Genome-wide Identification of the GRAS Gene Family of *Actinidia Arguta*

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Abstract

GRAS transcription factors play important roles in plant growth, development, and abiotic and biotic stress responses. In this study, the genome-wide identification of the transcription factor family of *Actinidia arguta* was carried out including an analysis of the physical and chemical properties, phylogenetic development, gene structure, collinearity between genes, and protein interactions. A total of 88 GRAS genes were identified in the genome of *Actinidia arguta* with protein lengths of 103-510 aa, a molecular mass of 11,603.25-22,457.96 kDa, and isoelectric points in the hydrophilic range between 4.45 and 6.50. From these genes, 67 were located in the nucleus and 21 in the chloroplast. The identified genes were further divided into eight subfamilies: SCR, HAM, DELLA, PAT1, SHR, SCL4/7, and *GIGR*. Members of the same subfamily had similar gene structures and conserved motifs. Motif 5 was highly conserved in the GRAS family. On the chromosomes of LG3, LG15, LG22, LG24, LG26 and LG28, there was a large number of tandem duplications of GRAS genes, with 64 pairs of genes orthologous with *Arabidopsis thaliana*. The analysis of protein interactions found that there were interactive relationships between SCL28 in the DLT subfamily and SCL14 in the LISCL subfamily and between SCL13 in the PAT1 subfamily and proteins of the LAS subfamily. Interactions were also observed between the SCL30, SCL33, and HAM4 proteins in the LISCL subfamily. This study, therefore, provides a reference for mining and verification within the GRAS genes in the *Actinidia arguta* genome.

Background

The GRAS gene family is classified as a plant-specific transcriptional regulatory family. The three earliest members of this family that were identified include gibberellic acid insensitive (GAI), repressor of GA1-3 mutant (RGA), and scarecrow (SCR), referring to the origin of its name [1–3]. According to the different functions of *Arabidopsis thaliana* GRAS transcription factors, The GRAS family is divided into eight subfamilies, namely, SCR, Hairy meristem (HAM), DELLA, LISCL, Short-root (SHR), Scarecrow-like 3 (SCL3), phytochrome A signal transduction 1 (PAT1), and LS. However, based on research on molecular signal recognition, the *Arabidopsis thaliana* GRAS transcription factors are divided into 10 subfamilies that include HAM, DLT, DELLA, AtPAT1, AtSCL3, AtSCL4/7, LISCL, AtLAS, AtSCR, and AtSHR. From a comparison and analysis of GRAS protein sequences in rice, poplar, tomato, and grape, a total of 13 subfamilies can be identified including the newly identified subfamilies Pt20, Os19, and Os4 [4].

A large number of studies have demonstrated that the GRAS family is involved in multiple processes including plant development, hormone signal transduction, and response to environmental signals [5]. For example, in *Arabidopsis thaliana*, SHR and SCR have been reported to regulate the formation of the cortex and endoderm. These genes have similar expression patterns in wheat roots, where they have also been shown to regulate the formation of the cortex and endoderm, suggesting that they may play an important role in regulating radial growth [5]. In *Arabidopsis*, the DELLA subfamily within the GRAS gene family consists of GAI, RGA, RGL1, RGL2, and RGL3. Among them, GAI, RGL1, and RGA are considered to be negative regulators of the gibberellin (GA) response, while SCL3 controls the steady-state of GA during root development [6]. In the study of Tartary buckwheat, it was found that the FtGRAS22 gene is related to

paclobutrazol metabolism [6]. GRAS proteins are also involved in abiotic and biotic stress responses. In the PAT1 subfamily, the three genes PAT1, SCL5 and SCL21 are positive regulators of phytochrome A signal transduction, while the major genes from the same subfamily participate in phytochrome B signal transduction and play key roles in hypocotyl elongation [7, 8]. Alfalfa qRT-PCR results have confirmed that MsGRAS51 is induced under drought stress and hormone (ABA, GA, and IAA) treatments, but its expression is down-regulated under salt stress [9]. At present, research on the GRAS gene family is becoming progressively extensive. However, the majority of this research is focused on crop species with relatively few studies on the MDH of *Actinidia arguta*. In this study, we identified the GRAS transcription factor family of *Actinidia arguta* using a bioinformatics approach and analyzed the physical and chemical properties, gene structure, conserved motifs, and expression patterns in different tissues under drought and salt stress in order to further understand the GRAS transcription factors in abiotic stress. This research can therefore provide a functional reference for further studies.

Materials And Methods

GRAS acquisition and prediction of physicochemical properties of *Actinidia chinensis*

The NCBI database (<https://www.ncbi.nlm.nih.gov/>) was used to download files and genome annotation from *Actinidia arguta* genome. The GRAS gene sequences from the *Arabidopsis thaliana* genome were downloaded from the TAIR database (<https://www.arabidopsis.org/>). Using the software TBtools, the *Arabidopsis thaliana* GRAS genes were compared with the *Actinidia arguta* genome using BLAST (E-value<1e-5) and the candidate genes of the GRAS family in *Actinidia arguta* were screened out. The candidate genes were submitted to the UniProt database (<https://www.uniprot.org/>) for batch comparison and only candidate genes that contained the GRAS conserved domains were used for further analysis. The online analysis software Expasy Proteomics (http://web.expasy.org/compute_pi) was used to analyze the physical and chemical properties of the GRAS transcripts. The online software Plant TFDB (<http://planttfdb.cbi.pku.edu.cn/prediction.php>) was then used to analyze the cis-acting elements of the 2,000 bp sequences upstream of the start codon of each GRAS gene.

Phylogenetic tree construction and gene structure analysis of GRAS genes in *Actinidia arguta*

The GRAS protein sequences of *Actinidia arguta* and *Arabidopsis thaliana* were imported into the MEGA 10.0 for comparison. These results were compared using the neighbor-joining method (NJ; execution parameter: Bootstrap method 1,000) to construct a phylogenetic tree. FigTree software was used to visualize the evolutionary tree. The online software NCBI-CDD (<https://www.ncbi.nlm.nih.gov/cdd/>) was used to predict the protein structures and detect the GRAS protein domain (E-value<0.01) in *Actinidia arguta*. The online software MEME (<http://meme-suite.org/>) was used to analyze the conservative motifs of the GRAS genes of *Actinidia arguta*, with the predicted number set to 10. The software TBtools was used to extract the relevant coding sequence (CDS) and untranslated region (UTR) of the *Actinidia arguta* GRAS gene from its genome annotation files [10]. Finally, TBtools software was used to combine the evolutionary tree, protein domains, gene conservative motifs, CDS, and UTR to construct an overall comparison diagram of the evolutionary relationships and structures of the GRAS genes.

Chromosome distribution and collinearity prediction of GRAS genes in *A. chinensis*

TBtools software was used to extract the GRAS gene location information from the *Actinidia arguta* genome file and gene annotation file to construct the physical map of the GRAS genes on the chromosomes. TBtools software was further used to calculate and plot the tandem duplication of GRAS on the chromosome of *Arabidopsis thaliana*, the collinearity genes existing between different chromosomes of this species, and the collinearity genes existing between *Arabidopsis thaliana* and *Vitis vinifera*.

Protein interaction network prediction and function enrichment analysis

The STRING database (<https://string-db.org/>) was used to construct a GRAS family interaction protein network (species model selection *Arabidopsis thaliana*) with the credibility value set to 0.4 and control the number of interacting proteins within 10. Functional annotation analysis was performed on different proteins in the predicted protein interaction network.

Results

Identification of the GRAS gene members and analysis of physical and chemical properties

Using the *Arabidopsis thaliana* GRAS gene and *Actinidia arguta* genome sequence alignment, the obtained genes were analyzed in the Swissport database. The genes with GRAS conserved domains were screened out. Finally, 88 GRAS genes were identified in the *Actinidia arguta* genome. The GRAS protein lengths in *Actinidia arguta* ranged from 103-510 aa, with an average length of 558 aa. The molecular mass ranged from 11,603.25-22,457.96 kDa, with an average molecular mass of 62,472.74 kD. The isoelectric points ranged from 4.45-6.50, indicating that these proteins were rich in acidic amino acids and were thus hydrophilic.. One GRAS transcription factor with an instability coefficient of <40 was considered to be a stable protein. Eighty0seven GRAS transcription factors with instability coefficients of >40 were identified as unstable proteins, suggesting that this protein family is not conserved. The average value of hydrophilicity (GRAVY) results demonstrated that all the proteins encoded by the GRAS gene of *Actinidia arguta* were hydrophilic. The subcellular locations of the GRAS transcription factors in *Actinidia arguta* were predicted using the BUSCA software. Of these, 67 were located in the nucleus and 21 in the chloroplast (Supplementary Table 1).

Analysis of promoter regions of the GRAS gene family

A total of 67 binding sites were predicted for the 88 GRAS genes of *Actinidia arguta*. These primarily included Sp1 and LAMP response elements involved in light response, LTR response elements involved in low-temperature responsiveness, and CGTCA-motif and TGACG-motif response elements involved in MeJA-responsiveness, which play key roles in the development of endosperm, cotyledons, and lateral roots. The MBS response element within the drought-inducible MYB binding site plays a key role in many

significant physiological and biochemical processes including stress response, plant growth, and development. Other elements identified included the ABRE response element involved in the abscisic acid response, a factor that affects the protein abundance of the positive regulator of abscisic acid signal, and the alkaline leucine zipper transcription factor ABRE-binding factor 2 (ABF2) that is considered to be the stress during vegetative growth and the main participants in the response. These six TFs were all related to plant resistance and metabolic regulation, confirming that this gene family is closely related to stress response, growth, and development (Supplementary Table 2 and Supplementary Figure 1).

Phylogenetic tree construction and gene structure analysis of the GRAS genes

In order to understand the evolutionary relationships between the different GRAS genes of *Actinidia arguta*, a phylogenetic tree of the *Actinidia arguta* and *Arabidopsis thaliana* GRAS genes was constructed. A total of 88 GRAS genes in *Actinidia arguta* were divided into eight subfamilies including LISCL, SCR, HAM, DELLA, PAT1, SHR, SCL4/7, and GIGR (Figure 1). The GRAS members of each subfamily have similar or related functions. In general, most subfamilies contained GRAS members from both species, suggesting that the differentiation of the GRAS genes occurred before the dicot-monocot differentiation. Furthermore, it was found that the GRAS genes of *Actinidia arguta* were unevenly distributed in the subfamilies from the topological structure of the phylogenetic tree. From the identified GRAS genes, the LISCL subfamily had the most members with 30, followed by the DELLA family with 27 members. The PAT1 and HAM families contained the least with only 9 members each. The diversity of gene structure as a possible basis for evolution can provide valuable information for the evolution of multi-gene families. The gene structure was analyzed based on the CDS and the genomic DNA sequences of each predicted gene. The gene structure of the same subfamily was principally the same and the conserved motifs of the same subfamily also had similarities (Figure 2). This suggests that the genes of the same subfamily may have similar functions. The maximum number of introns of the GRAS family genes in *Actinidia arguta* was 4. A total of 53 genes had no introns and the remaining 26 genes had 1 intron. Generally, GRAS genes from the same subfamily in *Actinidia arguta* had the same structure.

In order to reveal the diversity of the GRAS proteins in *Actinidia arguta* and further determine the relationships between the GRAS family members, MEME was used to predict the elements. A total of 10 conserved elements were identified from the candidate GRAS proteins of *Actinidia arguta* and the domain sequence identifiers (i.e. amino acid composition) of these conserved elements were presented in Figure 3. This study identified that all GRAS proteins in *Actinidia arguta* contained motif 5, indicating that it is highly conserved in the GRAS family. Furthermore, motifs 10, 6, 4, 1, 7 were highly conserved at the N-terminus of each subfamily member within SHR. The conserved motifs of ten members of PAT1, GIGR, and LISCL subfamilies showed good consistency, with a total of ten conserved elements (motifs 10, 6, 4, 1, 7, 9, 3, 2, 8 and 5). The DELLA and SCL4/7 subfamilies each had nine conserved elements (motifs 10, 6, 4, 1, 7, 9, 3, 2, 5 and 10, 6, 4, 1, 7, 9, 3, 8, 5, respectively). The closely related GRAS proteins in the phylogenetic tree contained the same conserved elements and some subfamilies had unique conserved elements, indicating that genes in the same subfamily may have similar functions. The analysis of the

gene structure and the position of conserved elements of the *Actinidia arguta* GRAS family determined that majority of the members were conserved in each subfamily.

Analysis of chromosome distribution and collinearity of GRAS genes in *Actinidia arguta*

The analysis of the GRAS gene positions on *Actinidia arguta* chromosomes demonstrated that genes were distributed on 29 chromosomes, with no significant correlation with the length of the chromosome (Figure 4). The largest number of GRAS genes (seven) was found on chromosome LG15. However, the lowest number of genes was found on the LG4, LG10, LG20, LG21, and LG29 chromosomes that each contained only one *GRAS* gene. The other chromosomes contained three to six GRAS genes. Tandem duplication of genes appeared on six of the chromosomes, especially on the chromosomes LG3, LG15, LG22, LG24, LG26, and LG28. There were eight pairs of *GRAS* gene duplications (the genes on the same chromosome are connected by blue lines, indicating that the related gene is a tandem repeat gene), which indicates that significant gene tandem duplication events have occurred in the GRAS family of *Actinidia arguta* and that the gene tandem duplication events play a key role in the expansion of the *GRAS* family in this species. The collinearity analysis between the chromosomes of *Actinidia arguta* identified 16 pairs of collinearity genes (represented with blue font) (Figure 5). These did not include Ac024553, Ac027128, Ac024554, Ac030278, and Ac030279 in the DELLA and PAT1 subfamilies. The other 83 belonged to different subfamilies, with many being paralogous as a result of gene duplications. Through the prediction of collateral homologous genes, we found that the LG4, LG17, LG21, and LG29 chromosomes had not undergone chromosomal segment duplication, unlike the other chromosomes. In order to further understand the evolutionary mechanisms, a collinearity diagram of the dicotyledon *Actinidia arguta* and *Arabidopsis thaliana* GRAS family was constructed (Figure 6). Finally, 64 pairs of orthologous genes were identified between *Actinidia arguta* and *Arabidopsis thaliana*, which is also in line with the evolutionary relationships between these two species.

Protein interaction network and functional enrichment analysis of the GRAS genes in *Actinidia arguta*

The STRING protein interaction database was used to predict the functional relationships and co-expression of the GRAS proteins in *Actinidia arguta* (species pattern selection *Arabidopsis thaliana*). The analysis of protein interactions found that there were interactive relationships between SCL28 in the DLT subfamily and SCL14 in the LISCL subfamily and between SCL13 in the PAT1 subfamily and proteins of the LAS subfamily. Interactions were also observed between the SCL30, SCL33, and HAM4 proteins in the LISCL subfamily. Therefore, it was hypothesized that these genes interact with each other in completing a physiological process (Figure 7). GO and KEGG enrichment analysis (Table 1) demonstrated that the *GRAS* genes in the interaction network participate in the formation of various cellular components, including the nucleus, nuclear speck, and spliceosome. The GRAS proteins were predicted to play roles in “sequence-

specific DNA binding”, “DNA-binding transcription factor activity”, and “nucleic acid binding”. The predicted pathways included “cell differentiation”, “multicellular organism development”, “mRNA splicing”, “negative regulation of seed germination”, “plant organ development”, and “leaf development”, as well as other development-related biological processes. The functional pathway of the KEGG enrichment primarily included the biological process of spliceosome and plant hormone signal transduction.

Table 2
Functional enrichment analysis of the GRAS genes.

Term description		False discovery rate
Cell component	nucleus	4.00E-25
	cell	2.26E-11
	nuclear speck	0.00042
	spliceosomal complex	0.0011
Molecular Function	sequence-specific DNA binding	2.11E-41
	DNA-binding transcription factor activity	2.9E-34
	nucleic acid binding	1.21E-29
Biology Process	negative regulation of gibberellic acid-mediated signaling pathway	0.00000412
	cell differentiation	0.0000671
	multicellular organism development	0.0000946
	mRNA splicing, via spliceosome	0.00086
	negative regulation of seed germination	0.0009
	plant organ development	0.0012
	negative regulation of the developmental process	0.0014
	shoot system development	0.0041
	regulation of shoot system development	0.0058
	tissue development	0.0071
	leaf development	0.0111
KEGG	Spliceosome	0.0027
	Plant hormone signal transduction	0.004

Discussion

With the advent of the high-throughput sequencing era and the sequencing of many genomes, a large amount of sequence information is available in the plant database. The use of bioinformatics technology to analyze genes has become the primary mechanism for breeders to mine gene functions. The functional verification analysis of genes has therefore become an important method in the field of plant gene research [22]. As an important tree species in the world, the *Actinidia arguta* genome sequence has been published. However, the underlying mechanisms of its resistance to external environmental and biological stresses remain still unclear. The GRAS genes are closely related to plant growth and stress resistance. The members of the gene family have been functionally verified and analyzed in multiple species [11, 12]. However, their function in *Actinidia arguta* has not been investigated to date.

The GRAS gene family is widely distributed in plants and the number of the family members is not correlated with the size of the genome [13]. In the process of biological evolution, plants mainly produce multiple members in gene families through extensive replication and diversification of their genomes [14]. In *Arabidopsis thaliana*, two tandem repeat events have been identified in the GRAS family [15]. Moreover, the tandem duplication of the GRAS gene family has been detected in tomato, *Oryza sativa*, poplar, pepper, and other species [16, 17]. This study identified 88 members of the GRAS gene family, lower than those of poplar (106) and apple (127) [18]. The lower number of gene family members may be due to the absence of a large-scale fragment duplication in the *Actinidia arguta* genome, as it is relatively conservative. Chromosome mapping analysis identified that the GRAS genes are widely distributed throughout the *Actinidia arguta* genome and that there were obvious tandem duplications, suggesting that tandem duplications play an important role in the amplification and evolution of this gene family.

Differences in gene structure play a vital role in the evolution of gene families. They are the adaptive process of speciation, which is more conducive to plants adapting to changes in the environment. Variations in gene structure can make more adaptable to the adverse conditions of the natural environment. In the same subfamily, *Actinidia arguta* SHR, PAT1, GIGR, and LISCL contained similar motifs. It was further hypothesized that members of the same subfamily may have similar functions. Using string to analyze the interacting proteins of the protein sequence and the *Arabidopsis thaliana* protein database as a reference, we identified that the differentially expressed genes (DEGs) in this family were similar to SCL28 in the *Arabidopsis thaliana* DLT subfamily and SCL14 in the LISCL subfamily and SCL13 in the PAT1 subfamily. There was an interactive relationship between genes in the LAS subfamily and SCL30, SCL33, and HAM4 genes in the LISCL subfamily. Therefore, it was hypothesized that they interact with each other in completing physiological process. Enrichment analysis showed that GRAS was involved in the biological process of plant hormone signal transduction. These results are consistent with previous studies demonstrating that GRAS family member proteins are related to abiotic stress response, specifically low temperatures, drought, salt, and others [19–21]. Prediction of the GRAS family protein interactions in *Actinidia arguta* is beneficial for further understanding of the functions of the GRAS family members.

Conclusions

Sequence alignment of the whole *Actinidia arguta* genome identified a total of 88 members of the GRAS family. These genes were grouped into eight different subfamilies with typical GRAS, LISCL, and DELLA conserved domains. The analysis of the physical and chemical indices, phylogenetic relationships, gene structure, and collinearity of the GRAS family of *Actinidia arguta* via bioinformatic methods demonstrated that chromosome fragment duplication and tandem duplication played important roles in the development of the 88 GRAS genes in *Actinidia arguta*.

Declarations

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Authors' contributions

Conceptualization, Z.L.; methodology, Z.L. and Y.S.; software, S.J.; validation, Y.R. and Y.W.; formal analysis, Z.L.; investigation, Y.S.; resources, S.J.; data curation, writing—original draft, Z.L.; writing—review and editing, Y.W.; project administration, Y.R.; funding acquisition, Y.W.; visualization, S.J.; All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

Data supporting the findings of this project are in the manuscript and its supplementary files.

Competing interests

The authors declare there are no competing interests.

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Supplementary Figure

Supplementary Figure 1 is not available with this version.

Figures

Figure 1

Phylogenetic tree of the GRAS gene family in *A. arguta* and *Arabidopsis*.

Different colors indicate and group together different subfamilies and different genes under the same color belong to the same subfamily.

Figure 2

Gene structure analysis of the *GRAS* genes in *A. arguta*.

Figure 3

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Gene motifs of the *GRAS* genes in *A. arguta*.

Figure 4

Chromosomal locations of the GRAS genes in *Actinidia arguta*.

Figure 6

Synteny analysis of the GRAS genes between *Actinidia arguta* and *Arabidopsis thaliana*.

Figure 7

Functional interaction network of the GRAS proteins.

Supplementary Files

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- [SupplementaryTable2cisactingelements.xlsx](#)