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Genome-Wide Identification, Expression and Interaction Analysis of GmSnRK2 and Type A PP2C Genes in Response to Abscisic Acid Treatment and Drought Stress in Soybean Plant

Xinjie Shen †, Hong Nan †, Yuzhuang Jiang, Yujia Zhou and Xuejun Pan *

College of Agriculture, Guizhou University,Guiyang 550025, China
* Correspondence: xipan@gzu.edu.cn
† These authors contributed equally to this work.

Abstract: As a typical ancient tetraploid, soybean (Glycine max) is an important oil crop species and plays a crucial role in supplying edible oil, plant protein and animal fodder worldwide. As global warming intensifies, the yield of soybean in the field is often strongly restricted by drought stress. SNF1-related protein kinase 2 (SnRK2) and type A protein phosphatase 2C (PP2C-A) family members are core components of the abscisic acid (ABA) signal transduction pathway in plants and have been suggested to play important roles in increasing plant tolerance to drought stress, but genetic information supporting this idea is still lacking in soybean. Here, we cloned the GmSnRK2s and GmPP2C-A family genes from the reference genome of Williams 82 soybean. The results showed that the expression patterns of GmSnRK2s and GmPP2C-As are spatiotemporally distinct. The expression of GmSnRK2s in response to ABA and drought signals is not strictly the same as that of Arabidopsis SnRK2 homologous genes. Moreover, our results indicated that the duplicate pairs of GmSnRK2s and GmPP2C-As have similar expression patterns, cis-elements and relationships. GmSnRK2.2 may have a distinct function in the drought-mediated ABA signaling pathway. Furthermore, the results of yeast two-hybrid (Y2H) assays between GmSnRK2s and GmPP2C-As revealed that GmSnRK2.17, GmSnRK2.18, GmSnRK2.22, GmPP2C5, GmPP2C7, GmPP2C10 and GmPP2C17 may play central roles in the crosstalk among ABA signals in response to drought stress. Furthermore, GmPP2C-As and GmSnRKs were targeted by miRNA and validated by degradome sequencing, which may play multiple roles in the crosstalk between ABA and drought signals and other stress signals. Taken together, these results indicate that GmSnRK2s and GmPP2C-As may play a variety of roles in the drought-mediated ABA signaling pathway.

Keywords: soybean; SnRK2; PP2C-A; abscisic acid; drought stress; protein interaction

1. Introduction

Plant hormones are a group of small organic signaling molecules that act as bridges to mediate the communication and subsequent responses of plants, both to endogenous molecular signal transduction and environmental adaptation. Along with the well-known six plant hormones, abscisic acid (ABA) has been suggested to play an essential role throughout the entire life of plants, including roles in seed dormancy and germination, bud dormancy, root development, stomatal movement, leaf senescence, vegetative development, flowering and fruit ripening [1–3]. In addition to its roles in various aspects of plant growth and development, ABA also serves as a key endogenous messenger in adapting to abiotic and biotic stress responses in plants [4]. Many studies have suggested that ABA plays essential roles in the response to multiple abiotic stresses, including drought, cold, salinity and heat stress [5–8]. Drought has been and continues to be a severe ecological problem worldwide, and strongly impacts crop yields and food security for humans [9]. ABA is the major stress-responsive hormone produced after drought signals are perceived. Plants have evolved sophisticated interconnected signaling pathways to overcome drought stress.
ABA is the key player in the plant response to drought stress. ABA is synthesized and accumulates in plant leaves, which can then promote stomatal closure, reduce transpiration and ensure water balance [10]. In the roots, ABA can promote root elongation so that the roots reach deep soil to obtain more water under drought conditions [11]. Moreover, in the roots, ABA can suppress the synthesis of reactive oxygen species (ROS) and ethylene, which act as root growth inhibitors to increase root length and density, specifically when the soil cannot hold water or when water is available deeper in the soil [1,12]. Furthermore, ABA can activate numerous cellular responses in plants through a series of signal transduction networks and pathways that interact with other drought-related phytohormones. ABA can also inhibit auxin signaling repressors (AXR3/AA17) and increase the expression of DR5 and IAA2, both of which act as auxin reporters to activate auxin signaling to transport auxin towards elongating root cells; in turn, auxin promotes root growth to take up deep water under drought conditions [13,14]. In addition to its interaction with auxin, ABA has also been reported to undergo negative crosstalk with gibberellin (GA), which can downregulate the DELLA protein PRO and upregulate ABA transport with AIT1.1, ultimately promoting guard cell responses under drought conditions [15]. Thus, ABA plays a vital role in the plant response to drought stress.

When plants are under drought conditions, the PYR/PYL/RCAR ABA receptor protein family members can perceive drought signals from the environment and then trigger ABA signal transduction through protein phosphorylation reactions in cells [16]. It has been well demonstrated that PYR/PYL/RACR and members of the type 2C protein phosphatase (PP2C) and SNF1-related kinase protein kinase 2 (SnRK2) families are core ABA signaling components. The classic method of ABA signal transduction can be described as follows: in the absence of ABA, the activity of SnRK2s is inhibited by physiological interaction with type A PP2Cs (PP2C-A) via the dephosphorylation of multiple Ser/Thr residues in an activation loop (Figure 1A); in the presence of ABA, the structure of the PYR/PYL/RCAR ABA receptors changes in response to binding ABA molecules, which enables the interaction of ABA receptors with PP2Cs and leads to suppression of PP2C-mediated dephosphorylation of SnRK2s (Figure 1B); and finally, SnRK2s are released from PP2C inhibition and are able to activate their downstream targets, such as ABA response element-binding factors (ABFs), ABA-INSENSITIVE (ABI) transcription factors, MYB transcription factors and ABA-responsive element-binding proteins (AREBs) [2,7,16–18]. In the model plant species Arabidopsis, the SnRK2 family can be divided into three groups [19]. Group I members (AtSnRK2.1, AtSnRK2.4, AtSnRK2.5, AtSnRK2.9, and AtSnRK2.10) are mainly involved in the response to osmotic stress and also increase drought tolerance, but do not respond to ABA signaling [19]. Group II members (AtSnRK2.7 and AtSnRK2.8) can respond to salt stress and weakly respond to ABA signals, and they can also improve drought tolerance [20,21]. Group III members (AtSnRK2.2, AtSnRK2.3, and AtSnRK2.6) have been proven to regulate ABA-induced stomatal movement and modulate seed germination and primary root growth [22,23]. The group A-type PP2Cs of Arabidopsis have been proven to act as central negative modulators of the ABA signaling pathway [24].

The identification of the ABA signal transduction core unit has greatly promoted advances in understanding plant ABA signal transduction under drought conditions, the utilization of ABA signaling mechanisms for manipulation of plant physiological structure responses, as well as for enhancement of the drought tolerance and water use efficiency of plants. The ABA signal transduction pathway can be activated in plants to regulate the expression of ABI11 and OST1 to generate H₂O₂, the secondary messengers, which can mediate stomatal closure to balance plant cell water contents under drought conditions [3,25]. A recent study showed that SnRK2.3 could phosphorylate and inactivate HD-ZIP (HAT1), potentially suppressing the expression of both ABA3 and NCED3, which negatively regulate ABA biosynthesis [26]. This leads to increased ABA biosynthesis, amplifying the ABA signal which increases plant resistance to drought stress. These results indicated that the core plant ABA signal transduction pathway is not a one-way and loop-locked signaling pathway but, rather, is a self-feedback and open-ended pathway [10,16].
ZIP (HAT1), potentially suppressing the expression of both ABA3 and NCED3, which negatively regulate ABA biosynthesis [26]. This leads to increased ABA biosynthesis, amplifying the ABA signal which increases plant resistance to drought stress. These results indicated that the core plant ABA signal transduction pathway is not a one-way and loop-locked signaling pathway but, rather, is a self-feedback and open-ended pathway [10,16].

Plant growth and stress responses are also carefully controlled by both the activity and the abundance of PP2Cs. Although stress induces ABA inactivation of PP2Cs, releasing SnRK2s and initiating the ABA signal transduction pathway, the expression levels of PP2Cs are actually upregulated by the ABA signal through the action of certain regulatory factors, such as ABFs; this creates a negative feedback loop control mechanism, with appropriate homeostatic levels maintained in order to desensitize the plant to high ABA levels [26,27]. Recent studies have suggested that PP2C-As are the central negative regulators of the ABA signal transduction pathway and can be induced in response to ABA and drought stress [28,29]. In addition to the interactions of some PP2C-As with SnRK2s which negatively regulate the ABA signaling pathway, the activity of some PP2C-As can also be enhanced by direct interaction with EAR1 proteins [30], indicating that PP2C-As also function under an open-loop and feedback regulatory mechanism to maintain their activity (Figure 1B). The PPC-A member ABI1 can directly dephosphorylate the N-terminus of SLAC1 to affect SLAC1-mediated stomatal closure under drought conditions [31,32].

Figure 1. ABA signal transduction model. (A) Normal condition. (B) Under drought condition. Black phosphate represent Dephosphorylation; Red phosphate represent phosphorylation.

Soybean (Glycine max L. Merr.) is an important source of protein and cooking oil for humans and fodder for animal husbandry. As global warming intensifies, soil drought is becoming increasingly severe and has an increasing impact on soybean yields. In plants, the ABA signal transduction pathway plays a central role in the response to drought stress. Although the SnRK2s and PP2Cs core ABA signaling components have been extensively studied in model plant species and in some crop plant species, the expression patterns of Mer PP2C-As and SnRK2s in different soybean tissues under ABA and drought treatment remain unclear. Moreover, the ABA- and drought-related interactions between PP2C-As and SnRK2s are poorly understood. In this article, we identified and evaluated the
expression members of the PP2C-As and SnRK2 families from the soybean genome under ABA and drought treatment. Moreover, we analyzed the possible interactions between PP2C-As and SnRK2s, both of which function in response to ABA and drought stress. This study presents the results of the first genome-wide analysis of the PP2C-A and SnRK gene families in response to ABA and drought treatment in soybean. Taken together, these results will broaden our insight into the roles of PP2C-A and SnRK2 genes in the drought-induced ABA signal transduction pathway and provide a foundation for further discoveries of new interaction factors and molecular regulatory mechanisms.

2. Results

2.1. Identification and Sequence Analysis of GmPP2C-A and GmSnRK2 Genes

Gene-specific primers were used to perform gene-specific PCR, resulting in the isolation of the full-length coding sequences of eighteen GmPP2Cs (GmPP2C1- GmPP2C18) and twenty-two GmSnRK2s (GmSnRK2.1-GmSnRK2.22) from the cDNA of soybean leaves. Phylogenetic analysis, comparing the full coding DNA sequences (CDSs) of these genes with those of homologous genes in Arabidopsis, revealed that the soybean PP2C and SnRK2 gene families could be clustered and grouped with the Arabidopsis PP2C and SnRK2 gene families, respectively. Additionally, protein sequence analysis suggested that the members of the soybean PP2C-As gene family (GmPP2C-As) have multiple key functional domains, including PPM-type phosphatase domains, Mn²⁺/-Mg²⁺-binding sites, and several protein activation sites (Figure 2A and Figure S1). The results of the protein sequence analysis of SnRK2s revealed that SnRK2s also have many key functional domains, such as protein kinase domains, protein kinase A catalytic subunit domains, Ser/Thr-protein kinase activation sites, activation loops and ATP-binding sites (Figures 2B and S2).

To explore the chemical and physical properties of the GmPP2C-A and GmSnRK2 proteins, the amino acid sequences encoded by these genes were profiled by ProtParam (http://web.expasy.org/protparam/, accessed on 16 July 2021) (Tables S1 and S2). The molecular weight (MW) of the GmPP2C-A genes varied from 26.6 to 60.82 KDa and from 25.64 to 41.08 KDa, respectively. The isoelectric point (pI) varied from 4.5 to 8.04 (GmPP2C-As) and from 4.69 to 8.33 (GmSnRK2s). These results suggested that, except for GmPP2C8 (pI: 7.55), GmPP2C13 (pI: 8.04) and GmSnRK2.12 (pI: 8.33), most members of the GmPP2C-As and GmSnRK2s were acidic. The instability index of the GmPP2C-A and GmSnRK2 proteins ranged from 37.34 to 67.40 and from 31.82 to 53.31, respectively, which suggested that the proteins of most members of these two gene families were unstable (aliphatic index > 40), except for GmPP2C16. Conversely, GmSnRK2.7, GmSnRK2.8, GmSnRK2.11, GmSnRK2.12, GmSnRK2.13, GmSnRK2.14, GmSnRK2.19, GmSnRK2.20, and GmSnRK2.22 were stable (aliphatic index < 40). The grand average of hydropathy (GRAVY) values of the GmPP2C-As and GmSnRK2s were all negative, indicating that the members of both gene families were hydrophilic and probably localized to the cytosol.

The nucleotide length and exon numbers were also analyzed. The length of the GmPP2C-A genes varied from 1434 to 3483 nt, with a mean of 1946 nt. The length of SnRK2s also ranged from 1353 to 2512 nt, with a median length of 1946 nt. Most genes in the group I, II, and III SnRK2s were shorter than 1900, 1700, and 2000 nt, respectively, with median lengths of 1863, 1560, and 1832 nt, respectively (Figure 3B). As described in Figure 3, the GmPP2C-A and GmSnRK2 genes presented distinct intron-exon distributions. The exon number of the GmPP2C-A genes ranged from three to seven, and most of them had four exons. However, the data indicated that, in terms of exon-intron composition, there were no significant changes among the same group of GmSnRK2 genes. For instance, all the group I, all the group III and the majority of the group II GmSnRK2s genes contained nine exons.
Figure 2. Phylogenetic analysis and distribution of the conserved motifs in GmSnRK2s and GmPP2C-As. (A) Phylogenetic relationships and conserved motifs in GmSnRKs and AtSnRK2s. (B) Phylogenetic relationships and conserved motifs in GmPP2C-As and AtSnRK2s. The ML phylogenetic tree shown was constructed via full-length protein sequences and with 1000 bootstraps. The conserved motifs were detected using MEME software and are represented by coloured boxes. Gm = G. max and At = A. thaliana.

2.2. Analysis of Stress-Related Cis-Elements of GmPP2C-A and GmSnRK2 Genes

To investigate and analyze the stress-related cis-element regulatory elements in the promoters of GmPP2C-As and GmSnRK2s, the genomic DNA sequences 2 kb upstream from the transcription initiation sites (TISs) were extracted and profiled via PlantCARE. The results obtained from the PlantCARE website showed that multiple stress-related regulatory elements are present within the promoter regions of these two families of genes (Figure 4A,B). For instance, thirteen GmPP2Cs (GmPP2C1, GmPP2C2, GmPP2C5, GmPP2C6, GmPP2C7, GmPP2C8, GmPP2C9, GmPP2C10, GmPP2C11, GmPP2C12, GmPP2C13, GmPP2C16, GmPP2C17, and GmPP2C18) and nineteen GmSnRK2s (GmSnRK2.2, GmSnRK2.3, GmSnRK2.4, GmSnRK2.5, and GmSnRK2.6 of group I, GmSnRK2.9, GmSnRK2.10, GmSnRK2.11, GmSnRK2.12, GmSnRK2.13, and GmSnRK2.14 of group II, and GmSnRK2.15, GmSnRK2.16, GmSnRK2.17, GmSnRK2.18, GmSnRK2.19, GmSnRK2.20, GmSnRK2.21, and GmSnRK2.22 of group III) had one or more the MYB-binding sites (MBSs), which are involved in drought inducibility. Moreover, the following had one or more ABA-responsive elements (ABREs): sixteen
genes. The results showed that GmPP2C10, GmPP2Cs, SnRK2.20, GmPP2C11, GmPP2C2, had one or more ABA-responsive elements (ABREs): sixteen binding sites (MBSs), which are involved in drought inducibility. Moreover, the following GmSnRK2.20, GmSnRK2.9, GmPP2C13, regulatory elements are present within the promoter regions of these two families of genes.

The results obtained from the PlantCARE website showed that multiple stress-related cis-elements were present within the promoters of GmPP2C-As. Moreover, cis-elements involved in the response to abiotic and biotic stresses, including zein, heat, fungi and low temperature, were also found within the upstream regions of GmPP2C-As and GmSnRK2s (Figure 4A,B). Notably, GmPP2C4, GmPP2C10, GmPP2C11, GmPP2C12, GmPP2C15, and GmPP2C16 as well as GmSnRK2.13 and GmSnRK2.15 contain cis-elements that function in response to wounding, which suggests that GmPP2C-As and GmSnRK2s might be associated with the response to biotic stress.

**Figure 3.** Length distribution (A) and exon numbers (B) of GmPP2C-A and GmSnRK2 genes.

**2.3. Chromosomal Distribution and Expansion Patterns of GmPP2C-A and GmSnRK2 Genes**

Soybean is a diploidy of ancient tetraploid plant, and soybean cells contain 20 chromosomes. In this work, we analyzed the distribution of GmPP2C-A and GmSnRK2 genes in the soybean genome. The results showed that the GmPP2C-A and GmSnRK2 genes were unevenly distributed on chromosomes 14 and 12, respectively (Figure 5). Across the soybean chromosomes, chromosomes 14 and 5 contained the most GmPP2C-As (three genes) and GmSnRK2s (four genes), respectively (Figure 5). The other chromosomes had one to three GmPP2C-A and GmSnRK2 genes. Chromosomes 3, 7, 10, 12, 16, and 20 carried no GmPP2C-A genes, and chromosomes 3, 9, 10, 13, 15, 16, 18, and 19 carried no GmSnRK2 genes. Furthermore, we also analyzed the differentially methylated (DMRs) GmPP2C-A and GmSnRK2 genes. The results showed that GmPP2C-A and GmSnRK2 genes were located in a few DMR regions during soybean domestication and improvement (Figure 5). Moreover, except for GmPP2C18, GmSnRK2.21 and GmSnRK2.21, all the GmPP2C-A and GmSnRK members were located in the high-GC-density regions in the soybean genome (Figure 5).
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Figure 4. Cis-elements in the promoters of GmPP2C-A (A) and GmSnRK2 (B) are related to hormone and stress responses. The bar indicates the number of cis-elements. The numbers 1, 2, 3, . . . represent the number of repeats of each cis-element, whereas 0 indicates absence of a particular cis-acting element. Letters a, b, c, . . . represent cis-elements. a: MBS (CAACTG, MYB-binding site involved in drought inducibility); b: ABRE (TACGTG, ABA response element); c: ARE (TGGTTT, anaerobic inducible); d: O2-site (GATGATGAT, cis-acting regulatory element involved in zein metabolism regulation); e: TC-rich repeats (ATTTTATCCA, defence and stress response element); f: CGTCA-motif (CGTCA, MeJA response element); g: HSE (AAAAAATTAC, heat stress response element); h: P-box (CCTTCTTC, GA response element); i: TCA-element (TCAGAAAAGG, salicylic acid response element); j: TGACG-motif (TGACG, MeJA response element); k: Box-W1 (TTGACC, fungal elicitor response element); l: GARE-motif (TCTGCGT, GA response element); m: long terminal repeat (LTR) (CCCCAAA, low-temperature response element); n: TGA-element (AAGCGAC, auxin response element); o: WUN motif (AAATTTCTCT, wound response element); p: ERE (ATTTCATAA, ethylene response element); q: CE3 (GATCGTGTGC, ABA and VPI response element); r: CCAAT-box (CAACGG, MYBHv1-binding site).

Figure 5. Chromosome distribution and synteny analysis of GmPP2C-A (A) and GmSnRK2 (B) genes. Chromosomes 1–20 are illustrated in different colours. The approximate locations of all the genes are shown with short red lines. The blue curves indicate segmentally duplicated genes. The tracks from outside to inside represent the gene density, methylated region distributions of Dos-DMR (process of soybean domestication, Dos-DMR), methylated region distributions of Imp-DMR (in the improvement process, Imp-DMR), and GC density.
Gene duplication events are critical factors involved in the expansion of gene families during genome expansion. The results showed that 10 and 16 paralogous gene pairs of GmPP2C-A (Figure 5A and Table S3) and GmSnRK2 (Figure 5B and Table S4) genes, respectively, were identified in soybean. To determine their duplication time, the nonsynonymous rate (Ka), synonymous rate (Ks) and the Ka/Ks ratios of paralogous gene pairs of GmPP2C-As and GmSnRK2s were analyzed (Tables S3 and S4). All the Ka/Ks ratios of GmPP2C-As and GmSnRK2s were less than 1, varying from 0.19 to 0.52 and from 0.02 to 0.24, respectively. These results suggested that the paralogous gene pairs of GmPP2C-As and GmSnRK2s were strongly selected during the expansion of the soybean genome. Moreover, we also estimated the duplication time. The duplication time of GmPP2C-As and GmSnRK2s varied from 7.5 to 80.2 million years ago (MYA) and from 6.1 to 46.4 MYA, respectively. These results indicated that all the duplication events of the two gene families occurred in the latest whole-genome duplication of soybean.

2.4. Gene Ontology (GO) Annotation

In order to further predict the functions of GmPP2C-A and GmSnRK2 genes, GO (gene ontology) annotation analyses were performed. A total of 22 distinct functional groups were determined: 15 involved in biological processes, 4 involved in cellular components and 3 involved in molecular functions (Figure 6). In biological processes, GO classifications of ‘reproductive process’, and ‘growth’ and ‘multi-organism process’ were specifically attributed in GmPP2C_GroupA and GmSnRK2 genes, respectively. Moreover, GmPP2C_GroupA genes could perform negative and positive regulation of biological processes, while GmSnRK2 members could only undertake positive regulation of biological processes. As for genes in the cellular component part, 18 GmPP2C-A genes were annotated with ‘membrane’, ‘organelle’, ‘cell part’, and ‘cell’, while 22 GmSnRK2 genes were associated with ‘organelle’, ‘cell part’, and ‘cell’. Under the molecular function term, 16, 8, and 2 genes were annotated with ‘catalytic activity’, ‘binding’, and ‘molecular function regulator’, respectively, while only 22 and 10 genes were annotated to have the designations ‘catalytic activity’ and ‘binding’. All these results indicate the multiple functions of GmPP2C-A and GmSnRK2 genes.

2.5. Prediction of GmPP2C-A and GmSnRK2 Genes Targeted by miRNAs

In order to obtain a deep understanding of the functional roles of GmPP2C-A and GmSnRK2 genes in soybean, we evaluated the potential regulation of these two gene families by miRNA targets prediction. The results showed that four miRNA-GmPP2C-As pairs and two miRNA-GmSnRK pairs were validated by degradome sequencing. These pairs included Gma-miR2606b: GmPP2C2 (Glyma.02G250200), Gma-miRN1266: GmPP2C3 (Glyma.05g197700), and Gma-miR9725: GmSnRK2.11 (Glyma.05G066700) (Figure 7). As for genes in the cellular component part, 18 GmPP2C-A genes were annotated with ‘membrane’, ‘organelle’, ‘cell part’, and ‘cell’, while 22 GmSnRK2 genes were associated with ‘organelle’, ‘cell part’, and ‘cell’. Under the molecular function term, 16, 8, and 2 genes were annotated with ‘catalytic activity’, ‘binding’, and ‘molecular function regulator’, respectively, while only 22 and 10 genes were annotated to have the designations ‘catalytic activity’ and ‘binding’. All these results indicate the multiple functions of GmPP2C-A and GmSnRK2 genes.

![Figure 6. GO enrichment analysis. (A) GO enrichment of GmPP2C-A genes. (B) GO enrichment of GmSnRK2 genes. According to the secondary terminology, the annotation results are divided into three ontology categories and distinguished by different colors.](image)
we measured the expression levels of GmPP2C-As in response to exogenous ABA treatment compared to those in the control roots (Figure 8A). Similar to the roots, the ABA and drought stress treatment.

Drought Stress

To explore the expression patterns of GmPP2C-As in response to exogenous ABA treatment and drought stress, we measured the expression levels of GmPP2C-As in the stems under ABA treatment (Figure 8A). In contrast to those in the roots and stems, the expression levels of all GmPP2C-As were measured in the root and leaf tissues at five time points: 0, 0.5, 1, 3 and 6 h. The results showed that all GmPP2C-As, except for GmPP2C3, were significantly upregulated at four time points (0.5, 1, 3 and 6 h) under ABA treatment compared to those in the control roots (Figure 8A). Similar to the roots, the expression levels of all GmPP2C-As (excluding GmPP2C3) were upregulated significantly in the stems under ABA treatment (Figure 8A). In contrast to those in the roots and

2.6. Expression Profiles of GmPP2C-As in Response to Exogenous ABA Treatment and Drought Stress

ABA has been proven to play a crucial role in plants in response to diverse environmental stresses. Along with its role in response to these environmental stresses, ABA has been demonstrated to act as a molecular signal in the drought signal transduction pathway. To explore the expression patterns of GmPP2C-As in response to drought stress and ABA, we measured the expression levels of GmPP2C-As by qRT-PCR in response to exogenous ABA and drought stress treatment.

The expression levels of GmPP2C-As were measured in the root and leaf tissues at five time points: 0, 0.5, 1, 3 and 6 h. The results showed that all GmPP2C-As, except for GmPP2C3, were significantly upregulated at four time points (0.5, 1, 3 and 6 h) under ABA treatment compared to those in the control roots (Figure 8A). Similar to the roots, the expression levels of all GmPP2C-As (excluding GmPP2C3) were upregulated significantly in the stems under ABA treatment (Figure 8A). In contrast to those in the roots and

Figure 7. Post-transcriptional regulation of GmPP2C-A and GmSnRK2 by degradome sequencing. (A) Cleavage features in GmPP2C2 by Gma-miR2606b. (B) Cleavage features in GmPP2C3 by Gma-miR1266. (C) Cleavage features in GmPP2C5 by Gma-miR1266. (D) Cleavage features in GmPP2C9 by Gma-miR1339. (E) Cleavage features in GmSnRK2.3 by Gma-miR1535. (F) Cleavage features in GmSnRK2.11 by Gma-miR9725.
stems, the expression levels of GmPP2C-As exhibited different patterns in the leaves. In terms of expression, of the 18 GmPP2C genes, GmPP2C2, GmPP2C4, GmPP2C6, GmPP2C7, GmPP2C8, GmPP2C10, GmPP2C13, GmPP2C14, GmPP2C16, GmPP2C17 and GmPP2C18 were upregulated in response to ABA treatment, while GmPP2C3 and GmPP2C11 were downregulated (Figure 8A). Taken together, these results suggested that most of the group A-type GmPP2C family genes could respond to ABA treatment but exhibited different expression patterns in different tissues of soybean seedlings.

When plants are under drought stress, ABA is always synthesized, increasing resistance to the stress conditions. In the present study, we also measured the expression levels of GmPP2C-As family genes in response to drought stress at five time points (0.5, 1, 3, 6 and 12 h) in the roots, stems and leaves. As shown in Figure 8B, following drought stress, GmPP2C1, GmPP2C2, GmPP2C4, GmPP2C6, GmPP2C7, GmPP2C8, GmPP2C9, GmPP2C10, GmPP2C11, GmPP2C13, GmPP2C14, GmPP2C15, GmPP2C17 and GmPP2C18 were upregulated significantly during drought stress compared to those of the control treatment (Figure 8B). Similar to ABA treatment, GmPP2C-As had similar expression patterns in the roots and stems under drought treatment (Figure 8B). In contrast to the results under ABA treatment, the expression levels of GmPP2C-As under drought treatment were similar in the leaves compared to that in the roots and stems (Figure 8B).

Taken together, these results suggested that most GmPP2C-As could respond to exogenous ABA and drought treatment, but exhibit different expression patterns in different tissues.
2.7. Expression Profiles of GmSnRK2s in Response to Exogenous ABA Treatment and Drought Stress

To expand our knowledge of the molecular function of GmSnRK2 genes in response to ABA treatment and drought stress, we measured their expression levels under exogenous ABA treatment and drought stress by qPCR in the roots, stems and leaves. In the roots, after ABA treatment, all GmSnRK2s except GmSnRK2.3, GmSnRK2.5, GmSnRK2.6, GmSnRK2.9, GmSnRK2.11, GmSnRK2.12, GmSnRK2.13, GmSnRK2.14, GmSnRK2.15, GmSnRK2.16 and GmSnRK2.17 showed differential expression during the time course of the treatment. The expression trends of GmSnRK2s in the stems were similar to those in the roots (Figure 8A). The expression levels of GmSnRK2s in the leaves were slightly different from those in the roots and stems, and the expression levels of GmSnRK2.18, GmSnRK2.20 and GmSnRK2.22 in the leaves were significantly higher than those in the roots and stems.

After drought stress treatment, most of the GmSnRK2 genes responded to drought stress (Figure 9B). The expression trends of GmSnRK2.1, GmSnRK2.8, GmSnRK2.9, GmSnRK2.11, GmSnRK2.12, GmSnRK2.18 and GmSnRK2.21 were gradually upregulated in roots, stems and leaves. Among these GmSnRK2 genes, the expression patterns of GmSnRK2.8 and GmSnRK2.22 were similar to those observed under exogenous ABA treatment, which suggested that GmSnRK2.8 and GmSnRK2.22 might play roles in the ABA-mediated drought stress response. These results suggested that the GmSnRK2 genes might play a variety of roles in the soybean ABA signaling pathway and response to drought stress.

Figure 9. Expression analysis of GmSnRK2s under ABA and drought treatments. (A) Expression analysis of the GmSnRK2s in the roots, stems and leaves of soybean under ABA treatment. (B) Expression analysis of the GmSnRK2s in the roots, stems and leaves of soybean under drought treatment. The expression levels in roots, stems and leaves before treatment (0 h) were used as controls and were assigned a value of 1. GmSKIP was used as an internal reference. Each point represents the mean value ± SE of three independent experiments performed in triplicate. The differences were statistically assessed using Student’s t test (**, p < 0.01; *, p < 0.05). The error bars in A and B represent the SEs of three replicates.
2.8. Soybean GmPP2C-As Interact with GmSnRK2s

In this work, we used a Y2H yeast system to analyze the interactions between GmPP2C-As and GmSnRK2s. Based on the results of the transcriptional activation effect of GmPP2C-As and GmSnRK2s, we tested the interactions between GmPP2C-As and GmSnRK2s (excluding GmPP2C14, GmPP2C15, GmPP2C16 and GmPP2C18). As shown in Figure 10, GmSnRK2s exhibited complex interactions with GmPP2C-As. GmSnRK2.7, GmSnRK2.8, GmSnRK2.10, GmSnRK2.14, GmSnRK2.17, GmSnRK2.18, GmSnRK2.20 and GmSnRK2.22 could interact with most of the GmPP2C-As, suggesting that, in conjunction with GmPP2C-As, these GmSnRK2s may play a basic role in the ABA signal transduction pathway. Other GmSnRK2s, such as group I GmSnRK2s (GmSnRK2.1-2.6), some group II GmSnRK2s (GmSnRK2.9, GmSnRK2.11, GmSnRK2.12, and GmSnRK2.13) and GmSnRK2.16, could interact with one to four GmPP2C-As. Notably, GmSnRK2.1 was found to interact with none of the GmPP2C-A genes: this indicated that GmSnRK2.1 may not respond to ABA and drought signals. Furthermore, all the tested GmPP2C-As could interact with most of the Group III GmSnRK2s (GmSnRK2.15-2.22), suggesting that soybean GmPP2C-As are involved in the ABA signal transduction pathway.

Figure 10. Physical interactions between GmPP2C-As and GmSnRK2s, determined using a Y2H system. A pGADT7 vector was used to express GmPP2C-As, and a pGBKT7 vector was used to express GmSnRK2s. The GmPP2C-AD and GmSnRK2-BD constructs were then co-transformed into yeast and selected on the basis of the ability of the yeast to grow on media lacking leucine (Leu) and tryptophan (Trp). The interaction was indicated by the ability of yeast to grow on selective media that included aureobasidin A (AbA) and X-α-Gal, but lacked Leu, Trp, histidine (His), and adenine (Ade). The interactions of the PacSnRK2-AD constructs with pGBKT7 were used as controls to test for yeast self-activation.
3. Discussion

ABA has been proven to play a crucial role in plants in response to various environmental stresses [1,2]. Along with its role in the response to these environmental stresses, ABA has been demonstrated to act as a molecular signal in the drought signal transduction pathway [3]. The PYL–PP2C–SnRK2 complex is the core component of the plant ABA signaling pathway [3,33]. Soybean is an important oil crop worldwide and is vital for understanding how soybean plants respond to drought stress through the ABA signaling pathway. Compared to previous studies [28], the latest version of GFF3 annotation file was used to determine PP2C and SnRK2 genes of Williams 82, which would be more accurate to reveal the structure and function of the two gene families. In addition, Zhang et al. and Fan et al. both only observed expression patterns of these genes at different tissues, and neither elaborated the mechanistic networks under drought stress related to the ABA signaling pathway [29,34]. In this study, we focus on the abundance of PP2C-A and SnRK2 members under ABA and drought treatments. These efforts together shed new light on the evolution and functional divergence of the two gene family in soybean under drought treatment. Moreover, we ultimately cloned and isolated 22 full-length GmSnRK2s and 18 full-length GmPP2C-As.

Based on a comparison with AtPP2C genes, all 18 GmPP2C-A genes are A-type genes, which are involved in the ABA signal transduction pathway. After exogenous ABA treatment, the expression patterns of GmSnRK2s were altered: most of group I and II, along with several group III GmSnRK2 genes (GmSnRK2.1, GmSnRK2.2, GmSnRK2.6, GmSnRK2.8, GmSnRK2.10, GmSnRK2.21, GmSnRK2.22), were significantly upregulated in response to ABA, while other expression patterns of the GmSnRK2 genes (GmSnRK2.2, GmSnRK2.3, GmSnRK2.5, GmSnRK2.11, GmSnRK2.12, GmSnRK2.13, GmSnRK2.15, GmSnRK2.17, GmSnRK2.18, and GmSnRK2.19) varied little in response to ABA treatment (Figure 9A). Interestingly, among the ten GmSnRK2s that did not respond to ABA signaling, with the exception of GmSnRK2.3, the remaining GmSnRK2s had no ABREs in their promoter regions (Figure 4B), which suggested that GmSnRK2.3 may be upregulated by other transcription factors induced by ABA signaling. Further studies should focus on elucidating the interacting proteins or upstream regulators of GmSnRK2.3 to discover a new signal transduction branch in the soybean ABA signaling pathway. Under drought treatment, most GmSnRK2 genes could be induced by drought signals, except for GmSnRK2.6 (Figure 9B). These results are somewhat consistent with our previous results [35] and indicate that GmSnRK2.6 might play a role in the response to ABA treatment, independent of its role in the drought stress signaling pathway in soybean. Zhang [36] reported that GmSnRK2.6 expression showed no difference according to a tissue-specificity expression analysis, and a possible reason is that GmSnRK2.6 may be an ABA-induced gene and not a constitutively expressed gene.

Previous studies have indicated that multiple stress-related cis-elements, such as ABREs and MBSs, or these elements coupled with other abiotic or biotic cis-elements are required for the expression of ABA- or drought-responsive genes [27,28]. In this study, we provided a comprehensive analysis of stress-related cis-elements in the promoter regions of GmPP2C-As and GmSnRK2s. The results shown in Figure 3 suggest that most of the GmPP2C-A and GmSnRK2 members have common ABA- and drought-related cis-elements, such as ABREs and MBSs; two exceptions are GmPP2C3 and GmPP2C15, which do not have those cis-elements. Interestingly, GmPP2C4 and GmPP2C14 did not have MBSs but could be significantly induced in the root and leaf tissues of soybean in response to drought treatment in our study (Figure 6B). Similar to those of GmPP2C4 and GmPP2C14, the expression levels of GmSnRK2.1, GmSnRK2.7 and GmSnRK2.8, whose promoter regions lack drought-related elements, were also significantly upregulated in response to drought signals (Figures 4B and 9B). One possible explanation for these findings is that other drought-related transcription factors might directly or indirectly regulate the promoters of these genes to increase the expression of GmPP2C4 and GmPP2C14 via drought signaling pathway crosstalk with other hormone- or stress-related signal transduction pathways. Wei et al. [37] demonstrated that GmWRKY54 could respond to drought signals and then
directly bind to the promoter of SRK2A to trigger ABA signal transduction to increase drought resistance. Further research could focus on identifying their upstream regulators or interacting proteins to discover novel molecular signaling mechanisms.

SnRK2 and PP2C proteins are known to interact to initiate the ABA signal transduction pathway \cite{36,38}. Here, we comprehensively analyzed the expression levels of GmPP2C-As and GmSnRK2s in response to ABA and drought signaling, as well as the interactions between GmPP2C-As and GmSnRK2s in soybean (Figure 11). Except for GmSnRK2.2 and GmSnRK2.5, which did not respond to ABA signals, most of the group I members of Gm-SnRK2s could respond to drought and ABA signals, which indicated that the gene functions of group I SnRK2s might differ between crop plant species and model plant species. The results of the expression patterns of group I and II GmSnRK2s in our work also support this view, and one possible reason is that SnRK2 genes have diverse spatiotemporal expression patterns in response to ABA and drought signals in soybean. In Arabidopsis, PP2C-A genes are involved in the ABA and drought signal response network \cite{24,29,38}. Our results were similar to those of previous studies and suggested that the functions of GmPP2C-As in the ABA and drought response signaling pathways may be conserved between model plant species and crop plant species. All the duplication events in GmPP2C-As were segmental duplication events (Figure 5A). Previous studies have demonstrated that the lack of tandem duplication events in PP2C family members might explain the genomic fractionation from transposon activities, relocating individual genes and driving their duplication \cite{39,40}. Notably, although duplicate pairs of GmSnRK2s and GmPP2C-As were located on different chromosomes, we found that they exhibited similar expression patterns in response to ABA and drought signals (Figure 11). It is possible that most duplicate GmSnRK2 and GmPP2C-A members have the same or similar amounts of ABA- or drought-responsive cis-elements in their promoter regions (Figures 5 and 11). To further understand gene function, we identify miRNAs potentially targeting GmSnRK2 and GmPP2C-A genes. The segmental duplicated genes (GmPP2C3 and GmPP2C5) divergent from 8.3 million years ago were both targeted by Gma-miRN1266, indicating the conservation of Gma-miRN1266-mediated regulation of the duplicated genes. However, GmPP2C2- and GmPP2C9-duplicated genes divergent from 73.5 million years ago were regulated by different miRNAs, suggesting that the functional divergence may have provided genetic sources with novel biological functions during the evolution to remove function redundancy. Interestingly, our results also revealed the broad expression spectrum profile of GmSnRK2s and GmPP2C-As members, indicating that they might play different roles in the regulation of plant growth and development. Our further research will focus on the functional verification of these genes in soybean.

In this study, using a Y2H system, we analyzed the interactions between GmSnRK2s and GmPP2C-As. We found that the interactions between GmPP2C-As and GmSnRK2s are extremely complex and do not strictly comply with the functional classification of Arabidopsis SnRK2 gene families, suggesting that the functions of GmPP2C-As and Gm-SnRK2s in the ABA signaling pathway in crop plant species such as soybean might differ from those in model plant species. Moreover, our results clearly showed that group III GmSnRK2s could interact with most GmPP2C-As, while group I GmSnRK2s interacted with few GmPP2C-As (Figure 11). Notably, GmSnRK2.2 neither responded to ABA signals nor interacted with any of GmPP2C-As and increased in expression in response to drought signaling, suggesting that GmSnRK2.2 might have a distinct function in interacting with other regulatory factors involved in ABA-mediated drought signal transduction; nonetheless, further research is needed. Interestingly, most duplicated pairs of GmSnRK2s had similar GmPP2C-A gene interaction targets, which indicated that the interactions between GmSnRK2s and GmPP2CAs are highly conserved, further suggesting that these genes have essential roles (Figure 11). Our results also showed that GmPP2C5, GmPP2C7, GmPP2C10 and GmPP2C17 could interact with group I/II/III GmSnRK2 members. This suggested that they may play a central role in the crosstalk among ABA signals in response to drought stress and are involved in different signaling pathways (Figure 11). Moreover, we found that GmPP2C5 is likely to be targeted by Gma-miRN1266, which further indicated that
GmPP2C5 may be involved in regulating the mevalonate pathway and increasing resistance to plant pathogens [41,42]. Future research should focus on determining whether GmPP2C5 represents the node of the crosstalk between ABA and the drought signaling pathway and other stress-related signaling pathways. Interestingly, three group III SnRK2 members (GmSnRK2.17, GmSnRK2.18 and GmSnRK2.22) could interact with all the tested GmPP2C-As. This suggested that they might be key regulators, that they definitively play crucial roles in regulating the drought-mediated ABA signaling pathway and that their functions may be similar to those of the Arabidopsis group III SnRK2 genes, such as regulating stomatal movement and modulating soybean plant growth and development [22,23].

Figure 11. Comprehensive analysis of the expression patterns in response to ABA, drought signals and the interactions between the GmPP2C-A and GmSnRK2 family members in soybean plants. The black solid line box represents the GmPP2C-As members that did not interact with GmSnRK2s, according to the Y2H assay results.

4. Materials and Methods

4.1. Plant Materials, Exogenous ABA and Drought Stress Treatments

For ABA treatment, seeds of the soybean cultivar ‘Williams 82’ were germinated in seed germination bags saturated with water. Eight days later, the seedlings were moved to a hydroponic system with half-strength Hoagland’s nutrient solution and grown in an incubator at 26 °C under a 16/8 (light/dark) photoperiod. After 21 days, the seedlings were treated with half-strength Hoagland’s nutrient solution that included 100 µM ABA (Sigma-Aldrich, Saint Louis, MO, USA) at four time points (0, 0.5, 1, 3 and 6 h), according to previous studies [43]. Seedlings treated with half-strength Hoagland’s nutrient solution were used as controls. For drought treatment, the seedlings were placed in the half-strength Hoagland’s nutrient solution with 20% PEG 6000 (26 °C; relative humidity (RH) of 50%) for six different durations (0, 0.5, 1, 3, 6 and 12 h); seedlings with half-strength Hoagland’s
nutrient solution only were used as controls. All the treatments included three biological replicates. Leaves and roots were collected at each time point, immediately frozen in liquid nitrogen and stored at $-80^\circ$C until further use.

4.2. Gene Cloning and Sequence Analysis

Primers for isolating the full-length coding sequences of GmPP2C-As and GmSnRK2s were designed using the transcript sequence database of Williams 82 from the SoyBase website (https://www.soybase.org/). A phylogenetic tree was constructed using MEGA 5.0 with the maximum likelihood (ML) method [44]; the bootstrap values were calculated for 1000 iterations. For promoter analysis, the 1500-bp upstream sequences of the genes were scanned for cis-elements via PlantCARE [45]. Gene Ontology (GO) annotation analysis was performed by eggNOG-mapper and used to predict the functions of the encoded proteins [46]. The GO annotations were then plotted by ggplot2 in R. The physical and chemical characteristics of GmPP2C-As and GmSnRK2s were calculated using the ProtParam website (http://web.expasy.org/protparam/). The exon and intron structures of GmPP2C-As and GmSnRK2s were generated and visualized by ggplot2. The motifs of each identified protein were analyzed by MEME suite software (version 4.12.0; http://meme-suite.org/tools/meme/) using the following parameter: maximum number of motifs, 6–10 [47].

4.3. Chromosomal Location and Gene Duplication Analysis

To understand the duplication events that occurred during the evolution of GmPP2C-As and GmSnRK2s, we used MCScanX to analyze the syntenic blocks within the soybean genome based on all-vs-all BLASTP alignments [48]. The Ka and Ks values of gene pairs were calculated using an “add_ka_and_ks_to_collinearity. Pl” script. The divergence times (T) were computed as $T = Ks/2r \times 10^{-6}$ MYA according to the approximate substitution rate $r = 6.5 \times 10^{-9}$. The chromosomal locations of GmPP2C-As and GmSnRK2s were generated based on genomic data. All the results (including gene positions) were visualized using Circos [49].

4.4. Differentially Methylated Region (DMR) Detection

DMRs were determined from a previous study [50]. The methylated region for the domestication process was determined by a comparison of the methylome data of wild soybean to that of landrace populations. For the improvement process, the methylome data of the landraces were compared to those of cultivars.

4.5. Prediction of GmPP2C-A and GmSnRK2 Genes Targeted by miRNAs

To predict genes that might be the targets of miRNAs, psRNATarget software was firstly used to align the sequences of all GmPP2C-A and GmSnRK2 members to miRNA sequences [51]. The PmiREN (https://www.pmiren.com/) was further employed to validate the miRNA–target pairs which were supported by degradome sequencing.

4.6. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the leaves, stems and roots using the TRIzol reagent (TransGen, Beijing, China) following the manufacturer’s procedure. The concentration and purity of the total RNA were analyzed with a NanoDrop spectrophotometer (TIANGEN, Beijing, China). cDNA was synthesized using the TransScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China) according to the manufacturer’s protocol.

4.7. qPCR Analysis

The primers used for qPCR were designed by using Primer Premier 5.0 software and are listed in Supplementary Table S5. For qPCR, Cq values were acquired using an Applied Biosystems Q3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). The total volume of each qPCR was 25 µL. The final concentration of primer in each PCR was 0.2 µM.
PCRs were performed with cDNA dilution using TB Green™ Premix kit (Takara, Shiga, Japan), and the means and corresponding standard errors were calculated. The qPCR conditions were as follows: 95 °C for 30 s, thirty-five PCR cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The relative quantification values were calculated using the \(2^{-\Delta\Delta Ct}\) method [52], and the soybean GmSKIP gene was used as the internal control. For each biological replicate (three in total for each experimental condition), four technical replicates were used.

4.8. Y2H Assays

For the interactions between GmPP2C-As and GmSnRKs, the full-length CDS of GmSnRK2s was cloned into the bait vector pGBKT7 (BD), and the full-length CDS of the GmPP2Cs was cloned into the prey vector pGADT7 (AD). The GmPP2C-AD and GmSnRK2-BD constructs were then cotransformed into yeast and selected on the basis of the ability of the yeast to grow on media lacking leucine (Leu) and tryptophan (Trp). The interaction was indicated by the ability of yeast to grow on selective media that included aureobasidin A (Aba) and X-α-Gal but lacked Leu, Trp, histidine (His), and adenine (Ade). The interactions of the PacSnRK2-AD constructs with pGBK7 were used as controls to test for yeast self-activation. The primers used to generate the various clones for the Y2H assays are listed in Supplementary Table S6.

5. Conclusions

Altogether, our study provides a new avenue for improving the understanding of the expression patterns of GmPP2CAs and GmSnRK2s in response to ABA and drought signals and the interactions between the two core regulatory components of the ABA signal transduction pathway in soybean plants. The results showed that the expression levels of GmSnRK2s and GmPP2C-As exhibited substantial spatiotemporal patterns. Notably, we found that the expression of GmSnRK2s in response to ABA and drought signals was not strictly the same as that of Arabidopsis SnRK2 homologous genes, which indicated that the function of soybean GmSnRK2s may differ from that in model plant species. Moreover, our results indicated that the duplicate pairs of GmSnRK2s and GmPP2C-As have similar expression patterns, cis-elements and interactions, which suggested that the functions of the GmSnRK2 and GmPP2C-A families were conserved after polyploidization occurred during the evolutionary history of soybean. GmSnRK2.2 may have a distinct function in the drought-mediated ABA signaling pathway. Furthermore, the results of our Y2H assay between GmSnRK2s and GmPP2C-As revealed that GmSnRK2.17, GmSnRK2.18, GmSnRK2.22, GmPP2C5, GmPP2C7, GmPP2C10 and GmPP2C17 may play central roles in the crosstalk among ABA signals in response to drought stress. Moreover, GmPP2C5 may play multiple roles in the crosstalk between ABA and drought signals and other stress signals. Taken together, our results provide worthwhile information for understanding the expression and interaction network of the GmSnRK2 and GmPP2C-A families. These findings also provide a scientific foundation for improving and breeding new varieties of soybean to adapt to drought stress conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113166/s1.

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Abbreviations

ABA  Abscisic acid
PYLs PYR/PYL/RCAR ABA receptors
SnRK2 Class III SNF–1–related protein kinase 2
PP2C-As Group A Type 2C protein phosphatase
ATP Adenosine triphosphate
TIS Transcription initiation site
MW Molecular weight
kDa Kilodalton
GRAVY Grand average of hydropathy
UTR Untranslated regions
ROS Reactive oxygen species
GA Gibberellin
IAA Indole–3–acetic acid
AbA Aureobasidin A
Leu Leucine
Trp Tryptophan
His Histidine
Ade Adenine
MYA Million years ago
MBS MYB–binding site involved in drought-inducibility
ABF ABA response element-binding factors
ABRE ABA responsive elements
ABI ABA INSENSITIVE transcription factor
AREB ABA-responsive element binding protein
ARE Anaerobic induce element
HSE Heat stress response element
ERE Ethylene response element
GARE Gibberellin response element
LTR Low-temperature response element
WUN-motif Wound-responsive element
O$_2$-site cis–acting regulatory element involved in zein metabolism regulation
TC-rich repeats Defense and stress response element
CGTCA-motif MeJA response element
P-box Gibberellin response element
Box-W1 Fungal elicitor response element
CE3 ABA and VP1 response element
CCAAAT-box MYBHv1 binding site
qPCR Quantitative real-time polymerase chain reaction
MeJA Methyl jasmonic acid
JA Jasmonic acid
SA Salicylic acid
PI Isoelectric point
Bp Base pair
Y2H Yeast two hybrid
GO Gene ontology
DMR Differentially methylated region
Dos-DMR Process of soybean domestication with differentially methylated region
Imp-DMR In the improvement process with differentially methylated region
ML Maximum Likelihood
