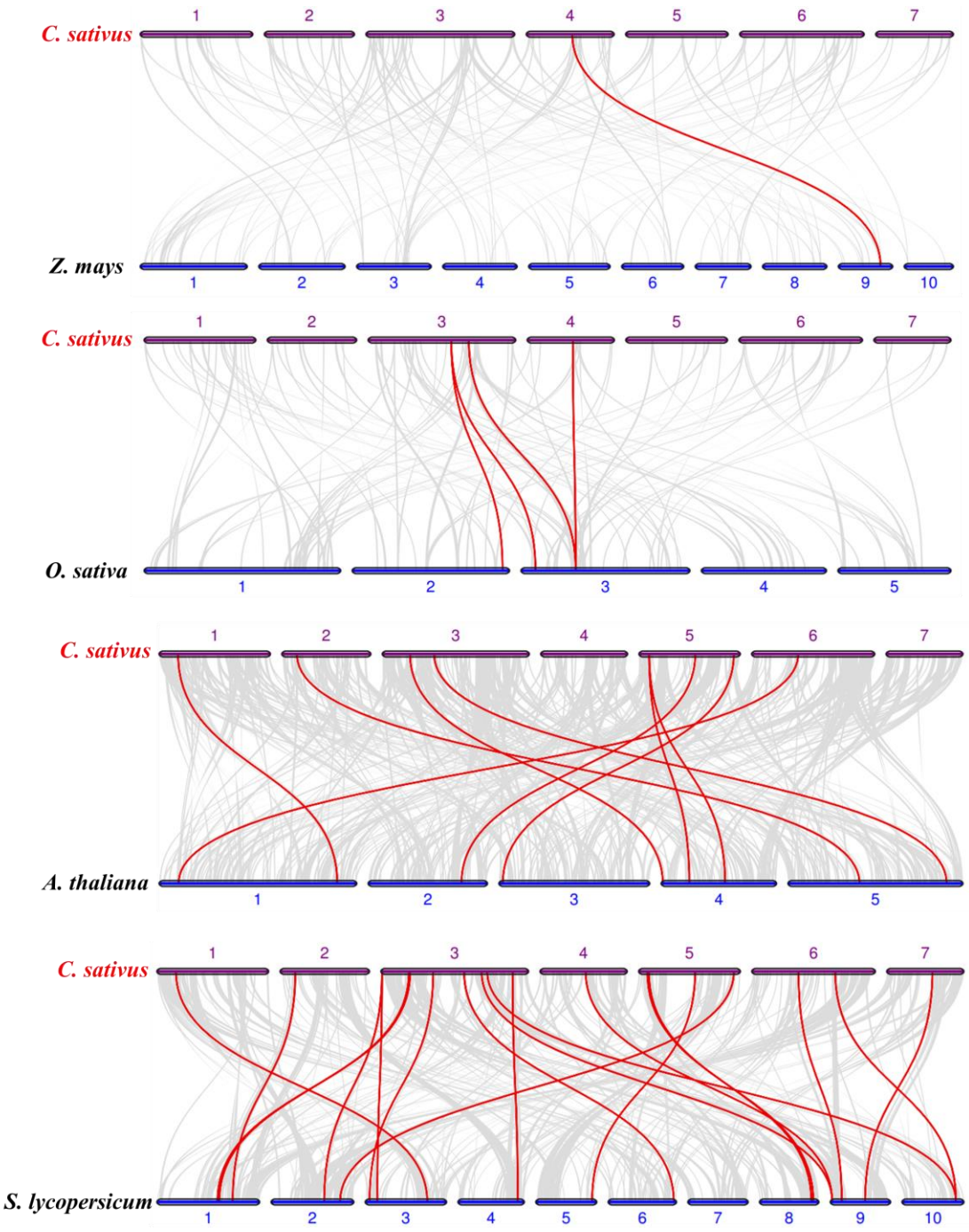

Figure 2. Synteny analysis of *TRMs* between cucumber and other plant species: The collinear blocks are marked by gray lines, while the collinear gene pairs with *TRM* genes are highlighted by red lines. *'C. sativus'*, *'Z. mays'*, *'O. sativa'*, *'A. thaliana'*, and *'S. lycopersicum'* indicate *Cucumis sativus*, *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, and *Solanum lycopersicum*, respectively.

3.4. Expression Profiles of CsTRM Genes in the Fruit

In cucumber and tomato, some *TRM* genes can regulate fruit shape [14,15,20]. To investigate the function of *CsTRMs* in fruit shape, we conducted expression analysis of *CsTRMs* using published RNA-seq data on fruits with different carpel numbers and lengths [48–50]. Relative to the South China type cucumber 32X (carpel number=3), the transcription levels of *CsTRMs* in the mutant Gui Fei Cui (GFC, carpel number=5) from 32X showed no significant changes (Figure 3A, Table S3), indicating that *CsTRMs* might not play a crucial role in regulating cucumber fruit carpel number. Compared to long fruit 408, there were 8 genes down-regulated in short fruit 409, namely *CsTRM5*, *6*, *10*, *11*, *14*, *21*, *26* and *27*(Figure 3B). Compared to empty vector/control transgenic plants WT,

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CsFUL1^{A}-OX-29 had a total of 12 genes down-regulated, namely *CsTRM1*, *2*, *5*, *6*, *7*, *10*, *12*, *13*, *16*, *20*, *21* and *27*; and 4 genes up-regulated, namely *CsTRM8*, *17*, *25*, *26* (Figure 3C). In *CsFUL1*^{A}-OX-29 versus empty vector/control plants and 409 versus 408, *CsTRM5*, *6*, *10*, *21*, and *27* were significantly down-regulated (Figure 3B, 3C), indicating that these genes play a crucial role in regulating fruit shape. However, the expression trend of *CsTRM26* in the two groups of long and short fruit materials is opposite (Figure 3B, 3C), which may be due to different genetic backgrounds of the materials.

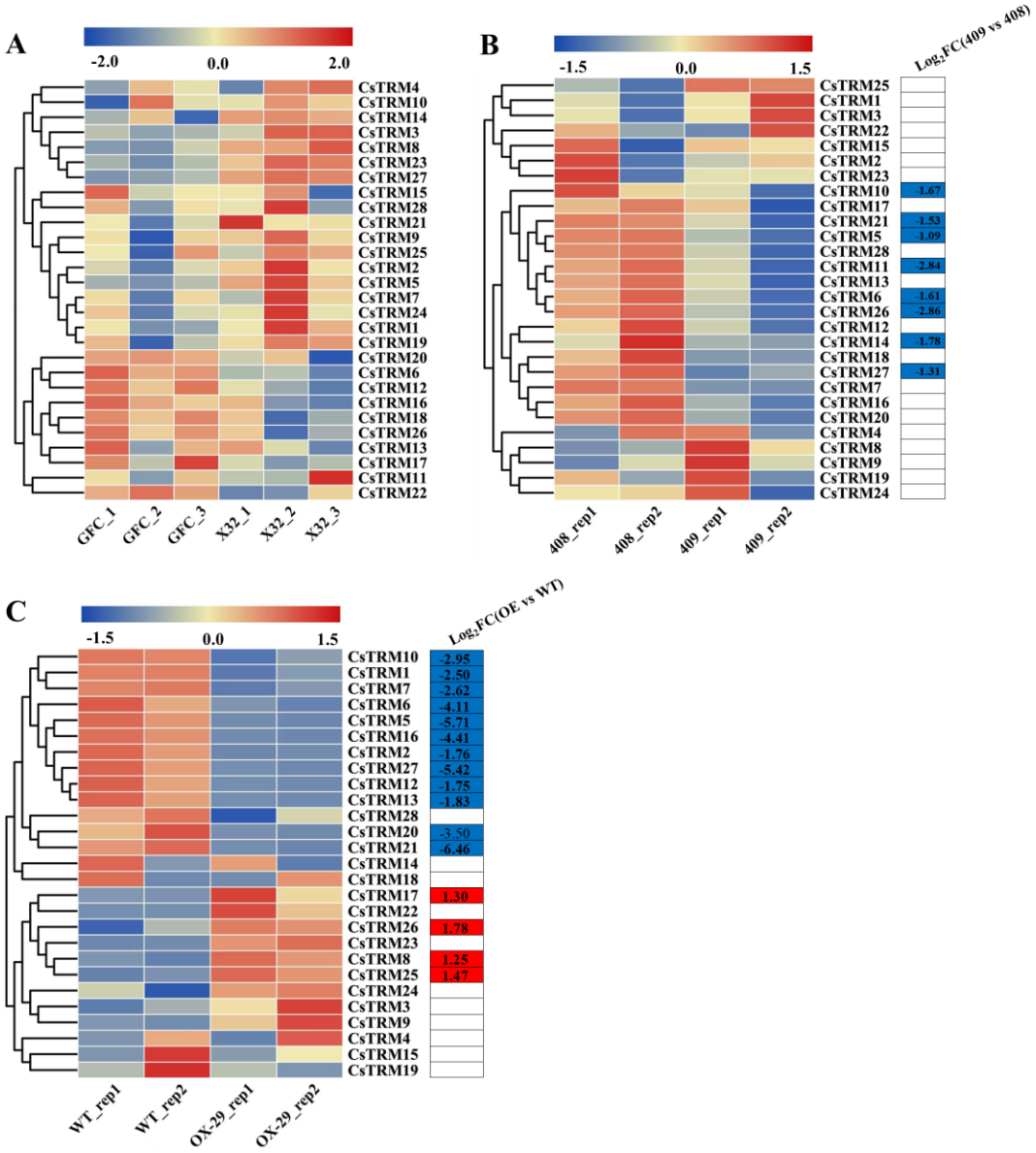

Figure 3. Expression analysis of *CsTRMs* in the fruit: The transcriptional levels of *CsTRM* genes in GFC (carpel number=5) and 32X (carpel number=3) (A), 408 (long fruit) and 409 (short fruit) (B), and WT and *CsFUL1*^{A}-OX (C) are shown on the heatmaps. A range of -2.0 to 2.0 and -1.5 to 1.5 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to red. GFC, mutant Gui Fei Cui (GFC) from South China type cucumber 32X. The carpel number changed from 3 in 32X to 5 in GFC, despite the number of other floral organs, such as sepal, petal and stamen remain unchanged. WT, empty vector/control transgenic plants. FC, fold-change.

3.5. Expression Profiles of CsTRM Genes under Abiotic and Biotic Stresses

TRM gene family members are often localized to microtubules, microtubules are involved in immune responses and stress tolerance. We analyzed the comprehensive expression patterns of

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CsTRM genes under different stresses, including salt, heat, downy mildew (DM, *Pseudoperonospora cubensis*), gray mold (GM, *Botrytis cinerea*) and powdery mildew (PM, *Podosphaera fusca*) based on public transcriptome information [51–55], to further explore the roles of *CsTRM* genes under different stresses.

First, we analyzed the roles of *CsTRM* genes under salt stress (Table S4). The transcriptomic data were presented as a heatmap (Figure 4A). We observed that the expression levels of *CsTRM4*, *8* and *14* considerably increased in response to NaCl stress, and four genes exhibited the opposite trend with exposure to NaCl stress, they are *CsTRM5*, *11*, *21* and *24* (Figure 4A). Under the conditions treated with Silicon (Si) only, the expression of *CsTRM3* and *CsTRM14* was upregulated, whereas the expression of *CsTRM11*, *21* and *24* was downregulated. The expression of *CsTRM14* was upregulated under both individual NaCl treatment and individual Si treatment, while the expression of *CsTRM11*, *21* and *24* was downregulated. Previous research has demonstrated that the application of Silicon (Si) can enhance plant growth when subjected to salt stress. After treatment with Si, the gene expression levels of *CsTRM11*, *14* and *24*, which exhibited significant changes under salt stress, returned to normal levels; *CsTRM5*, *8* and *21* showed only slight regression, while the expression level of upregulated *CsTRM4* showed a slight increase. We also analyzed the responses of *CsTRM* genes to heat stress (Figure 4B, Table S4). At three hours after high-temperature treatment, *CsTRM1*, *11*, *16*, *18*, *21*, *22* and *26* were downregulated, while *CsTRM3*, *8* and *20* were upregulated. At six hours after heat stress, the expression of *CsTRM16* and *CsTRM22* showed no significant difference compared to the 0 hour heat treatment, while the changes in other differentially expressed genes were consistent with the 3 hour heat treatment. Specifically, the genes upregulated at three and six hours after heat stress were nearly identical (Figure 4B), indicating their potential significant roles in conferring thermostolerance. Additionally, *CsTRM3*, *8*, *11* and *21* were differentially expressed in responding to the treatments of heat and NaCl, with consistent trends.

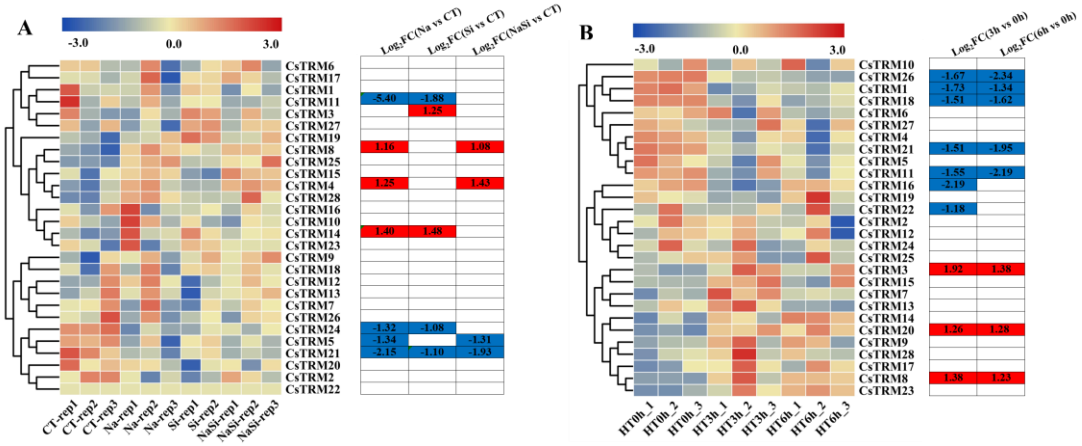

Figure 4. Expression profiles of *CsTRM* genes in response to various abiotic stress treatments: The transcriptional levels of *CsTRM* genes in response to salt (A) and heat (B) stresses are shown on the heatmap. A range of -3.0 to 3.0 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to red. CT, control treatment; HT, heat treatment; HT0h, heat treatment for 0 h (hours); HT3h, heat treatment for 3 h; HT6h, heat treatment for 6 h; FC, fold-change.

To explore the potential functions of *CsTRMs* in the resistance to biotic stresses, we performed expression analyses of *CsTRMs* using the published RNA-Seq data of cucumber seedlings inoculated with PM for 48 h, GM for 96 h and with DM for 8 days [53–55]. After inoculation with PM, a total of 4 genes were differentially expressed in the susceptible cucumber line D8 leaves compared with the control, the expression of *CsTRM14*, *21* and *27* were upregulated, while *CsTRM20* were downregulated; and a total of 4 genes were differentially expressed in the resistant cucumber line SSL508-28 leaves compared with the control, the expression of *CsTRM4*, *14* and *27* were upregulated, while *CsTRM21* were downregulated (Figure 5A). In the susceptible and the resistant cucumber line

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affected by PM, *CsTRM14* and *CsTRM27* had similar expression trends, while *CsTRM21* had opposite expression trends (Figure 5A). After 96 hours of GM inoculation, cucumber seedlings showed significant downregulation of 14 *CsTRM* genes compared to the uninoculated control, namely *CsTRM1*, *2*, *5*, *6*, *7*, *10*, *11*, *13*, *14*, *16*, *20*, *21*, *27* and *28*, and significant upregulation of 3 genes, namely *CsTRM3*, *18* and *26* (Figure 5B). In the transcriptomic data from cucumber seedlings inoculated with DM, only 5 TRMs genes exhibited significant changes in expression (Figure 5C). *CsTRM1*, *7*, *14* and *28* were upregulated at a minimum of one treatment timepoint, while *CsTRM* 8 were downregulated at 6 days post inoculation (dpi) and 8 dpi. *CsTRM28* were upregulated at 2 dpi, 3 dpi, 4 dpi, 6 dpi and 8 dpi (Figure 5C), indicating its significant role in responding to the DM. In summary, the expression of *CsTRM14* was significantly upregulated in cucumber seedlings inoculated with PM, BC, and DM, indicating its broad-spectrum role in responding to biotic stress.

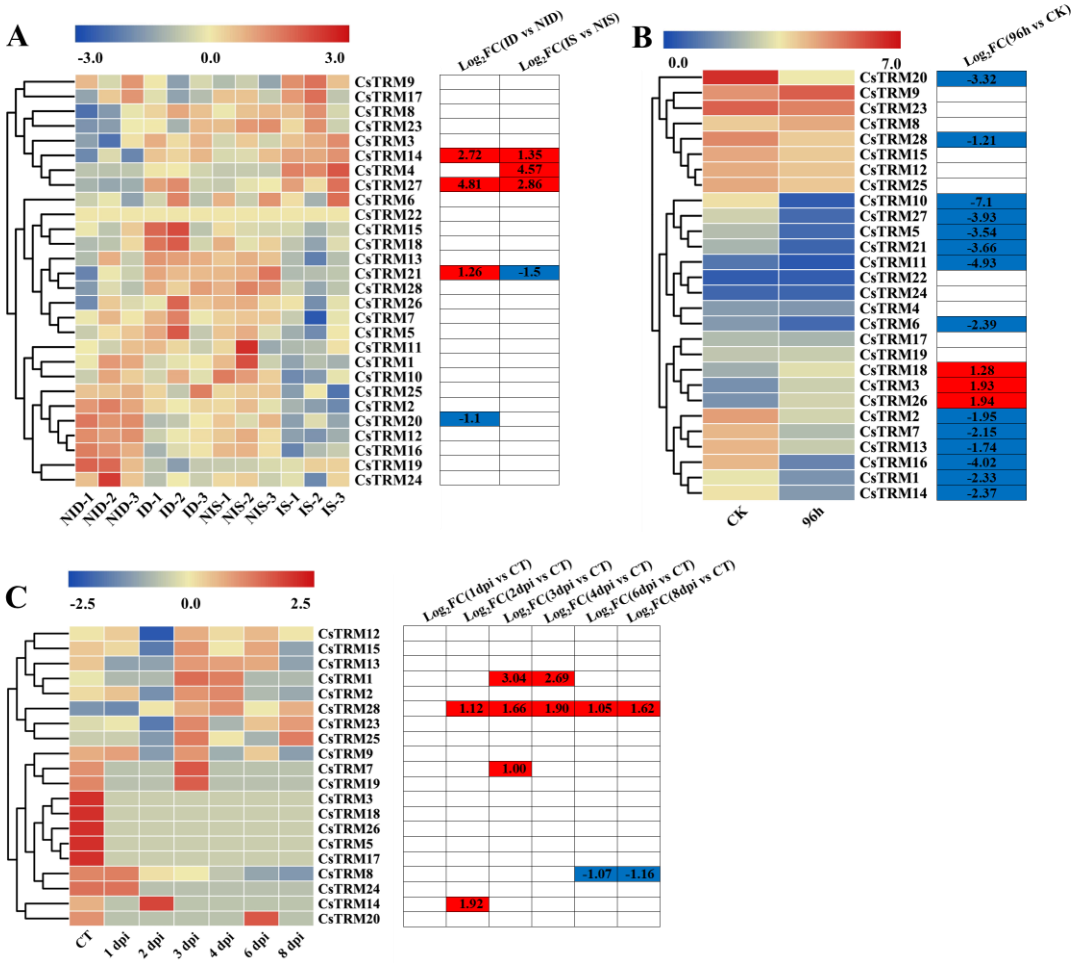

Figure 5. Expression analysis of *CsTRMs* under biotic stresses: The transcriptional levels of CsTRM genes after infection with powdery mildew (PM) for 48 h (A), gray mold (GM) for 96 h (B), and with downy mildew (DM) for 1-8 days post-inoculation (C) are shown on the heatmaps. A range of -3.0 to 3.0, -2.5 to 2.5 and 0.0 to 7.0 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to red. ID, PM-inoculated susceptible cucumber line D8 leaves; NID, non-inoculated D8 leaves; IS, PM-inoculated resistant cucumber line SSL508-28 leaves; NIS, non-inoculated SSL508-28 leaves; CT, without inoculation; DPI, days post inoculation; FC, fold-change.

4. Discussion

Researches demonstrated that a single reference genome is inadequate for capturing the diversity within a species [58]. Hence, we conducted a comprehensive analysis to identify and characterize the TRM family in 13 different cucumber varieties. Although in the previous study, 28

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TRM family were identified [56], in this study, a novel member, *CsTRM29* which is only present in PI183967, was discovered (Table 1). Moreover, only 5 *CsTRMs* have the same protein length among 13 cucumber accessions, and all the identified TRM proteins have amino acid variations including insertions, deletions, single amino acid changes and frame shifts（Table S1）. Some *CsTRMs* underwent changes not only in gene structure but also in conserved motifs (Figure 1). Therefore, in this study, we found rich variations occurred in *CsTRM*s from the pan-genomes of 13 cucumber accessions, and these variations will provide a base for discovering TRM genes with novel functions, which will accelerate the breeding of new cucumber varieties, just as the things are performed with pan-genomics [44].

It is widely recognized that there exists a correlation between gene expression and gene function. The cucumber fruit typically have three fused carpels [59], the carpel number is an important fruit trait that affects fruit shape, size and internal quality [48]. In the lines with different carpel numbers, there were no significant differences observed in the expression of *CsTRMs* (Figure 3A), suggesting that *CsTRMs* might not play a critical role in regulating the number of carpels in cucumber fruits. However, in the short-fruited lines (409 and *CsFUL1*^{A}-OX-29), *CsTRM5*, *6*, *10*, *21*, and *27* were significantly down-regulated (Figure 3B, 3C), indicating that these genes might play crucial roles in regulating cucumber fruit length. Interestingly, the expression of *CsTRM26* is lower in the short-fruited line 409 than in the long-fruited line 408, but higher in the short-fruited line CsFUL1A-OX-29 than in the wild type. This could be due to differing genetic backgrounds or the possibility that CsTRM26 does not regulate cucumber fruit length.

So far, TRMs have been reported to be functional in plant organ growth, but not in plant response to stresses. But an increasing number of researches suggested that apart from their crucial roles in mechanical architecture and cell division, microtubules are also implicated in plants adaptation to severe environmental conditions [60]. Since some TRMs are microtubule-binding proteins, they might participate in stress responses. Therefore, in this study, we analyzed the expression patterns of *CsTRMs* under certain stress conditions. Many *CsTRM* genes showed expression changes at varying degrees under different stress conditions (Figures 4 and 5). Under salt and heat stress conditions, the expression of *CsTRM3* and *CsTRM8* was significantly upregulated, while *CsTRM11* and *CsTRM21* were significantly downregulated (Figure 4), however, under inoculation with PM, BC, or DM, the expression of *CsTRM14* was significantly increased, while the expression of *CsTRM21* showed significant changes after inoculation with PM and BC(Figure 5). These results might indicate that different *CsTRMs* respond to abiotic or biotic stresses. Remarkably, *CsTRM21* plays a crucial role in regulating fruit shape (Figure 3B,C) and in responding to biotic stresses (Figures 4 and 5). Therefore, this study provided not only a base for the function of CsTRMs in stress tolerance, but also a cross talk point between organ growth and biotic stresses.

5. Conclusions

In this study, we performed pan-genome-wide identification of the TRM gene family in cucumber. In total, 29 members were identified, including a novel member, *CsTRM29* which is only present in PI183967. Only 5 of the CsTRMs have consistent protein lengths among the 13 accessions. All CsTRM proteins showed amino acid variations. Furthermore, Transcriptomic data of fruits with different shapes indicate that *CsTRM*s play a significant role in regulating fruit length but not in controlling carpel number. And transcriptomic data under different stress conditions revealed the differences and similarities in the stress-induced expression of *CsTRM*s in response to abiotic and biotic stresses, and *CsTRM14* was found to response to salt stress, powdery mildew, gray mold and downy mildew. Notably, *CsTRM21* plays a role in regulating both fruit shape and resistance. In conclusion, this study provides a reference for investigating the potential role of TRMs for fruit shape and stress resistance in cucumber.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, Z.R., L.W. and C.C.; methodology, L.Z. and K.W.; validation, L.Z.; software, L.Z., K.W., Z.W. and S.C.; formal analysis, L.Z. and K.W.; investigation, L.Z. and K.W.; data curation, L.Z. and K.W.; writing-original draft preparation, L.Z.; writing-review and editing, Z.R.; supervision, L.W. and Z.R.; project administration, Z.R., L.W. and C.C.; funding acquisition, Z.R. and L.W. All authors have read and agreed to the published version of the manuscript.

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