Identification and Expression Analysis of Cytokinin Metabolic Genes in Soybean under Normal and Drought Conditions in Relation to Cytokinin Levels

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Abstract

Cytokinins (CKs) mediate cellular responses to drought stress and targeted control of CK metabolism can be used to develop drought-tolerant plants. Aiming to manipulate CK levels to improve drought tolerance of soybean cultivars through genetic engineering of CK metabolic genes, we surveyed the soybean genome and identified 14 CK biosynthetic (isopentenyltransferase, GmIPT) and 17 CK degradative (CK dehydrogenase, GmCKX) genes. Comparative analyses of GmIPTs and GmCKXs with Arabidopsis counterparts revealed their similar architecture. The average numbers of abiotic stress-inducible cis-elements per promoter were 0.4 and 1.2 for GmIPT and GmCKX genes, respectively, suggesting that upregulation of GmCKXs, thereby reduction of CK levels, maybe the major events under abiotic stresses. Indeed, the expression of 12 GmCKX genes was upregulated by dehydration in R2 roots. Overall, the expressions of soybean CK metabolic genes in various tissues at various stages were highly responsive to drought. CK contents in various organs at the reproductive (R2) stage were also determined under well-watered and drought stress conditions. Although tRNA-type GmIPT genes were highly expressed in soybean, cis-zeatin and its derivatives were found at low concentrations. Moreover, reduction of total CK content in R2 leaves under drought was attributable to the decrease in dihydrozeatin levels, suggesting a role of this molecule in regulating soybean’s responses to drought stress. Our systematic analysis of the GmIPT and GmCKX families has provided an insight into CK metabolism in soybean under drought stress and a solid foundation for in-depth characterization and future development of improved drought-tolerant soybean cultivars by manipulation of CK levels via biotechnological approach.

Introduction

Soybean (Glycine max L.), which is one of the major legume crops native to East Asia, provides an abundant source of oil and protein-rich food for both human and animal consumption. The growth and productivity of soybean are adversely affected by a number of environmental stresses [1,2]. Among the adverse environmental factors commonly encountered by soybean, drought is considered the harshest, affecting all stages of plant growth and development. Drought stress typically results in significant yield losses and a reduction of seed quality for soybean [2,3].

Generally, in response to drought stress, plants activate a wide range of defense mechanisms that function to increase tolerance to water limiting conditions [4]. The early events of a plant’s adaptation to drought stress are the stress signal perception and subsequent signal transduction, leading to the activation of various physiological and metabolic responses [4–9]. In Arabidopsis, it has been reported that the signaling processes activated under water limiting conditions involve the conversion of stress signal perception to stress-responsive gene expression. The cytokinin (CK)-related two-component system (TCS), which consists of CK receptor histidine kinases (AHKs), His-containing phosphotransferases (AHPs) and response regulators (ARRs), function as molecular switches during stress responses. The utilization of CK receptor mutants as a central tool to study CK functions has led to the suggestion that CKs might mediate osmotic stress responses [5,10]. Recent analyses of CK-deficient plants have demonstrated that CKs may act as negative regulators through CK signaling in response to drought and salt stresses [11–13].

CKs are produced in large quantities in proliferating tissues, such as root and shoot apical meristems, young leaves and immature seeds [14]. In Arabidopsis, the rate-limiting step of CK
biosynthesis is catalyzed by the isopentenyltransferases (IPTs), which consist of ATP/ADP IPTs and tRNA IPTs. Studies on the biosynthetic pathways for these compounds have clarified that the ATP/ADP IPTs control the biosynthesis of isopentenyladenines (iP) and trans-zeatin (Z)-type CKs, whereas tRNA IPTs are responsible for the synthesis of cis-zeatin (Z)-type CKs [14,15]. On the other hand, CK degradation is catalyzed by the CK dehydrogenases (CKXs), which have distinct biochemical characteristics. For instance, in *Arabidopsis*, the AtCKX1 and AtCKX3 and AtCKX2 and AtCKX4 pairs possess similar function and substrate specificity as demonstrated by gain-of-function studies [16,17]. Functional analyses of IPTs, CKXs and CK-related TCS members in *Arabidopsis* using both gain- and loss-of-function approaches, have suggested that CKs control many biological processes, such as development, growth and cell division, in addition to responses to environmental stimuli [18]. CKs have been shown to negatively regulate root growth but positively regulate shoot growth in both vegetative and reproductive stages [16,19,20]; however, excessive overproduction of CKs above a threshold may cause stunted plant growth and abnormal tissue development [21–25]. Drought stress accelerates leaf senescence, which is associated with a decrease in CK content and suppression of CK signaling [26–29]. Strong lines of evidence have indicated that appropriate manipulation of CK levels may enhance tolerance to drought stress [30–32]. An overproduction of CKs during plant maturation, just prior to the onset of senescence, significantly increased drought tolerance with minimal yield loss due to a delay of drought-induced senescence associated with a pre-programmed increase in CK levels [33–35]. On the other hand, reduction of CK levels by the overexpression of *CKX* genes in roots promotes primary root elongation and root branching, resulting in an increase in root biomass that subsequently improves drought tolerance of transgenic plants [12].

Taking into account the importance of CKs and CK signaling in the regulation of stress tolerance, which provides multiple biotechnological strategies for agronomy, we have previously identified and characterized expression profiles of each TCS member in soybean seedlings under dehydration stress [36,37]. In this report, we have identified and systematically characterized all of the IPT and CKX encoding genes in soybean. We have found that a large number of putative *GmIPT* and *GmCKX* genes may be the result of genome duplication. Since there is a wealth of structural and functional information for *Arabidopsis* IPTs and CKXs, we performed sequence analyses and phylogenetic relationship studies of IPTs and CKXs of soybean and *Arabidopsis* to classify the functions of *GmIPT* and *GmCKX* proteins in CK metabolism based on their sequence architecture. To clarify the regulation of CK metabolism in soybean during normal growth and drought stress, we have analyzed the expression patterns of *GmIPT* and *GmCKX* genes under normal and drought stress conditions in a tissue-specific fashion. Expression profiles of *GmIPT* and *GmCKX* genes were examined in various tissues of both dehydrated young seedlings and soil-dried plants at vegetative and flowering stages. Additionally, we have investigated the correlation between the drought-stress-dependent alterations of CK metabolic gene expression and CK biosynthesis by determining the endogenous CK levels in soybean drought-treated leaf tissue.

**Materials and Methods**

**Identification and annotation of the soybean *GmIPT* and *GmCKX* genes and in silico analyses**

*Arabidopsis* IPTs and CKXs were applied as seed sequences to identify the GmIPT and GmCKX proteins in soybean (Glyma 1.0 version) using reciprocal blast as previously described [37]. Proteins, whose encoding sequences containing start and stop codons, were selected for further analysis. Selected protein sequences were searched against the PFAM database to confirm the presence of domain signatures. Protein sequence alignments were performed with a gap open penalty of 10 and gap extension penalty of 0.2 using ClustalW implemented in MEGA software [38,39]. Unrooted phylogenetic trees were constructed using the neighbor-joining method. The confidence level of monophyletic groups was estimated using a bootstrap analysis of 10,000 replicates. Only bootstrap values higher than 40% are displayed next to the branch nodes.

Tandem duplicates were defined as those genes located within 20 loci from each other. Segmental duplications were identified by synteny analysis using an online tool [http://chibba.agtec.uga.edu/duplication/] [40].

To identify cis-regulatory motifs in the promoter regions of *GmIPT* and *GmCKX* genes, previously reported abiotic-stress related cis-elements[4] were used to search against the 1,000-bp upstream sequence of the transcriptional start site of each *GmIPT* or *GmCKX* gene using MEGA 4 software [39].

**Plant growth, dehydration and drought treatments and tissue collections**

Growth and dehydration treatment of young soybean seedlings were performed as previously described [41]. Briefly, 12-day-old plants were removed from soil and roots were gently washed to remove soil. Plants were subsequently transferred onto filter paper and allowed to dry for 2 h and 10 h under 60% relative humidity, 25 °C and 10 μmol m⁻² s⁻¹ photon flux. For drought treatment, soybean plants (cv. Williams 82) were grown in pots (3 plants per 6-liter pot) containing Supermix (Supermix A, Sakata, Japan). Water was given to each pot once a day under greenhouse conditions (continuous 30°C temperature, photoperiod of 12 h/12 h, 80 μmol m⁻² s⁻¹ photon flux density and 50% relative humidity). Soybean plants at V6 stage (28 days after sowing, containing 7 trifoliate leaves) were withheld from watering to initiate the drought treatment. Water was provided to the well-watered control plants to maintain the volumetric soil moisture content (SMC) at 40–45%. At the sixth day of water withholding, where the SMC was below 5% and the soybean plants contained 7 fully open trifoliate leaves and a half-open 8th trifoliate leaf (Figure S1A), soybean leaves were separately collected from each trifoliate leaf. The 3rd, 5th and 7th trifoliate leaves (counted from the bottom-up) were used for determination of the stress severity by measuring leaf relative water content (Figure S1B and S1C). At the same time, trifoliate leaves 4th, 6th and 8th were quickly frozen in liquid nitrogen and stored at −80°C for the isolation of RNA for qRT-PCR. All of the samples were collected in four biological replicates.

To collect the root and leaf tissue samples at reproductive stages (R2), the soybean plants were allowed to grow in a semi-hydroponic manner. The plants were grown in the pots as described above and the roots were allowed to outgrow through the soil layers to reach the water tray as seen in Figure S2. The hydroponic parts of auxiliary roots were cut while remained underwater. For dehydration treatment, the roots were removed from the water and kept on filter paper and allowed to dry at room temperature for 5 h. Control water-treated and dried roots were then collected and quickly frozen in liquid nitrogen and stored at −80°C until further use. All of the samples were collected in three biological replicates. Fully open flowers were collected during the R1 to R2 period in three biological replicates. Full pods were collected in three biological replicates during the R4 stage, of...
which all of the collected pods ranged from 10 to 20 mm in length. R3 seeds were collected during the R3 stage when the seeds were approximately 3 mm in length.

For the collection of well-watered and drought-treated leaves at the R2 reproductive stage, we measured the chlorophyll content of the 3rd trifoliate leaves (counting down from the growing shoots) and marked the leaves with similar chlorophyll indexes. From each trifoliate leaf, we collected one side-leaf under normal conditions (well-watered, SMC of 30%). These leaves were quickly cut into two halves, one for CK measurement and the other for RNA purification, and both were frozen in liquid nitrogen and stored in −80°C until further use. Three leaves were also collected from the other two 3rd trifoliate leaves of similar chlorophyll index to determine leaf relative water content under well-watered conditions (91 ± 1%). The plants were then allowed to undergo drought treatment by withholding water until the SMC reached 5%. From the same 3rd trifoliate leaves, from which one side-leaves had been collected as control well-watered samples, the other side-leaves were collected as drought-treated samples and prepared as described above for CK measurement and RNA purification. Three remaining leaves of the two trifoliate leaves that were previously used for measuring relative water content under well-watered conditions were also collected separately to determine leaf relative water content under drought conditions (32 ± 2%). All of the samples were collected at mid-day (11AM-1PM) and in three biological replicates.

RNA isolation, DNase treatment and cDNA synthesis for qRT-PCR

RNAs were purified using Trizol reagent (Invitrogen) according to a manufacturer-recommended protocol. DNase I treatment and cDNA synthesis were performed as previously described [41].

qRT-PCR and statistical analysis of the data

Primers for qRT-PCR were designed as previously described [41]. The CTP2 gene was used as a reference gene in the expression profiling of soybean genes [42]. qRT-PCR reactions and data analyses were performed according to previously published methods [41]. Delta-Ct method was used to calculate initial amount of target genes. When appropriate, a Student’s t-test (one tail, unpaired, equal variance) was used to determine the statistical significance of the differential expression patterns between tissues and/or between treatments.

Cytokinin analysis

Cytokinins were extracted and purified according to Dobrev and Kaminek [43] using reverse phase and ion exchange chromatography. Derivatives of I2 Cks were determined from retention time and the mass spectra of unlabeled standards and response ratio of their I2 counterparts. HPLC-MS analysis was performed as described by Dobrev et al. using an HPLC (Ultimate 3000, Dionex) coupled to hybrid triple quadrupole / linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) set in selected reaction monitoring mode.

Results and Discussion

Identification and annotation of the GmIPT and GmCKX genes in soybean

We used a previously described genome-wide analysis method in soybean [37] to identify 17 putative GmIPT and 20 putative GmCKX genes which encode IPTs and CKXs proteins, respectively. Manual inspection led to the removal of several truncated sequences and we finally obtained a list of 14 and 17 putative soybean GmIPT and GmCKX genes, respectively. All of the GmIPT proteins were predicted to contain one or two IPP transferase domains (PF01715) and all of the GmCKX proteins contained the CK-binding (PF09265) and FAD-binding (PF01365) domains. Sequence features of the GmIPTs and GmCKXs are summarized in Tables 1 and 2.

Among the 14 GmIPTs, three members, namely GmIPT02, GmIPT03 and GmIPT14, contain a large number of exons (10, 9 and 11, respectively), while five GmIPT members lack introns. The remaining six GmIPT genes possess two or three exons (Figure 1A, Table 1). This feature is also observed in genes encoding IPTs from Arabidopsis (Figure 1A, box). The group of IPT genes that lack introns or contain a small number of exons might have evolved from a prokaryotic ancestor and eventually some of the genes started gaining introns through the intronization process. Similar to their Allipt2 and Allipt9 counterparts in Arabidopsis, the GmIPT genes that contain high numbers of exons belong to the tRNA-dependent isopenotenyltransferase group. Additionally, the rest of the GmIPTs were predicted to have at least one glycosylation site, except GmIPT01, 05 and 11. Each of the GmIPTs also possesses a signal peptide that directs it to various locations, such as mitochondria, chloroplast and other secretory pathways (Table 1).

The homology of soybean GmCKX proteins to their Arabidopsis counterparts was in the range from 43% to 68% (Table 2). The GmCKX genes contain 4 to 7 exons and encode proteins of 429 to 551 amino acid residues (Figure 1B, Table 2). Each of the GmCKXs contains an FAD-binding domain and a CK-binding domain with extremely high confidence (E-values < 3.4E-11 for FAD-binding domain and < 6.9E-67 for CK-binding domain). The GmCKX proteins were also predicted to harbor one to seven predicted sites for glycosylation, e.g. modifications which are responsible for the enhancement of enzyme activity [45], as well as a signal peptide that direct their transport.

Phylogenetic analyses of GmIPT and GmCKX proteins

To uncover the evolutionary relationships among the soybean and Arabidopsis IPTs, as well as the soybean and Arabidopsis CKXs, we performed phylogenetic analyses using the neighbor-joining method implemented in MEGA software [39]. The protein sequences of the IPTs and CKXs from both species are highly similar (Figure 2). Among the GmIPTs clustered with either AtIPT2 (GmIPT02 and GmIPT03) or AtIPT9 (GmIPT14), which are known to code for tRNA-dependent IPTs. As shown in Figure 2A, the tRNA-dependent IPTs and the ADP/ATP-dependent IPTs were highly diverse. It is also evident that the two species maintained different sets of IPTs, as well as the soybean and Arabidopsis CKXs. We observed that the Ckx genes from both species are highly similar (Figure 2). Three of the GmCKXs clustered with either AtCKX07, which was recently reported to act on CKXs from Arabidopsis, and AtCKX09 (GmCKX14), which are known to code for tRNA-dependent CKXs from Arabidopsis and soybean (Figure 2B). The GmCKX15, 16 and 17 were clustered with AtCKX07, which was recently reported to act on tRNA-dependent CKXs as a preferred substrate for degradation [46].

In addition, we observed that there are more CKX than IPT enzyme encoding genes (17 versus 14) in soybean, whereas the opposite was found in Arabidopsis (7 versus 9). This could be an advantage of evolution as more CKXs would enable soybean plants to robustly and precisely reduce CK content for a better response to adverse environmental conditions.
Table 1. Soybean genes encoding putative IPT enzymes and their properties.

| Gene name | Chromosome locus | Number of exons\textsuperscript{a} | Domain feature (IPPT E-value)\textsuperscript{b} | Family\textsuperscript{c} | Length (aa) | Identity (%d) | Glycosylation sites\textsuperscript{e} | TargetP\textsuperscript{f} |
|-----------|-----------------|--------------------------------------|----------------------------------------|-----------------|-------------|----------------|-------------------------------|-----------------|
| GmIPT01   | Glyma10g41990   | 2                                    | 2.30E-19 - AtIPT1/AtIPTB/AtIPT6       | 308             | 46.7/43.5/42.8 | 0              | M/5                           |
| GmIPT02   | Glyma11g19330   | 10                                   | 2.10E-44 - AtIPT2                      | 470             | 52          | 4              | C/5                           |
| GmIPT03   | Glyma12g09140   | 9                                    | 4.90E-20 - AtIPT2                      | 321             | 36.2        | 2              | –/5                           |
| GmIPT04   | Glyma03g30850   | 3                                    | 3.40E-21 4.80E-09 AtIPT3               | 296             | 48.5        | 2              | C/4                           |
| GmIPT05   | Glyma10g03060   | 3                                    | 1.70E-20 5.40E-11 AtIPT3               | 315             | 46.7        | 0              | –/4                           |
| GmIPT06   | Glyma02g16750   | 10                                   | 9.90E-19 2.90E-11 AtIPT3               | 338             | 45.4        | 1              | –/4                           |
| GmIPT07   | Glyma19g33680   | 2                                    | 5.20E-22 6.00E-10 AtIPT3               | 283             | 46.1        | 1              | C/5                           |
| GmIPT08   | Glyma17g02080   | 1                                    | 1.40E-15 3.20E-10 AtIPT5               | 311             | 51.1        | 2              | –/3                           |
| GmIPT09   | Glyma15g11040   | 2                                    | 4.20E-18 1.00E-09 AtIPT5               | 342             | 48.1        | 4              | S/5                           |
| GmIPT10   | Glyma07g38620   | 2                                    | 3.10E-17 9.40E-112 AtIPT1/AtIPT6       | 256             | 36.2/34.5   | 0              | –/3                           |
| GmIPT11   | Glyma18g53460   | 3                                    | 3.70E-18 2.70E-10 AtIPT3/AtIPT7        | 211             | 29.5        | 1              | –/3                           |
| GmIPT12   | Glyma08g48020   | 1                                    | 2.40E-18 - AtIPT3/AtIPT7               | 246             | 32.3/31.5   | 2              | –/2                           |
| GmIPT13   | Glyma13g27990   | 3                                    | 6.50E-07 - AtIPT5                      | 338             | 57.2        | 1              | –/3                           |

\textsuperscript{a}Minus signs represent genes located on opposite strand.
\textsuperscript{b}PFAM e-values for having the IPP transferase protein domain (PF01715) some GmIPTs contain more than one IPP domain.
\textsuperscript{c}Closest homologs from Arabidopsis.
\textsuperscript{d}Percentage of identical amino acids with the closest Arabidopsis homologs.
\textsuperscript{e}Glycosylation sites were predicted with NetNGly (http://www.cbs.dtu.dk/services/NetNGlyc/); 1-5, highest to lowest possibility.
\textsuperscript{f}Localization predicted with TargetP (http://www.cbs.dtu.dk/services/TargetP/); M, mitochondria; C, chloroplast; S, secretory pathways; “–”, not known location; 1-5, highest to lowest possibility.

Table 2. Soybean genes encoding putative CKX enzymes and their properties.

| Gene name | Chromosome locus | Number of exons\textsuperscript{a} | FAD_binding\textsuperscript{b} | CK-binding\textsuperscript{b} | Family\textsuperscript{c} | Length (aa) | Identity (%d) | Glycosylation sites\textsuperscript{e} | TargetP\textsuperscript{f} |
|-----------|-----------------|--------------------------------------|-------------------------------|-----------------|-----------------|-------------|----------------|-------------------------------|-----------------|
| GmCKX01   | Glyma19g31620   | 5                                    | 1.50E-22 2.20E-112 AtCKX1/AtCKX6 | 544             | 59.1/60.8     | 0              | C/5                           |
| GmCKX02   | Glyma03g28910   | 7                                    | 1.20E-21 2.70E-69 AtCKX1/AtCKX6    | 551             | 49.2/49.7     | 4              | C/5                           |
| GmCKX03   | Glyma09g07190   | 6                                    | 1.10E-21 4.00E-93 AtCKX2           | 533             | 45           | 6              | –/5                           |
| GmCKX04   | Glyma09g07360   | 6                                    | 1.40E-18 1.30E-118 AtCKX3           | 536             | 54.8        | 2              | –/5                           |
| GmCKX05   | Glyma13g16420   | 7                                    | 1.80E-12 1.70E-102 AtCKX3           | 429             | 42.1        | 1              | –/3                           |
| GmCKX06   | Glyma13g16430   | 5                                    | 1.10E-23 1.00E-114 AtCKX3           | 535             | 54.3        | 1              | S/3                           |
| GmCKX07   | Glyma15g18560   | 5                                    | 2.60E-20 5.90E-118 AtCKX3           | 543             | 53.9        | 2              | S/5                           |
| GmCKX08   | Glyma17g06220   | 5                                    | 4.30E-23 1.60E-116 AtCKX3           | 535             | 55.8        | 2              | S/1                           |
| GmCKX09   | Glyma17g06230   | 6                                    | 1.60E-20 4.90E-102 AtCKX3/AtCKX4    | 528             | 46.8/46.6    | 6              | –/5                           |
| GmCKX10   | Glyma06g31810   | 5                                    | 1.40E-22 9.40E-120 AtCKX5           | 518             | 64          | 1              | S/2                           |
| GmCKX11   | Glyma04g03130   | 5                                    | 3.20E-22 3.30E-120 AtCKX5           | 458             | 60.8        | 1              | –/4                           |
| GmCKX12   | Glyma09g35950   | 6                                    | 1.50E-20 1.10E-113 AtCKX6           | 534             | 68.6        | 3              | S/1                           |
| GmCKX13   | Glyma11g20860   | 5                                    | 5.20E-21 2.80E-114 AtCKX6           | 552             | 64.4        | 6              | M/4                           |
| GmCKX14   | Glyma12g01390   | 6                                    | 2.20E-21 6.90E-67 AtCKX6            | 442             | 55.5        | 1              | –/4                           |
| GmCKX15   | Glyma04g05840   | 6                                    | 3.40E-11 1.90E-101 AtCKX7           | 494             | 58.6        | 2              | –/2                           |
| GmCKX16   | Glyma14g11280   | 4                                    | 7.40E-29 1.20E-103 AtCKX7           | 513             | 61.5        | 3              | –/2                           |
| GmCKX17   | Glyma17g34330   | 4                                    | 3.10E-26 5.90E-103 AtCKX7           | 513             | 58.6        | 2              | –/2                           |

\textsuperscript{a}Minus signs represent genes located on opposite strand.
\textsuperscript{b}PFAM e-values for having the indicated protein domains.
\textsuperscript{c}Closest homologs from Arabidopsis.
\textsuperscript{d}Percentage of identical amino acids with the closest Arabidopsis homologs.
\textsuperscript{e}Glycosylation sites were predicted with NetNGly (http://www.cbs.dtu.dk/services/NetNGlyc/); 1-5, highest to lowest possibility.
\textsuperscript{f}Localization predicted with TargetP (http://www.cbs.dtu.dk/services/TargetP/); M, mitochondria; C, chloroplast; S, secretory pathways; “–”, not known location; 1-5, highest to lowest possibility.

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Our analysis has indicated that soybean genes encoding GmIPTs and GmCKXs are distributed on various chromosomes, with exception of chromosomes I, V, XVI and XX (Figure 3). Gene duplication is also of interest because it is the source of genetic material for diversification [47]. Thus, we subsequently investigated the duplication patterns of GmIPT and GmCKX genes. We defined the genes as tandem duplicates if they were located within 20 loci from each other. For segmental duplicates, we analyzed the synteny blocks using an online tool (http://chibba.agtec.uga.edu/duplication/) [40]. We found three tandem duplicated pairs among the GmCKXs (Figure 3), namely GmCKX03 and GmCKX04 on chromosome IX; GmCKX05 and GmCKX06 on chromosome XIII; and GmCKX08 and GmCKX09 on chromosome XVII. No tandem duplication was found among the GmIPTs. Segmental duplicates were found in both GmIPT and GmCKX gene families. As evidenced by synteny analysis, four pairs of GmIPTs were formed by segmental duplication: GmIPT04 (Chr. III) and GmIPT07 (Chr. XIX); GmIPT10 (Chr. VII) and GmIPT08 (Chr. XVII); GmIPT12 (Chr. VIII) and GmIPT11 (Chr. XVIII); and GmIPT13 (Chr. XIII) and GmIPT09 (Chr. XV) (Figure S3A). Among 17 GmCKX genes, five pairs were formed by segmental duplication: GmCKX02 (Chr. III) and GmCKX01 (Chr. XIX); GmCKX11 (Chr. IV) and GmCKX10 (Chr. VI); GmCKX12 (Chr. IX) and GmCKX14 (Chr. XII); GmCKX05 (Chr. XIII) and GmCKX06 (Chr. XIII); and GmCKX08 (Chr. XVII) and GmCKX09 (Chr. XVII), the last two pairs being first formed by tandem duplication and then by segmental duplication (Figure 3; Figure S3B). The differences in duplication patterns of the two gene families may reflect their contrasting functions in regulating CK levels in soybean plant.

Stress-inducible *cis*-regulatory elements in the promoter regions of GmIPT and GmCKX genes

*Cis*-regulatory elements, which are located in the upstream regions of genes and act as the binding sites for TFs, have essential roles in determining the tissue-specific or stress-responsive expression patterns of genes. Over the years, extensive promoter analyses have identified a number of stress-responsive *cis*-elements, which are important molecular switches involved in the transcriptional regulation of a dynamic network of gene activities.
controlling abiotic stress responses [4,48,49]. Increasing evidence has demonstrated a positive correlation between multi-stimulus responsive genes and cis-element density in upstream regions [50-52]. Therefore, in order to identify possible candidates among GmIPT and GmCKX genes which are involved in abiotic stress responses in soybean plants, we performed a search for the existence of the eleven known stress-responsive cis-elements in the 1000-bp promoter region upstream of the transcription start site of each GmIPT and GmCKX encoding gene. As shown in Table S1, three of the known abiotic-stress responsive cis-elements were found in the promoters of GmIPT gene family members. Specifically, MYCR (MYC recognition site) was found in five IPT members (GmIPT04, 05, 08, 11 and 12), ZFHDR (zinc finger homeodomain recognition site) was found in GmIPT01 and ICEr2...
(Inducer of CBF expression 2 recognition site) was found in GmIPT07. Among the promoters of the GmCKX family, we detected five abiotic-stress responsive cis-elements, which are ABRE (ABA-responsive element), MYBR (MYB recognition site), MYCR, ZFHDR and OSE2 (Table S1). Although distributed on promoters of both GmIPTs and GmCKXs, on the average there were 0.4 and 1.2 abiotic stress-inducible cis-elements per promoter of genes encoding GmIPT and GmCKX, respectively. This evidence suggested that upregulation of GmCKXs, thereby reduction of CK levels, could be the major event in soybean plants under stress. Evidence suggested that upregulation of GmIPT and GmCKX, respectively. This upregulation of GmCKXs, thereby reduction of CK levels, could be the major event in soybean plants. Evidence suggested that upregulation of GmIPT and GmCKX, respectively. This upregulation of GmCKXs, thereby reduction of CK levels, could be the major event in soybean plants under stress.

Expression of GmIPT and GmCKX genes in vegetative organs at early and late developmental stages under normal growth conditions

Tissue-specific and development stage-related expression data are useful in the identification of genes that are involved in defining the precise nature of individual tissues in a given developmental stage. Moreover, the mechanisms controlling the response to drought stress may be associated with root- and/or shoot-related traits. For instance, suppression of shoot-growth and/or promotion of primary root growth are considered morphological adjustments enabling plants to adapt better to drought stress [11]. The upregulated CKX genes, either in Arabidopsis or soybean, may contribute to faster degradation of the accumulated CKs upon exposure to environmental stresses, thereby enhancing plant adaptation to adverse stress conditions.

Cytokinin Metabolism in Soybean under Drought

Expression of GmIPT and GmCKX genes in vegetative organs at early and late developmental stages under dehydration/drought stress

Among the IPT transcripts expressed in remarkably high abundance, the GmIPT08 transcript was consistently increased in the leaves and young seedling shoots under drought or dehydration conditions (Figures 5A, 6A and 7A). The induction level of this transcript was the highest in young seedling shoots (~300-fold, Figure 5A). In the V6 leaves, the induction levels were correlated with the age of the trifoliate leaves; the older the leaf is the higher the induction (Figure 6A). In R2 leaves, GmIPT08 was the only gene whose expression was significantly induced (~7-fold) by drought (Figure 7A). Other GmIPT genes, which were significantly induced by drought in the V6-stage leaves, are GmIPT09 and 13, and the degree of induction of these genes was higher in the younger trifoliate leaves (Figure 6A). As for the drought-repressible IPT genes identified in the leaf tissues, the GmIPT05 transcripts were repressed by drought in the leaves of various stages (Figures 5A, 6A and 7A). In the R2-stage leaves, expression of almost all highly expressed GmIPT genes, except that of GmIPT08, was repressed by drought. The most substantial repression was observed in case of the GmIPT07 gene (Figure 7A). In the roots of young seedlings, the most abundant GmIPT08 transcript (Figure 4A) was slightly induced by dehydration after 2 h of treatment, being subsequently repressed after 10 h of dehydration (Figure 5A). In the R2 auxiliary roots, the transcripts with high abundance did not change significantly upon dehydration. These data are in contrast with the observation in root hairs where all major transcripts, such as GmIPT01, 07, 08, 09 and 13, were induced upon 3-h dehydration (Figure 7A).

As for the GmCKX genes, five GmCKXs (GmCKX01, 02, 06, 13 and 18) were found to be severely reduced at transcriptional level by drought in the V6 leaves (Figure 6B), and four of which, except GmIPT08 was found to be the major transcript in the roots of young seedlings while GmIPT02 mRNA had the highest abundance in the roots of R2 plants (Figure 4B). Taken together, these data indicated that soybean requires different IPT genes for the biosynthesis of CKs in different organs and at different developmental stages. CKXs are the key enzymes involved in the regulation of CK levels in plants for the maintenance or reestablishment of CK homeostasis. In order to determine which GmCKX gene(s) may play important regulatory roles in specific organ(s) of the soybean plants, we measured the GmCKX transcripts in various tissues/ organs. Among 17 GmCKX genes, GmCKX13, 15 and 16 were highly expressed in all tissues examined, while the expression of four other GmCKXs, GmCKX05, 07, 10 and 11, was hardly detected. At the same time, GmCKX14 transcript was only found in young seedling tissues (Figure 4B). In soy leaves, GmCKX12, 13, 14 and 16 were detected as major transcripts in young seedling shoots, GmCKX13, 14, 15 and 16 mRNAs were the most abundant in the V6 leaves and GmCKX12 and 16 expression levels were the highest in R2 leaves (Figure 4B). As for root tissues, GmCKX12 and 14 were the major transcripts in the young seedling roots, while GmCKX09 and 16 were most abundant in the R2 auxiliary roots and root hairs. On the other hand, GmCKX15 was the major transcript in R2 auxiliary roots only (Figure 4B). Additionally, the major transcripts of GmIPT and GmCKX genes determined in our study were also highly expressed in various tissues as reported by Libault and co-workers [54] using Illumina transcriptome sequencing (Figure S4), suggesting a good correlation between the qRT-PCR and Illumina transcriptome sequencing methods in expression profiling.
GmCKX06, were also significantly downregulated in R2 leaves under drought (Figure 7B). Nevertheless, in young seedling shoots, the expression of GmCKX01 and 02 was induced by dehydration (Figure 3B). In addition, the GmCKX14 transcript was found to be significantly induced by drought in various tissues at different stages. Furthermore, most of the GmCKXs were induced by drought in the R2 roots (Figure 7B). Among 17 GmCKX genes, only GmCKX16, whose encoded protein might be involved in degradation of αβ-type CKs as its AtCKX7 ortholog [46], was significantly upregulated by drought in the roots of the R2 stage soybean plants (Figure 7B).

Expression of GmIPT and GmCKX genes in reproductive tissues under normal growth conditions

Strong lines of evidence have also suggested that CKs play an important role in the development of reproductive organs and seed yield. Disruption of CKX3 and CKX5 genes in Arabidopsis resulted in higher CK levels, which subsequently led to larger inflorescences and floral meristems, increased size of the WUSCHEL expression domain, supernumerary ovules and increased seed yield of the dckx3,5 double mutant plants [55]. An increase in CK accumulation caused by a null mutation in the OsCKX2 gene was also shown to enhance the size of inflorescence meristems and increase the number of reproductive organs, resulting in enhanced grain yield [56]. Therefore, to gain an insight into the CK metabolism in reproductive organs, we analyzed the expression of CK metabolic genes in flowers, full pods and R5 seeds. Results shown in Figure 8A indicated that GmIPT02 is ubiquitously expressed in all three reproductive tissues examined while five other GmIPTs (GmIPT03, 05, 08, 10 and 12) were not expressed. GmIPT02 was the major transcript in flowers, GmIPT01 and 02 expressed in the greatest abundance in full pods while GmIPT01, 02 and 11 were the most abundant transcripts in R5 seeds. The variation in the GmIPT transcript levels in flowers, pods and R5 seeds suggested that each of these organs required different GmIPT genes for CK biosynthesis. GmIPT02 and GmIPT11 might play the most important role in flowers and R5 seeds, respectively, while GmIPT01 and 02 appear to be equally important in full pods as judged by their abundant expression.

The expression levels of GmCKX genes were also determined in flowers, pods and R5 seeds (Figure 8B). In flowers, five out of 17 GmCKX mRNAs were dominant, including GmCKX04, 07, 08, 12 and 16. Unlike in flowers, GmCKX08 transcript was in the highest abundance measured in pods. In R5 seeds, GmCKX08 was still the...
Figure 5. Expression profiles of GmIPTs and GmCKXs in the roots and shoots of 12-day-old soybean seedlings under normal and dehydration conditions. (A) GmIPT genes. (B) GmCKX genes. Black bars; expression under normal condition (0 h); gray bars, expression under 2 h dehydration; white bars, expression under 10 h dehydration.
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most highly abundant transcript, while the second most abundant one was GmCKX16. It is noteworthy to mention that the expression of nine out of 17 GmCKXs was hardly detected in the three reproductive tissues examined (Figure 8B), including GmCKX13, which were highly expressed in various vegetative tissues (Figure 4B). Our data suggested that GmCKX08 is perhaps the major regulator of CK levels in reproductive organs. On the other hand, in flower tissues, the concerted action of at least five GmCKXs is required for maintaining CK homeostasis.

In addition, our expression data presented in Figures 4, 5, 6, 7, and 8 also indicated that a few duplicated gene pairs have undergone expression divergence (GmIPT08 and GmIPT10; GmCKX03 and GmCKX06; and GmCKX12 and GmCKX14), whereas the majority of the duplicated pairs have not changed their expression patterns (GmIPT04 and GmIPT07; GmIPT11 and GmIPT12; GmIPT09 and GmIPT13; GmCKX01 and GmCKX02; GmCKX08 and GmCKX09; and GmCKX10 and GmCKX11). The data also showed that although the majority of GmIPT and GmCKX

Figure 6. Expression profiles of GmIPTs and GmCKXs in V6 trifoliate leaves (4th, 6th and 8th) under normal and drought conditions. (A) GmIPT genes. (B) GmCKX genes. Black bars; expression under normal condition; white bars, expression under drought condition.
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Figure 7. Expression profiles of GmIPTs and GmCKXs in the leaves, roots and root hairs of soybean plants at reproductive stage under normal and drought conditions. (A) GmIPT genes. (B) GmCKX genes. Black bars; expression under normal condition; white bars, expression under drought condition.

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genes exhibited similar stress-responsiveness to that of their respective Arabidopsis orthologous genes, several GmIPT and GmCKX genes have undergone transcriptional divergence during evolution (Table S3).

**CK metabolites in various organs of soybean plants under normal and drought conditions**

To gain an overall image of CK functions in the development of soybean plants, we quantified the CK metabolites in various tissues/organs collected from soybean plants at various stages as described in Materials and Methods (Table S4). The most remarkable result is that compared with the levels of \( tZ \)-type and \( iP \)-type CKs, the levels of \( cZ \)-type CKs were significantly lower in almost all of the tissues/organs examined (Figure 9; Table S4), despite the fact that RNA-type GmIPT encoding genes were found to be highly expressed (Figure 4). These data are similar to the situation observed in Arabidopsis [11,19]. Our results added up to a recent study by Gajdosova et al. [46] which indicated that \( cZ \)-type CKs occur ubiquitously across plant kingdom and their abundance is, perhaps, correlated with life strategy rather than with evolutionary complexity. In the vegetative organs, such as leaves, the total content of CKs, as well as the CK compositions, also varied with age. For example, the total CK content in young R2 leaves was higher than that in fully developed R2 leaves. This increase was mainly result of an increase in the DZR and DZRP (Figure 9, left panel), suggesting that the DZ-type CKs are also significantly produced in young leaves of soybean, in addition to dormant seeds and apical buds as observed in bean [57,58].

Increasing evidence suggests that several CK receptor kinases, such as the AHK3 of Arabidopsis or the ZmHK2 of maize, have affinity to DZ-type CKs [59-61] suggesting that the DZ-type CKs might have biological functions in plants. Previously, we showed that in soybean there are two GmHKs, the GmHK12 and 13, which have high homology to the AHK3 [37]. Taken together, the DZ-type CKs might be biologically active in soybean and their variation in levels may reflect their active roles in regulating plant growth and development of soybean plants.

In the R2 plants, the levels of \( tZ \) and \( tZR(P) \) were higher in young leaves than in the more mature fully expanded leaves. The mature leaves accumulated more \( tZ \) deactivation products (\( tZ(R)O-glucosides \)). It appears that CK metabolites are organ-specific. For example, \( tZ \), its riboside and their immediate precursors (\( tZRPs \)) are abundant in roots, especially in root tips and hairs, which are the primary sites of CK biosynthesis. \( tZ \), the most physiologically active CK in the stimulation of cell division, is also relatively abundant in other rapidly growing tissues (e.g. young leaves, flowers and pods). In addition, flowers and pods also contain very high levels of \( tZR \) and \( tZRP \)s as well (Figure 9, left and middle panels). On the contrary, \( tZRP \)s were not detected in fully developed leaves (Figure 9, left panel) at reproductive stage. Physiologically inactive \( tZOG \) and \( tZROG \) were more abundant in mature leaves and were less abundant in young leaves, flowers and pods. These data suggest that a large amount of \( tZ \)-type CKs are in storage forms in these organs. CK O-glucosides were undetectable in roots. Pods exhibited the highest level of total CKs, 5- to 10-fold higher than that found in other organs. These
Conclusions
Research in the last several years has indicated that CKs play an essential role in the regulation of plant adaptation to various environmental stresses, including drought [63]. Repression of CK metabolism under adverse stress conditions, which leads to a downregulation of CK signaling, is known as one of the mechanisms used by plants to adapt to adverse environmental conditions [32]. The results of this study provided the first insight into the previously uncharacterized CK metabolic genes encoding GmIPTs, which are involved in the rate-limiting step of CK biosynthesis. In addition, we also investigated GmCKXs, encoding the main CK degrading enzymes, which contribute to maintenance or reestablishment of CK homeostasis. Throughout our investigation, we placed a particular emphasis on their tissue-specific and/or drought-responsive expression. Collectively, these data enable us to understand the molecular mechanisms regulating CK homeostasis in various tissues/organisms at different developmental stages under both normal and drought stress conditions.

In addition, appropriate modulations of CK levels, based upon the knowledge of mechanisms regulating CK metabolism and CK homeostasis, represent promising approaches for the genetic engineering of drought-tolerant economically important crops [32,63]. A reduction in CK content in roots by the constitutive overexpression of a CKX gene in a root-specific manner can improve drought tolerance by enhancing root biomass [12]. On the other hand, an increase in CK content just prior to the onset of senescence was also shown to improve leaf longevity and photosynthetic capacity under drought stress, thereby enhancing drought tolerance without yield penalties [33,64]. Therefore, our study has generated a solid foundation for the identification of candidate genes for future studies which aim to manipulate CK metabolism to appropriate levels and ultimately contribute to the development of improved drought-tolerant transgenic soybeans.

Supporting Information
Figure S1  Drought treatment of soybean plants grown in pots at the V6 stage. (A) Three soybean plants were grown in each pot to V6 stage (four weeks). The V6 plants (containing 7 trifoliate leaves, unifoliate leaves still remained) were withheld from watering; during this time, volumetric soil moisture content (SMC) and room relative humidity were recorded. (B) At the 6th day after withholding water, the leaves were collected from both well-watered and drought-stressed plants. Trifoliate leaves 3rd, 5th and 7th were used for measuring leaf relative water content, while trifoliate leaves 4th, 6th and 8th were used for RNA extraction. After the leaves were collected, the drought-stressed plants were re-watered and monitored to ensure that all drought-treated plants...
survived after drought treatment. Figure S1C shows well-watered and drought-stressed soybean plants just prior to collecting the leaves.

(DOC)

Figure S2 Growth of soybean plants under semi-hydroponic conditions. Soybean plants were allowed to grow under semi-hydroponic conditions for the collection of root tissues. Detached roots were used for dehydration treatment.

(DOC)

Figure S3 Synteny analysis of GmIPT and GmCKX genes. (A) Synteny analysis revealed evidence of the segmental duplication among several GmIPT genes in soybean. (B) Synteny analysis revealed evidence of the segmental duplication among several GmCKX genes in soybean.

(DOC)

Figure S4 Clustering analysis of tissue-specific expression profiles of GmIPT and GmCKX genes. (A) Expression data (normalized Illumina-Solexa read numbers) collected from Libault et al. (2010) [54]. (B) Expression data from our study. Both two data sets showed that GmIPT02 was highly expressed among the tissues examined and that GmCKX04, 07, 08, 12 and 16 were highly expressed in flowers, suggesting a good agreement between our qRT-PCR data and the data derived from Illumina-Solexa cDNA-sequencing study.

(DOC)

Table S1 Number of abiotic-stress inducible cis-elements in the promoters of GmIPTs and GmCKXs.

(DOC)

Table S2 Primers used for qRT-PCR.

(DOC)

Table S3 Drought/dehydration-responsiveness of the soybean and Arabidopsis IPT and CKX genes.

(DOC)

Table S4 CK contents in various soybean tissues under normal and drought stress conditions. (A) Concentration of individual CK metabolites in various soybean tissues. (B) CK contents in various soybean tissues in group of compounds.

(DOC)

Author Contributions

Conceived and designed the experiments: L-SPT. Performed the experiments: DTL RN YW MT RV. Analyzed the data: DTL L-SPT. Contributed reagents/materials/analysis tools: MS LHH KY-S LS L-SPT. Wrote the paper: DTL L-SPT. Revised the manuscript: DTL L-SPT.

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