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Comprehensive Analysis of GASA Family Members in the Peanut Genome: Identification, Characterization, and Their Expressions in Response to Pod Development

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Abstract: The gibberellic acid-stimulated Arabidopsis (GASA) gene family is essential for plant growth and development, hormone level control, and phytohormone signal transmission. Different plants have been shown to contain numerous GASA homologs. However, there is no knowledge about these proteins in peanuts. In the current study, we performed a thorough bioinformatics and expression analysis and found 20, 22, and 40 GASA genes by genome-wide analyses of *A. hypogaea* L., *A. duranensis*, and *A. ipaensis*, respectively. We analyzed and predicted the physical properties of these genes. Based on the results of our phylogenetic analysis, the evolutionary tree constructed from the 40 AhGASA proteins was divided into seven categories, forming a total of 14 gene pairs. According to our observations, tandem duplication is a significant factor in the expansion of the GASA gene family. AhGASA was unevenly distributed on 20 chromosomes, and 17 tandem duplicated genes were identified. A co-lineage analysis with the A/B subgenome identified 69 linear/parallel homologous gene pairs. A cis-element analysis revealed that the AhGASA protein is crucial for hormone responsiveness. In materials with different size traits at various stages of peanut pod development, transcriptomics and RT-qPCR analyses revealed that AhGASA genes are expressed at various levels and are tissue-specific. This finding suggests that some AhGASA genes may be involved in controlling peanut pod size. This study suggests that GASA genes are crucial for controlling the development of peanut pods and provides the first systematic identification and analysis of GASA genes in peanut. These findings will help future research into the function of the GASA gene in the cultivated peanut.

Keywords: expression profile; GASA; genome-wide; peanut; plant gene families; pod development

Citation: Wu, Y.; Sun, Z.; Qi, F.; Zhao, M.; Dong, W.; Huang, B.; Zheng, Z.; Zhang, X. Comprehensive Analysis of GASA Family Members in the Peanut Genome: Identification, Characterization, and Their Expressions in Response to Pod Development. *Agronomy* **2022**, *12*, 3067. https://doi.org/10.3390/agronomy12123067

Academic Editor: MaoTeng Li

Received: 20 October 2022
Accepted: 1 December 2022
Published: 3 December 2022

1. Introduction

The GASA (gibberellic acid stimulated Arabidopsis) gene family is vital among plants; the expression of its members is induced by gibberellin. According to [1–3], GASA genes are involved in the regulation of growth and development processes as well as hormone levels in plants and phytohormone signal transduction.

First discovered in a tomato mutant in 1992 [4], GASA proteins have since been found in *Arabidopsis thaliana* [1,5], *Moso Bamboo* [6], apple [3], rice [7], wheat [8], *Glycine max* [9], *Solanum tuberosum* [10], grapevine [11], Sorghum bicolor [12], and cotton [13]. The majority of the tiny, cysteine-rich proteins that GASAs encode are found on the cell wall. An N-terminal signal peptide and a conserved C-terminal region with 12 cysteine residues are

two common features of GASA proteins. According to Sun's research, crucial peptide residues that are deleted or altered may result in the loss of biological functions [14]. This shows that the C-terminal domain has undergone very little change during its history and has significant biological functions.

GASA proteins have an impact on seed development, flower and root growth, and blooming period. For instance, *AtGASA2/3/5* and *AtGASA14* are implicated in Arabidopsis flower induction and ABA signaling [1]. *AtGASA4* promotes blooming in Arabidopsis, but *AtGASA5* represses or delays flowering. Similarly, GEG (the gerbera homolog of GASA 1 gene) slows petal development, whereas PRGL (proline-rich and GASA-like) stimulates gerbera petal development. More significantly, the overexpression of the *AtGASA4* gene increased the size, weight, and yield of seeds in Arabidopsis. Additionally, *VvGASA2/7* is involved in the development of grapevine (*Vitis vinifera* L.) seeds [11], *TaGASR7* is linked to grain length in wheat [15], and *OsGASA* increases rice grain size and length [7]. These findings demonstrate that GASA has enormous potential to contribute to the growth of fruits and increase crop breeding yield. Considering that this gene family may be heavily involved in the regulation of phytohormone signal transduction networks, the GASA protein also influences phytohormone response, which is primarily regulated by gibberellin and is also involved in the regulation of phytohormones such as BR, auxin, and abscisic acid [16–18]. Phytohormones are crucial for managing seed development, size, and quality in agriculture [19,20]. Gibberellin, a vital regulator of the growth and development of plants, is mainly found in vigorous growth sites and is involved in the lengthening of stems, the germination of seeds, the lengthening of hypocotyls, the ripening of fruits, the expansion of leaves, and the transition of plants from vegetative growth to flowering [21,22]. The placement of genes within the cell can also reveal information about their function. As with *OsGASR1* and *OsGASR2*, the majority of the discovered GASA proteins are found in the cell wall or apoptosome and have important roles in protein trafficking and localization [18,23,24]. Similar to *AtGASA4* and *AtGASA6*, they relocate to the nucleus in the absence of a signal peptide, where they are often detected at the cell periphery [16].

The cultivated peanut (*Arachis hypogaea* L.) is a significant source of edible oil and protein and is one of the most important cash crops [25–27]. The seed oil and protein from peanuts are important in fighting malnutrition and ensuring food security. In recent years, numerous peanut gene families, such as small auxin-upregulated RNAs (SAURs) [28], late embryogenesis abundant proteins (LEA) [29], the WRKY gene family [30], and the phytochrome-interacting factors (PIFs) gene family [31], have been described and investigated. One of the key agronomic characteristics of crops is their seed size. Larger seeds typically produce larger plants which are better able to compete for nutrients and light and to withstand stress [32]. In our comprehensive time-series comparative transcriptome analysis, we focused on the important roles of gibberellin, auxin, brassinolide, cytokinin, and abscisic acid in regulating seed size development in peanut and initially constructed regulatory networks for key factors [33]. The enrichment results disclosed a large number of genes in the gibberellin regulatory pathway, particularly the GASA gene family. However, to our knowledge, there is no report of a bioinformatics analysis of the GASA gene family at the genome-wide level. There is no information regarding the *GASA* gene family in peanut studies, despite the fact that *GASA* genes play significant roles as plant growth regulators, especially in seed size and weight. Overall, research on peanut seed size is still in its infancy, and there is a lack of new genes with important utilization value at present. Determining the members of the peanut *GASA* family and their possible roles will thus surely be helpful.

Therefore, in this study, GASA family genes are identified from the genomes of *A. hypogaea* L., *A. duranensis*, and *A. ipaensis*, and the distribution of each gene on chromosomes, the structural characteristics of the genes, and differences between cultivated and wild peanuts as well as phylogenetic relationships are analyzed. The possible functions of some AhGASA genes were inferred by a bioinformatics analysis and temporal transcrip-

tomics with differences in pod size traits, combined with RT-qPCR assays of peanut shells and seeds at different developmental periods and using different tissues. To the best of our knowledge, this research is the first comprehensive analysis of cultivated peanut GASA genes. Rarely have reports of this gene family in oilseed crops been made. The results of this study will serve as the basis for more in-depth research on the peanut GASA gene and the identification of important genes involved in the control of peanut pod size.

2. Materials and Methods

2.1. Screening and Identification of the GASA Family in the Peanut

To determine the GASA gene family members, as well as the *Arachis hypogaea* cv. Tifrunner protein, CDS, and genome sequences, Tifrunner was downloaded from PeanutBase (https://peanutbase.org/data/v2/Arachis/hypogaea/) (accessed on 15 March 2022), a database of peanut-related information. The reported GASA protein sequences of Arabidopsis were downloaded from TAIR (https://www.arabidopsis.org/) (accessed on 15 March 2022), and then, the local cultivar peanut genome database was constructed for a BlastP search with the Arabidopsis GASA protein sequences as the query object. The threshold $E \leq 1e^{-5}$ was set, number of hits $\geq$ 500, and num of hits $\geq$ 250. In order to identify the conserved domain and eliminate genes that lack the GASA domain, the genomic, CDS, protein, and 1.5 kb upstream promoter areas of the GASA genes were extracted and subjected to a query to the NCBI database. Based on the conserved structural domains of GASA proteins, further analysis was performed using the Pfam database. The physicochemical properties of the AhGASA gene, such as protein isoelectric point (PI) and molecular weight (MW), were analyzed using the online tool Expasy-Protparam (https://web.expasy.org/compute_pi/) (accessed on 20 March 2022). We used the online prediction tool WOLF-PSORT (https://wolfpsort.hgc.jp/) (accessed on 20 March 2022) to study the subcellular distribution of SNAT proteins. Using ExPASy (http://web.expasy.org/protparam/) (accessed on 25 March 2022), AhGASA protein sequence properties are predicted.

2.2. Phylogenetic Analysis of GASA Proteins

To further investigate the evolutionary relationships of the cultivated peanut GASA genes, we constructed an evolutionary tree of cultivated peanut-intraspecific GASA genes. To investigate the evolutionary relationships among AhGASA genes, the protein sequences of cultivated peanut AhGASA were entered into MEGA7 [34] and multiple sequence alignment analysis was performed using ClustalW. The intraspecific evolutionary tree was constructed using the maximum likelihood method with the following parameters: bootstrap replication: 1000; model and method: JTT model + G + I; Missing Data Treatment: partial deletion. The resulting phylogenetic tree was illustrated using the website Evolview [35].

2.3. Conserved Motif and Gene Structure Analyses

Using the entire genome annotation file of the domesticated peanut retrieved from PeanutBase, the gene structure of AhGASA members was ascertained. The online MEME predicts the motifs of the genes [36]. Using the Gene Structure Display Server (GSDS) program [37], we mapped the exon/intron structures of exons/introns using the CDS and genome sequences of the cultivated peanut genome. The evolutionary relationships, gene structure, and motif composition of GASA were mapped using TBtools [38].

2.4. Chromosome Localization, Duplications, and Evolutionary Analysis of AhGASAs

The physical location of the chromosome positions of two peanut wild species and one peanut cultivar variety were mapped using TBtools software. We downloaded the genome-wide annotation files of *A. hypogaea* L., *A. duranensis*, *A. ipaensis*, *Medicago truncatula* L., and *Glycine max* L., and used MCScanX to identify node and tandem repeat gene pairs in the

cultivated peanut genome and genomic co-linear gene pairs. We used TBtools software to visualize chromosomal location and gene duplication for members of five species of GASAs.

2.5. Promoter Analysis of AhGASAs

Approximately 2000 bp upstream sequences of AhGASAs were used to better understand the potential functions of the promoters. PlantCARE [39] was used to identify cis-regulatory elements present in the promoters of genes associated with stress responses and hormonal effects. We predicted cis-acting elements in the AhGASA gene's promoter region using the PlantCARE website.

2.6. Plant Materials

In this study, we determined the gene transcript levels in the following varieties: the large-grain variety YH15 with different pod length and width, seed length and width, and 100-seed weight phenotypes; the small-grain variety W1202; the long-pedigree line S181523; and the short-pedigree line S181517 from the RIL population, obtained by crossing them. Pods at the early stage of development and shells and seeds at mid- and late pod development were sampled separately; these five samples are subsequently referred to as Pod-25, Shell-35, Seed-35, Shell-45, and Seed45. The sampling was performed three times and samples were immediately frozen in liquid nitrogen and stored at −80 °C until further use. The raw data for RNA-seq were downloaded from NCBI (SRA accession number: PRJNA847769).

2.7. Gene Expression Analysis and Functional Annotation of AhGASAs

The output obtained after the comparison was processed by featureCounts [40] and the count values were converted into FPKM. The DEGseq2 [41] method was used to identify DEGs, and the screening criteria were set as p-value < 0.05 and $|\log2^{(\text{foldchange})}| > 1$. To analyze the potential functions of genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used. The KEGG enrichment analysis was performed using the OmicShare (https://www.omicshare.com/tools/) (accessed on 15 April 2022), and the Phyper of R version 3.6.3 was used to determine the p-value. Significant enrichment was defined as functional when p-value < 0.05.

2.8. Validation of Gene Expression

To further verify the reliability of AhGASA expression in transcriptome data, the mRNA expression levels of seven genes selected from AhGASAs were measured by RT-qPCR using the same batch of tissue samples. First, the total RNA was extracted using the TaKaRa MiniBEST Plant RNA Extraction Kit (Takara Bio Inc., Kusatsu, Shiga, Japan). Then, the RNA extracted from the pods was reverse-transcribed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit to cDNA (TaKaRa). Primers were designed using Oligo7 and checked for primer specificity using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (accessed on 1 May 2022). Gene-specific primers for RT-qPCR were synthesized at BGI (Beijing, China), were listed in Table S1. Subsequently, RT-qPCR was performed using the ABI QS5 RT-qPCR detection system (Applied Biosystems, USA) and Power Up™ SYBR™ Green Master Mix (A25742, Applied Biosystems, Foster City, CA, USA). The reaction system was configured using 5 µL 2X PowerUp SYBR Green Master Mix, 0.5 µL forward primer, 0.5 µL reverse primer, and 100 µg cDNA for RT-qPCR experiments. An ABI QS5 RT-qPCR system was used under the following procedure: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min in a 10 µL volume. At least three technical duplicates were run for each gene in each pool, and the entire sample preparation was conducted under cover and on ice. *ADH3* was used as the reference gene. Gene expression differences among samples were calculated by the $2^{-\Delta\Delta Ct}$ method, and a correlation analysis was performed using GraphPad Prism 8 to assess the agreement between RT-qPCR results and RNA-seq data [42].

3. Results

3.1. Composition and Protein Characteristics of the Peanut GASA-Gene Family

Fifteen AtGASA protein sequences were used as search criteria to search for GASA genes in the genomes of *A. hypogaea* L., *A. duranensis*, and *A. ipaensis*. After a homologous gene search and the elimination of protein sequences without conserved structural domains using Pfam, 20 members of the AdGASA-like family were identified in the AA subgenome, 22 members of the AiGASA-like family were identified in the BB subgenome, and 40 members of the AhGASA-like family were identified in cultivars. We simplified the gene names to *AdGASA1–AdGASA20*, *AiGASA1–AiGASA22*, and *AhGASA1–AhGASA40*, according to their positions on the chromosomes. The number of AhGASA genes was higher in this study compared to those reported in Arabidopsis, apple, potato, and soybean. Among all members of the AhGASA family, the C-terminus was found to consist of 50 amino acids (11 of which are cysteine residues) that form a conserved structural domain. In a multiple sequence comparison of the core structural domain of cultivated peanut, 13 amino acids were extremely conserved, 13 amino acids were extremely conserved in apple, 18 amino acids were extremely conserved in soybean, and 20 amino acids were extremely conserved in Trichoderma (Figure S1).

The physical characteristics of these genes, such as Gene ID, isoelectric point, protein molecular weight, protein length, and subcellular localization, were then examined and predicted (Table S2). AhGASA proteins have an isoelectric point between 6.06 (*AhGASA18*) and 9.70 (*AhGASA22*). All proteins have more positively charged amino acids than negatively charged amino acids, so the isoelectric point is almost always greater than 8. Protein lengths range between 60 (*AhGASA5*) and 202 (*AhGASA22*) amino acids, with *AhGASA22* and *AhGASA3* proteins having 202 and 198 amino acids, respectively, which differ significantly from other proteins. Most AhGASA proteins are stable, but *AhGASA5* has an instability index as high as 94.88. Studies have shown that this stability is closely related to the lifetime of proteins involved in cellular enzymatic reactions. The aliphatic index ranged between 27.67 (*AhGASA5*) and 100.57 (*AhGASA18*), and most of the proteins were hydrophilic; only three were hydrophobic. Subcellular localization predicts 36 genes in the cell wall, 2 genes in the nucleus, 1 gene in the chloroplast, and 1 gene in the endoplasmic reticulum. The predicted tertiary structure of AhGASA protein was found to be mainly composed of 2-4 α-helix and 2-3 random coils (Figure 1). Combined with the classification of the phylogenetic tree, the tertiary structure of the proteins in its III. IV. V clusters is more consistent and differs significantly from the tertiary structure of the proteins in its I. II. VI.VII clusters in terms of α-helices. In addition, this protein family's preponderance of random coils contributes to its structural flexibility.

3.2. Phylogenetic Analysis of GASAs

To further investigate the evolutionary relationships of the AhGASA gene family, we constructed an intraspecific evolutionary tree of AhGASA genes (Figure 2). According to the evolutionary relationships of genes and protein structural features, cultivated peanut GASA genes can be divided into seven branches. Branches I, II, and VII contained nine AhGASAs; branches III, IV, and V contained two AhGASAs; and branch VI contained seven AhGASAs. In the phylogenetic tree, two genes were clustered together to form gene pairs, forming a total of 14 gene pairs. In each pair, one gene is from the A subgenome and one is from the B subgenome. AhGASA tends to occur in clusters with AdGASA and AiGASA, laterally indicating that *A. duranensis* and *A. ipaensis*, as ancestors of tetraploid cultivars, are closely linked in their genetic evolution.

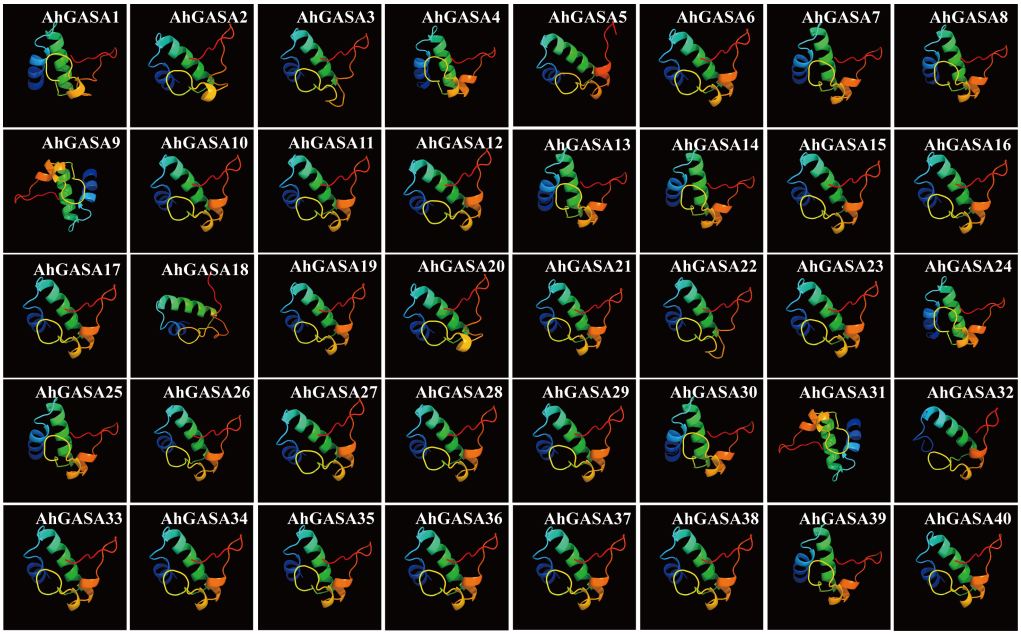

Figure 1. Predicted dimensional structures of AhGASA protein.

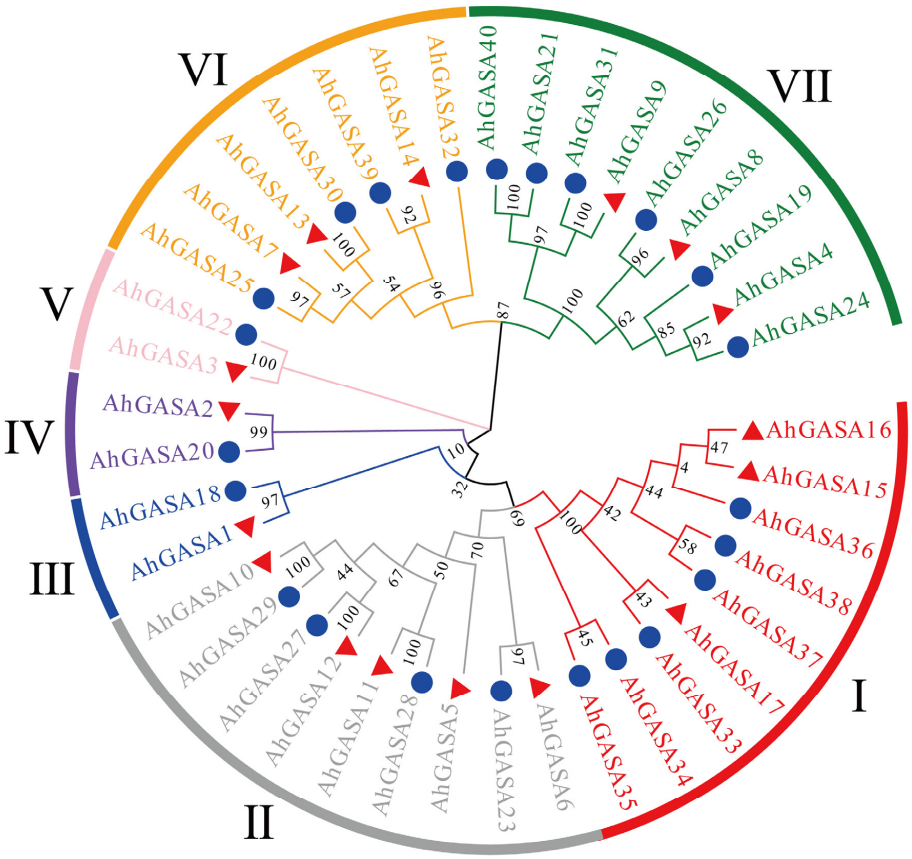

Figure 2. Phylogenetic tree of GASA proteins in *A. hypogaea* cv. Tifrunner. The subgroups of GASAs are shown using seven different colors. Triangles represent the AA subgenome and circles represent the BB subgenome. Numbers near the tree branches indicate bootstrap values and I–VII represent the evolutionary tree branches.

3.3. Correlation Analysis of AhGASA Gene Structure and Motif Composition

To further understand the possible structural evolution of AhGASA, we constructed a phylogenetic tree and analyzed the gene structure and motif association of AhGASA members. For clear labeling, each phylogenetic group is shown in a different color (Figure 3). We can see that related genes are grouped in the same set of phylogenetic trees based on the gene structure and phylogenetic tree results. The number of exons of each gene is one to six. In most cases, two genes in the gene pair had similar exon–intron structures and lengths, such as *AhGASA34/35* and *AhGASA12/27*, while some gene pairs differed in structure, such as *AhGASA1/18*. *AhGASA6*, *AhGASA5*, and *AhGASA30* which had only one exon. In contrast, *AhGASA18* had six exons. The number of exons varied by branch; however, most AhGASA members in the same branch had the same exon–intron structure. On an evolutionary basis, we discovered that the AhGASA gene structure is closely related to phylogeny. The motifs of each protein ranged from 1 to 20. The predicted sequences of AhGASA proteins were mainly concentrated in the C-terminal of GASA proteins. In addition, two genes in most gene pairs had the same motif composition, which implies that they function similarly at the protein level. Additionally, most AhGASA proteins contained Motif1 and Motif2, a conserved structural domain shared by the AhGASA gene family. Clade I consisted mainly of motifs 1, 2, 3, and 4, and *AhGASA17* in branch I also contained motif 16. Clade II consisted mainly of motifs 1, 2, and 5, and also contained motifs 4, 6, 12, 13, and 14, but all had motif 1. Clade III, IV, and V consisted of three gene pairs, and the gene pair motifs were similar in structure; additionally, all contained motifs 1 and 2. Clade VI had a more conservative motif composition, consisting of motifs 1, 2, 5, and 8, and differed only in *AhGASA32*. Most AhGASA motifs in Clade VII were irregular in composition, and the structure was similar between gene pairs, consisting mainly of motifs 1, 2, 5, and 9. Motifs 3, 5, and 7 were located in the N-terminal of the protein and contained a large amount of the hydrophobic amino acid leucine. Interestingly, the *AhGASA5* and *AhGASA32* genes differed not only in gene structure from other protein sequences in the same branch, but also in motif composition. It is speculated that it may have undergone mutations during the evolutionary process, resulting in changes in gene structure and function.

3.4. Chromosome Location and Gene Duplication of GASAs

To investigate the chromosomal distribution and gene duplication of GASA gene family members, we mapped the physical location of these genes on the chromosomes of cultivated peanut versus wild peanuts. The A subgenome of the cultivated peanut is derived from *A. duranensis*, with 20 genes localized on eight chromosomes and the number of GASA genes on each chromosome ranging from 1 to 4. The absence of GASAs on chromosomes A02 and A04 suggests that gene loss may have contributed to species evolution. *A. duranensis* had two tandem replications on chromosome A06, three tandem replications on chromosome A08, and three tandem replications on chromosome A09 (Figure 4A). The B subgenome of the cultivated peanut from *A. ipaensis*, with 22 genes localized on 10 chromosomes, was unevenly distributed, with the number of GASA genes on each chromosome ranging from 1 to 6. *A. ipaensis* had three tandem replications on chromosome B07 and five tandem replications on chromosome B09 (Figure 4B).

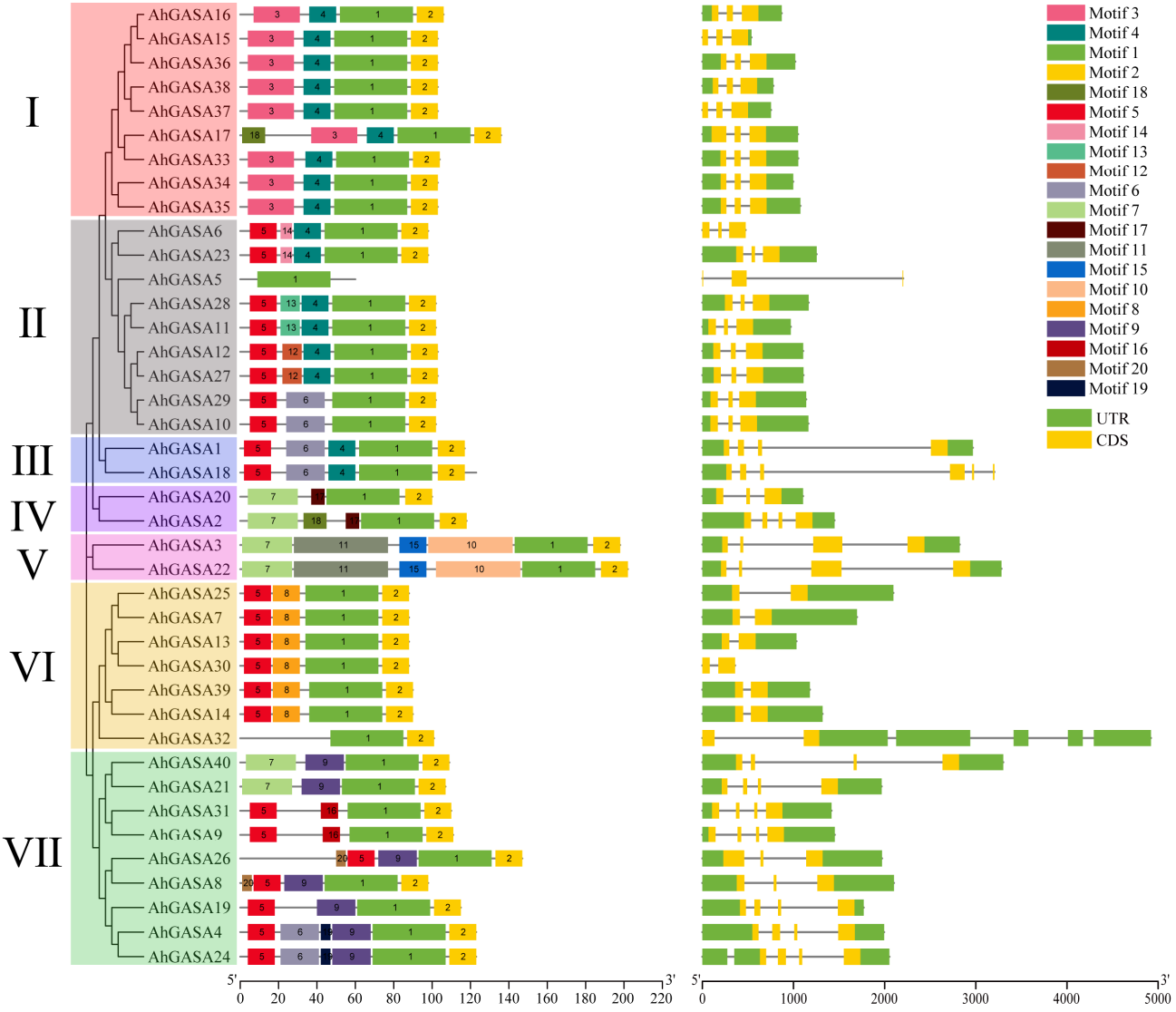

Figure 3. The evolutionary relationship of AhGASAs in *A. hypogaea* L., the motif composition, and gene structure association analysis. The numbers 1 to 20 correspond to motif 1 to motif 20.

Tandem and segmental replication contribute to the generation of gene families during evolution. In *A. hypogaea* L., 40 genes were localized on 17 chromosomes, with the number of AhGASA genes on each chromosome in the range of 1–7, 17 genes in the A subgenome, and 23 genes in the B subgenome. The amount of GASA in the A/B subgenome varied, as did the placement of genes on the chromosomes. Interestingly, the majority of members were scattered on both chromosomal ends. Chromosomes chr02, chr04, and chr10 had no AhGASA (Figure 4C). Chromosomes chr03/A03, chr05/A05, chr06/A06, chr08/A08, chr09/A09, chr11/B01, chr12/B02, chr13/B03, chr14/B04, chr15/B05, chr18/B08, and chr20/B10 had the same number of genes, while the number of genes on the other A/B chromosomes differed, probably due to duplication or loss of GASA members during evolution. Similar to the wild diploid ancestor, tandem duplications occurred on chr06, chr08, chr09, chr17, and chr19, and a total of 17 tandem duplicated genes were found, forming five gene clusters and corresponding to physical locations on the chromosomes.

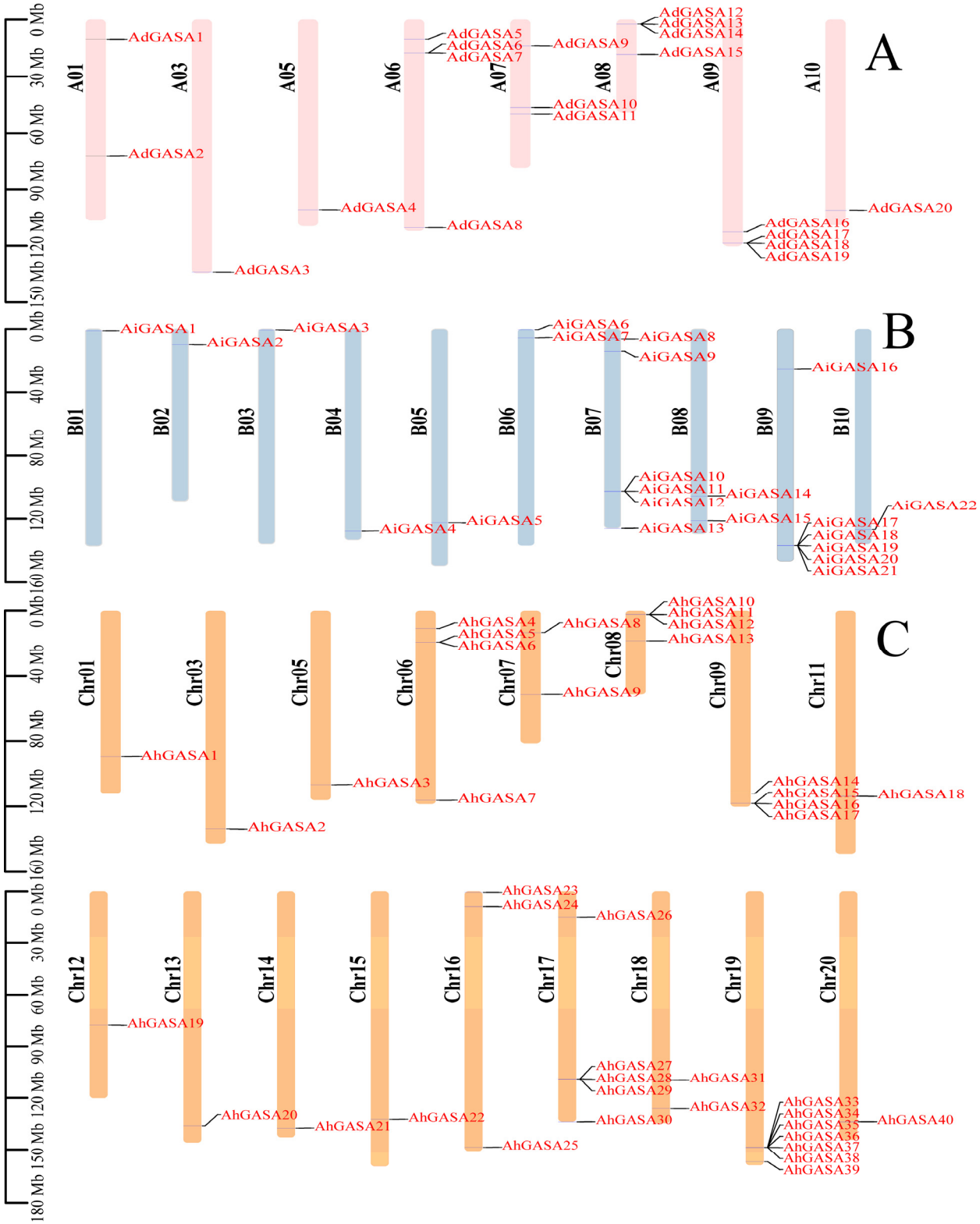

Figure 4. Chromosome localization and gene replication of GASAs. GASAs were distributed in (**A**) *A. duranensis*, (**B**) *A. ipaensis*, (**C**) *A. hypogaea* L., and tandem repeats occurred in the gene pairs connected by wires during evolution.

3.5. Duplication Events of GASAs and Collinearity Analysis

In plant species, the expansion and evolution of gene families is significantly influenced by the occurrence of gene duplication events, also known as segmentation or

crosstalk. The majority of plants have experienced ancient whole-genome duplication events or polyploidy, and whole-genome duplication results in duplicated regions in which all genes are often reproduced on a vast scale, as opposed to single genes or multiple genes. Large-scale whole-genome duplication (WGD) and small-scale tandem and fragmental duplication between species can be identified from co-linear fragments and can be used as data for tree species inference. Homology analysis of GASA genes between the cultivated peanut and wild peanut (*A. hypogaea* L., *A. duranensis*, and *A. ipaensis*) was performed to visualize the locus relationships of the three peanut GASA genes (Figure 5).

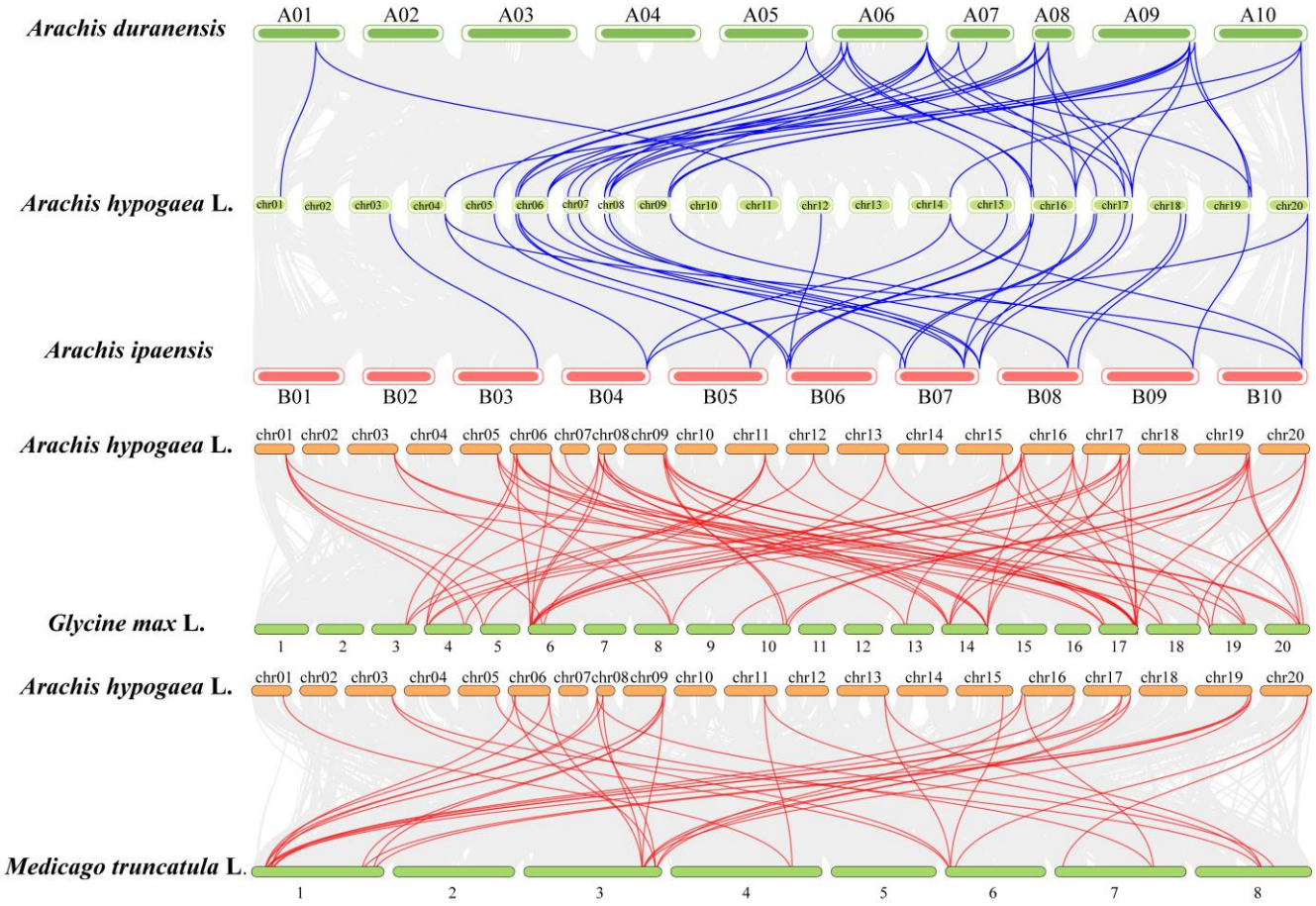

Figure 5. Synteny of GASA genes in the different genomes of *A. duranensis*, *A. ipaensis*, *Medicago truncatula* L., *Glycine max* L., and *A. hypogaea* L.

Comparing the peanut genome and the A/B subgenome, a total of 69 linear/parallel homologous gene pairs were identified, and the number of common linear gene pairs between *A. duranensis* and *A. ipaensis* was 39 and 30, respectively (Table S3). As shown in Figure 5, the co-linear gene pairs between *A. hypogaea* L. and *A. duranensis* were unevenly distributed on all 20 chromosomes, and there were no co-linear gene pairs on chromosomes A02, A03, and A04. In addition, the number of co-linear gene pairs between peanut and *A. ipaensis* was smaller than that of *A. hypogaea* and *A. duranensis*, and no co-linear gene pairs were seen on chromosomes B01 and B02. The covariance results indicated the presence of several highly conserved loci in the A and B subgenomes of tetraploid cultivated peanut, which also resulted from the cross between two tetraploids and two diploids. In addition, comparative synteny analysis was performed between *A. hypogaea* L., *M. truncatula* L., and *Glycine max* L. in order to define the evolutionary trend of GASA genes in legume species. A total of 72 (Table S4) and 38 (Table S5) homologous gene pairs were identified in *A. hypogaea* L., *Medicago truncatula* L., and *Glycine max* L.

3.6. Analysis of Cis-Acting Elements in AhGASA Promoters

Promoters can regulate the onset and level of gene expression by interacting with transcription factors. Cis-acting elements are found in the gene's promoter region and can be used as a guide for tissue specificity and stress response in various situations. Therefore, the analysis of the AhGASA promoter region can help to explore the potential functions of the gene. To explore the role played by gibberellin in peanut growth and development, we analyzed the cis-acting elements in the upstream sequence of the promoter. Cis-acting elements play an important role in developmental regulation. Members of the GASA gene family respond primarily to light and, to a lesser extent, to gibberellin. These promoters also contain hormone-response components for low temperature, defense, and stress, in addition to response components for abscisic acid, growth hormone, methyl jasmonate, and salicylic acid, which are connected to plant growth and development. The results show that a large number of cis-acting elements were detected in the promoter region, and 22 cis-acting elements associated with phytohormones, light response, tissue-specific expression, and abiotic stresses were selected for further analysis (Table S6 and Figure 6A,C). It is worth noting that the cis-acting elements associated with plant hormones are the cis-acting element involved in the abscisic acid responsiveness, cis-acting element involved in salicylic acid responsiveness, cis-acting element involved in gibberellin-responsiveness, and cis-acting regulatory element involved in auxin responsiveness, etc. AhGASA, as gibberellin-stimulating proteins, is a class of cysteine-rich polypeptides that play a role in gibberellin regulation and interact with other plant hormones.

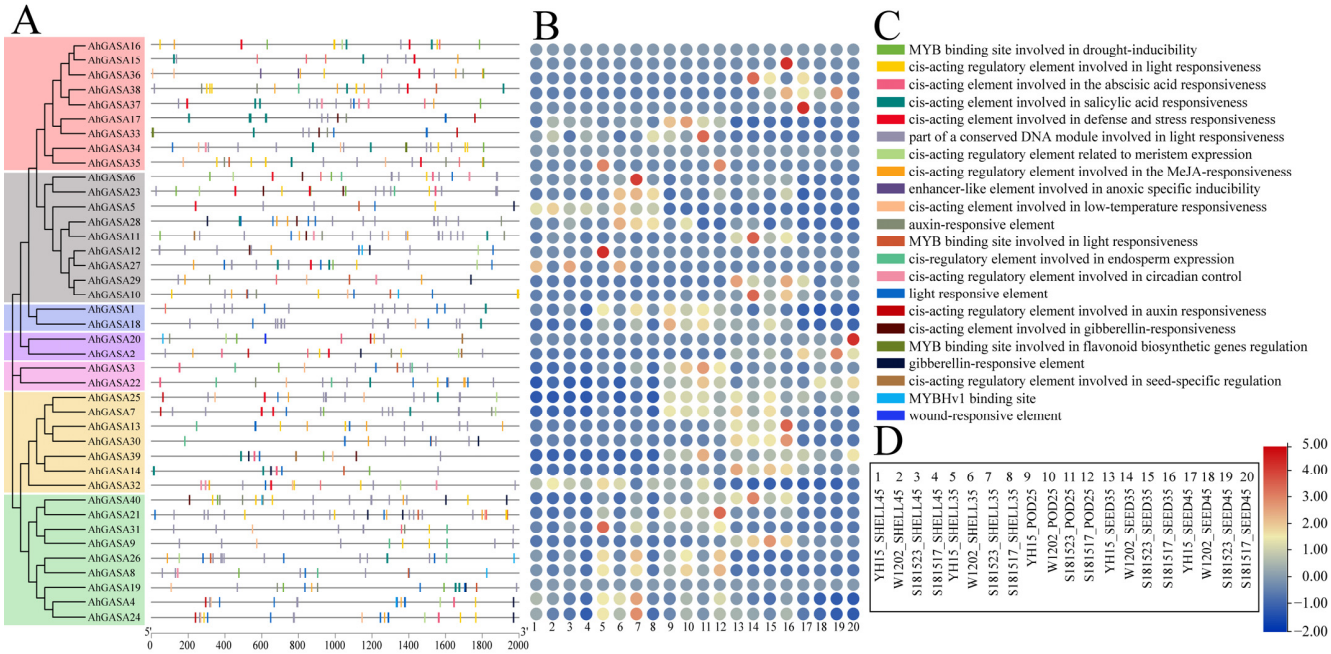

Figure 6. Expression pattern and promoter analysis of the GASA gene family in *A. hypogaea* L. (**A**) The phylogenetic tree and promoter analysis of AhGASAs. (**B**) The expression patterns of AhGASAs under different developmental stages of peanut pod. (**C**) The cis-acting elements associated with phytohormones, light response, tissue-specific expression, and abiotic stresses. (**D**) Different stages of peanut pod development.

More than half of the hormone receptor genes had abscisic acid response elements, 15 genes contained growth hormone response elements, 13 contained gibberellin response elements, and 18 contained salicylic acid response elements. It has been shown that the gibberellin MYB protein is a positive regulator of the gibberellin signaling cascade response, as we found in the AhGASA promoter region that MYB binding site, involved in drought-inducibility, the MYB binding site, involved in flavonoid biosynthetic genes regulation, and the MYB binding site, involved in light responsiveness, consistent with previous

studies. There are three elements associated with light response and more than half of the genes are involved in light response elements. The cis-acting elements associated with tissue specificity are: cis-acting regulatory element involved in seed-specific regulation, cis-regulatory element involved in endosperm expression and cis-regulatory element involved in endosperm expression. *AhGASA20*, *AhGASA2*, and *AhGASA39* were found to contain cis-acting regulatory elements involved in seed-specific regulation, with *AhGASA20* and *AhGASA2* in the same gene pair on the evolutionary tree. It is noteworthy that the AhGASA gene family plays a significant role in controlling the growth and development of peanut pods, which will be further explained by gene expression levels. Furthermore, abiotic stress response elements include defense- and stress-response elements, injury-response elements, drought-induced elements, and low-temperature response elements. The cis-acting elements associated with abiotic stress are: cis-acting element involved in low-temperature responsiveness, MYB binding site involved in drought-inducibility and cis-acting element involved in defense and stress responsiveness.

3.7. Expression Analysis of AhGASAs under Different Developmental Stages of Peanut Pod Using RNA-seq

Currently, the expression pattern of the GASA gene family in seeds is relatively clear in Arabidopsis, but the expression of the AhGASA gene family in peanut pods is unknown. To determine the expression of FPKM of the AhGASA gene family at each developmental pod stage (early, middle, and mid-late stages) and construct a gene expression heat map (Figure 6B,D), we used the large-fruited variety YH15 and the small-fruited variety W1202, which have different phenotypes of pod and seed size, length, and width, as well as the long-grained extreme family S181523 and the short-grained extreme family S181517 in the RIL population (obtained by crossing them) to perform a comparative transcriptome analysis. To exclude the interference caused by background noise during the analysis, the DEGs obtained by comparing both S181523 and S181517 with those obtained by comparing both YH15 and W1202 at the same period of down-regulated DEGs were taken as intersection sets, and 1788, 763, 827, 325, and 457 differentially expressed genes (DEGs) were obtained at shell-35, shell45, pod-25, seed35, and seed45 periods, respectively. Among them, *arahy.V4B8PK* (*AhGASA7*), *arahy.VES4LD* (*AhGASA25*), *arahy.F1TUFL* (*AhGASA40*), *arahy.FJ610L* (*AhGASA21*), and *arahy.VJ6NUA* (*AhGASA31*) of the AhGASA gene family were included.

Further analysis of the expression patterns of the 40 AhGASAs gene families revealed that 14 genes were not expressed at all time points, suggesting that they may be pseudogenes or not expressed in pod organ development. Excluding the 14 unexpressed genes, most of the 26 genes were expressed at the highest level at the Pod-25. Among them, during the Pod-25 period, the expression of large-grain genes in *AhGASA6, AhGASA4, AhGASA23, AhGASA24, AhGASA26*, and *AhGASA8* was lower than that of the small-grain type, while the expression patterns of *AhGASA18, AhGASA1, AhGASA14*, and *AhGASA7* showed the opposite trend. During seed maturation, the expression of the AhGASA gene family as a whole tended to decline. Among them, the expression of genes in *AhGASA18, AhGASA1, AhGASA14, AhGASA7* and *AhGASA2* was higher in the large- than the small-grain type, while *AhGASA6* and *AhGASA23* showed the opposite trend. Similar to the seed tissue development pattern, the AhGASA gene family had a declining tendency in expression from the shell-35 to the shell-45 period. Except for *AhGASA5*, gene expression was higher in the large- than in the small-grain type, especially *AhGASA1, AhGASA6* and *AhGASA18*. The KEGG enrichment results show that 16 AhGASA genes were significantly enriched to metabolic pathways (ko01100), and 12 genes were significantly enriched in the Glycosylphosphatidylinositol (GPI)-anchor biosynthesis (ko00563) pathway (Table S7).

3.8. Expression Analysis of AhGASAs under Different Developmental Stages of Peanut Pod Using RNA-seq

For a better understanding of the possible regulatory roles of the AhGASA family genes in the peanut regulation of pod growth, seven representative genes from YH15,

W1202, S181523, and S181517 were selected to verify whether their expression levels could be induced under different materials and developmental stages by RT-qPCR. The PCR expressions were consistent with the RNA-seq data (Figure 7). The results of the RT-qPCR analysis validated the findings obtained from the RNA-seq data.

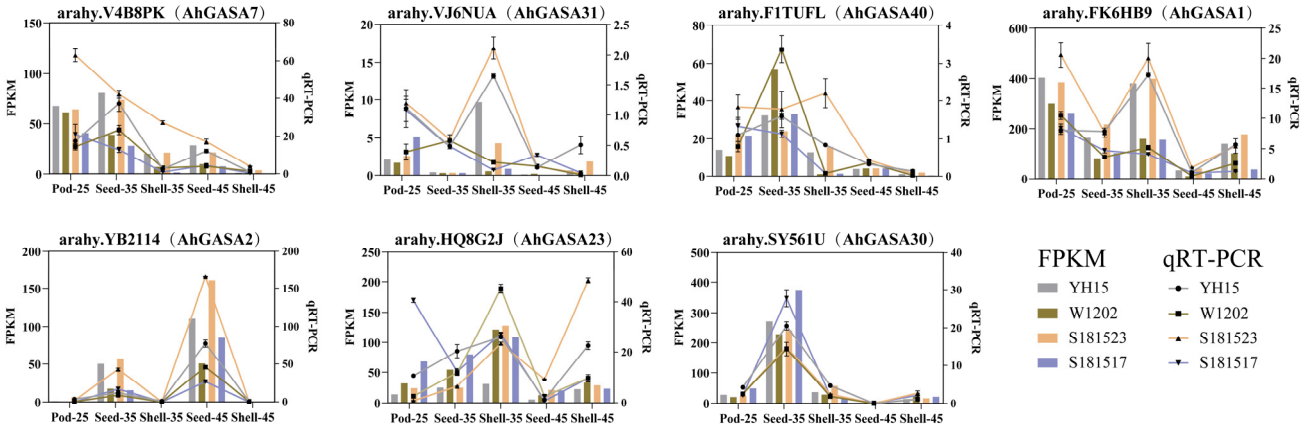

Figure 7. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) validation of 7 GASA genes. The line chart shows RT-qPCR relative expression, and the column diagram shows RNA-seq FPKM.

4. Discussion

GASA genes play an important role in a variety of physiological and developmental processes in plants, including seed formation, floral organ development, phytohormone signaling, stress response, and pathogen defense [43]. Notably, the members of the GASA gene family have a variety of crucial roles in the growth and development of plants seed. For example, the overexpression of the *AtGASA4* gene increases seed size in Arabidopsis [1], *TaGASR7* has been associated with grain length in wheat [15], and *OsGASA* was shown to increase grain size and length in rice [7]. In bioinformatic analyses of the GASA gene family, changes in expression patterns at various seed developmental stages have received little attention. Such changes have only been investigated in grapes and have not been documented in peanut or oilseed crops. To address peanut yield enhancement, it is necessary to increase peanut yields, as seed size is a key agronomic feature and a key factor influencing output. We conducted systematic bioinformatics to thoroughly investigate and track GASA gene family characterization, intergenic evolution, and tissue-specific transcriptomic changes in pod development in light of the significance of the GASA gene family to plant growth and development. The genomes of Wild diploidx [44] and cultivated allotetraploid species [26,27,45] have been published, providing more accurate data for the whole-genome analysis of peanut gene family.

4.1. Genome-Wide Identification and Characteristics of GASA Gene Families in Cultivated Species

Based on the BLASTP search method, we identified 40 GASA genes in cultivated peanut using the Arabidopsis GASA gene as a query; the number of AhGASA genes was higher than the wild species ancestral species AdGASA and AiGASA and higher than GmGASA, OsGASA, VvGASA, PtGASA, GhGASA, MdGASA, and StGASA [7,9–11,13,46]. A conservative sequence analysis confirmed that the identified AhGASA contained 12 cysteine residues at the C-terminus (Figure 3) [5,16,47].

This study examined the many properties of the GASA protein family, including molecular weight, isoelectric point, and number of exons and introns. Low-molecular-weight proteins were found in the newly discovered AhGASA genes, supporting previous investigations that have shown that GASA genes can produce proteins with reduced molecular weights. The C-terminal region of conserved amino acid sequences keeps all 12 cysteine residues which are essential to changing their protein structure and biological function. This led us to hypothesize that redox-active cysteines significantly influence how

well GASA proteins protect against reactive oxygen species [48,49]. The AhGASA protein was discovered to have a comparable structure and motif (motif 1), which is in line with earlier discoveries about GmGASA and GhGASA. Further investigation revealed that most genes belonging to the same group share the same exon–intron composition, indicating a connection between the genetic makeup of the GASA domain and its evolutionary history. It was shown that 40 AhGASA genes were present on 17 *A. hypogaea* chromosomes (Figure 4C), while no copies of any AhGASA genes were present on three *A. hypogaea* chromosomes, Chr02, Chr04, and Chr10. Similar results were reported in *Malus domestica*, *Glycine max*, rice, *Vitis vinifera* L., *Populus trichocarpa*, and cotton, with the irregular allocation of GASA genes.

4.2. Evolutionary and Syntenic Relationships among GASA Genes

The phylogenetic and covariance relationships among GASA genes were analyzed. First, we constructed a phylogenetic tree of GASA protein sequences in the cultivated peanut (Figure 2). The GASA gene family in cotton, Populus trichocarpa, Grapevine, rice, Glycine max, and Malus domestica was divided into three subgroups, and we performed a more detailed division of the identified AhGASA genes into seven subgroups (I~VII) based on the phylogenetic analysis of the GASA genes. On an evolutionary basis, we discovered that the AhGASA gene structure is closely related to phylogeny. The two genes that make up the majority of gene pairs were shown to share the same motif composition, which suggests that their functions at the protein level are comparable. In addition, the majority of AhGASA proteins had the conserved structural domains Motif1 and Motif2, which are shared by all members of the AhGASA gene family. There is a possibility that the specific functions of the AhGASA gene family can be deduced from the conserved nature of the genes that make up each branch of the AhGASA gene family.

Changes in gene amplification may be caused by functional variety, and the discovery and characterization of new genes, which, in turn, may trigger evolutionary processes. However, evolutionary diversity among groups may not always represent functional differences. In rice, *OsGASA4* and *OsGASA6* are clustered into different subgroups, although they have similar physiological functions such as GA induction and ABA and SA repression [7]. The same has been found in strawberry, where *FaGAST1* and *FaGAST2* have different expression patterns but similar physiological functions, although they belong to different branches of the developmental tree [50]. However, gene function in the AhGASA gene family is associated with phylogenetic diversity. For example, *AhGASA4*, *AhGASA9*, and *AhGASA31*, which belong to the same branch in the developmental evolutionary tree, are jointly annotated to *AtGASA6*, whose overexpression advances flowering time in Arabidopsis and whose expression is positively regulated by growth hormone, BR, cytokinin, and GA and negatively regulated by ABA [51]. *AhGASA3*, *AhGASA20*, and *AhGASA22* are jointly annotated to *AtGASA14*, which exert the same biological function by regulating ROS accumulation to regulate leaf expansion and abiotic stress resistance [14]. Thus, we discovered that clusters of AhGASA genes in the same branch may have some homology in evolutionary processes and specific functional associations; phylogenetic diversity does not always imply functional differences, and closely related individuals of AhGASA genes in branches of the evolutionary tree may have a common ancestor. According to the data provided by chromosomal localization, the origin of *A. duranensis* and *A. ipaensis* as cultivated peanut is extremely similar to that of *A. hypogaea* L. Moreover, tandem duplication was an important factor in the expansion of the AhGASA gene family, and 17 tandem duplicated genes were identified.

4.3. AhGASA Gene Expression Profiles and Potential Functions

It has been demonstrated that the GASA gene family, which is controlled by gibberellin, participates in the phytohormone signaling process, as well as being engaged in the regulation of growth and development [1–3]. Plant hormones such as BR, growth hormone, and abscisic acid, in addition to gibberellin, also control the expression of the

GASA gene, indicating that this gene family may be heavily involved in controlling plant hormone signal transduction networks.

Based on transcriptome sequencing data and RT-qPCR results, we investigated the expression patterns of the AhGASA gene family in the large-grain peanut variety YH15 and the small-grain peanut W1202, and the extreme families S181523 and S181517 in the RIL population constructed with both as parents, at different developmental stages [52]. The RT-qPCR and transcriptome sequencing data showed slight differences in expression levels, but the results were largely in agreement (Figure 7). The expression of the AhGASA gene family in branch I was small at all periods of peanut pod development, implying that it does not play an important function in pod development. However, the expression patterns of *AhGASA6*, *AhGASA23*, and *AhGASA5* are still worth discussing. *AhGASA6*, *AhGASA23*, and *AhGASA5* are genetically evolutionarily related, with expression rising and then falling during seed development and gradually rising during shell development, annotated to *AtGASA1* and *AtGASA11*, respectively. It has been shown that *GASA1* is responsible for seed size in the development of almond seeds [33], and *GASA11* are highly expressed during all stages of seed development in seeded cultivars compared to seedless cultivars [11]. Interestingly, the expression of *AhGASA1* and *AhGASA18* in branch III was higher in the large-grain than in the small-grain peanut at all periods of pod development, presumably playing an important role in pod development. Both *AhGASA1* and *AhGASA18* were annotated to the gibberellin-stimulated-like protein SN2 (snakin-2) gene, which plays a regulatory role in tuber shoot growth by regulating lignin biosynthesis and peroxide accumulation; however, a role in legume seed development has not been reported. The expression patterns of *AhGASA2* and *AhGASA20* in branch IV differed widely, i.e., *AhGASA2* expression gradually increased with seed development and was higher in the large-grain than in the small-grain peanut at the seed-35 and seed-45 periods. The expression of most genes in branch VI increased and then decreased from the pod-25 to the seed-35 periods, differing by a gradual decrease in expression during shell development. Looking at branch seven revealed that similar evolutionary tree branches had similar expression patterns. For example, *AhGASA8* and *AhGASA26*, which are expressed at higher levels during the pod-25 period and are significantly more expressed in large-grain material than in small-grain material during shell development, were annotated to RSI-1, which is a member of the GASA gene family that includes regulators involved in cell and organ elongation; RSI-1 has been identified as a regulator of lateral root development [53]. The expression of *AhGASA4* and *AhGASA24* rises with seed maturation and gradually decreases with hull development. The cis-elements present in the respective promoters of the AhGASA genes were investigated in order to further investigate whether the expression of AhGASA genes is controlled by a variety of hormones or signals. We found that the majority of GASA promoters featured hormone-responsive elements that are commonly connected with plant growth and development. These elements may be involved in the complex regulatory mechanisms that regulate gene expression.

5. Conclusions

A total of 40 AhGASA, 20 AdGASA, and 22 AiGASA genes were discovered, and AhGASA genes were shown to contain a conserved GASA structural domain. Most AhGASA proteins are stable, have similar physical and chemical properties, and have a protein tertiary structure consisting of an α-helix and random coils. According to our phylogenetic analysis, AhGASA genes can be divided into seven classes, with each containing 14 gene pairs from the A/B subgenome. Through gene structure and evolutionary research, tandem duplication was identified as a crucial element in the expansion of the GASA gene family. AhGASA is dispersed unevenly over 20 chromosomes containing 17 tandem duplication genes. An analysis of cis-acting elements indicated that the AhGASA protein is crucial for hormone regulation. In addition, in materials with different size traits at various stages of peanut pod development, transcriptomics and RT-qPCR analyses revealed that AhGASA genes are expressed at various levels and are tissue-specific. Overall, the

whole genome-wide level discovery of the GASA gene in cultivated- and wild-peanut provides comprehensive information for future studies. The data presented in this paper may contribute to our understanding of the molecular mechanisms of GASA in peanut and other oilseed crops and help to elucidate the key role it plays in pod development, an important trait.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123067/s1. Figure S1: Sequence logo analysis of the conserved AhGASA domains. Each stack represents their amino acids. Table S1: RT-qPCR primers for validation of RNA-Seq data. Table S2: The physical properties of AhGASA genes, including Gene_ID, sequences, theoretical pI, molecular weight, length, instability index, aliphatic index, grand average of hydropathicity, and subcellular localization. Table S3: The common linear gene pairs between *A. duranensis* and *A. ipaensis*. Table S4: The common linear gene pairs between *A. hypogaea* L., and *Glycine max* L. Table S5: The common linear gene pairs between *A. hypogaea* L., and *M. truncatula* L. Table S6: There are 21 cis-acting elements linked to phytohormones, light response, and tissue-specific expression. Table S7: Results of the KEGG enrichment analysis.

Author Contributions: Formal analysis, Y.W., Z.S. and F.Q.; Data curation, Y.W. and Z.S.; validation, Y.W. and M.Z.; writing—original draft preparation, Y.W.; writing—review and editing, W.D. and B.H.; supervision, Z.Z. and X.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Shennong Seed Industry Laboratory First-class Subject Project (SN01-2022-03), the National Key R&D Program Project (2022YFD1200400), the Special Project for National Supercomputing Zhengzhou Center Innovation Ecosystem Construction (201400210600), Major Science and Technology Projects of Henan Province (201300111000), China Agriculture Research System (CARS-13), Henan Provincial Agriculture Research System, China (S2012-5), and Henan Provincial R&D Projects of Inter-regional Cooperation for Local Scientific and Technological Development Guided by Central Government (YDZX20214100004191).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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