Identification, Evolutionary and Expression Analysis of PYL-PP2C-SnRK2s Gene Families in Soybean

Zhaohan Zhang 1,2, Shahid Ali 1,2, Tianxu Zhang 1,2, Wanpeng Wang 1,2 and Linan Xie 1,2,*

1 College of Life Sciences, Northeast Forestry University, Harbin 150040, China; zhangzhaohan@nefu.edu.cn (Z.Z.); shahidali@nefu.edu.cn (S.A.); tianxuzhang@nefu.edu.cn (T.Z.); wangwanpeng@nefu.edu.cn (W.W.)
2 Key Laboratory of Saline-Alkali Vegetative Ecology Restoration, Ministry of Education, College of Life Science, Northeast Forestry University, Harbin 150040, China
* Correspondence: linanxie@nefu.edu.cn

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Abstract: Abscisic acid (ABA) plays a crucial role in various aspects of plant growth and development, including fruit development and ripening, seed dormancy, and involvement in response to various environmental stresses. In almost all higher plants, ABA signal transduction requires three core components; namely, PYR/PYL/RCAR ABA receptors (PYLs), type 2C protein phosphatases (PP2Cs), and class III SNF-1-related protein kinase 2 (SnRK2s). The exploration of these three core components is not comprehensive in soybean. This study identified the GmPYL-PP2C-SnRK2s gene family members by using the JGI Phytozome and NCBI database. The gene family composition, conservation, gene structure, evolutionary relationship, cis-acting elements of promoter regions, and its coding protein domains were analyzed. In the entire genome of the soybean, there are 21 PYLs, 36 PP2Cs, and 21 SnRK2s genes; further, by phylogenetic and conservation analysis, 21 PYLs genes are classified into 3 groups, 36 PP2Cs genes are classified into seven groups, and 21 SnRK2s genes are classified into 3 groups. The conserved motifs and domain analysis showed that all the GmPYLs gene family members contain START-like domains, the GmPP2Cs gene family contains PP2Cc domains, and the GmSnRK2s gene family contains S_TK domains, respectively. Furthermore, based on the high-throughput transcriptome sequencing data, the results showed differences in the expression patterns of GmPYL-PP2C-SnRK2s gene families in different tissue parts of the same variety, and the same tissue part of different varieties. Our study provides a basis for further elucidation of the identification of GmPYL-PP2C-SnRK2s gene family members and analysis of their evolution and expression patterns, which helps to understand the molecular mechanism of soybean response to abiotic stress. In addition, this provides a conceptual basis for future studies of the soybean ABA core signal pathway.

Keywords: soybean; PYL; PP2C; SnRK2; ABA signal pathway; differential expression; gene family

1. Introduction

Soybean (Glycine max L. Merr) is an essential leguminous crop that provides the human diet with an essential protein source, livestock feed, and industry biodiesel [1]. However, the sustainability of soybean yields is challenged by expected changes in the climate with prolonged drought, salinity, and high temperature in many areas of the world [2]. Many innate survival mechanisms have evolved to respond to these adverse conditions, a crucial metabolic process called abscisic acid accumulation [3]. Abscisic acid (ABA) is a key regulator for plant stress-adaptation; the ABA signaling pathways are made up of many important turnover mechanisms in response to ever-changing environmental conditions [4]. The PYL-PP2C-SnRK2s family of proteins are the main members of the ABA core signaling pathway.
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and play an important role in response to abiotic stress-mediated by ABA. PYL is located at the top of
the ABA signal transduction pathway, mainly regulated by inhibiting type 2C protein phosphatase
(PP2C) [5]. In the absence of ABA, PYL protein does not bind to PP2C, so PP2C activity is very
high, thus preventing the activation of SNF1-Related protein kinases2 (SnRK2) and its downstream
factors; in the presence of ABA, PYL binds to PP2C and inhibits its activity [6,7]. The inhibited PP2C
release SnRK2, phosphorylated SnRK2 activating the ABA response element-binding factor (ABFs),
and initiates a downstream response to stress [8,9]. ABA signal transduction pathway plays a key
role in plant response to drought and salt stresses [10,11]. At present, the PYL family of the model
plant Arabidopsis thaliana has been studied comprehensively. 14 PYL proteins with highly conserved
amino acid sequences have been identified in A. thaliana [12]. Some PYL protein functions have been
successfully verified, such as overexpression of AtPYR1, AtPYL1, AtPYL2, and AtPYL3 genes that
can improve drought resistance and water use efficiency of A. thaliana [13]. In A. thaliana, the PYR1
and PYL2 are the key receptors for stomatal closure induced by abscisic acid [14]. Overexpression of
AtPYL5 reduces the transpiration rate and reduce water loss, oxidative stress damage, and increase
photosynthesis rate under drought stress [15]. Overexpression of AtPYL4, AtPYL8, and AtPYL13
genes can enhance drought tolerance in A. thaliana [16–19]. Although the soybean GmPYLs gene
family members are already identified [20], the bioinformatics analysis and expression characteristics
of the soybean GmPYLs gene family have not been reported. The SnRK2 is the key component of
ABA signal transduction pathways; each SnRK2 protein contains three functional domains, such as a
conserved Ser/Thr kinase domain, adenosine triphosphate (ATP) binding domain [21], and a diverse
regulatory C-terminal. The members of the SnRK2s family have been identified in various plant species,
including Triticum aestivum (TaSnRK2.3, 2.4, 2.7, and 2.8) [22,23], Oryza sativa (SAPK1–10) [24,25],
A. thaliana (AtSnRK2.1–2.10), G. max (GmSnRK2.1–2.22) [26]. Different studies demonstrated ABA
hypersensitivity after PP2C loss-of-function mutant, indicating its important role in ABA signaling
pathway. PP2C proteins belong to monomer enzymes, and their functions depend on Mg²⁺ and
Mn²⁺. The catalytic domain of PP2C proteins is located at either the C-terminus or N-terminus in
eukaryotes [27]. Further study has shown that the catalytic domain regions of eukaryotic PP2C
proteins are conserved, while the non-catalytic domain regions have diverse amino acid sequences [28].
However, to the best of our knowledge, bioinformatics analysis of PYL-PP2C-SnRK2s gene families in
soybean is not comprehensive.

To further explore soybean genome information at the whole genome level, and elaborate
the structure and function of GmPYL-PP2C-SnRK2s gene families, in the present study,
the GmPYL-PP2C-SnRK2 family in soybean, their phylogenetic relationship, chromosomal distribution,
gen structure, protein motifs, and expression patterns in various tissues have helped to better
understand the molecular mechanisms and the role of core components in the ABA signaling pathway.
Furthermore, this study should help the breeder to manipulate and improve the yield and quality of
soybean under different stress conditions.

2. Results

2.1. Identification and Nomenclature of GmPYL-PP2C-SnRK2s Gene Families

To identify the GmPYL-PP2C-SnRK2s gene family members in soybean genome using A. thaliana
and O. Sativa PYL-PP2C-SnRK2s gene family as queries, we named the homologous soybean
genomes of each GmPYL-PP2C-SnRK2s by searching Blastp against NCBI, a total of 21 GmPYLs, 36
GmPP2Cs, and 21 GmSnRK2 genes. The genes were named GmPYL1~GmPYL21, GmPP2C1~GmPP2C36,
and GmSnRK2.1~GmSnRK2.21, respectively. Furthermore, the length, amino acid sequence of genes,
and position of these genes on chromosomes were identified (Table S1). The coding sequence length
of the 21 GmPYLs genes varies from 534 to 801 bp, but the encoded protein length ranges from 177 to 266
amino acids. The coding sequence length of 36 GmPP2Cs genes varies between 732 to 2163 bp, and the
protein length ranges from 243 to 720 amino acids. The coding sequence length of 21 GmSnRK2s
genes varies from 1011 to 1113 bp, and the length of the encoded protein is almost the same, ranging from 336 to 370 amino acids. The number of PYL-PP2C-SnRK2s did not vary substantially between A. thaliana (PYL:14, PP2C:12, SnRK2:10) and O. sativa (PYL:13, PP2C:12, SnRK2:10), but the amount of PYL-PP2C-SnRK2 in G. max (PYL:21, PP2C:36, SnRK2:21) was significantly higher than that in A. thaliana and O. sativa.

2.2. Phylogenetic Analysis of GmPYL-PP2C-SnRK2s Gene Families

To classify and determine the evolutionary relationship of PYL-PP2C-SnRK2s genes, the amino acid sequences of PYLs, PP2Cs, and SnRK2s proteins from G. max, A. thaliana, and O. sativa were selected to construct three phylogenetic Maximum Likelihood trees. The name and ID of PYL-PP2C-SnRK2s genes in O. sativa and A. thaliana are shown in Table S2. According to the branching characteristics and bootstrap values of phylogenetic trees, a total of 48 PYLs from these three species were clustered into three subgroups (I–III), including 21 GmPYLs (G. max), 14 AtPYLs (A. thaliana), and 13 OsPYLs (O. sativa) (Figure 1A). A total of 60 PP2Cs from these three species were clustered into ten subgroups (I–X), including 36 GmPP2Cs (G. max), 12 AtPP2Cs (A. thaliana), and 12 OsPP2Cs (O. sativa) (Figure 1B). And a total of 41 SnRK2s from these three species were clustered into three subgroups (I–III), including 21 GmSnRK2s (G. max), 10 AtSnRK2s (A. thaliana) and 10 OsSnRK2s (O. sativa) (Figure 1C). Meanwhile, GmPP2C17, GmPP2C18, and GmPYL19 were phylogenetically close to AtPP2C9 and AtPYL10, and GmPP2C13 and GmPP2C14 were phylogenetically close to AtPP2C9. Furthermore, GmSnRK2.4 and GmSnRK2.19 were phylogenetically close to AtSnRK2.3. There may be similarities in gene structure, protein structure, and functional domain among the same group of genes PYLs, PP2Cs, and SnRK2s in A. thaliana and G. max (Figure 1).

![Figure 1](http://gsds.gao-lab.org)  
**Figure 1.** Phylogenetic trees of PYLs (A), PP2Cs (B), and SnRK2s (C) genes in G. max, A. thaliana, and O. sativa by using the complete protein sequences. The phylogenetic trees were constructed, the Maximum Likelihood was adopted using MEGA 10.0 software, with 1000 bootstrap replicates. In GmPYLs and GmSnRK2s genes, the group I–III, and in GmPP2C genes, I–X use different colors to distinguish them.

2.3. Evolutionary Conservation and Gene Structure of GmPYL-PP2C-SnRK2s

We used the GENE Structure display website (http://gsds.gao-lab.org) to draw GmPYL-PP2C-SnRK2s gene structure online, and show 16 GmPYLs genes have no intron, except GmPYL15, GmPYL16, GmPYL17, GmPYL18, and GmPYL19 (Figure 2A) [29]. In the GmPP2C family, 14 genes have 4 introns, while GmPP2C14 has 10 introns, GmPP2C13 lacks the UTR region. Almost all GmSnRK2s have 9 introns; only GmSnRK2.12 has 10 introns.
Figure 2. The gene structure and conserved motifs of GmPYLs (A), GmPP2Cs (B), and GmSnRK2s (C) based on phylogenetic relationships. All motifs were identified using the MEME website with the complete amino acid sequences of GmPYLs, PP2Cs, and SnRK2s. For example, in A, the phylogenetic tree (left) was constructed using the MEGA10.0 program with the complete amino acid sequences of the 21 GmPYLs proteins; Exon and intron structure analyses of GmPYLs genes were performed by the GENE Structure website (middle). The white boxes, black boxes, and the black lines indicate UTRs region, exons, and introns. The length of the amino acid, exon, and intron can be inferred by the ruler at the bottom—conserving motif distribution map of the GmPYLs gene performed by TBtools (right). The 10 predicted motifs are represented by different colored boxes, Motif LOGO showing below.
For conservative motif analysis, used MEME website (http://meme-suite.org/) showed that 10 conserved motifs (motif1~motif10) were identified in GmPYL-PP2C-SnRK2s proteins, respectively [30]. The width, sites, and E-value of GmPYL-PP2C-SnRK2s proteins and their conserved motifs are shown in Table S3. The conserved motif’s width, approximately 8–50 amino acids, conserved motif LOGO, is shown in Figure 2. Genes in the same branch of the phylogenetic tree have a similar conservative motif.

Using “Batch SMART” plug-in in the TBtools software (Version 1.055) upload amino acid sequence, we found that all GmPYLs genes include the Polyketide_cyc2 domain, some GmPYLs genes contain Polyketide_cyc and Pfam: Bet_v_1 domain, only GmPYL14 contains Pfam: peroxidase domain, which indicates that GmPYL14 may have different functions in the GmPYLs gene family (Figure 3A). All 36 GmPP2Cs genes contain PP2Cc domains (Figure 3B). Each GmSnRK2s gene contains a major serine-threonine (Ser/Thr) kinase catalytic domain (S_TKc) (Figure 3C). The phosphatase domain of the PP2C-type consists of 10 beta-strand segments and 5 alpha-helix segments and comprises a pair of detached subdomains. The first is a small beta-sandwich with strand beta1 packed against strands beta2 and beta3; the second is a larger beta-sandwich in which a four-stranded beta-sheet pack against a three-stranded beta-sheet with flanking alpha-helices. And each GmSnRK2 contains a major serine-threonine (Ser/Thr) kinase catalytic domain (S_TKc). The catalytic domain of serine/threonine protein kinases is predominantly alpha-helical and displays functional and structural similarity with the ATP-grasp fold and PIPK. Anti-parallel beta-sheets form the alpha and beta folds. It is a phosphotransferase that contains a conserved catalytic center. There is a glycine-rich area of residues near a lysine residue involved in ATP binding at the N-terminal end. There is an aspartic acid residue in the center of the STKc catalytic domain that drives the catalytic enzyme (Figure 3C), SCOP d1ihma domain is distinctive to GmPP2C1, GmPP2C28. Furthermore, the coiled-coil region is differential to GmSnRK2.7, GmSnRK2.14, and GmSnRK2.15 nearly all genes contain a low complexity region.

![Figure 3](https://example.com/figure3.jpg)

**Figure 3.** Analysis of the conserved domains GmPYLs (A), GmPP2Cs (B), and GmSnRK2s (C) by using the SMART website. All GmPYLs gene contains Pfam: Polyketide_cyc2 domain. All GmPP2Cs genes contain PP2Cc domain, and further, each GmSnRK2s contains a major serine-threonine (Ser/Thr) kinase catalytic domain (S_TKc).
2.4. Chromosomal Localization and Collinearity Analysis of GmPYL-PP2C-SnRK2s Gene Families

Based on the positioning information for chromosomes in the JGI Phytozome database (Table S1), the result drawing with MapChart software is shown in Figure 4. Among the 20 chromosomes of soybean, 21 GmPYLs genes were located on chromosomes 1–4, 6–9, and 13–18. There are three PYLs genes on chromosome 1, and two PYLs genes on chromosomes 6, 7, 13, and 14; further, the rest of the chromosomes containing a single gene of the PYL family. The 36 GmPP2Cs genes are located on chromosomes 2–4, 6, 7, 9–14, and 17–20. There are five genes of GmPP2Cs on chromosome 6, and the remaining genes are located on other chromosomes. 21 GmSnRK2s genes are located on chromosomes 1, 2, 4–8, 11, 12, 14, 17, and 20. The chromosomes 1, 2, 7, 8, 11, and 17 contain two genes, and the rest of the chromosomes contain one gene. Besides, collinearity and replication analysis of all genes was carried out using TBtools software. The results showed that most of the GmPYLs, GmPP2Cs, and GmSnRK2s genes contained a collinear relationship, indicating that most of the GmPYLs, GmPP2Cs, and GmSnRK2s genes had fragment replication, which led to an increase in the number of genes (Figure 5, Table S4). The results showed that GmPYLs, GmPP2Cs, and GmSnRK2s genes had complex genomic replication events, and most of the genes were retained.

Figure 4. Chromosomal localization of GmPYLs, GmPP2Cs, and GmSnRK2s genes. 21 GmPYLs genes were distributed on chromosomes 1–9 and 11–18, the 36 GmPP2Cs genes are located on chromosomes 2–4, 6, 7, 9–14, and 17–20; further, the 21 GmSnRK2s genes are located on chromosomes 1, 2, 4–8, 11, 12, 14, 17 and 20, respectively. The chromosome numbers are indicated at the top of each vertical bar. The ruler indicates the length of the chromosome/Mb.
After 14 days of seedlings, abscisic acid (ABA), preceded by the response element of methyl jasmonate (MeJA) (Figure 6, Table S5).

The GmPYL-PP2C-SnRK2s gene families mainly contains cis-acting elements related to stress, growth, development, and plant hormones. The GmPYL-PP2C-SnRK2s gene families contain five hormone response elements. It includes the abscisic acid (ABA) response element, gibberellin response element, auxin response element, MeJA response element, and salicylic acid response element. Between them, mostly, the response elements belong to the abscisic acid (ABA), preceded by the response element of methyl jasmonate (MeJA) (Figure 6, Table S5).

Figure 5. Collinearity analysis of GmPYLs (A), GmPP2Cs (B), and GmSnRK2s (C) gene families. In each circle, red, green, and blue lines showing that most of the GmPYLs, GmPP2Cs, and GmSnRK2s genes existed collinear relationship.

2.5. Analysis of GmPYLs, GmPP2Cs, GmSnRK2s Genes Promoter Sequence

To predict the function of GmPYLs, GmPP2Cs, and GmSnRK2s gene family, the cis-acting elements of each GmPYL-PP2C-SnRK2s genes’ upstream 2 kb sequences from the transcription start site (TSS) were analyzed. The results show that GmPYL-PP2C-SnRK2s gene families have a wide range of potential mechanisms in response to abiotic stress. The promoter region of GmPYL-PP2C-SnRK2s gene families mainly contains cis-acting elements related to stress, growth, development, and plant hormones. The GmPYL-PP2C-SnRK2s gene families contain five hormone response elements. It includes the abscisic acid (ABA) response element, gibberellin response element, auxin response element, MeJA response element, and salicylic acid response element. Between them, mostly, the response elements belong to the abscisic acid (ABA), preceded by the response element of methyl jasmonate (MeJA) (Figure 6, Table S5).

Figure 6. The composition of cis-acting regulatory elements in the promoters of GmPYLs (A), GmPP2Cs (B) and GmSnRK2s (C). The five hormone response elements contained in each gene family promoter include the ABA response element, GA response element, IAA response element, MeJA response element, and the SA response element.
We found eight potential elements to be involved in the regulatory mechanisms of ABA, MeJA, IAA (auxin), GA (gibberellin), and SA (salicylic acid), such as ABRE, TCA-element, AuxRR-core, GARE-motif, CGTCA/TGACG-moif, P-box, TATC-box, and TGA-element. In addition, the four elements (such as ARE, MBS, TC-rich repeats and LTR) were involved in reacting to various stresses, such as drought, low temperature, salinity and anaerobic induction. Various types and numbers of cis-elements have been found in GmPYLs, GmPP2Cs, GmSnRK2s promoters, indicating that they participate in various regulatory mechanisms during plant growth and development (Figure 6, Table S5).

2.6. Tissue-Specificity of GmPYL-PP2C-SnRK2s Genes Expression

The young seedling of the soybean is more vulnerable to abiotic stress. The two representative soybean cultivars, Williams82 and Jack, were used to extract the total RNA from meristem, unifoliate leaves, epicotyl, hypocotyl, and root at VC stage (After 14 days of seedlings, two unifoliate leaves unroll). Each sample produces 6.0-Gb sequencing data, which is sequenced by a high-throughput transcriptome method to investigate the expression patterns of GmPYL-PP2C-SnRK2s gene families. The analysis of differentially expressed genes among two varieties showed that the GmPYLs gene family contained 2, 6, 4, 7, 6 highly significant differentially genes (p ≤ 0.01) and 1, 1, 2, 3, 0 the significant differentially genes (0.01 < p ≤ 0.05) in the meristem, unifoliate leaves, epicotyl, hypocotyl, and roots. The GmPP2Cs gene family contains 12, 14, 8, 16, 16 highly significant differentially genes and 5, 3, 9, 5, 3 significant differentially genes in the meristem, unifoliate leaves, epicotyl, hypocotyl, and roots, respectively. The GmSnRK2s gene family contains 6, 8, 3, 7, 7 highly significant differentially genes, 3, 4, 3, 3, 2 significant differentially genes in the meristem, unifoliate leaves, epicotyl, hypocotyl, and roots respectively (Figure 7). Among them, three genes in the GmPYLs gene family, GmPYL4, GmPYL10, and GmPYL11, were significantly different in unifoliate leaves, hypocotyls, and roots. There are three genes in the GmPP2Cs gene family, which are GmPP2C2, GmPP2C8, and GmPP2C12, are significantly different in epicotyls, hypocotyls, and roots, the Venn diagram is shown in Figure 8. There is no apparent overlap between the two varieties with significant differences in expression among GmPYLs, GmPP2Cs, and GmSnRK2s gene families. The p-value is between 0.01 and 0.05, showing a significant difference between the two groups, while the p-value is ≤ 0.01, showing a highly significant difference between the two groups (Table S6).

![Figure 7](image1.png)  
**Figure 7.** Differentially expressed genes between Williams82 and Jack cultivars in each tissue. Showing the highly significant differential expression (p-value ≤ 0.01) (A). Showing the significant differential expression (0.01 < p-value ≤ 0.05) (B). The different color shows different tissue. M, meristem; U, unifoliate leaves; E, epicotyl; H, hypocotyl; R, roots.
The tissue-specificity of GmPYL-PP2C-SnRK2s gene families' expression is shown in Figure 9, and the TPM (Transcript Per Million) values are shown in the supplementary file (Table S7). In Williams82 and Jack cultivars, the expression of GmPYL15 and GmPYL16 genes were significantly higher in all tissue. Similarly, GmPP2C1, GmPP2C25, GmPP2C28, GmSnRK2.1, GmSnRK15, GmSnRK17 and GmSnRK21 genes were highly expressed in all tissue. It is suggested that these nine genes may play an important regulatory role in response to stress during the early developmental stage.

Figure 8. The Venn diagrams represent GmPYLs (A), GmPP2Cs (B), and GmSnRK2s (C), respectively. Showing the number of highly significant differentially expressed genes between Williams82 and Jack, and different circles represent different tissues. The number of overlapping or non-overlapping regions represents the number of genes. M, meristem; U, unifoliate leaves; E, epicotyl; H, hypocotyl; R, roots.

Figure 9. Differentially expressed genes (DEG) in different tissues. GmPYLs gene family expression level in Williams82 (A) and Jack (B). GmPP2Cs gene family expression level in Williams82 (C) and Jack (D). GmSnRK2s gene family expression level in Williams82 (E) and Jack (F). From blue to red represents a gradual increase in value. The left cluster tree is classified according to the level of expression. A horizontal row indicates the expression of the same gene in different tissues. The column represents the expression of different genes in the same tissue.
Among them, three genes in the GmPYLs gene family, GmPYL16, GmPYL2, and GmPYL14 were significantly different in the unifoliate leaves, hypocotyl, and roots. Five genes of the GmPP2Cs gene family were significantly different in the apical meristem, hypocotyl, unifoliate leaves, and in the root, among which the two genes GmPP2C11 and GmPP2C29 were significantly different in the root, while GmPP2C31, GmPP2C34, and GmPP2C5 had a highly significant difference in the apical meristem, hypocotyl and in unifoliate leaves.

Four genes of the GmSnRK2s gene family were significantly different in the meristem, hypocotyl, and root, among which the two genes, GmSnRK2.5 and GmSnRK2.16, significantly different in the root, while the GmSnRK2.2 and GmSnRK2.7 are significantly different in meristem and epicotyls.

3. Discussion

The GmPYL-PP2C-SnRK2s genes are involved in plant abiotic stress responses, which has been documented in many species, as the core genes of the ABA core signaling pathway. In the past 20 years, researchers have revealed how plants react to stress by producing the plant hormone ABA through the physiological and molecular study of the model plant *A. thaliana* under stress conditions. ABA induces various regulatory mechanisms, including gene expression reprogramming, closing stomata, osmotic adjustment, and finally achieving adaptive growth and development [31–33]. Studying and implementing the molecular mechanisms on essential crops to obtain plant materials with high-stress resistance and sustainable water supply meet global food demand [34]. The ABA signal transduction included three core components of the GmPYL-PP2C-SnRK2s gene family [35]. In the presence of ABA, ABA binds with the receptor PYL to form a complex, which binds to PP2C and inhibits the catalytic activity of PP2C by inserting the door ring into the active crack of PP2C. PYL-mediated inhibition of PP2C activates SnRK2 kinases by activating cycloserine autophosphorylation, which transmits ABA signals by phosphorylating downstream transcription factors [8]. The phytohormone ABA plays an important role in plant growth and development, leaf senescence, and lateral root growth [36,37]. In this study, soybean genome and transcriptome data were used to comprehensively analyze the GmPYLs, GmPP2Cs, and GmSnRK2s gene families, which provides a theoretical basis for studying the mechanism of soybean ABA pathway core genes responding to abiotic stress and affecting growth and development [38,39]. Consistent with *A. thaliana* and *O. sativa* [40], the GmPP2Cs gene family is larger than GmPYLs and GmSnRK2s.

The PYL-PP2C-SnRK2s gene families support the evolutionary relationship among the three selected species. Among them, the gene location of the GmSnRK2s is the same as that of previous studies [26], but compared to other GmSnRK2s, GmSnRK2.6 lacks one intron, three motifs, and some amino acid residues. In addition, GmSnRK2.6 expression showed no difference at each time point. We analyzed the domains of these 22 GmSnRK2 genes and found that all of them contained S_TKc domains except GmSnRK2.6, the GmSnRK2.6 contains the Pfam: kinase domain. Considering these conditions, GmSnRK2.6 is not included in the analysis of our article. Through phylogenetic analysis, the genes of GmPYLs, GmPP2Cs, and GmSnRK2s families were divided into 3, 10, and 3 groups. In the phylogenetic trees of *G. max*, *A. thaliana* and *O. sativa*, the conserved motifs in the same branch are highly similar, indicating that the same group of genes may have similar functions. For example, multiple genes in *A. thaliana* (AtPYR1, AtPYL1, AtPYL2, AtPYL3) all promote ABA-induced stomatal closure [41–43].

SnRK2.6 might influence the expression of RD22 and RD29B, which are ABA-responsive genes in *A. thaliana* [44]. SnRK2.2, SnRK2.6, and SnRK2.3 are redundant SnRK2s activated by ABA and play important roles in seed development and dormancy regulation [45]. Different studies have shown that PP2C plays an important role in abiotic stress response in plants, especially in the ABA signaling pathway [46,47]. The collinear analysis showed that GmPYL1, GmPYL2, GmPYL3, and GmPYL4 genes were homologous to the *A. thaliana* ABA-induced stomatal closure genes, it implies the possible similarity in function. All GmPYLs genes contain Pfam: Bet_v_1, Pfam:Polyketide_cyc and Pfam: Polyketide_cyc2 domains. Among them, Pfam: Polyketide_cyc and Pfam: Polyketide_cyc2 are
members of the START domain superfamily, and their protein folding has a conservative ligand binding mode to adapt to a variety of catalytic activity and non-catalytic regulatory functions. Pfam: Bet_v_1 is the birch pollen protein domain [12], homologous with Pfam: Polyketide_cyc domain [48], overlap with each other in their position. These domains are important for PYL used as the direct receptor of ABA, which combines ABA and PP2C and negatively regulates ABA's core signal pathway [49]. As a core component of ABA signaling, all GmPP2Cs genes contain the PP2Cc domain. In soybean, GmSnRK2s genes contain the S_TK domain, which performs an important function in responses to hyperosmotic stress, high salinity, and dehydration [50,51].

The cis-acting element analysis results showed that in group III, GmPYL17, GmPYL18, and GmPYL19 genes were located at the same branch of the phylogenetic tree, and all contained auxin response elements. Previous studies have shown that AtPYL8 promotes the transcription of auxin response genes independent of the ABA core signal pathway, relieves the ABA core signal pathway’s inhibition on lateral root growth, and promotes lateral root growth [43]. Phylogenetic analysis showed that GmPYL17, GmPYL18, and GmPYL19 genes were firmly related to the A. thaliana AtPYL8 gene. It is speculated that GmPYL17, GmPYL18, and GmPYL19 genes may regulate similar signal pathways in soybean. Besides, all GmPYLs, GmPP2Cs, and GmSnRK2s genes contain at least one plant hormone response element, and plant hormone signals are the key to regulating drought or water deficiency responses [52]. Therefore, GmPYL-PP2C-SnRK2s gene families may play an important role in regulating drought or water deficiency responses [53].

Furthermore, previous studies showed that ABA might induce the expression of jasmonate (JA) biosynthetic genes and then increased the concentrations of JA partially based on the function of ABI5 with clade A protein phosphatases type 2C (PP2Cs), consequently leading to the degradation of JAZ proteins [54]. According to Mustilli et al. [55], 10 members of the SnRK2 family in the A. thaliana genome, of which ABA strongly induces SnRK2.2, SnRK2.3, and SnRK2.6/OST1. SnRK2.6/OST1 is expressed in stomatal guard cells related to stomatal closure induced by ABA, CO2, and methyl jasmonate (MeJA) [55]. In this study, more than half of the GmPP2Cs and GmSnRK2s genes contain MeJA response elements, indicating that these genes may be related to stomatal closure.

The differences in expression patterns of GmPYL-PP2C-SnRK2s gene families in different soybean tissues indicated that there were functional differences among GmPYL-PP2C-SnRK2s gene families. As key genes in ABA’s core signal pathway, GmPYLs, GmPP2Cs, and GmSnRK2s are involved in various abiotic stress responses. It is reported that overexpression of AtPYR1, AtPYL1, AtPYL2, and AtPYL3 genes in the A. thaliana group I can improve drought stress tolerance [13,56]. It can be inferred that group I GmPYL1, GmPYL2, GmPYL3, GmPYL4, GmPYL5, GmPYL6, GmPYL7, and GmPYL8 genes have similar functions, which may also be related to enhance the drought stress tolerance in soybean. The transcriptome data and the previous research data [57] showed the same gene expression patterns, which proves the reliability of the tested results. In the present study, the family structure, evolutionary relationship, and expression pattern of GmPYLs, GmPP2Cs, and GmSnRK2s genes were comprehensively analyzed, which provided a theoretical basis for the directional study of PYL-PP2C-SnRK2s genes in soybean.

4. Materials and Methods

4.1. Plant Materials

Two soybean cultivar, Williams82, and Jack were used as a plant material grown in a greenhouse with (26 °C, light 14 h/dark 10 h) growth conditions. After 14 days of seedlings (VC stage, two unifoliate leaves unroll), the healthy plant’s meristem, unifoliate leaves, epicotyl, hypocotyl, and root were collected separately, and three independent replicas for each sample were frozen into liquid nitrogen. The total RNA was extracted from each tissue using TRIzol (TIANGEN Biotech, Beijing, China) following the manufacturers’ procedure. The total RNA’s quantity/quality was measured with a Nanophotometer Spectrophotometer (Implen, München, Germany). The whole genomes and amino
acid sequences of A. thaliana, G. max, and O. sativa were obtained from JGI Phytozome [58] and Ensembl databases (http://plants.ensembl.org/index.html).

4.2. Identification of GmPYL-PP2C-SnRK2s Gene Families

The AtPYL-PP2C-SnRK2s gene family information were obtained by using the TAIR database (https://www.arabidopsis.org/) for A. thaliana. The JGI Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html) was used to compare the homology of amino acids, the candidate soybean PYL-PP2C-SnRK2 proteins with high homologous correlation with A. thaliana PYL-PP2C-SnRK2 protein. Furthermore, through the NCBI database’s Blast function, the GmPYL-PP2C-SnRK2s genes were identified and given specific names.

4.3. Phylogenetic Analysis

The amino acid sequences of GmPYLs, GmPP2Cs, and GmSnRK2s proteins from G. max, A. thaliana, and O. sativa were selected to construct a Maximum Likelihood using MEGA 10.0 software, use “find best DNA / protein models (ML)” program to test the most suitable model. “Jones-Taylor-Thornton (JTT) model” and “Gamma Distributed (G)” rates among sites were selected. The robustness of each node in the tree was determined using 1000 bootstrap replicates, and then the default parameter for the remaining parameters was selected [59].

4.4. Analysis of Gene Structure and Their Conservation

The gene structure of GmPYLs, GmPP2Cs, and GmSnRK2s was analyzed online by the GENE Structure display (GSDS 2.0) website (http://gsds.gao-lab.org/). Upload genomic sequence and coding sequence with FASTA format, and then generate gene structure. MEME website (http://meme-suite.org/) and TBtools software (version 1.055) were used to analyze the conserved motif of soybean GmPYLs, GmPP2Cs, and GmSnRK2s protein. The MEME website uses Classic mode to perform motif discovery on protein datasets: site distribution select Zero or One Occurrence Per Sequence (zoops). Generated conserved motif files are visualized with TBtools [60]. Using the plug-in “Batch SMART” of TBtools, it links to the SMART website (http://smart.embl-heidelberg.de/), adopted to determine whether the PYL-PP2C-SnRK2s domain existed in the candidate PYL-PP2C-SnRK2s genes [61].

4.5. Chromosomal Location and Collinearity Analysis

The chromosome mapping information of GmPYLs, GmPP2Cs, and GmSnRK2s gene families was obtained from the NCBI database. The chromosome mapping map was drawn by MapChart software [62], and the collinear relationship of GmPYL-PP2C-SnRK2s gene families was analyzed by using MCScanx and TBtools software. To further elaborate the replication events of the GmPYL-PP2C-SnRK2s gene families, and identify orthologous and paralogous genes.

4.6. Analysis of Promoter Sequence

The 2000 bp genomic sequences upstream of the transcription start site (TSS) of GmPYL-PP2C-SnRK2s gene families were extracted from the NCBI database, and the cis-acting elements in the promoter region were analyzed online by PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [63].

4.7. Analysis of Expression Patterns

The expression data were obtained by high-throughput transcriptome sequencing, and the sequencing platform was Illumina Hiseq2500 at Shanghai Biozeron Biological Technology Co., Ltd. Each sample produces 6.0-Gb sequencing data, which is sequenced by double-terminal (PE) sequencing with a reading length of 150 bp. Post-trimming reads were aligned to G. max reference genome (Phytozome v11.0, https://phytozome.jgi.doe.gov/pz/portal.html, accessed on 16 July 2016).
The transcriptome dataset of soybean (accession number: SRP038111) was uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. The data used in the chart are the TPM values commonly used in transcriptome analysis, which will be used to analyze the tissue specificity and in different cultivars expression of GmPYL-PP2C-SnRK2s gene families. The mapping of these RNA-seq reads was done using TBtools (version 1.055) with the default settings. Quantification of gene expression was done using the TPM algorithm. The clustered heatmap of resulting TPM values per tissue for GmPYL-PP2C-SnRK2s was generated using the heatmap function of TBtools (version 1.055). Logarithm TPM data, set base = 2 and log with = 1, and cluster rows.

5. Conclusions

It is therefore concluded that there are 21 PYLs genes, 36 PP2Cs genes, and 21 SnRK2s genes present in the entire genome of the soybean. Gene structure, conserved sequence, and domain analysis have shown that members of the GmPYL-PP2C-SnRK2 gene families are evolutionarily relatively conservative. Complex genomic replication events have occurred; most genes have been retained. Identified GmPYL-PP2C-SnRK2s gene families contain START-like domain, PP2Cc domain, and S_TK domain. The expression pattern of these genes has tissue specificity and differences among varieties. In summary, these findings indicate the potential role of GmPYL-PP2C-SnRK2 gene families in plant growth and development, and multiple stress responses.

Supplementary Materials: The supplementary materials are available online at http://www.mdpi.com/2223-7747/9/10/1356/s1, Figure S1: The Venn diagrams showing the number of significant differentially expressed genes between Williams82 and Jack. Table S1 GmPYL-PP2C-SnRK2s Genes screening, naming and location, Table S2. The accession numbers and gene names of PYL-PP2C-SnRK2 gene families in A. thaliana and O. sativa, Table S3. Width, Sites, E-value of GmPYL-PP2C-SnRK2s conserved motif, Table S4. The genes that contain a collinear relationship and the location of GmPYL-PP2C-SnRK2s on scaffolds, Table S5. Prediction of cis-elements of GmPYL-PP2C-SnRK2s gene family. Table S6. The P-value of T-test of GmPYL-PP2C-SnRK2s gene families DEG Between Williams82 and Jack. Table S7. TPM values of GmPYL-PP2C-SnRK2s all genes in two varieties.

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