Genome-wide identification and expression analysis of gibberellin synthesis related genes during pod development in peanut (#86034)

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Genome-wide identification and expression analysis of gibberellin synthesis related genes during pod development in peanut

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Background. Gibberellin (GA) plays an important role in regulating peanut growth and development. GA20ox and GA3ox are the key enzymes known to be involved in GA biosynthesis. These enzymes encoded by a multigene family belong to the 2OG-Fe (II) oxygenase superfamily. To date, no genome-wide comparative analysis of peanut AhGA20ox and AhGA3ox-encoding genes has been performed, and the roles of these genes in pod development of peanut are not clear. **Methods.** A whole-genome analysis of the AhGA20ox and AhGA3ox gene families in peanut were identified, and their gene structure, phylogenetic analysis, chromosomal localization, promoter and protein-protein interaction network were analyzed using multiple bioinformatics methods. qRT-PCR was performed to examine the expression pattern of *AhGA20ox* and *AhGA3ox* genes at different stages of peanut pod development.**Results.** In this study, a total of 15 *AhGA20ox* and 5 *AhGA3ox* genes were identified in peanut genome, which were randomly distributed across the twenty chromosomes. Phylogenetic analysis divided these members of AhGA20ox and AhGA3ox families into three main groups. The conserved pattern of gene structure, cis-elements, and protein motifs further confirmed their evolutionary relationship in peanut. In addition, the expression analysis of *AhGA20ox* and *AhGA3ox* genes at various pod developmental stages in peanut suggested their differential expression pattern during pod development. The strong expression of *AhGA20ox1/AhGA20ox4*, *AhGA20ox12/AhGA20ox15*, *AhGA3ox1* and *AhGA3ox4/AhGA3ox5* in S1-stage indicated that these genes could have a key role in controlling peg elongation and growth. Furthermore, the expression of *AhGA20ox* and *AhGA3ox* also suggested a diverse pattern in different peanut tissues including leaves, main stems, flowers and inflorescences.

Noticeably, the expression of *AhGA20ox9/AhGA20ox11* and *AhGA3o4/AhGA3ox5* were up-regulated in the main stem, whereas the expression of *AhGA3ox1* and *AhGA20ox10* were enhanced in the inflorescence. Similarly, the expression levels of *AhGA20ox2/AhGA20ox3*, *AhGA20ox5/ AhGA20ox6*, *AhGA20ox7/AhGA20ox8*, *AhGA20ox13/AhGA20ox14* and *AhGA3ox2/ AhGA3ox3* were high in the flower tissues, suggesting that these genes might be involved in the regulation of flower development. Our findings provide a strong basis for deciphering the GA-induced molecular mechanisms governing plant growth and development, as well as for elucidating the functional characteristics of the AhGA20ox and AhGA3ox genes in peanut.

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ABSTRACT

Background. Gibberellin (GA) plays an important role in regulating peanut growth and development. GA20ox and GA3ox are the key enzymes known to be involved in GA biosynthesis. These enzymes encoded by a multigene family belong to the 2OG-Fe (II) oxygenase superfamily. To date, no genome-wide comparative analysis of peanut AhGA20ox and AhGA3ox-encoding genes has been performed, and the roles of these genes in pod development of peanut are not clear.

Methods. A whole-genome analysis of the AhGA20ox and AhGA3ox gene families in peanut were identified, and their gene structure, phylogenetic analysis, chromosomal localization, promoter and protein-protein interaction network were analyzed using multiple bioinformatics methods. qRT-PCR was performed to examine the expression pattern of *AhGA20ox* and *AhGA3ox* genes at different stages of peanut pod development.

Results. In this study, a total of 15 *AhGA20ox* and 5 *AhGA3ox* genes were identified in peanut genome, which were randomly distributed across the twenty chromosomes. Phylogenetic

analysis divided these members of AhGA20ox and AhGA3ox families into three main groups. The conserved pattern of gene structure, cis-elements, and protein motifs further confirmed their evolutionary relationship in peanut. In addition, the expression analysis of *AhGA20ox* and *AhGA3ox* genes at various pod developmental stages in peanut suggested their differential expression pattern during pod development. The strong expression of *AhGA20ox1/ AhGA20ox4*, *AhGA20ox12/AhGA20ox15*, *AhGA3ox1* and *AhGA3ox4/AhGA3ox5* in S1-stage indicated that these genes could have a key role in controlling peg elongation and growth. Furthermore, the expression of *AhGA20ox* and *AhGA3ox* also suggested a diverse pattern in different peanut tissues including leaves, main stems, flowers and inflorescences. Noticeably, the expression of *AhGA20ox9/AhGA20ox11* and *AhGA3o4/AhGA3ox5* were up-regulated in the main stem, whereas the expression of *AhGA3ox1* and *AhGA20ox10* were enhanced in the inflorescence. Similarly, the expression levels of *AhGA20ox2/AhGA20ox3*, *AhGA20ox5/ AhGA20ox6*, *AhGA20ox7/AhGA20ox8*, *AhGA20ox13/AhGA20ox14* and *AhGA3ox2/ AhGA3ox3* were high in the flower tissues, suggesting that these genes might be involved in the regulation of flower development. Our findings provide a strong basis for deciphering the GA-induced molecular mechanisms governing plant growth and development, as well as for elucidating the functional characteristics of the AhGA20ox and AhGA3ox genes in peanut.

Keywords: *Arachis hypogaea*; Gibberellin biosynthesis; Gene expression analysis; Pod development

INTRODUCTION

Gibberellins (GAs) are the endogenous hormones of diterpenes with the largest variety and the widest physiological function. GAs are widely involved in various stages of plant growth and development (*Binenbaum, Weinstain & Shani, 2018*), such as seed germination (*Xu et al., 2020*), stem elongation (*Zhang, Wang & Huang, 2021*), shade response (*Yang & Li, 2017*), flowering regulation (*Bao, Hua & Shen, 2020*), and fruit development (*Hu et al., 2018*). So far, 136 GAs with definite structures have been identified in plants, bacteria and fungi. Although there are many kinds of GAs, only a few GAs have physiological effects on plant development, such as

60 GA_1, GA_3, GA_4 and GA_7 (*Giacomelli et al., 2013; MacMillan, 2001*).

61 The GA20 oxidase (GA20ox) and GA3 oxidase (GA3ox) are key rate-limiting enzymes in
62 GA synthesis, catalyzing successive steps of GA biosynthesis to produce bioactive GA. At the
63 later stage of GA synthesis, GA20ox catalyzed inactive GA12 and GA53 to remove C-20 and
64 convert them into GA9 and GA20. Subsequently, GA9 and GA20 were catalyzed by GA3ox to
65 produce bioactive GA4 and GA1 through 3β-hydroxylation (*Hedden, 2020; Salazar et al., 2018*).
66 It was found that GA20ox and GA3ox are the members of 2OG-Fe (II) oxygenase superfamily
67 and are generally encoded by multiple genes in plants. Rice *sd1* (semi-dwarf 1) gene encodes
68 GA20ox2 in the gibberellin biosynthesis pathway, and mutation of this gene can inhibit GA
69 biosynthesis and lead to semi-dwarfing in rice (*Sasaki et al., 2002*). In addition, the *ga3ox1*
70 single mutant of *Arabidopsis thaliana* showed semi-dwarfism while the GA*3ox1* and GA*3ox2*
71 double mutants were more significantly dwarfed than the *ga3ox1* single mutant (*Mitchum et al.,*
72 *2006*).

73 Fruit setting is a key process in agricultural production and is usually triggered by ovule
74 fertilization. Plant hormones play an important role in fruit development, especially IAA and GA,
75 which are the main hormones promoting fruit development (*Liu et al., 2018*). DELLA protein is
76 a key negative regulator of GA signaling pathway and acts as a fruit growth inhibitor prior to
77 fertilization. In pollinated ovaries, the increased transcription level of *GA20ox* promotes the
78 increase of GA content, leading to the degradation of DELLA protein through the 26S
79 proteasome pathway, thus releasing the inhibitory effect of DELLA protein on fruit development
80 (*Fuentes et al., 2018*). Further studies have shown that the application of GA, independent of
81 pollination and fertilization, can promote fruit-setting and paraphyletic outcomes in some crops,
82 as has been demonstrated in pears, apricots, strawberries and grapes. After GA3 treatment, the
83 expressions of *ARF2* and *ARF8* were inhibited, suggesting that GA-induced parthenogenesis
84 might be caused by the downregulation of *ARF2* and *ARF8* (*Maaike, Mariani & Vriezen, 2009*).

85 Peanut, a leguminous dicotyledonous plant, rich in protein, oil, vitamins and other nutrients,
86 has become one of the world's top five oil crops (*Toomer, 2018*). The development of the pod

directly determines the yield and quality of peanut. After flowering and fertilization, the fertilized egg only divides several times to form the proembryo and then stops dividing. While the stalk of the ovary extends continuously with the unexpanded ovary after fertilization to form a peg growing toward the ground. The peg expands horizontally after the ovules are buried in the soil (*Zhang et al., 2016*). The development of peanut pod is regulated by a variety of hormones such as auxin, GA, brassinosteroids (BR), abscisic acid (ABA), ethylene and cytokinin. In the early stage of peanut pod development, auxin promotes the elongation and growth of peg, while cytokinin regulates the cell division of peg (*Edgar, 2003*). GA promoted not only the elongation and growth of peg in the early stage of pod development, but also nutrient accumulation in the middle and late stage of peanut pod development. In addition, ethylene and ABA were involved in the accumulation of peanut pod at the later stage of development. Taken together, these studies suggested that various hormones regulate the development of peanut pod (*Kumar et al., 2019*). However, the genome-wide identification and function of the AhGA20ox and AhGA3ox families in peanut have not been reported. The functions of GA20ox and GA3ox family genes in pod development of peanut are not clear.

In the current study, all members of the AhGA20ox and AhGA3ox gene families were identified in peanut genome, and their gene structure, chromosomal localization, promoter and protein-protein interaction network were analyzed using multiple bioinformatics resources. The phylogenetic analysis was also carried out to investigate their evolutionary relationship with other plant species. In order to explore the regulation of GA synthesis related genes on peanut pod development, qRT-PCR was performed to examine the expression pattern of *AhGA20ox* and *AhGA3ox* genes at different stages of peanut pod development. The results will further enrich the concept of hormonal regulation of fruit development. In addition, the expression patterns of *AhGA20ox* and *AhGA3ox* genes in different tissues of peanut cultivar JH8 were also studied by qRT-PCR, which laid a foundation to study the functions of *AhGA20ox* and *AhGA3ox* genes.

MATERIALS AND METHODS

Plant materials and treatment conditions

The peanut cultivar JH8 with high oleic acid developed by our lab is used in this study. Stems, leaves, flowers, inflorescences and pods at different stages of development were collected from plants grown in the field. All the materials were frozen immediately in liquid nitrogen and stored at −80℃ for RNA extraction and gene expression analysis.

Identification of AhGA20ox and AhGA3ox gene family members in peanut

All sequences were downloaded from four databases: TAIR (Arabidopsis Information Resource, http://www.arabidopsis.org/), Rice (Rice Information Resource, http://www.rice.plantbiology.msu.edu/), Soybean Genome Annotation Project Database (http://www.phytozome.net/soybean/) and NCBI Genome Database (https://www.ncbi.nlm.nih.gov/) (*Honi et al., 2020*). The *Arabidopsis* GA20ox and GA3ox gene sequences were first downloaded from the TAIR website. Subsequently, gene sequences of AhGA20oxs and AhGA3oxs were searched against the NCBI genome database using the amino acid sequences of five Arabidopsis GA20oxs and four GA3oxs as queries, respectively. The resulting sequences were further validated in SMART (http://smart.embl.de) and Pfam (http://pfam.xfam.org) to obtain all members of AhGA20ox and AhGA3ox encoding genes in peanut genome. The predicted physical properties of the GA20ox and GA3ox proteins, such as protein isoelectric point (pI), molecular weight (Mw), amino acid number (aa), were analyzed using the ProtPram tool in ExPASy (https://www.expasy.org/).

Phylogenetic analysis of AhGA20ox and AhGA3ox in peanut

Multiple sequence alignment of AhGA20ox and AhGA3ox amino acid sequences from peanut and homologous sequences from *Arabidopsis thaliana*, rice, and soybean was performed using the DNAMAN software (Vers7; Lynnon Corporation, Montreal, QC, Canada) using the default settings. Then, phylogenetic analysis was performed by AhGA20ox and AhGA3ox protein sequences from peanut together with other plant species by using neighbor-joining method in MRGA 7.0 with bootstrap value of 1000. Finally, iTOL (https://itol.embl.de/) online software.

Chromosomal distribution and gene structure analysis of AhGA20ox and AhGA3ox-

encoding genes in peanut

The loci of *GA20ox* and *GA3ox* genes were downloaded from the genome annotation file obtained from the Peanutbase in order to obtain chromosome location information. Then, the chromosome map was generated using Mapchart (version2.2) (http://mg2c.iask.in/mg2c_v2.1/) software. For gene structure analysis, the online tools GSDS (version2.0) (http://gsds.cbi.pku.edu.cn) was employed to analyze the genomic sequences *AhGA20ox* and *AhGA3ox* genes for the exon and intron distribution.

Analysis of the conserved protein motifs and cis-acting elements

The online webserver of MEME software was used to analyze the conserved protein motifs of peanut AhGA20ox and AhGA3ox family members. The occurrence of top 8 conserved protein motifs in AhGA20ox and AhGA3ox sequences were further screened and analyzed.

The 2000 bp sequence upstream of the start codon of the *AhGA20ox* and *AhGA3ox* genes was downloaded from the peanut genome database, and the cis-acting elements were predicted and analyzed by PlantCARE (http://bioinformatics.psb.ugent.be/).

AhGA20ox and AhGA3ox protein interaction networks prediction

The online webtool of STRING network (https://string-db.org/), was utilized to predict the functional protein interaction network of AhGA20ox and AhGA3ox proteins. All possible interacting proteins including experimental and hypothetical proteins constituting a hierarchical network with AhGA20ox and AhGA3ox proteins were further classified and shown graphically in STRING-generated network.

Expression analysis of *AhGA20ox* and *AhGA3ox* gene family members in peanut

Total RNA was extracted by using FastPure Plant Total RNA isolation kit (AG RNAex Pro Reagent, Changsha, China). The extracted RNA was used as template for reverse transcription reaction using Hiscrip Ⅱ Q RT SuperMix for qPCR (Vazyme, Nanjing, China).

The primers of qPCR were designed by Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) (Table S1). An ABI 7500 real-time PCR instrument (Thermo Fisher Scientific, Waltham, USA) and ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) was

used for subsequent quantitative fluorescence reaction. The reaction mixture consisted of 10 μL ChamQ SYBR qPCR Master Mix, 0.5 μL of each primer (10 μM), 2 μL cDNA template, and 7 μL RNase-free H_2O. Reaction conditions include pre-denaturation (95℃, 30s), cyclic reaction (95℃, 10s; 60 ℃, 30 s; 40 cycles) and other parameters retain the default values. According to $2^{-\Delta\Delta CT}$, and the relative expression of target genes in different samples was quantitatively analyzed.

RESULTS

Identification and physicochemical characterization of AhGA20ox and AhGA3ox genes in peanut

Based on the amino acid sequences of five Arabidopsis GA20oxs and four GA3oxs, candidate gibberellin-dioxygenases genes were explored through searching against the NCBI genome database using BLASTP (e-value ≦0.001) methods. After removing redundant sequences and confirming the presence of gibberellin-dioxygenases domains by SMART and Pfam, 20 Gibberellin-dioxygenases genes were finally retained and used for further analysis, including 15 GA20ox genes (AhGA20ox1-15) and 5 GA3ox (ZmGA3ox1-5) genes, respectively. Further analysis showed that the length of amino acid encoded by AhGA20ox genes varied from 363 aa (AhGA20ox15) to 428 aa (AhGA20ox4), and the molecular weight was 41.3 kDa-49.2 kDa. The isoelectric point (pI) values were between 5.14 (AhGA20ox10) and 7.09 (AhGA20ox8). The amino acid length of GA3oxs varied from 352 aa (AhGA3ox2) to 375 aa (AhGA3ox1), the molecular weight is 40 kDa -41.7 kDa, and the pI values varied from 6.49 (AhGA3ox3) to 8.11 (AhGA3ox1) (Table 1).

Phylogenetic analysis of AhGA20ox and AhGA3ox in peanut

In order to further analyze the phylogenetic relationship of GA20ox and GA3ox gene family, the phylogenetic tree was constructed using the protein sequences of GA20ox and GA3ox from peanut, *Arabidopsis thaliana*, rice, and soybean (Table S2). The results showed that the GA20oxs and GA3oxs were divided into three groups (group A-C). The largest group was group C with 32 members of GA20ox and GA3ox gene family, including all AhGA20ox family members. While groups A was the smallest ones only with four members of rice GA20ox family

(Fig. 1). Group B contains 17 members, all of which were members of the GA3ox family. Moreover, most of the genes related to GA synthesis in the same species were clustered together. Further analysis revealed that the GA20ox and GA3ox family members in peanut are closely related to those in soybean, but distantly related to those in rice (Fig. 1).

Gene structure and conserved motifs of AhGA20ox and AhGA3ox genes

The exon-intron diversity of the gene family members plays a crucial role in the evolution of multiple gene families. The exon-intron structure analysis of 15 *AhGA20ox* and 5 *AhGA3ox* genes showed that except that *AhGA20ox4* had four exons and three introns, the other *AhGA20ox* genes contain three exons and two introns. Furthermore, *AhGA20ox4* had one longest intron. All *AhGA3ox* genes contain two exons and one intron (Fig. 2).

The conserved motifs of AhGA20ox and AhGA3ox proteins in peanut were predicted to further understand their sequence diversity. In the predicted eight motifs, the E value of each motif was significant, and the length of motif was 36-50 conserved amino acids. Motif 1, motif 2, motif 3 and motif 5 were distributed in all AhGA20ox and AhGA3ox sequences (Fig. 3), suggesting that these four motifs may be the core conserved domain of AhGA20ox and AhGA3ox. This indicated that the AhGA20ox and AhGA3ox families were highly conserved and presumably had some degree of functional redundancy. Further analysis showed that members of AhGA3ox gene family presented in group B (Fig. 1). In addition, motif 8 exists only in the AhGA3ox gene family (Fig. 3). All members of the AhGA20ox gene family exist in the group C (Fig. 1), while motif 6 and 7 exist only in the AhGA20ox gene family (Fig. 3). Therefore, it can be inferred that motif 6 and 7 are conserved domains specific to the group C.

AhGA20ox and AhGA3ox genes contain key cis-acting elements

The analysis of conserved cis-regulatory units in the promoter region of *AhGA20ox* and *AhGA3ox* genes were investigated using the 2 kb sequence upstream of the start codon. The results showed the presence of widely known eight key cis-elements in the promoter of *AhGA20ox* and *AhGA3ox* genes (Fig. 4, raw data is shown in Table S3). The most abundantly presented cis-elements mainly included gibberellin-responsive unit (TATC-box), light-

responsive units such as G-box (TACGTG), GATA-motif (AAGATAAGATT), and GARE-motif (TCTGTTG). Similarly, hormone-responsive units such ABA responsive-motif (ACGTG), low-temperature responsive units (TATC-box), transcription regulatory units such as (TATA-box) and MYB-responsive motifs (TAACTG) were also identified. The occurrence of these well-known cis-elements suggested that *AhGA20ox* and *AhGA3ox* genes are strongly linked to plant growth, development, and tolerance to varied stresses, as well as other crucial signaling pathways in peanut.

Chromosome mapping of *AhGA20ox* and *AhGA3ox* genes

Chromosomal mapping of *AhGA20ox* and *AhGA3ox* genes based on peanut genome information showed that *AhGA20ox* and *AhGA3ox* genes were distributed on 14 chromosomes. *AhGA20ox1* and *AhGA20ox2* were localized at chr.2. Similarly, *AhGA20ox9* and *AhGA20ox10* were localized at chr.5, whereas *AhGA20ox12* and *AhGA3ox2* were mapped to chr.8. *AhGA20ox13* and *AhGA3ox4* was positioned at chr.9, while *AhGA20ox3* and *AhGA20ox4* were found at chr.12. *AhGA20ox14* and *AhGA3ox5* were located at chr.19. Notably, most of *AhGA20ox* and *AhGA3ox* genes located at the distal ends of chromosomes (Fig. 5, raw data is shown in Table S4).

Interactive protein network of *AhGA20ox* and *AhGA3ox* encoding proteins

We investigated the protein–protein interaction (PPI) network of the *AhGA20ox* and *AhGA3ox* encoding proteins by employing the STRING database. The major interacting partners of AhGA20ox AhGA3ox proteins were predicted as Fe2OG dioxygenase domain containing protein, which is a key component of iron-ascorbate dependent oxidoreductase family. This Fe2OG enzyme is known to catalyze a wide range of oxidative reactions crucial to plant metabolisms. In addition, other proteins such as ABC transporter, and protein kinase were also co-associated with the AhGA20-ox and AhGA3-ox proteins. (Fig. 6). The prediction of PPI network of GA-ox-encoding proteins in peanut provides important insights into understanding the orchestrated regulatory mechanism underlying gibberellin biosynthesis.

Expression analysis of *AhGA20ox* and *AhGA3ox* genes during different pod developmental

stages

The spatio-temporal expressions of *GA20ox* and *GA3ox* genes during peanut pod development were investigated using quantitative real-time polymerase chain reaction (qRT-PCR). Peanut pod development was S1divided into six stages including green or purple aerial-grown pegs (Stage 1, S1), white pegs that had been embedded in the soil for approximately 3 days and in which pod enlargement was not detected (Stage 2, S2); pegs that had been buried in the soil for approximately 9 days and in which pod enlargement had been initiated (Stage 3, S3); pegs that had been buried in the soil for approximately 12 days (Stage 4, S4); pegs that had been buried in the soil for approximately 20 days (Stage 5, S5); pegs that had been buried in the soil for approximately 30 days (Stage 6, S6) (Fig. 7).

Both *AhGA20ox* and *AhGA3ox* were expressed ubiquitously at different stages of pod development, but had different expression patterns at different stages. The expressions of *AhGA20ox2/AhGA20ox3*, *AhGA20ox6/AhGA20ox5*, *AhGA20ox7/AhGA20ox8* and *GA20ox10* gradually increased with the development of peanut pod, reaching the highest level at S6 (Fig. 8, raw data is shown in Table S5). Interestingly, *AhGA20ox2/AhGA20ox3, AhGA20ox6/ AhGA20ox5* and *AhGA20ox10* were closer evolutionarily than other AhGA20ox members, implying a potential functional redundancy or synergistic effect of these AhGA20ox genes. On the contrary, *AhGA20ox15/ AhGA20ox12* and *AhGA3ox1* gradually decreased with the development of peanut pod, indicating different members of the *GA20ox* and *AhGA3ox* family have different functions in pod development. *AhGA20ox1/AhGA20ox4*, *AhGA20ox12/AhGA20ox15*, *AhGA3ox1* and *AhGA3ox4/ AhGA3ox5* showed high expression levels in S1 (Fig. 6), suggesting that these genes may be involved in regulating the elongation of the peg. Other genes may be involved in regulating the expansion and growth of peanut pod, even though they showed different expression patterns during the expansion and growth of peanut pod. For example, *AhGA3ox1* and *AhGA3ox2/AhGA3ox3* showed high expression levels in S2, and *AhGA20ox13/ AhGA20ox14* showed the highest expression level in S3. The

expression levels of *AhGA20ox1/AhGA20ox4* and *AhGA20ox19/11* were the highest in S4 (Fig. 8, raw data is shown in Table S5).

Expression analysis of *AhGA20ox* and *AhGA3ox* genes in different tissues of peanut

We analyzed the expressions of *AhGA20ox* and *AhGA3ox* genes in peanut leaves, main stems, flowers and inflorescences. The results showed that *AhGA20ox* and *AhGA3ox* were expressed in different tissues, but the expression patterns were differential. The expression levels of *AhGA20ox9/ AhGA20ox11* and *AhGA3ox4/AhGA3ox5* were the highest in the main stem, and the expression levels of *AhGA20ox12/AhGA20ox15* were the lowest in the main stem (Fig. 9, raw data is shown in Table S6). Except *AhGA20ox9/AhGA20ox11*, *AhGA3ox1* and *AhGA3ox4/AhGA20ox5*, other genes showed high expression levels in peanut flowers (Fig. 7), implying that these genes may be involved in the regulation of flower development. *AhGA20ox2/AhGA20ox3*, *AhGA20ox5/AhGA20ox6*, *AhGA20ox7/AhGA20ox8, AhGA20ox13/AhGA20ox14* and *AhGA3ox2/AhGA3ox3* were highly expressed in inflorescence (Fig. 7). *AhGA20ox1/ AhGA20ox4*, *AhGA20ox12/AhGA20ox15* and *AhGA3ox1* showed high expression levels in leaves (Fig. 9, raw data is shown in Table S6). These results indicated that different genes may play different roles in specific tissues or organs.

DISCUSSION

Gibberellin is one of the important hormones that regulate plant growth and development. The proteins encoded by the *GA20ox* and *GA3ox* genes play a key role in GA biosynthesis. In recent years, *GA20ox* and *GA3ox* genes have been identified in many higher plants, such as 9 members of Arabidopsis (*Han & Zhu, 2011*), 10 members of rice (*Han & Zhu, 2011*), 14 members of soybean (*Han & Zhu, 2011*), 13 members of grape (*He et al., 2019*), 9 members of *Phyllostachys edulis* (*Ye et al., 2019*) and 14 members of maize (*Ci et al., 2021*). In this study, a total of 15 AhGA20ox and 5 AhGA3ox family members were identified from peanuts. The evolutionary properties of the gibberellin oxidase gene family in Arabidopsis, rice, soybean and peanut showed that functionally different GA3ox and GA20ox clusters were distributed in separate groups. AhGA20ox and AhGA3ox genes in peanut were more closely related to those in

soybean, while far related to those in rice. In addition, the DIOX_N and 2OG-FeII_Oxy superfamily domains contained in the protein sequences of GA20ox and GA3ox are conserved domains shared by all species (*Honi et al., 2020*).

SD1 (*OsGA20ox2*) is a gene of the rice Green Revolution. It was found that the mutant of this gene causes semi-dwarf of rice. It catalyzes the conversion of GA53, a precursor of gibberellin synthesis, to GA20 (*Sasaki et al., 2002*). In this study, OsGA20ox2 and AhGA20ox14 had the highest protein sequence similarity with 48.98%, followed by GA20ox9 and GA20ox11 with 48.73% and 48.48%, respectively. According to qRT-PCR analysis, the expression levels of *GA20ox9/ GA20ox11* in the main stem of were significantly higher than those in other tissues. Therefore, it was speculated that *GA20ox9/ GA20ox11* may be the key genes involved in regulating the development of main stem of peanut.

Although the biosynthetic pathway of GA has been widely studied, the evolutionary analysis of this gene family has not been reported in details. It was found that members of the *GA20ox* and *GA3ox* gene families have a certain degree of functional redundancy, but the expression of each gene is spatio-temporal and tissue specific, and the function is different. For example, there are five *GA20ox* genes in *Arabidopsis thaliana*, in which *AtGA20ox1* and *AtGA20ox2* are expressed in vegetative growth phase, the former is mainly regulated by biological clock and the latter is mainly regulated by far-red light. *AtGA20ox3* is expressed in the outer epidermis, seeds and fruits (*Rieu et al., 2008; Phillips et al., 1995*), *AtGA20ox4* is expressed in roots, and *AtGA20ox5* is expressed in fruits (*Xu et al., 1995*). *AtGA3ox1* and *AtGA3ox2* are mainly active in germination and vegetative growth, while *AtGA3ox3* and *AtGA3ox4* are mainly active in reproductive growth (*Mitchum et al., 2006*). Our results also showed that the expression patterns of *AhGA20ox* and *AhGA3ox* were different in different tissues and pod development stages of peanut. For example, *AhGA20ox1/AhGA20ox4*, *AhGA20ox12/AhGA20ox15*, *AhGA3ox1* and *AhGA3ox4/AhGA3ox5* may be involved in regulating the elongation and growth of peanut pods, Other *AhGA20ox* and *AhGA3ox* genes may be involved in the expansion and accumulation of peanut pod in the middle and late stages of

development. Therefore, the expression of *AhGA20ox* and *AhGA3ox* family members was not only functionally complementary, but also spatio-temporal specific, indicating that the regulation of GA on peanut plant growth and development was not the result of a single gene. Therefore, more experiments are needed to further study the exact function of these genes and the gene regulation mechanism in peanut.

CONCLUSIONS

In this study, we identified 15 GA20ox and 5 GA3ox family members in peanut, which were scattered on 14 chromosomes and could be clustered into three groups. The expression analysis showed that *AhGA20oxs* and *AhGA3oxs* were differentially expressed in different tissues and pod of different developmental stages, suggesting their association with growth and developmental of these processes in peanut. These results contribute to have a better understanding the roles of *GA20ox* and *AhGA3ox* genes encoding the key enzymes involved in GA biosynthesis in peanut.

ADDITIONAL INFORMATION

Data Availability

The raw measurements are available in the Supplemental Files.

Figure Legend

Fig. 1 Phylogenetic analysis of GA2ox and GA3ox protein in *Arabidopsis Thaliana* (At), rice (Os), soybean (Gm) and peanut (Ah). The phylogenetic tree was constructed using neighbor-joining method in MRGA 7.0 with bootstrap value of 1000. The numbers represent The scale of the evolutionary tree. Gene accession numbers of the sequences used in this tree are listed in Table S2.

Fig. 2 Gene structure organization of peanut *GA20ox* and *GA3ox* family members. Exons (CDS) and UTR are represented by yellow boxes and blue boxes, respectively, and grey lines between exons represents introns.

Fig. 3 Distribution of conserved motifs in GA20ox and GA3ox family members. The

sequence information of motifs marked different colors is represented at the bottom.

Fig. 4 The organization of cis-acting elements in the promoter region of *AhGA20ox* and *AhGA3ox* genes in peanut. Different colors were used to indicate different elements.

Fig. 5 Chromosome mapping of *AhGA20ox* and *AhGA3ox* genes. Fifteen AhGA20ox and 5 AhGA3ox genes were unevenly distributed on the 14 chromosomes, with the exception of chr. 01, 06, 10, 11 and 16. The location on the chromosome of each AhGA20ox and AhGA3ox gene was indicated on the right side of the respective chromosome. The scale bar for chromosome length was showed at the left of all chromosomes.

Fig. 6 The prediction of protein– protein interaction network of AhGA20ox and AhGA3ox encoding proteins. Nodes represent proteins, and lines indicate that they have interaction relationship between proteins.

Fig. 7 The morphology peg and pod at different developmental stages. S1-S6 means different development stages of pod in peanut.

Fig. 8 Expression analysis of *AhGA20ox* and *AhGA3ox* genes in different stages of peanut pod development. The heatmap was generated with the qRT-PCR values of 15 AhGA20ox and 5 AhGA3ox genes using the online tool, TBtools, and the color scale beside the heat map indicates gene expression levels, low transcript abundance indicated by blue color and high transcript abundance indicated by red color. Fifteen AhGA20ox and 5 AhGA3ox genes were classifed into three groups Group I, *AhGA20ox15/12/4/1*and *AhGA3ox 1/4/5*; Group II, *AhGA20ox2/3/5/6 /10* ; Group III, *AhGA20ox7/8/9/11/13/14* and *AhGA3ox 2/3*.

Fig. 9 Expression analysis of *AhGA20ox* and *AhGA3ox* genes in different tissues of peanut plants. The heatmap was generated with the qRT-PCR values of 15 AhGA20ox and 5 AhGA3ox genes using the online tool, TBtools, and the color scale beside the heat map indicates gene expression levels, low transcript abundance indicated by blue color and high transcript abundance indicated by red color. Fifteen AhGA20ox and 5 AhGA3ox genes were classifed into two groups Group I, *AhGA20ox9/11*and *AhGA3ox 1/4/5*; Group II, *AhGA20ox1-AhGA20ox9*, *AhGA20ox10*, *AhGA20ox12-AhGA20ox15* and *AhGA3ox 2/3*.

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Figure 1

Phylogenetic analysis of GA2ox and GA3ox protein in *Arabidopsis Thaliana* (At), rice (Os), soybean (Gm) and peanut (Ah)

The phylogenetic tree was constructed using neighbor-joining method in MRGA 7.0 with bootstrap value of 1000. The numbers represent The scale of the evolutionary tree. Gene accession numbers of the sequences used in this tree are listed in Table S2.

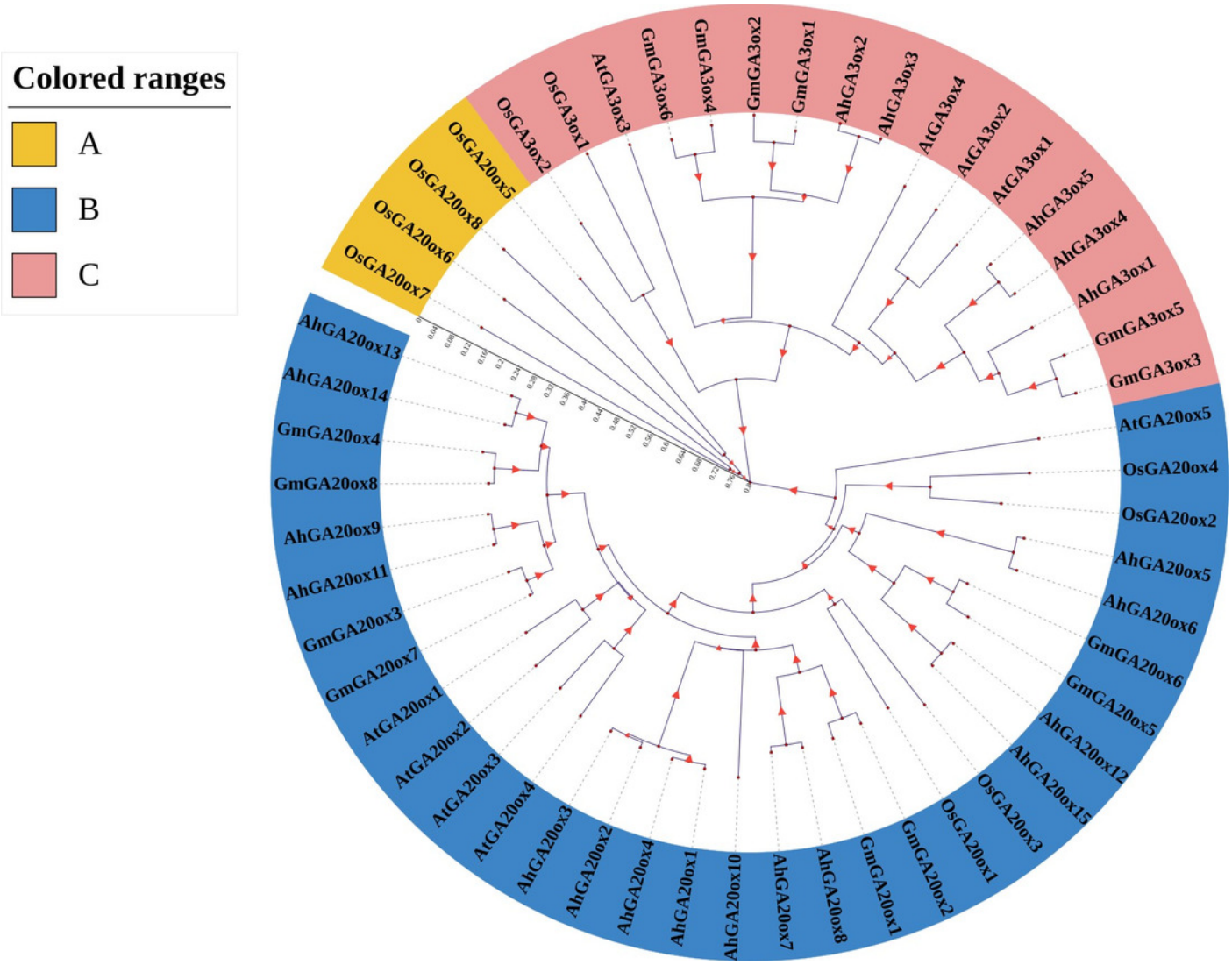

Figure 2

Gene structure organization of peanut *GA20ox* and *GA3ox* family members

Exons (CDS) and UTR are represented by yellow boxes and blue boxes, respectively, and grey lines between exons represents introns.

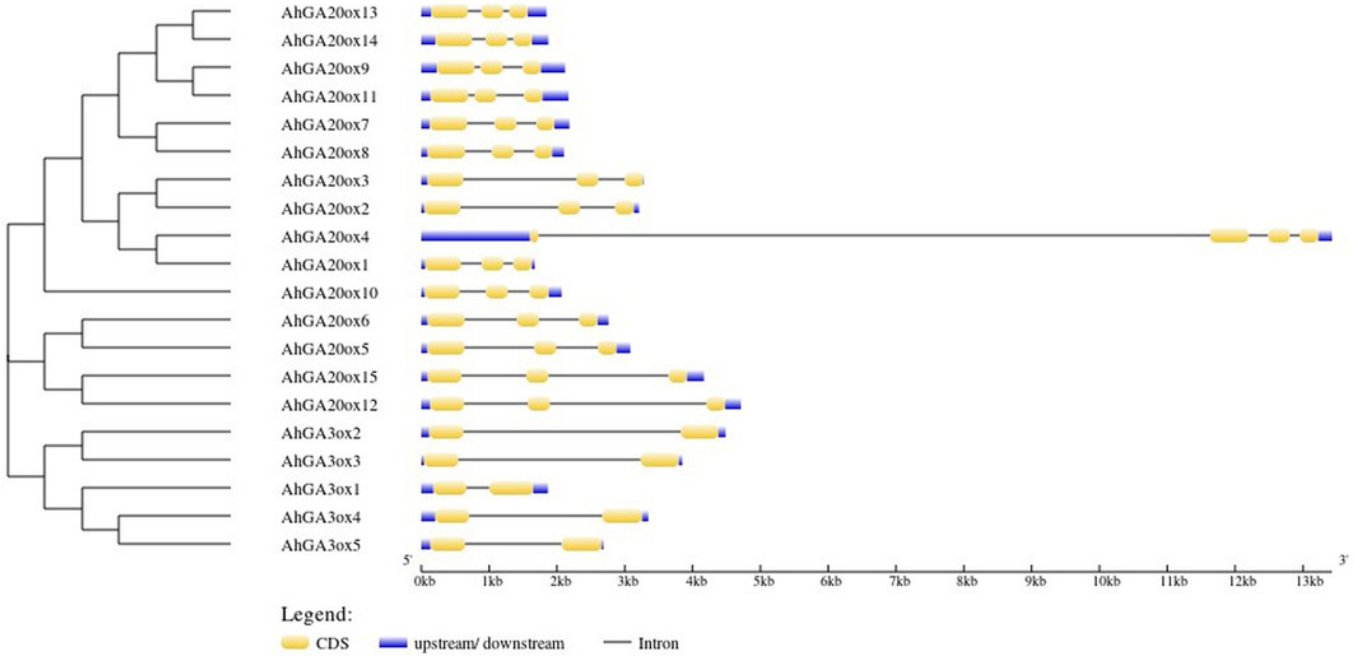

Figure 3

Gene structure organization of peanut *GA20ox* and *GA3ox* family members

The sequence information of motifs marked different colors is represented at the bottom.

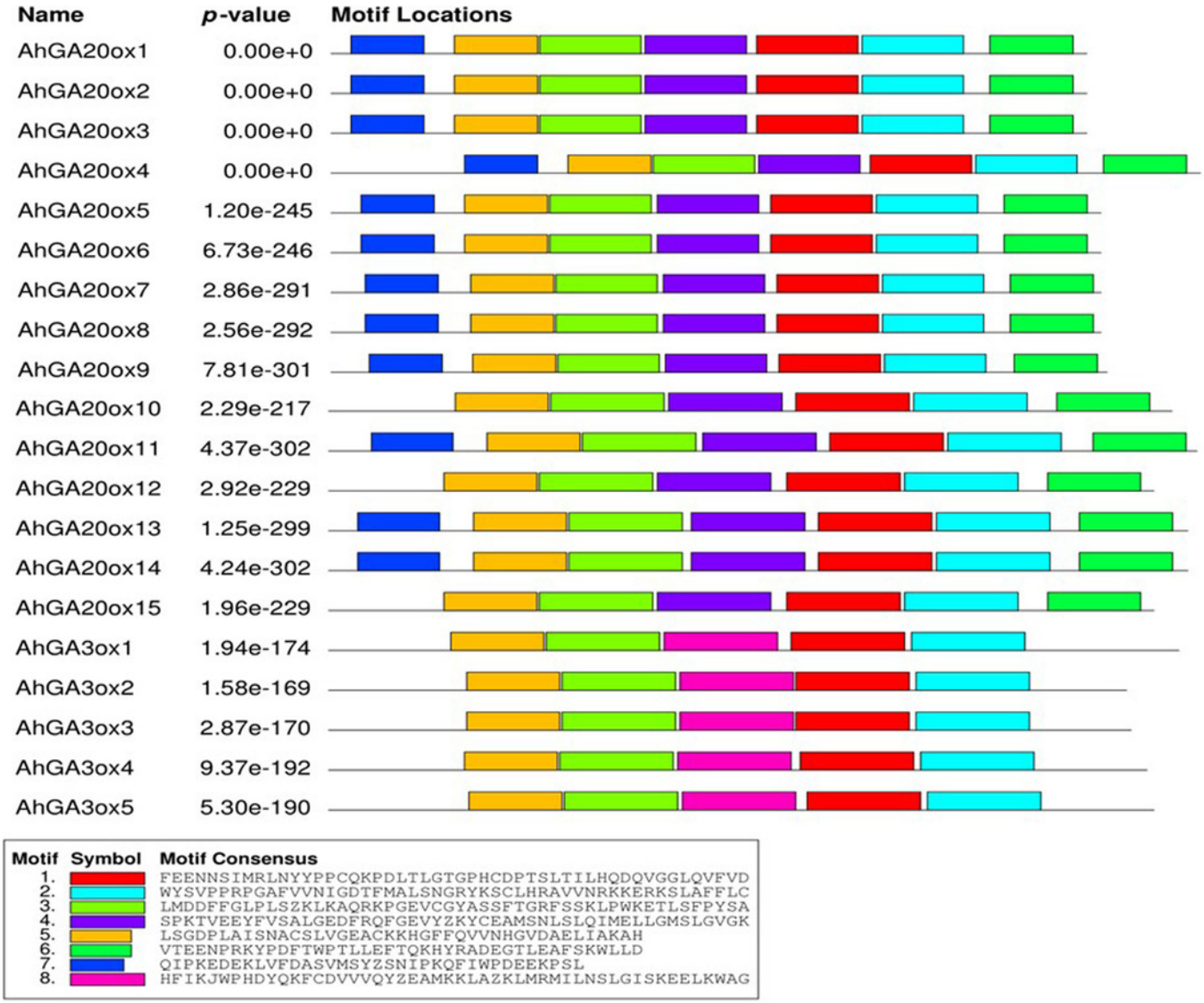

Figure 4

The organization of cis-acting elements in the promoter region of *AhGA20ox* and *AhGA3ox* genes in peanut.

Different colors were used to indicate different elements.

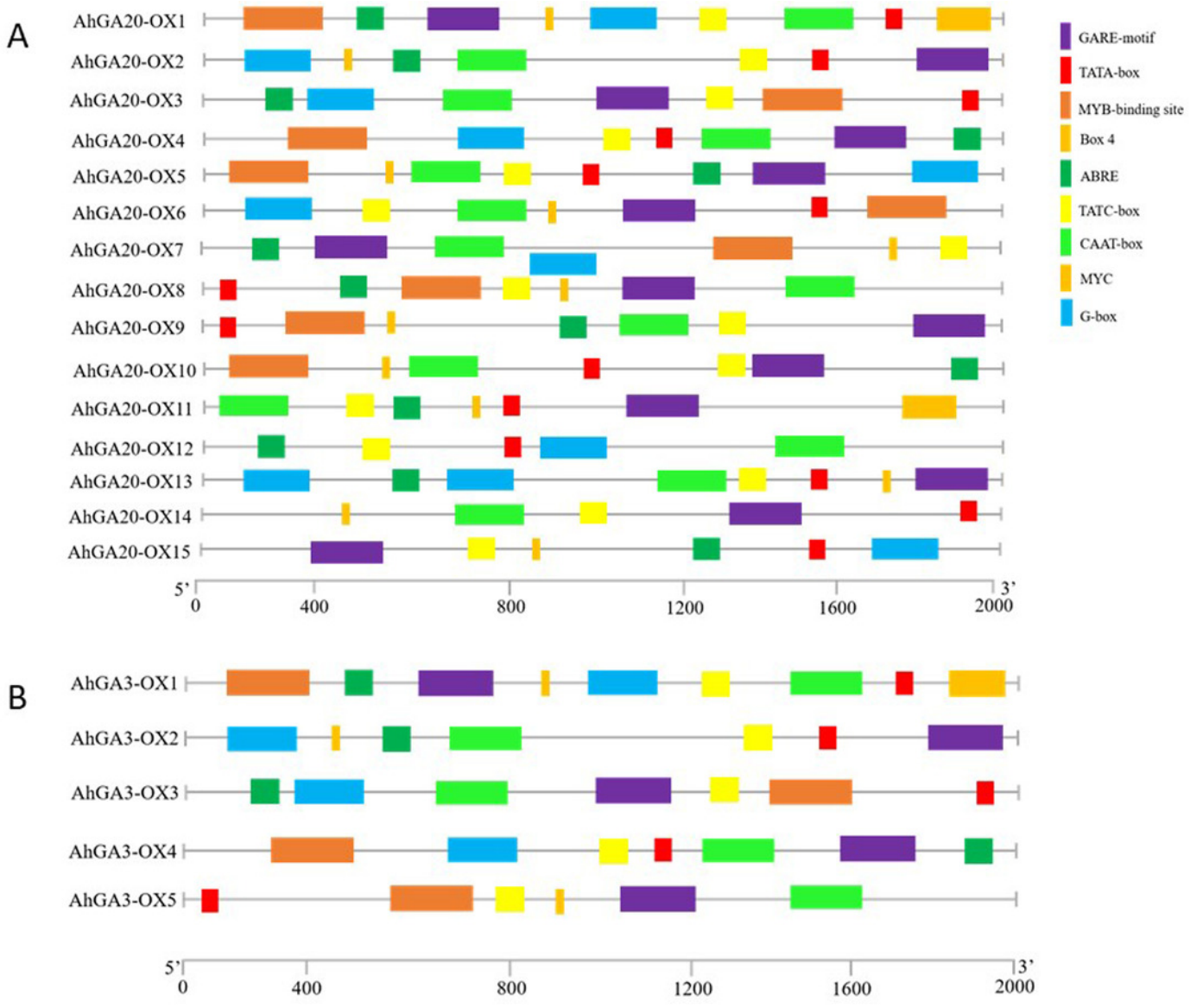

Figure 5

Chromosome mapping of *AhGA20ox* and *AhGA3ox* genes.

Fifteen AhGA20ox and 5 AhGA3ox genes were unevenly distributed on the 14 chromosomes, with the exception of chr. 01, 06, 10, 11 and 16. The location on the chromosome of each AhGA20ox and AhGA3ox gene was indicated on the right side of the respective chromosome. The scale bar for chromosome length was showed at the left of all chromosomes.

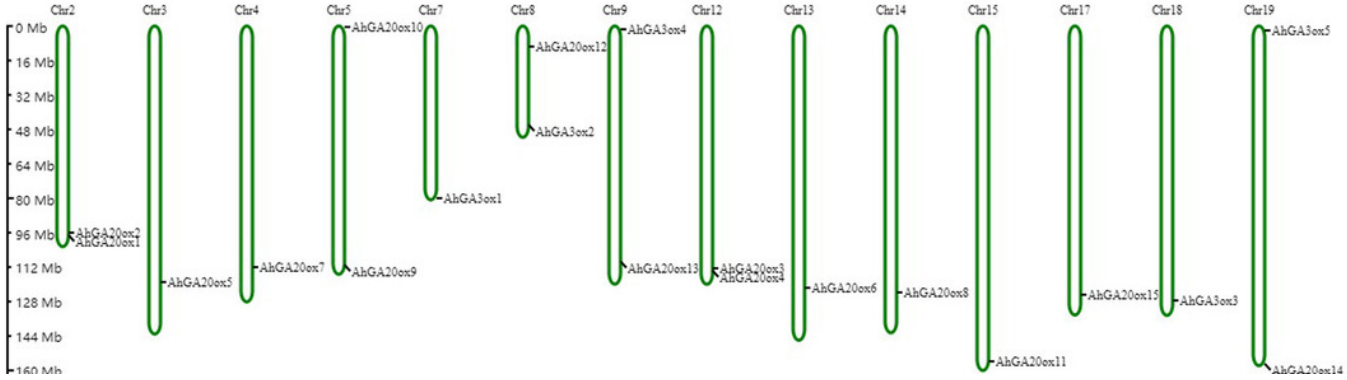

Figure 6

The prediction of protein– protein interaction network of AhGA20ox and AhGA3ox encoding proteins.

Nodes represent proteins, and lines indicate that they have interaction relationship between proteins.

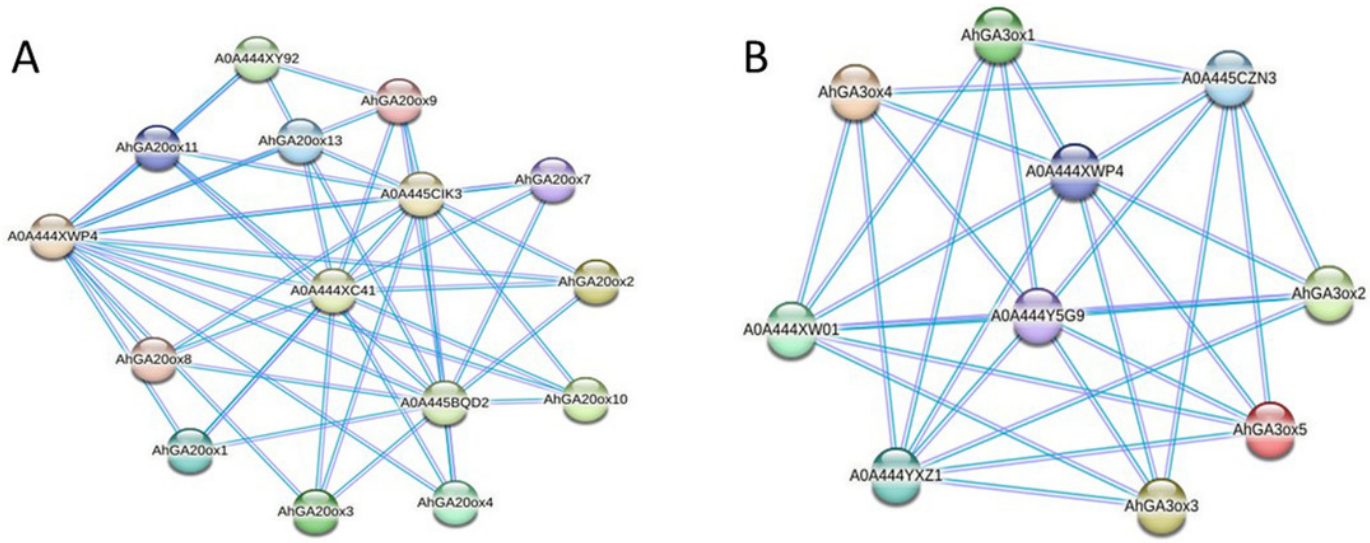

Figure 7

The morphology peg and pod at different developmental stages.

S1-S6 means different development stages of pod in peanut.

Figure 8

Expression analysis of *AhGA20ox* and *AhGA3ox* genes in different stages of peanut pod development.

The heatmap was generated with the qRT-PCR values of 15 AhGA20ox and 5 AhGA3ox genes using the online tool, TBtools, and the color scale beside the heat map indicates gene expression levels, low transcript abundance indicated by blue color and high transcript abundance indicated by red color. Fifteen AhGA20ox and 5 AhGA3ox genes were classifed into three groups Group I, *AhGA20ox15/12/4/1*and *AhGA3ox 1/4/5*; Group II, *AhGA20ox2/3/5/6 /10* ; Group III, *AhGA20ox7/8/9/11/13/14* and *AhGA3ox 2/3*.

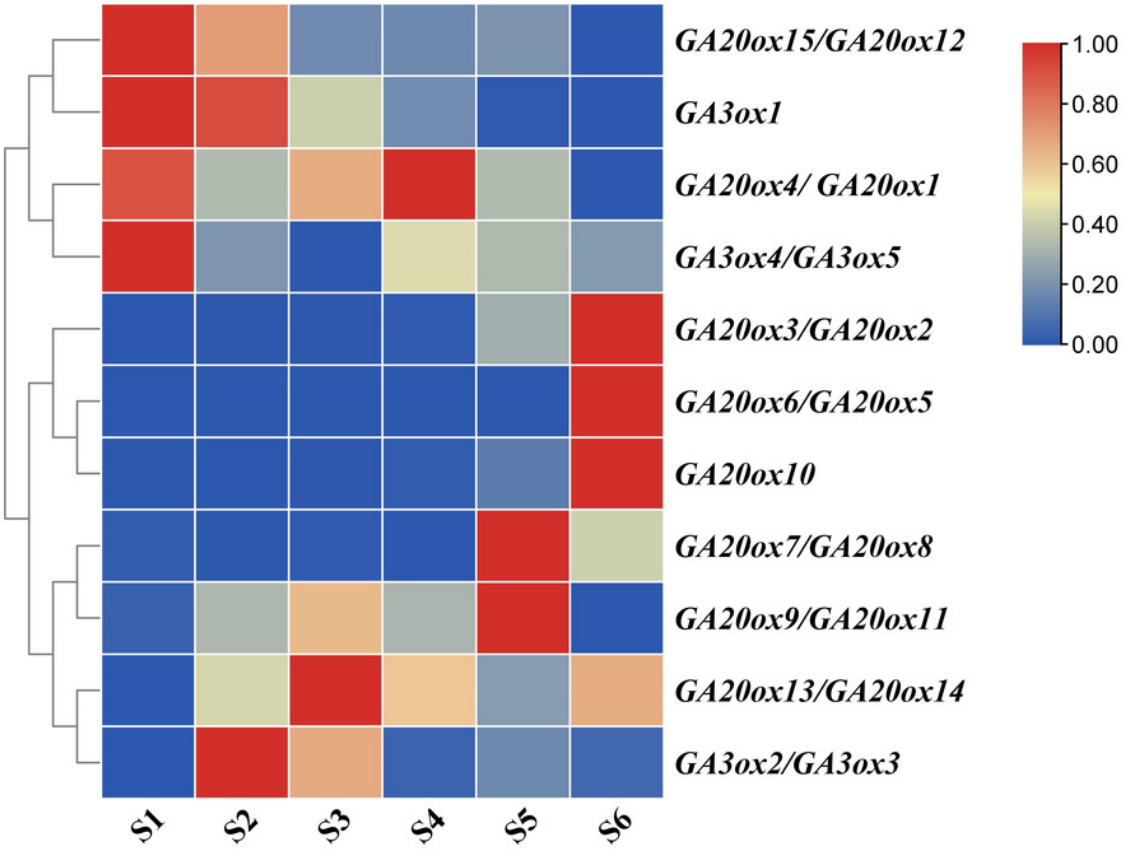

Figure 9

Expression analysis of *AhGA20ox* and *AhGA3ox* genes in different tissues of peanut plants.

The heatmap was generated with the qRT-PCR values of 15 AhGA20ox and 5 AhGA3ox genes using the online tool, TBtools, and the color scale beside the heat map indicates gene expression levels, low transcript abundance indicated by blue color and high transcript abundance indicated by red color. Fifteen AhGA20ox and 5 AhGA3ox genes were classifed into two groups Group I, *AhGA20ox9/11*and *AhGA3ox 1/4/5*; Group II, *AhGA20ox1-AhGA20ox9*, *AhGA20ox10*, *AhGA20ox12-AhGA20ox15* and *AhGA3ox 2/3*.

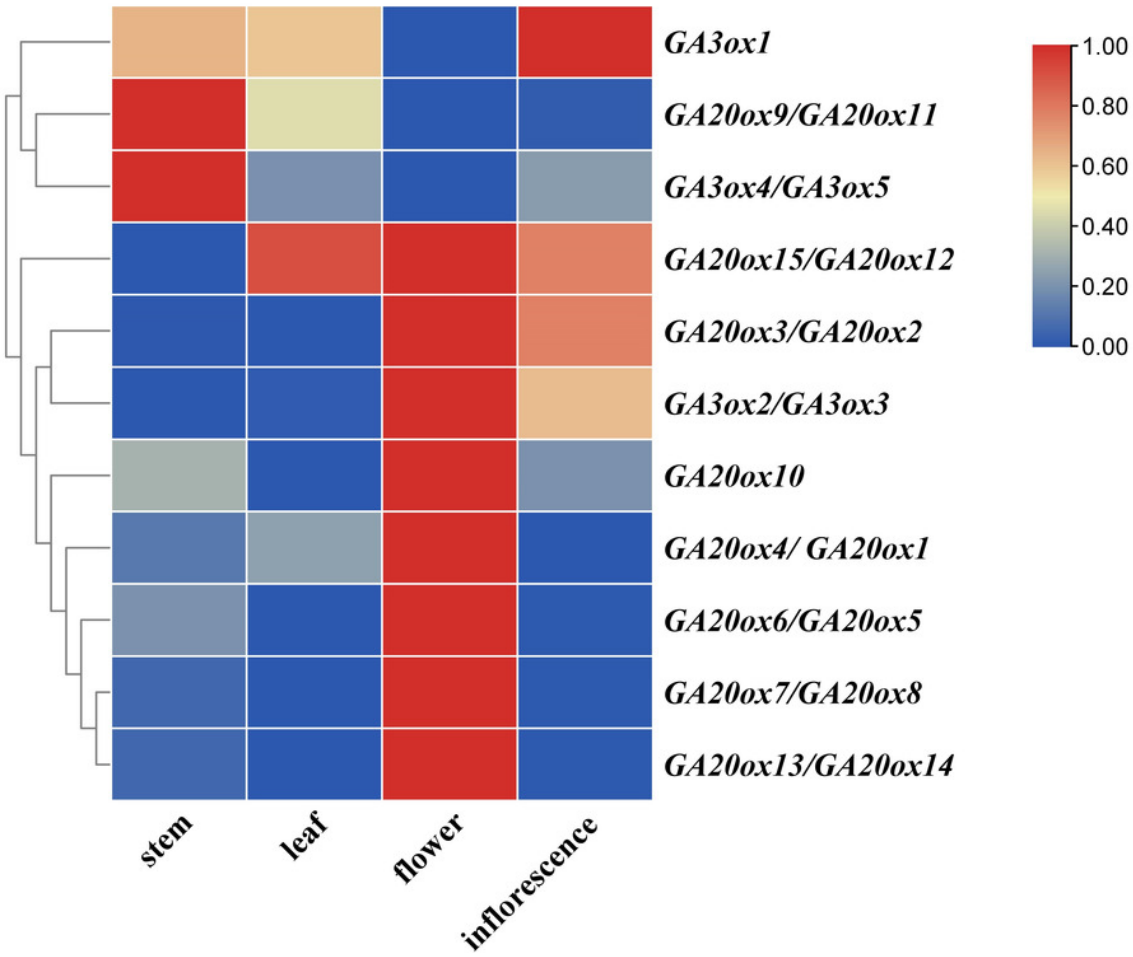

Table 1(on next page)

Physicochemical properties of AhGA20ox and AhGA3ox family members in peanut

Table 1 Physicochemical properties of AhGA20ox and AhGA3ox family members in peanut.

Gene name	Gene accession number	Chrome	Length of CDS	Length of peptide	PI	MV
AhGA20ox1	LOC112755206	2	1119	372	6.14	42612.51
AhGA20ox2	LOC112754423	2	1119	372	5.70	42603.44
AhGA20ox3	LOC112728370	12	1119	372	5.70	42531.33
AhGA20ox4	LOC112728437	12	1287	428	6.20	49169.01
AhGA20ox5	LOC112791095	3	1140	379	6.16	42955.71
AhGA20ox6	LOC112733062	13	1140	379	5.73	42927.69
AhGA20ox7	LOC112794404	4	1140	379	6.76	43758.31
AhGA20ox8	LOC112740586	14	1140	379	7.09	43671.10
AhGA20ox9	LOC112802766	5	1149	382	5.80	43477.38
AhGA20ox10	LOC112799975	5	1116	371	5.14	42256.14
AhGA20ox11	LOC112751696	15	1149	382	6.27	43374.34
AhGA20ox12	LOC112706011	8	1092	363	6.94	41469.38
AhGA20ox13	LOC112711704	9	1137	378	6.16	42856.01
AhGA20ox14	LOC112779497	19	1137	378	6.16	42909.95
AhGA20ox15	LOC112767252	17	1092	363	6.94	41371.32
ATGA20ox1	AT4G25420	4	1428	377	5.77	43224.26
ATGA20ox2	AT5G51810	5	1358	378	4.90	33520.78
ATGA20ox3	AT5G07200	5	1323	380	6.90	43437.39
ATGA20ox4	AT1G60980	1	1568	376	7.14	43133.35
ATGA20ox5	AT1G44090	1	1810	385	8.04	43161.15
GmGA20ox1	Glyma03g02260	3	1149	383	6.15	43484.61
GmGA20ox2	Glyma07g08950	7	1191	397	6.50	44987.53
GmGA20ox3	Glyma09g27490	9	1149	383	5.63	43363.39
GmGA20ox4	Glyma10g38600	10	1098	366	5.64	41163.03
GmGA20ox5	Glyma13g09460	13	1128	376	6.27	42797.56
GmGA20ox6	Glyma14g25280	14	1047	349	6.37	39525.06
GmGA20ox7	Glyma16g32550	16	1026	342	5.85	38535.98
GmGA20ox8	Glyma20g29210	20	1152	384	5.85	43327.47
OsGA20ox1	Os03g63970	3	1854	372	5.98	42255.71
OsGA20ox2	Os01g66100	1	3123	389	5.73	42513.19
OsGA20ox3	Os07g07420	7	2744	367	5.75	40494.75
OsGA20ox4	Os05g34854	5	6929	444	6.70	47634.91

OsGA20ox5	Os03g42130	3	2008	352	5.17	39293.06
OsGA20ox6	Os04g39980	4	2123	300	5.51	32102.33
OsGA20ox7	Os08g44590	8	2380	383	5.96	41831.59
OsGA20ox8	Os04g55070	4	3640	326	5.34	35797.44
AhGA3ox1	LOC112703711	7	1128	375	8.11	41693.86
AhGA3ox2	LOC112707838	8	1059	352	7.30	40020.57
AhGA3ox3	LOC112769088	18	1065	354	6.49	40322.80
AhGA3ox4	LOC112709815	9	1086	361	8.07	40659.64
AhGA3ox5	LOC112777276	19	1095	364	8.08	41025.03
AtGA3ox1	At1g15550	1	1077	358	6.34	40161.81
AtGA3ox2	At1g80340	1	1044	347	6.56	38782.41
AtGA3ox3	At4g21690	4	1050	349	6.16	39210.76
AtGA3ox4	At1g80330	1	1068	355	5.49	39152.51
GmGA3ox1	Glyma04g07520	4	1026	342	6.62	38420.91
GmGA3ox2	Glyma06g07630	6	1044	348	6.18	39059.39
GmGA3ox3	Glyma13g43850	13	1059	353	8.52	39245.13
GmGA3ox4	Glyma14g16060	14	1044	348	6.45	38768.46
GmGA3ox5	Glyma15g01500	15	1062	354	7.72	39313.07
GmGA3ox6	Glyma17g30800	17	1053	351	6.65	39126.85
OsGA3ox1	Os05g08540	5	1155	385	5.95	41555.12
OsGA3ox2	Os01g08220	1	1122	374	6.47	40572.22

4