Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2021.014396

ARTICLE

Genome-Wide GRAS Gene Family Analysis Reveals the Classification, Expression Profiles in Melon (*Cucumis melo* L.)

Yanfei Bi[1], Bin Wei[1], Ying Meng[2], Zhongzhao Li[2], Zhenghui Tang[1], Feng Yin[3] and Chuntao Qian[2,*]

[1]Jiangsu Changshu National Agricultural Science and Technology Park, Suzhou, 215500, China

[2]College of Horticulture, Nanjing Agricultural University, Nanjing, 210095, China

[3]Changshu Agricultural Science and Technology Development Co., Ltd., Suzhou, 215500, China

[*]Corresponding Author: Chuntao Qian. Email: ctqian@126.com

Received: 23 September 2020 Accepted: 24 January 2021

ABSTRACT

Melon (*Cucumis melo*), belonging to the Cucurbitaceae family, is a globally important economic crop. GRAS (GAI, RGA, SCR) genes, which are a type of transcription factor, play a critical role in plant growth and development, including processes such as radial root patterning, light signalling, abiotic/biotic stress, axillary shoot meristem formation, and phytohormone (gibberellin) signal transduction. In this study, the GRAS family in melon was analysed comprehensively with respect to chromosomal location, motif prediction, gene structure, and expression pattern. A total of 37 GRAS genes were first identified in melon, after which a phylogenetic tree was built with the GRAS genes of three model species (*Arabidopsis*, rice, and sacred lotus) and were divided into nine groups based on the findings of previous studies. Motif and gene structure analysis showed typical conserved domains in all melon GRAS and similar structures in the same subfamilies. The expression analysis of GRAS genes done using RNA-seq data, showed that these genes were differentially expressed in different melon leaves under powdery mildew stress. Furthermore, the real-time quantitative PCR for GRAS genes revealed gene expression corresponding to powdery mildew stress. Our results provide useful information for a better understanding of GRAS genes and provide the foundation for additional functional exploration of the melon GRAS gene family in the powdery mildew stress response.

KEYWORDS

Genome-wide identification; GRAS; melon; stress response

1 Introduction

Melon (*Cucumis melo* L.) is a eudicot diploid species ($2n = 24$) from the Cucurbitaceae family, which includes other well-known crops such as watermelon, cucumber, and pumpkin. The Cucurbitaceae family is of great economic significance, second only to the Solanaceae family, and among them, melon is one of the most important crops. Melons are planted in multiple climatic environments, and approximately 32 million tons of melons were produced worldwide in 2017 (Food and Agriculture Organization, http://faostat.fao.org). The melon genome was originally sequenced in 2012 [1], and the sequences were improved in 2018 [1,2] along with the development of genetic and genomic resources [3] that greatly advanced molecular-level

research focusing on the genomic characterization of important agronomic traits related to abiotic or biotic stress [4–6], fruit ripening [7–9], and sex determination [10].

Melon is susceptible to powdery mildew disease (PM) during the later stage of development. PM is a kind of fungal disease of melon caused by *Golovinomyces cichoracearum* or *Podosphaera xanthii* (Px), which is widespread and can cause serious yield losses in melon, and PM has severely hindered the development of melon industry [11–14].

Transcription factors play a significant role in plant development and stress response, and GRAS is a group of transcription factor genes that are widespread in plants. Many studies have shown that GRAS genes respond to plant development and biotic or abiotic stress; these studies have been carried out in model species such as *Arabidopsis thaliana* [15], *Oryza sativa* [16], *Solanum lycopersicum* [17], *Nelumbo nucifera* [18], and *Nicotiana tabacum* [19].

The acronym GRAS originated from the characteristic letters of the first known three members, GAI (gibberellic acid insensitive) [20], RGA (repressor of GA1–3 mutant) [21], and SCR (scarecrow) [22]. Normally, GRAS genes contain one or two structural domains, with a protein length of 400–700 amino acid residues [23]. In previous studies, GRAS family members were further divided into 8–13 branches based on phylogenetic relationships and on common features in different species [15–19,24]. Because of their highly homologous gene structures, GRAS genes from the same branches may have similar functions [17], as has been supported by similar gene expression patterns observed in *Arabidopsis*, rice, and *Populus* [24]. Since then, many GRAS genes have been identified in plants, including 34 from *A. thaliana* [16], 60 from *O. sativa* [24], 38 from *N. nucifera* [18], 53 from *S. lycopersicum* [15], 21 from *N. tabacum* [19] and 106 from *Populus tremula* [24].

Sequencing and annotation of the melon genome provide an opportunity to identify all the melon GRAS genes [1,2]. In this study, 37 GRAS genes were identified from the melon genome, and analyses of their structure, phylogeny, chromosomal distribution, conserved motifs, and stimulation in response to powdery mildew were performed. The results provide insights into the evolution of the melon GRAS genes and their functions in resisting powdery mildew (PM). Identification and characterization of these GRAS genes may provide opportunities to improve the PM resistance of melon.

2 Materials and Methods

2.1 Source of Gene Sequences

All melon protein sequences were downloaded from Melonomics (https://www.melonomics.net/), which is the melon genomic database. The *Arabidopsis thaliana GRAS* protein sequences were downloaded from TAIR (https://www.arabidopsis.org), *Oryza sativa GRAS* protein sequences were from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/downloads_gad.shtml), and *N. nucifera* GRAS protein sequences were from the lotus genome database (http://lotus-db.wbgcas.cn/).

2.2 Identification of the GRAS Gene Family in Melon

To identify the GRAS gene in the melon genome (downloaded from https://www.melonomics.net/, genome version:V3.6.1), the HMM profile for GRAS genes was first constructed using 132 protein sequences from *O. sativa*, *A. thaliana,* and *N. tetragona*, and it was used to scan out candidate GRAS genes in the melon genome using HMMER 3.0 [25]. Meanwhile, all melon protein sequences were searched against 132 downloaded GRAS proteins to identify candidate GRAS genes using blastp with the *e*-value set to $1e^{-10}$ [26]. Finally, candidate GRAS genes acquired from two identification methods were combined and used to identify GRAS genes using pfamscan [27,28]. Consequently, genes including the PF03514.13 pfam domain were finally recognized as GRAS genes in melon. The subcellular locations of

these GRAS genes were then predicted by ProtComp 9.0 (http://linux1.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc) using the default parameters.

2.3 Chromosomal Location and Phylogeny Analysis of the GRAS Gene Family in Melon

The physical positions of the CmGRAS genes in the chromosomes were located using in-house scripts and drawn using MG2C v2 (http://mg2c.iask.in/mg2c_v2.1/). ClustalW2 [29] was used to perform multiple sequence alignments, and MEGA 7 [30] was used to construct an MP phylogenetic tree based on the amino acid sequences of melon GRAS genes and 132 protein sequences from *O. sativa*, *A. thaliana*, and *N. tetragona* with 500 bootstrap replicates.

2.4 Motif Analysis and Gene Structure Visualization

MEME [31] was used to analyse the conserved motifs in the melon GRAS genes, with the largest number of motifs to seek in GRAS sequences set to 10. Gene structures were visualized using GSDS 2.0 [32].

2.5 Analysis of CmGRAS Gene Expression in Response to Powdery Mildew

The transcriptome data of melon in resistant and susceptible leaves after pathogen inoculation were downloaded from NCBI (SRA identifier: SRP095589) to observe the expression patterns in response to powdery mildew [33]. Raw data were quality controlled by fastp [34] to remove reads containing adapter, reads containing poly-N, and low-quality reads. The clean reads were mapped to the melon reference genome using Tophat [35], and FPKM was calculated using Cufflinks [36] using the default parameters. CmGRA expression profile heatmaps were drawn using the R package pheatmap [37].

2.6 Plant Materials and RT-qPCR Experiment

We selected two plant varieties as experimental materials. They were the powdery mildew (1) disease-resistant (i.e., Elizabeth) and (2) susceptible (i.e., Zhaojun 1) varieties. Muskmelon seeds with full grains and uniform size (26.07 ± 0.82 g/1,000 grain weight for Elizabeth, and 17.22 ± 0.61 g/1,000 grain weight for Zhaojun 1) were selected and then soaked in water at 30°C for 24 h to accelerate germination. The young plants were sowed in a 72-hole burrow plate filled with the following matrix = peat: vermiculite: perlite = 3 V:1 V:1 V). When the seedlings grew to 2 leaves and 1 heart, plants of the two varieties with the same growth conditions were selected to wash their root. Then the plants were transplanted into a water culture plastic box (50 cm × 35 cm × 15 cm). Plants were planted in each box and cultured in Hoagland nutrient solution. The culture conditions were as follows: day temperature 28°C/night temperature 18°C, light 16 h/dark 8 h; relative humidity: $75 \pm 5\%$. The nutrient solution was changed every 2 days. Approximately 100 PM x strains were collected from the farm using the single sporangiophore transfer method. When the seedlings grew to 3 leaves and 1 heart, the strains were inoculated onto seedlings with two or three unfolded leaves. The leafs of muskmelon seedlings were taken for quantitative analysis at 0 h, 24 h, 72 h and 168 h, respectively.

The Primer3 software (http://bioinfo.ut.ee/primer3/) was used to design the RT-qPCR primers. RT-qPCR analysis was used to analyze the GmGRASs. Using the actin gene as an internal control, the standard RT-qPCR with SYBR Premix Ex Taq II (TaKaRa) was repeated at least three times on a CFX96 Real Time System (BioRad). The relative expression profiles were analyzed through the $2-\Delta\Delta CT$ method with the samples in 0 h point used as controls, then the relative mRNA expression information was obtained [38]. Student's *t*-test was used for significance analysis. The Y samples were used as controls.

3 Results and Discussion

3.1 Identification of GRAS Family Genes and Chromosomal Locations in Melon

In total, 132 GRAS protein sequences from *A. thaliana*, *O. sativa*, and *N. nucifera* were collected to build a hidden Markov model (HMM), and the search against the melon genome was performed on the model using HMMER 3.0 [25]. Meanwhile, to improve the sensitivity of identifying GRAS homologs, the blastp program included in blast+ 2.29 [26] with an *e*-value set to $1e^{-5}$ was used to find GRAS homologs against 132 collected GRAS proteins in the melon genome. In total, 158 candidate GRAS genes were identified. These candidate melon GRAS proteins were scanned against the Pfam A database using pfamscan, with an *e*-value of $1e^{-5}$, to filter out genes without the GRAS domain PF03514.13 [27,28]. Finally, only 37 melon proteins were identified as GRAS and were labelled as CmGRAS1, CmGRAS2, and so forth (Tab. 1). The length of 37 melon GRAS genes ranged from 1445 to 4234 bp, with an average length of approximately 2382 bp. Of the 37 CmGRAS genes, all were mapped to 12 melon chromosomes (Fig. 1); chromosome 08 contained the greatest number of CmGRAS genes (6%, 16.2%), whereas chromosome 10 and chromosome 12 contained only one CmGRAS gene. The subcellular locations of these GRAS genes were predicted as follows: cytoplasmic (13.51%), extracellular (10.81%), and nuclear (75.68%).

3.2 Classification and Phylogenetic Analysis of the CmGRAS Genes

Based on multiple alignments of melon with *Arabidopsis*, rice, and lotus GRAS proteins, a phylogenetic tree was constructed using the maximum parsimony (MP) method, with a bootstrap value of 500. The number of GRAS genes in melon was higher than that in *Arabidopsis* (34), but less than in rice (60) and in the sacred lotus (38) [18,24]. In previous studies, GRAS genes were divided into nine branches in plants, and the phylogenetic tree generated in this study supported the formal classification. As shown in Fig. 2, among 37 CmGRAS genes, 2, 1, 3, 5, 4, 6, 4, 6, and 6 were found in the SCL4/7, LAS, SCR, SCL 3/28, DELLA, HAM, SCL9, PAT1, and SHR branches, respectively. GRAS genes have been reported to be involved in the development, regulation, and signal transduction [16,20]. In the melon genome, most GRAS genes (31/37) were clustered in six branches, particularly the DELLA and PAT1 branches, whose members in *Arabidopsis* have been found to be related to signal transduction in the cytoplasm and to the negative regulation of GA signalling [20,21].

3.3 Conserved Motifs and Structure of the CmGRAS Genes

Using GRAS phylogenetic relationship data (Fig. 3a), we identified structural features of the melon GRAS, including conserved motifs and the locations of UTRs, exons, and introns. Using the multiple em for motif elicitation (MEME), we identified 10 conserved motifs in the melon GRAS (Fig. 3c). Of the 37 GRAS genes, 14 contained all 10 motifs, and all melon GRAS genes had more than 7 motifs. The gene structures (Fig. 3b) revealed that only eight CmGRAS genes had more than one exon (8/37). In addition, 59.4% (22/37) of CmGRAS genes had no introns, which is less than those of tomato (77.4%), sacred lotus (73.7%), and *Arabidopsis* (67.6%), but is higher than those of rice (55%) and *Populus* (54.7%), supporting the notion that most GRAS genes in plants are intronless [15,18,24].

Table 1: CmGRAS gene data

Gene symbol	Gene locus	Linkage group	Start	End	Gene length	Subgroup	Molecular weight (Da)
CmGRAS1	MELO3C015155.2.1	chr02	7082721	7084954	2233	SCL3/28	53127.09
CmGRAS2	MELO3C025987.2.1	chr11	13281910	13285163	3253	RAT1	61092.18
CmGRAS3	MELO3C005298.2.1	chr09	20268181	20270373	2192	DELLA	64910.28
CmGRAS4	MELO3C012447.2.1	chr10	406016	408553	2537	SCL3/28	52571.39
CmGRAS5	MELO3C008170.2.1	chr03	1388416	1391236	2820	SCL3/28	75401.86
CmGRAS6	MELO3C022854.2.1	chr09	13565275	13567338	2063	DELLA	65926.23
CmGRAS7	MELO3C023684.2.1	chr01	6999153	7002533	3380	RAT1	64083.46
CmGRAS8	MELO3C014133.2.1	chr06	37237905	37241168	3263	RAT1	60833.81
CmGRAS9	MELO3C018144.2.1	chr04	9719324	9721895	2571	RAT1	65846.13
CmGRAS10	MELO3C025282.2.1	chr08	26917790	26922024	4234	SCR	92420.94
CmGRAS11	MELO3C025288.2.1	chr02	18706903	18708838	1935	DELLA	58283.97
CmGRAS12	MELO3C007444.2.1	chr08	2821624	2824827	3203	RAT1	60045.76
CmGRAS13	MELO3C020206.2.1	chr06	16711217	16713346	2129	DELLA	61747.08
CmGRAS14	MELO3C014056.2.1	chr06	36380727	36382983	2256	SCL3/28	73470.12
CmGRAS15	MELO3C009253.2.1	chr04	33346095	33348937	2842	SCL9	84940.2
CmGRAS16	MELO3C020619.2.1	chr12	1580739	1583344	2605	SCL9	79434.95
CmGRAS17	MELO3C016202.2.1	chr07	22097078	22098702	1624	SCR	45095.11
CmGRAS18	MELO3C017561.2.1	chr07	25139041	25141212	2171	SCL4/7	65010.01
CmGRAS19	MELO3C005834.2.1	chr09	24653783	24655857	2074	SCL3/28	65382.57
CmGRAS20	MELO3C025904.2.1	chr11	20986593	20989187	2594	SCL9	81961.68
CmGRAS21	MELO3C007630.2.1	chr08	4248779	4250685	1906	SHR	55611.15
CmGRAS22	MELO3C007823.2.1	chr08	5594244	5596338	2094	SCL4/7	71780.83
CmGRAS23	MELO3C026237.2.1	chr02	26532069	26533604	1535	SCL3/28	56770.25
CmGRAS24	MELO3C021146.2.1	chr11	31778627	31781562	2935	HAM	81876.82
CmGRAS25	MELO3C011591.2.1	chr03	14354929	14356374	1445	HAM	53762.53
CmGRAS26	MELO3C013947.2.1	chr06	35048408	35050956	2548	HAM	84196.02
CmGRAS27	MELO3C022440.2.1	chr11	33713267	33715768	2501	SHR	52208.2
CmGRAS28	MELO3C014029.2.1	chr06	36015359	36018109	2750	LAS	47579.73
CmGRAS29	MELO3C012729.2.1	chr01	23618252	23620017	1765	SCR	55121.07
CmGRAS30	MELO3C007619.2.1	chr08	4150398	4151919	1521	SHR	52477.22
CmGRAS31	MELO3C020907.2.1	chr11	2962878	2966672	3794	SHR	58288.84
CmGRAS32	MELO3C017547.2.1	chr07	25018504	25020266	1762	HAM	57138.79
CmGRAS33	MELO3C017548.2.1	chr07	25032967	25034695	1728	HAM	59429.31
CmGRAS34	MELO3C018789.2.1	chr01	2825699	2828025	2326	SHR	48445.09
CmGRAS35	MELO3C015921.2.1	chr01	31778526	31780829	2303	SCL9	66154.24
CmGRAS36	MELO3C024355.2.1	chr01	36292861	36294610	1749	SHR	62272.68
CmGRAS37	MELO3C008036.2.1	chr08	7062943	7064454	1511	HAM	57500.72

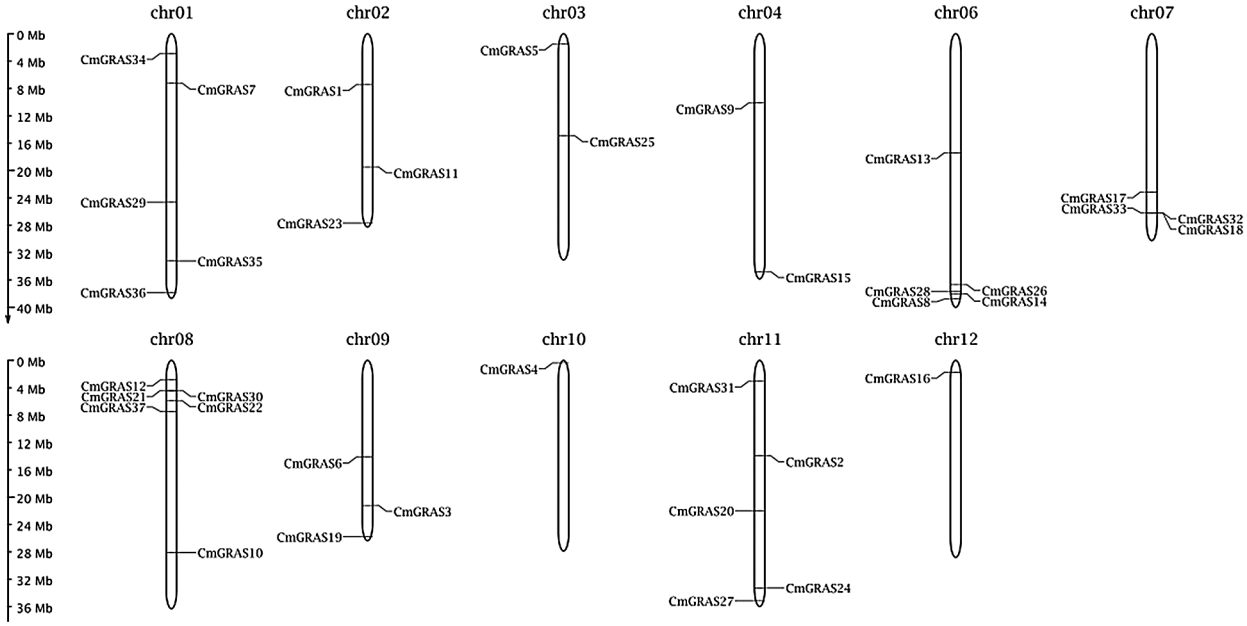

Figure 1: Distribution of CmGRAS genes within the melon chromosomes. Vertical bars represent the chromosomes within the melon genome. The chromosome number is indicated at the top of each chromosome. The scale on the left is in millions of bases (Mb) and indicates the physical length of each linkage group. The positions of each CmGRAS gene are represented by black lines

3.4 Cis-element Analysis of the Promoter Regions of the CmGRAS Genes

Analysis of the 2 kb upstream regions (from the translation start site) of CmGRAS genes revealed the presence of various regulatory elements that are associated with development and abiotic/biotic stress signalling. As shown in Fig. 4, the regulatory elements involved in the regulation of growth and development processes, such as the Dof (DNA binding with one finger) binding site, appear to be enriched in CmGRASs. Stress-responsive cis-regulatory elements identified in this study included basic helix-loop-helix (bHLH) binding site, basic region/leucine zipper motif (bZIP) binding site, and WRKY binding site. Other regulatory elements were also identified, such as the C2H2 zinc finger transcription factor (involved in the occurrence of plant leaves and the regulation of floral organs) binding site and lateral organ boundaries domain (LBD, involved in the development and formation of plant lateral organs) binding sites. In particular, the promoters of 37 CmGRASs contained MBS elements ranging from 1 to 14 copies (Tab. S1), indicating the importance of MYB transcription factors in regulating CmGRASs.

3.5 Gene Ontology Analysis of GRAS Family Genes

To better understand the functions of CmGRAS family genes, gene ontology (GO) analysis was performed. As shown in Tab. S2, 35 CmGRASs proteins were annotated in terms of molecular function. In total, 31 CmGRASs were annotated as DNA-binding transcription factor activity. 35 CmGRASs were annotated in the category of biological process. The annotated CmGRASs were involved in a variety of biological processes. All CmGRASs took part in regulation of transcription, DNA-templated. In addition, six CmGRASs could participate in response to gibberellin. This is consistent with the study that GRAS family are major players in gibberellin (GA) signalling [16]. Based on the biological process analysis, the main functions of CmGRASs were to bind regulation of transcription, DNA-templated and participate in some other biological processes such as leaf development and regulation of nitrogen utilization. In addition, 35 CmGRASs were annotated in a cellular component. The results showed that CmGRASs were located on the cell nucleus, which was related to their function of transcription factor activity.

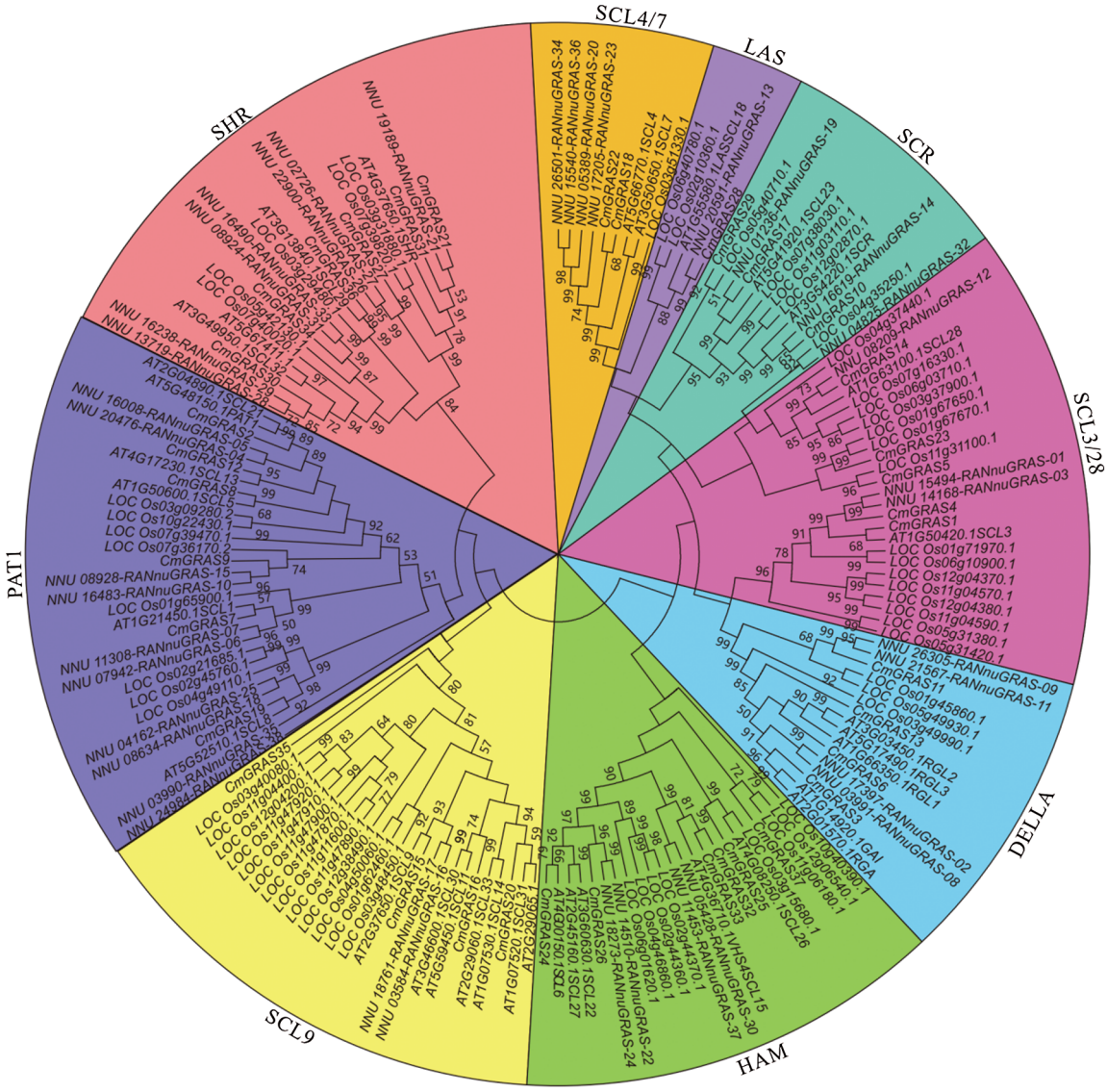

Figure 2: Phylogenetic analysis of the GRAS proteins in melon and 3 other plants. Multiple sequence alignments of GRAS amino acid sequences were performed using ClustalX, and the phylogenetic tree was constructed with MEGA 7 using the maximum parsimony (MP) method and 500 bootstrap replicates. The tree was divided into nine phylogenetic subgroups: SCL4/7, LAS, SCR, SCL 3/28, DELLA, HAM, SCL9, PAT1, and SHR branches. Bootstrap values were set to ≥50%

GO enrichment analysis was performed to investigate the function of CmGRASs. As shown in Fig. S1 and Tab. S3, 37 GO terms showedenrichment in CmGRASs. Transcription, DNA-templated and regulation of transcription, DNA-templated are the top 2 enriched GO terms, which was related to their function of transcription factors. In addition, some other biological processes, such as response to xenobiotic stimulus and positive regulation of signaling receptor activity were also enriched. It indicated that CmGRASs play an important role in plant stress resistance and biological regulation.

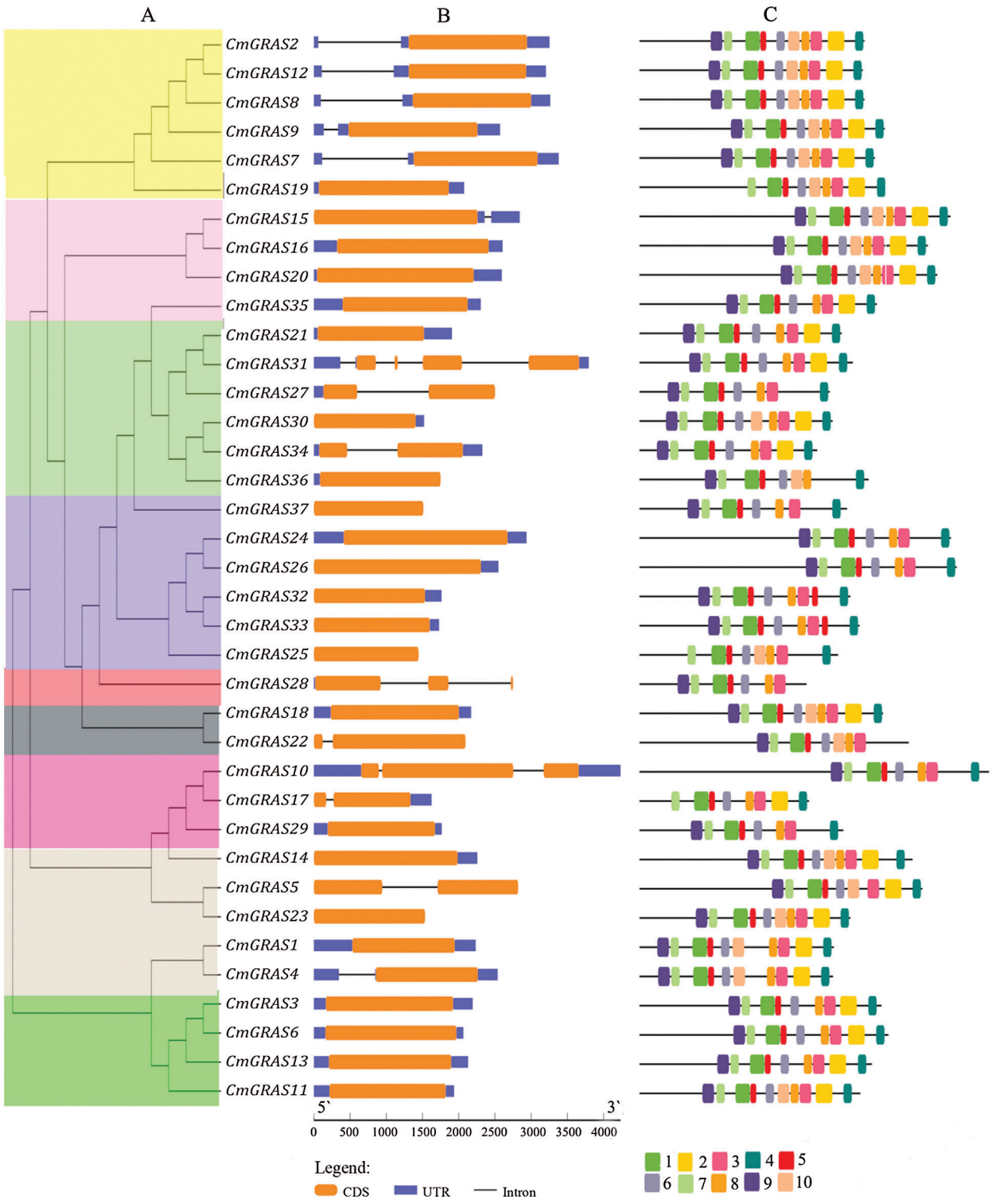

Figure 3: Structure of CmGRAS genes. (A) phylogenetic tree, with coloured regions representing the SCL4/7, LAS, SCR, SCL 3/28, DELLA, HAM, SCL9, PAT1, and SHR branches, as shown in Fig. 2. (B) gene structure, with the brown rectangle representing exon, blue rectangles representing UTR, and blue lines corresponding to introns; protein lengths are shown at the bottom. (C) motif logo, with the amino acid composition of each motif

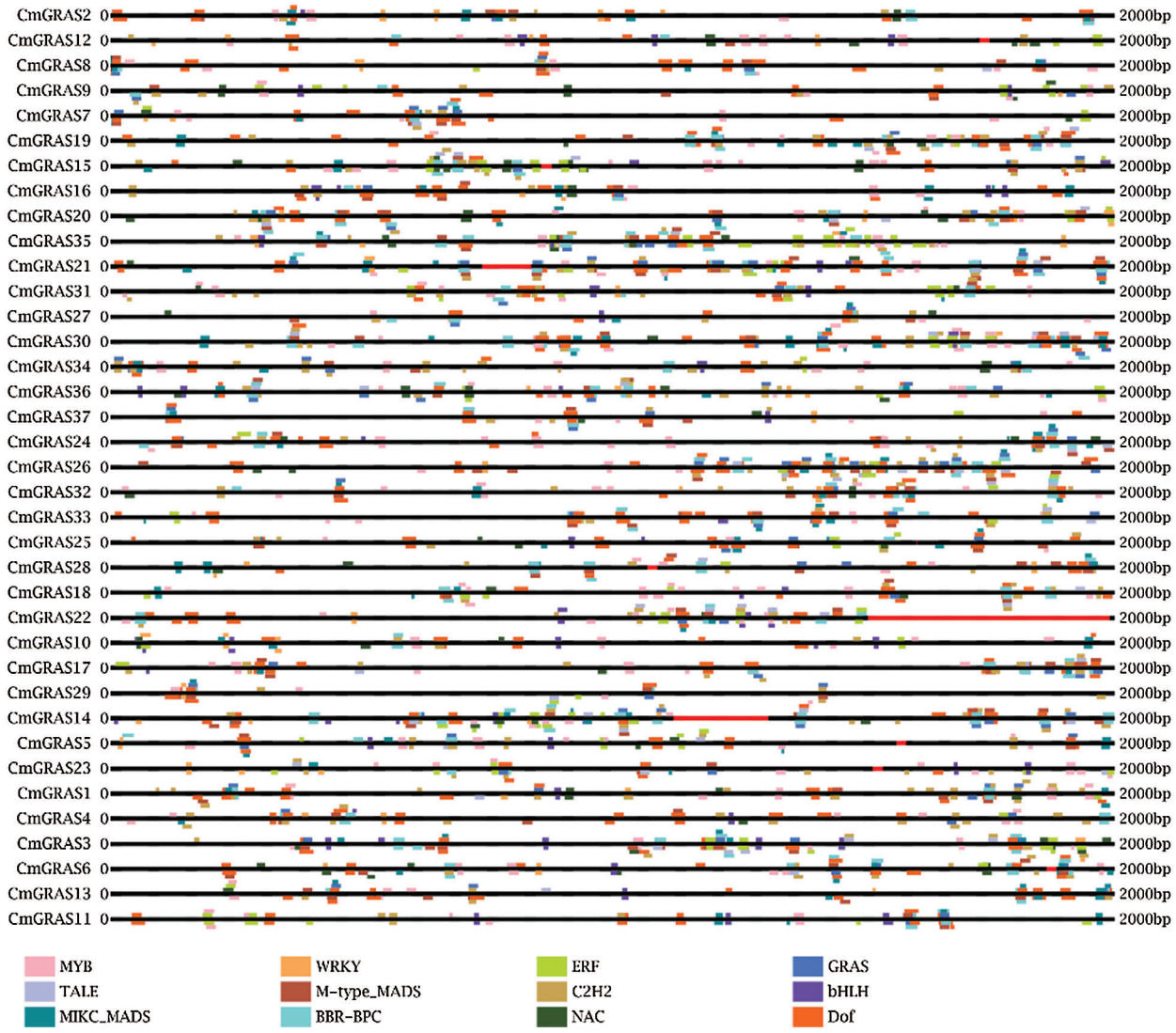

Figure 4: The cis-elements identified in more than ten CmGRAS genes

3.6 Expression Patterns of CmGRAS in Response to Powdery Mildew

In this study, we analysed the expression levels of GRAS genes in two types of melon leaves (PM-resistant type MR-1 and susceptible type Topmark), with pathogen inoculation using the published RNA-seq data [33]. Generally, a similar expression pattern was observed in the same branch of GRAS genes; for instance, most members of the DELLA and PAT1 branches exhibited a higher expression level than the GRAS genes of other branches, whereas the SHR subfamily showed a notably low expression level in all samples (Fig. 5).

However, few PM-resistance genes have been identified in melon. Recent studies have demonstrated that GRAS genes are involved in the responses to the interactions with fungi in plants such as *Lotus japonicus* and *Petunia hybrid* [39–41]. There is compelling evidence that GRAS are plant transcription factors that regulate resistance to PM in melon. Interestingly, GRAS genes were expressed differentially at different time points after inoculation. For example, members of the SCL3/28 subfamily, such as CmGRAS14, and members of the SCL4/7 subfamily, such as CmGRAS18, showed higher expression in the susceptible cultivar than in the resistant type at 168 h post-inoculation (Fig. 5), indicating that they may play a critical role in the response to PM.

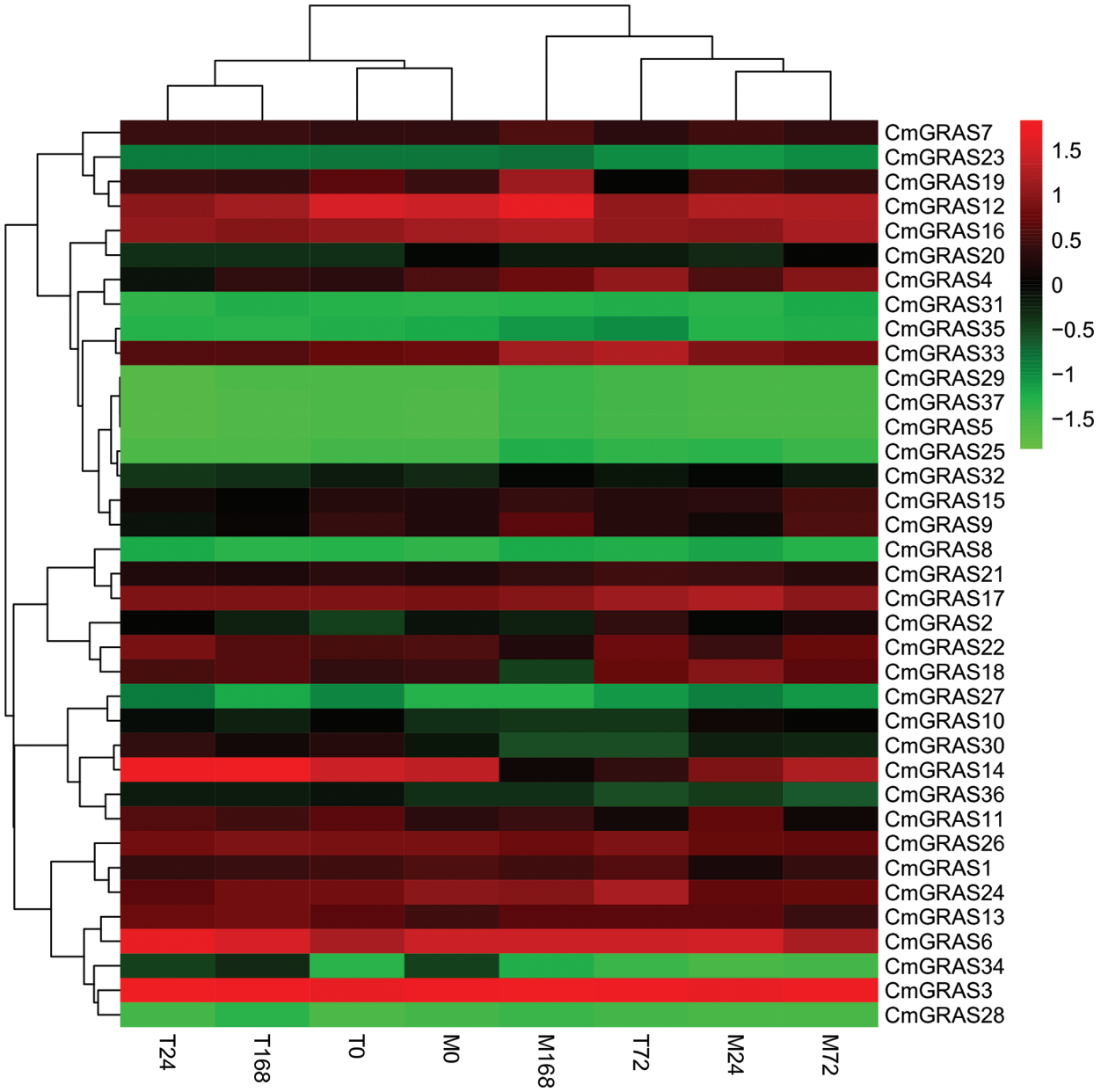

Figure 5: Expression profile analysis of CmGRAS genes in two melon types (PM-resistant and PM-susceptible) inoculated with *Podosphaera xanthii* (Px) at different time points. T represents the susceptible cultivar "TopMark" and M represents the resistant cultivar "MR-1"; the numbers 0, 24, 72 and 168 represent the time (in h) of Px infection. Transcriptome data (fragments per kilobase million; FPKM) were used to measure the expression levels of CmGRAS genes. The coloured scale used for the different expression levels is shown

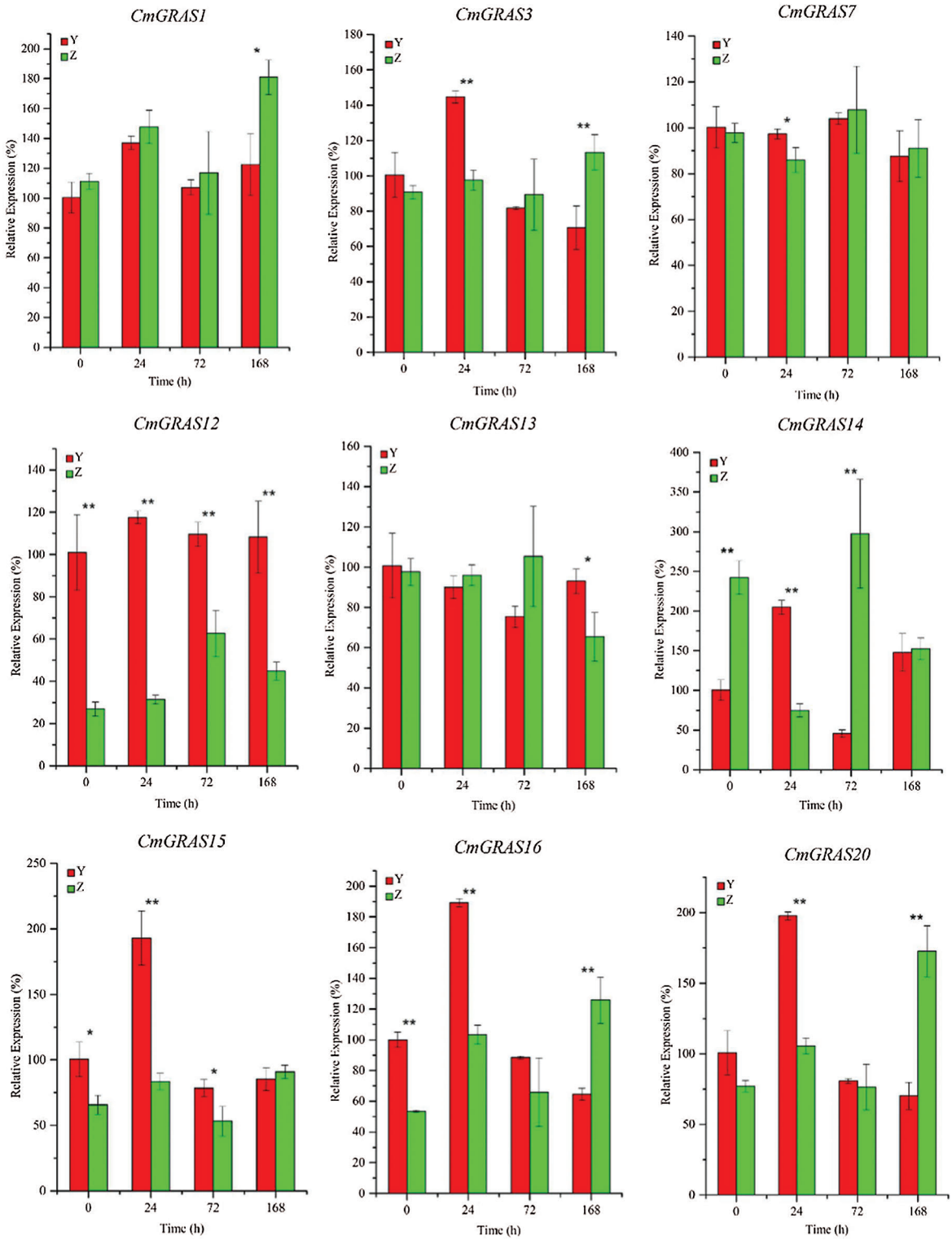

Figure 6: (Continued)

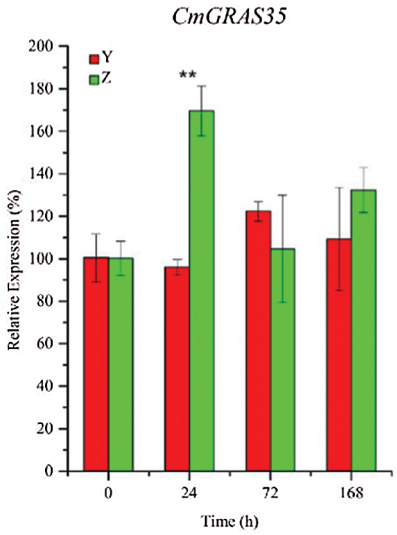

Figure 6: Relative expression profiles of CmGRASs at different time points. Error bars represent means ± SD (n = 3). Three independent experiments were performed for each sample. The significant levels were labeled with asterisk "*" indicated p value < 0.05, and "**" indicated p value < 0.01

To further confirm the expression of CmGRASs in other powdery mildew resistant and sensitive materials, ten CmGRASs were further analyzed using qRT-PCR (Fig. 6). As shown in Fig. 6, there has differential expression genes between powdery mildew resistant and powdery mildew sensitive varieties in different time points. It indicates that CmGRASs has a stress response under pathogen invasion. By GO enrichment analysis, the CmGRASs were enrichment in the GO term of response to xenobiotic stimulus. These results indicated that CmGRAS family is an important gene resource for the improvement of powdery mildew resistant in *Cucumis melo* L.

In this study, a total of 37 melon GRAS genes were identified, and we focused on the GRAS genes involved in the response to powdery mildew infection. Additionally, we investigated the characteristics of melon GRAS genes, such as physical distribution, classification, and gene structure. In these different stages of RNA-seq data, the differential expression patterns of CmGRAS genes showed that these genes play different roles in melon powdery mildew responses. Additionally, CmGRAS gene expression analysis revealed that some were markedly upregulated or downregulated in response to powdery mildew. In recent decades, many studies have focused on the functions of GRAS genes, and various investigations have revealed that GRAS genes are related to plant growth, development, and stress response in *Solanum lycopersicum*, *Gossypium hirsutum*, *Medicago truncatula*, and *Vitis vinifera* [15]. Our results also revealed significant differences in the powdery mildew stress-induced GRAS expression, indicating the involvement of these GRAS genes in powdery mildew stress tolerance in melon. Accordingly, our study establishes a structural and functional framework for investigating melon GRAS proteins. Although the melon genome has already been sequenced, identification of melon GRAS genes and investigation of their functions, such as powdery mildew response, are still in their early stages. Our results will facilitate further studies on the functions of GRAS genes important in the abiotic stress response and the development of molecular breeding programs that can be used to enhance abiotic stress tolerance in melon.

4 Conclusion

We identified 37 GRAS gene family genes in melon genome for the first time, and revealed their structure and evolutionary characteristics. In addition, we revealed the response pattern of this gene family when melon was infected by powdery mildew. Our results provide useful information for a better

understanding of GRAS genes and provide the foundation for additional functional exploration of the melon GRAS gene family in powdery mildew stress response.

Acknowledgement: Yanfei Bi and Bin Wei performed the data analyses and wrote the manuscript. Ying Meng and Zhongzhao Li contributed to analysis and manuscript preparation. Zhenghui Tang and Feng Yin contributed to the conception of the study. Chuntao Qian designed the experiment and revised the manuscript.

Funding Statement: This work was supported by the Science and Technology Project of Suzhou (SNG2017083) and the Science and Technology Project of Changshu (CN201701).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Garcia-Mas, J., Benjak, A., Sanseverino, W., Bourgeois, M., Mir, G. et al. (2012). The genome of melon (*Cucumis melo* L.). *Proceedings of the National Academy of Sciences, 109(29),* 11872–11877. DOI 10.1073/pnas.1205415109.
2. Ruggieri, V., Alexiou, K. G., Morata, J., Argyris, J., Pujol, M. et al. (2018). An improved assembly and annotation of the melon (*Cucumis melo* L.) reference genome. *Scientific Reports, 8(1),* 1–9. DOI 10.1038/s41598-018-26416-2.
3. Ezura, H., Fukino, N. (2009). Research tools for functional genomics in melon (*Cucumis melo* L.): Current Status and Prospects. *Plant Biotechnology, 26(4),* 359–368. DOI 10.5511/plantbiotechnology.26.359.
4. Jiao, Z., Sun, J., Wang, C., Dong, Y., Xiao, S. et al. (2018). Genome-wide characterization, evolutionary analysis of WRKY genes in cucurbitaceae species and assessment of its roles in resisting to powdery mildew disease. *PLoS One, 13(12),* e0199851. DOI 10.1371/journal.pone.0199851.
5. Joobeur, T., King, J. J., Nolin, S. J., Thomas, C. E., Dean, R. A. (2004). The fusarium wilt resistance locus Fom-2 of melon contains a single resistance gene with complex features. *The Plant Journal, 39(3),* 283–297. DOI 10.1111/j.1365-313X.2004.02134.x.
6. Natarajan, S., Kim, H. T., Thamilarasan, S. K., Veerappan, K., Park, J. I. et al. (2016). Whole genome re-sequencing and characterization of powdery mildew disease-associated allelic variation in melon. *PLoS One, 11(6),* e0157524. DOI 10.1371/journal.pone.0157524.
7. Pech, J. C., Bouzayen, M., Latché, A. (2008). Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Science, 175(1–2),* 114–120. DOI 10.1016/j.plantsci.2008.01.003.
8. Ríos, P., Argyris, J., Vegas, J., Leida, C., Kenigswald, M. et al. (2017). ETHQV 6.3 is involved in melon climacteric fruit ripening and is encoded by a NAC domain transcription factor. *The Plant Journal, 91(4),* 671–683. DOI 10.1111/tpj.13596.
9. Pereira, L., Santo Domingo, M., Ruggieri, V., Argyris, J., Phillips, M. A. et al. (2020). Genetic dissection of climacteric fruit ripening in a melon population segregating for ripening behavior. *Horticulture Research, 7(1),* 1–18. DOI 10.1038/s41467-020-16924-z.
10. Ge, C., Zhao, W., Nie, L., Niu, S., Fang, S. et al. (2020). Transcriptome profiling reveals the occurrence mechanism of bisexual flowers in melon (*Cucumis melo* L.). *Plant Science, 301*, 110694. DOI 10.1016/j.plantsci.2020.110694.
11. Gao, C., Sun, J., Dong, Y., Wang, C., Xiao, S. et al. (2020). Comparative transcriptome analysis uncovers regulatory roles of long non-coding RNAs involved in resistance to powdery mildew in melon. *BMC Genomics, 21(1),* 125. DOI 10.1186/s12864-020-6546-8.
12. Hong, Y. J., Hossain, M. R., Kim, H. T., Park, J. I., Nou, I. S. (2018). Identification of two new races of *podosphaera xanthii* causing powdery mildew in melon in South Korea. *The Plant Pathology Journal, 34(3),* 182. DOI 10.5423/PPJ.OA.12.2017.0261.

13. Polonio, Á., Pineda, M., Bautista, R., Martínez-Cruz, J., Pérez-Bueno, M. L. et al. (2019). RNA-Seq analysis and fluorescence imaging of melon powdery mildew disease reveal an orchestrated reprogramming of host physiology. *Scientific Reports, 9(1),* 1–16. DOI 10.1038/s41598-019-44443-5.
14. Jones, J. G., Korir, R. C., Walter, T. L., Everts, K. L. (2020). Reducing chlorothalonil Use in fungicide spray programs for powdery mildew, anthracnose, and gummy stem blight in melons. *Plant Disease, 104(12),* 3213–3220. DOI 10.1094/PDIS-04-20-0712-RE.
15. Huang, W., Xian, Z., Kang, X., Tang, N., Li, Z. (2015). Genome-wide identification, phylogeny and expression analysis of GRAS gene family in tomato. *BMC Plant Biology, 15(1),* 1–18. DOI 10.1186/s12870-015-0590-6.
16. Pysh, L. D., Wysocka-Diller, J. W., Camilleri, C., Bouchez, D., Benfey, P. N. (1999). The GRAS gene family in arabidopsis: Sequence characterization and basic expression analysis of the SCARECROW-like genes. *The Plant Journal, 18(1),* 111–119. DOI 10.1046/j.1365-313X.1999.00431.x.
17. Tian, C., Wan, P., Sun, S., Li, J., Chen, M. (2004). Genome-wide analysis of the GRAS gene family in rice and arabidopsis. *Plant Molecular Biology, 54(4),* 519–532. DOI 10.1023/B:PLAN.0000038256.89809.57.
18. Wang, Y., Shi, S., Zhou, Y., Zhou, Y., Yang, J. et al. (2016). Genome-wide identification and characterization of GRAS transcription factors in sacred lotus (*Nelumbo nucifera*). *PeerJ, 4*, e2388. DOI 10.7717/peerj.2388.
19. Chen, Y. Q., Tai, S. S., Wang, D. W., Ding, A. M., Sun, T. T. et al. (2015). Homology-based analysis of the GRAS gene family in tobacco. *Genetics and Molecular Research, 14(4),* 15188–15200. DOI 10.4238/2015. November.25.7.
20. Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J. et al. (1997). The arabidopsis GAI gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes & Development, 11(23),* 3194–3205. DOI 10.1101/gad.11.23.3194.
21. Silverstone, A. L., Ciampaglio, C. N., Sun, T. P. (1998). The arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *The Plant Cell, 10(2),* 155–169. DOI 10.1105/tpc.10.2.155.
22. Di Laurenzio, L., Wysocka-Diller, J., Malamy, J. E., Pysh, L., Helariutta, Y. et al. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the arabidopsis root. *Cell, 86(3),* 423–433. DOI 10.1016/S0092-8674(00)80115-4.
23. Bolle, C. (2004). The role of GRAS proteins in plant signal transduction and development. *Planta, 218(5),* 683–692. DOI 10.1007/s00425-004-1203-z.
24. Liu, X., Widmer, A. (2014). Genome-wide comparative analysis of the GRAS gene family in *populus*. *Arabidopsis and Rice. Plant Molecular Biology Reporter, 32(6),* 1129–1145. DOI 10.1007/s11105-014-0721-5.
25. Mistry, J., Finn, R. D., Eddy, S. R., Bateman, A., Punta, M. (2013). Challenges in homology search: HMMER3 and convergent evolution of coiled-coil regions. *Nucleic Acids Research, 41(12),* e121–e121. DOI 10.1093/nar/gkt263.
26. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J. et al. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics, 10(1),* 1–9. DOI 10.1186/1471-2105-10-421.
27. Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y. et al. (2014). Pfam: The protein families database. *Nucleic Acids Research, 42(D1),* D222–D230. DOI 10.1093/nar/gkt1223.
28. Li, W., Cowley, A., Uludag, M., Gur, T., McWilliam, H. et al. (2015). The EMBL-eBI bioinformatics web and programmatic tools framework. *Nucleic Acids Research, 43(W1),* W580–W584. DOI 10.1093/nar/gkv279.
29. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A. et al. (2007). Clustal W and clustal X version 2.0. *Bioinformatics, 23(21),* 2947–2948. DOI 10.1093/bioinformatics/btm404.
30. Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7. 0 for bigger datasets brief communication. *Molecular Biology and Evolution, 33(7),*1870–1874. DOI 10.1093/molbev/msw054.
31. Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E. et al. (2009). MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Research, 37(2),* W202–W208. DOI 10.1093/nar/gkp335.
32. Hu, B., Jin, J., Guo, A. Y., Zhang, H., Luo, J. et al. (2015). GSDS 2.0: An upgraded gene feature visualization server. *Bioinformatics, 31(8),* 1296–1297. DOI 10.1093/bioinformatics/btu817.

33. Zhu, Q., Gao, P., Wan, Y., Cui, H., Fan, C., Liu, S. et al. (2018). Comparative transcriptome profiling of genes and pathways related to resistance against powdery mildew in two contrasting melon genotypes. *Scientia Horticulturae, 227*, 169–180. DOI 10.1016/j.scienta.2017.09.033.
34. Chen, S., Zhou, Y., Chen, Y., Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics, 34(17),* i884–i890. DOI 10.1093/bioinformatics/bty560.
35. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R. et al. (2013). Tophat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology, 14(4),* 1–13. DOI 10.1186/gb-2013-14-4-r36.
36. Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G. et al. (2010). Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology, 28(5),* 511–515. DOI 10.1038/nbt.1621.
37. Kolde, R., Kolde, M. R. (2015). Package 'pheatmap'. *R Package, 1(7),* 790. https://mran.microsoft.com/snapshot/2017-09-01/web/packages/pheatmap/pheatmap.pdf
38. Guilfoyle, T., Hagen, G., Ulmasov, T., Murfett, J. (1998). How does auxin turn on genes?. *Plant Physiology, 118(2),* 341–347. DOI 10.1104/pp.118.2.341.
39. Xue, L., Cui, H., Buer, B., Vijayakumar, V., Delaux, P. M. et al. (2015). Network of GRAS transcription factors involved in the control of arbuscule development in *lotus japonicus*. *Plant Physiology, 167(3),* 854–871. DOI 10.1104/pp.114.255430.
40. Rich, M. K., Courty, P. E., Roux, C., Reinhardt, D. (2017). Role of the GRAS transcription factor ATA/RAM1 in the transcriptional reprogramming of arbuscular mycorrhiza in *petunia hybrida*. *BMC Genomics, 18(1),* 1–14. DOI 10.1186/s12864-017-3988-8.
41. Hartmann, R. M., Schaepe, S., Nübel, D., Petersen, A. C., Bertolini, M. et al. (2019). Insights into the complex role of GRAs transcription factors in the arbuscular mycorrhiza symbiosis. *Scientific Reports, 9(1),* 1–15. DOI 10.1038/s41598-019-40214-4.

Appendix

Figure S1: The GO enrichment analysis of CmGRASs.

Table S1: Regulatory elements in the 2 kb upstream regions of the CmGRAS genes.

Table S2: The GO annotation of all CmGRASs.

Table S3: The enrichment score, gene counts and qvalue of GO enrichment analysis.

Table S4: Primer sequence.

Table S5: GenBank accession number.