Genome-wide Identification and Expression Analysis of GRAS Genes in Cucumber

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Abstract  GRAS transcription factors regulate various biological processes in plant growth, development and stress responses. Cucumber (Cucumis sativus L.) is an economically important vegetable crop. However, the biological functions of GRAS gene family remain largely unknown in cucumber. In order to explore the potential function of the GRAS gene family in cucumber, the whole genomic identification of CsGRAS family was performed, and the gene structure, protein structure, characteristic, subcellular locations, phylogenetic relationship and tissue expression pattern were analyzed by bioinformatics. Here, a total of 37 GRAS members were identified in cucumber genome, most of the CsGRAS proteins are neutral or acidic protein, which are encoded by a single exon. Phylogenetic analysis divided CsGRAS members into 16 subfamilies, with each having distinct conserved domains and functions. 37 GRAS genes are unevenly distributed on the 7 chromosomes of cucumber and contain 1 pair of tandem repeat genes and 3 pairs of fragment repeat genes. Gene expression analysis in various tissues demonstrated that most CsGRAS genes showed tissue-specific expression, uncovering their potential function in cucumber growth and development. This study provided a comprehensive analysis of the cucumber GRAS gene family and laid a foundation for further studying the roles of GRAS gene family members in cucumber.

Keywords  Cucumber; GRAS gene family; Bioinformatics analysis; Expression pattern

GRAS proteins, an important family of transcriptional regulators, play an important role in the growth and development of plants and are named after the acronyms of three initially identified members: Gibberellic acid insensitive (GAI), Repressor of GA1 (RGA) and Scarecrow (SCR) (Bolle, 2004). GRAS proteins are composed of 400–700 amino acid residues, and contain several highly-conserved motifs at their C-termini, such as LHRI (Leucine heptad repeat I), VHIID, LHRRII (Leucine heptad repeat II), PFYRE and SAW (Lee et al., 2008). Except for two conserved N-terminal motifs (DELLA and TVHYNP) characterized only for the members of DELLA subgroup, N-termini of other GRAS proteins display large differences, which may determine functional specificity of such regulatory proteins (Sun et al., 2011).

GRAS proteins are widespread in plants. 34, 60, 53, 106, 48, 43, 34 and 127 members were identified in Arabidopsis thaliana, Oryza sativa, tomato (Solanum lycopersicum), Populus, Chinese cabbage(Brassica rapa), Vitis vinifera, Amborella trichopoda and Malus domestica, respectively(Tian et al., 2004; Ma et al., 2010; Abarca et al., 2014; Song et al., 2014; Huang et al., 2015; Lu et al., 2015; Cenci and Rouard, 2017; Zhang et al., 2017). GRAS family members in two model plants, Arabidopsis and rice, were classified into eight distinct subfamilies, namely DELLA, HAM (HAIRY MERISTEM), LISCL (Lilium longiflorum SCR-like), PAT1 (phytochrome A signal transduction), LS (LATERAL SUPPRESSOR), SCR, SHR (SHORT ROOT) and SCL3(Tian et al., 2004). However, the number of subfamily was ranged from 8 to 17 in other plants, such as 11 subfamilies have been identified in sweet orange (Zhang et al., 2019), 13 in Populus trichocarpa (Liu and Widmer, 2014), 16 in Solanum lycopersicum (Huang et al., 2015) and 17 in Amborella trichopoda (Cenci and Rouard, 2017).

The diverse structure of GRAS proteins determine its role in various physiological processes during plant growth and development, such as meristem formation, root development, gibberellin acid signal transduction,
gametogenesis, phytochrome signal transduction and the response to stresses (Cenci and Rouard, 2017). Considering the fact that amino acid sequences have difference in each subfamily, each group might possess distinct functions. Members of the PAT1 subfamily play an important role in light signal transduction, such as SCL3 mainly function as positive regulator mediating phytochrome-B (phyB) signaling pathway (Bolle et al., 2000), PAT1, SCL5 and SCL21 participate in phyA signaling pathway (Torres-Galea et al., 2006); DELLA subfamily members contain DELLA conserved domains and play a negative regulatory role in gibberellin signaling pathway (Sun and Gubler, 2004); SCR and SHR subfamily members are involved in regulating root and leaf growth and development, such as AtSHR and AtSCR regulating root and shoot radial organization via a SCR/SHR complex (Cui et al., 2007; Helariutta et al., 2000); LS subfamily members are related to the development of axillary meristem initiation; HAM subfamily members play an irreplaceable role in maintaining the shoot meristem (Li et al., 2003).

Cucumber (Cucumis sativus L.), the important economic crops, is one of the most widely grown and consumed vegetable crops due to crispy taste, rich nutrition, and diverse eating methods in China. The accomplishment of whole genome sequencing provides a platform for us to conduct genome-wide analysis for an entire gene family in cucumber (Huang et al., 2009). By far, transcription factor families, such as MADS-box, WOX and LTP have been characterized in cucumber (Hu and Liu, 2012; Gu et al., 2020; Wang et al., 2020). However, the GRAS transcription factor family closely related to cucumber growth and development has not been identified and analyzed in detail. In this study, a total of 37 CsGRAS genes were identified at the genome-wide level using bioinformatics technology, their gene structure, protein structure and phylogenetic relationships were analyzed, and their chromosomal locations and expression patterns in different tissues were studied. The present study provides data support to further illuminate molecular functions of GRAS genes in regulation of cucumber growth and development.

1 Results
1.1 Genome-wide identification of GRAS gene family in cucumber
To identify GRAS genes in cucumber, a BLASTP search was performed using the A. thaliana, O. sativa and S. lycopersicum GRAS proteins as queries and a total of 39 candidate protein sequences were obtained. Using Pfam and SMART to analyze the candidate protein sequences, 37 cucumber GRAS transcription factor family members were finally identified. Each gene was named based on its location on the chromosome (Table 1).

1.2 Gene structures analysis
The gene structure of cucumber GRAS family members was analyzed using GSDS (Figure 1). The results showed that the length of 37 CsGRAS genes was between 1.0 and 4.0 Kb. Among them, 28 GRAS genes did not have any introns, 8 had one intron, and only one (CsGRAS5) had two introns.

1.3 Protein sequences and conserved motifs analysis
The analysis of cucumber GRAS protein sequence characteristics showed that the length of CsGRAS proteins varied greatly, with length ranging from 300 to 900 aa, the shortest was 363 aa (CsGRAS8), the longest was 857 aa (CsGRAS18). Their molecular weights ranged from 41.84 to 92.74 kDa, and their isoelectric points varied from 4.84 to 7.63. Most of the CsGRAS proteins were neutral or acidic protein, except that the isoelectric point of the protein encoded by CsGRAS37 was greater than 7.0, which was an alkaline protein. Subcellular localization analysis showed that all 37 CsGRAS proteins were located in the nucleus (Table 1).

The protein conserved motifs of cucumber GRAS gene family members were analyzed using MEME. A total of 10 potential conserved motifs (named motif 1-10) were identified, with more motifs locating at C-terminus than at N-terminus (Figure 2). Most CsGRAS proteins (57%) contained all conserved motifs, CsGRAS4 and CsGRAS19 did not contain motif 9, and CsGRAS27 only contained motifs 1, 5, 6 and 9. Moreover, the motifs from the same subfamily nearly hold the similar patterns. For example, all members of the SCL3 subfamily contained motifs 9, 5, 1, 6, 8, 8, 4, 7 and 3, while members of the SCL4 / 7 subfamily contained motifs 5, 1, 6, 8, 10, 4, 7, 2 and 3.
1.4 Phylogenetic analysis of the GRAS transcription factor family

To explore the evolutionary relationships of the GRAS gene family in cucumber, a total of 184 GRAS proteins comprising 34 from Arabidopsis, 60 from rice, 53 from tomato and 37 from cucumber, were used to construct an phylogenetic tree. The results showed that the relationship between CsGRAS genes and SIGRAS members was close (Figure 3). According to the evolutionary relationship, 184 GRAS genes were divided into 17 subfamilies. The distributions of candidate CsGRASs among the different subfamilies were as follows: HAM(4), LISCL(3), SHR(3), NSP1(1), SCL32(2), PAT(6), DELLA(4), SCR(3), SCL3(2), DLT(1), SCL4/7(2), LS(1), NSP2(2), MIG1(1), RAD1(1), RAM1(1). Of these, PAT1, LISCL, HAM and DELLA subfamily contained the most members.
No members of the SCLA subfamily were identified in cucumber, indicating that the genes of this subfamily may have been specifically deleted in cucumber.

Figure 1 Gene structures and evolutionary relationship analysis of cucumber GRAS genes

Figure 2 Protein structure of cucumber GRAS genes
1.5 Chromosomal location analysis

The distribution of cucumber GRAS gene on chromosome was analyzed using MapChart software. The results showed that 37 CsGRAS genes were unevenly distributed across 7 chromosomes of cucumber. Among those members, Chr6 occupied the largest number of GRAS genes (n=10), followed by Chr3 (n=7) and the least was Chr2 (n=2) (Figure 4). A total of 1 pair of tandem repeat genes (CsGRAS15 & CsGRAS16) and 3 pairs of segmental repeat genes (CsGRAS3 & CsGRAS10, CsGRAS3 & CsGRAS27, CsGRAS10 & CsGRAS27) were detected in cucumber GRAS genes.

![Figure 3 Phylogenetic analysis of GRAS protein family in Arabidopsis, tomato, rice and cucumber](image)

![Figure 4 Chromosome location of cucumber GRAS genes](image)
1.6 Expression of GRAS genes in different tissues

To gain insights into the role of the GRAS genes, RNA-seq data of 37 CsGRAS genes from different tissues were downloaded and analysed. The results showed that the expression of CsGRAS genes was tissue-specific. CsGRAS29 and CsGRAS33 were only highly expressed in leaves; CsGRAS24 and CsGRAS30 were highly expressed in roots, indicating that they might play a role in root development; CsGRAS16 and CsGRAS19 were predominantly expressed in male flowers, indicating that they might be involved in the regulation of male flowers development; genes such as CsGRAS11, CsGRAS20 and CsGRAS21 were expressed in all tissues, while CsGRAS6, CsGRAS35 and CsGRAS28 were not expressed in all tissues (Figure 5).

Figure 5: The tissue expression of cucumber GRAS genes

2 Discussion

In this study, a total of 37 GRAS genes were identified in cucumber genome (Table 1). Compared with Vitis vinifera (43), Brassica rapa (48), Capsicum annuum (50), Solanum lycopersicum (53), Oryza sativa (60), Populus (106) and Malus domestica (127) (Tian et al., 2004), the cucumber GRAS gene family members are relatively small. The variation of GRAS gene number might be related to gene duplication events or genome size. This study detected one pair of tandem duplicated CsGRAS genes and 3 pairs of segmental duplicated CsGRAS genes. However, 15 SIGRAS members were identified as tandem duplications in tomato, two pairs of tandem duplicated CaGRAS genes and 10 pairs of segmental duplicated CaGRAS genes were identified in pepper (Lu et al., 2015; Liu et al., 2018). Moreover, cucumber genome size (350 Mb) was significantly smaller than the tomato genome (900 Mb) and pepper genome (3.48 Gb), indicating that the relatively small number of members of cucumber GRAS gene family might be related to the small genome of cucumber and the low occurrence of chromosome replication events.

Analysis of the cucumber GRAS gene structures revealed that most CsGRAS genes (28) contained just one exon (Figure 1). The high percentage of such intronless GRAS genes was detected as 67.6%, 54.7% and 83.3% in Arabidopsis, Populus and Chinese cabbage (Tian et al., 2004; Lee et al., 2008; Song et al., 2014), respectively, evidencing again that the GRAS proteins were highly conserved among those plant species. The GRAS proteins contained 5 conserved domains at the C-terminus, all of which had important functions. Based on the identified 10 conserved motifs in cucumber, most CsGRAS protein (57%) with all conserved motifs. And the types, numbers and order of conserved motifs encoded by members of the same subfamily were very consistent, such as members of the SCL3 family contained motif 9, 5, 1, 6, 8, 4, 3, 7 and 3, while members of the SCL4/7 family contained motif 5, 1, 6, 8, 10, 4, 7, 2 and 3 (Figure 2).
To explore the function of those CsGRAS genes, we analyzed the expression patterns of CsGRAS members in different tissues and found that the expression of CsGRAS genes was tissue-specific (Figure 5). CsGRAS19 was highly expressed in male flowers, and in Arabidopsis, its homologous genes AtPAT1 and SCL13 were involved in phyA and phyB signaling pathway, which indicated that the function of CsGRAS19 might have been differentiated during evolution. CsGRAS24 and CsGRAS30 from the SHR subfamily were highly expressed in roots, which was consistent with the function of the homologous gene AtSHR that regulated radial growth of roots (Cui et al., 2007), indicating that the SHR genes in cucumber may also be involved in root development. CsGRAS11, CsGRAS20 and CsGRAS21 from the DELLA subfamily were highly expressed in all tissues, while in Arabidopsis, DELLA subfamily members played a negative regulatory role in the process of gibberellin signal transduction (Sun and Gubler, 2004). Therefore, CsGRAS11, CsGRAS20 and CsGRAS21 may play a key role in the gibberellin signal transduction. However, CsGRAS6, CsGRAS28 and CsGRAS35 from the RAM1, NAP2 and SCR subfamilies were not expressed in all tissues, indicating that these genes may have lost their functions during evolution.

In general, our study identified the cucumber GRAS gene family at the genome-wide level using bioinformatics technology, analyzed its gene structure, conserved motifs and evolutionary relationships, determined its distribution on the chromosome and the expression patterns in different tissues. The present study provides a theoretical basis for further exploring the functions of cucumber GRAS members.

3 Materials and Methods
3.1 Identification of GRAS genes in cucumber
The identification and analysis of cucumber GRAS genes were performed using the Chinese Long V3.0 genome sequence data of cucumber deposited at Cucurbit Genomics Data website (http://cucurbitgenomics.org/organism/20). Arabidopsis GRAS genes were obtained from TAIR (https://www.Arabidopsis.org/), whereas rice GRAS genes were downloaded from RGAP (http://rice.plantbiology.msu.edu). The tomato GRAS information was obtained from SGN (https://solgenomics.net/). Meanwhile, all AtGRAS, OsGRAS and SIGRAS proteins were used as queries to search for putative counterparts in cucumber by using BLASTP. The Pfam and SMART tools were used to verify the screened protein sequence (ID: PF03514.11) and deleted candidate sequences that were not conserved in the GRAS domain. Cucumber GRAS family members were named according to their position on the chromosome.

3.2 Gene structure analysis
The cucumber genome database (http://cucurbitgenomics.org/organism/20) was used to download the DNA and CDS sequences of CsGRAS genes, and the gene structures were analyzed and visualized using the Gene Structure Display Server online tool (http://gsds.cbi.pku.edu.cn/). The multiple sequence alignments of the amino acid sequences of CsGRAS family members were performed using MAFFT v5.3 under the default settings. The phylogenetic tree was constructed using the neighbour joining (NJ) method in MEGA 7.0 based on the multiple sequence alignment of CsGRAS family members under the default settings. The reliability of the statistical analysis was improved by performing bootstrap analysis with 1000 replicates to assess statistical support for each node.

3.3 Protein sequences and conserved motifs analysis
The biochemical properties of the CsGRAS proteins were predicted using the ExPASy Proteomics Server (http://expasy.org/) and their subcellular localization was investigated using Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). The conserved motifs in CsGRAS proteins were identified with MEME 4.9.1 (http://meme-suite.org/), and visualized with WebLogo (http://weblogo.berkeley.edu/logo.cgi).

3.4 Phylogenetic analysis
The multiple sequence alignments of the amino acid sequences of AtGRAS, CsGRAS, OsGRAS and SIGRAS family members were performed using MAFFT v5.3 under the default settings. The phylogenetic tree was constructed using the neighbour joining (NJ) method in MEGA 7.0 based on the multiple sequence alignment of AtGRAS, CsGRAS, OsGRAS and SIGRAS family members under the default settings. The reliability of the statistical analysis was improved by performing bootstrap analysis with 1000 replicates to assess statistical support for each node. The tree file was visualized with Evolview (http://120.202.110.254:8280/evolview).
3.5 Genome distribution analysis
The gene loci of CsGRAS genes were extracted from the cucumber genome database (http://cucurbitgenomics.org/organism/20). The chromosomal locations of CsGRAS genes were visualized with MapChart software.

3.6 Transcriptome data analysis and Gene expression heatmap
The FPKM (Fragments per kilobase of exon per million reads mapped) values of 7 tissue transcriptome data of root, stem, leaf, female flower, male flower, ovary and tendril were downloaded from the cucumber genome database(http://cucurbitgenomics.org/). Based on the FPKM value, TBtools software was used to visualize the expression profile of the CsGRAS genes.

Authors’ contributions
Tang Rui is the experimental design and executor of the experimental research; Tang Rui completed the data analysis and the writing of the first draft of the paper; Han Ni, Hu Yajing and Wang Lixue participated in the experimental design and analysis of the experimental results; Ren Zhonghai and Wang Lina conceive and person in charge, guide experiment design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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