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Genome-wide Analysis of Phosphoenolpyruvate Carboxylase Gene Family and Their Response to Abiotic Stresses in Soybean

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Phosphoenolpyruvate carboxylase (PEPC) plays an important role in assimilating atmospheric CO₂ during C₄ and crassulacean acid metabolism photosynthesis, and also participates in various non-photosynthetic processes, including fruit ripening, stomatal opening, supporting carbon–nitrogen interactions, seed formation and germination, and regulation of plant tolerance to stresses. However, a comprehensive analysis of PEPC family in *Glycine max* has not been reported. Here, a total of ten PEPC genes were identified in soybean and denominated as *GmPEPC1-GmPEPC10*. Based on the phylogenetic analysis of the PEPC proteins from 13 higher plant species including soybean, PEPC family could be classified into two subfamilies, which was further supported by analyses of their conserved motifs and gene structures. Nineteen *cis*-regulatory elements related to phytohormones, abiotic and biotic stresses were identified in the promoter regions of *GmPEPC* genes, indicating their roles in soybean development and stress responses. *GmPEPC* genes were expressed in various soybean tissues and most of them responded to the exogenously applied phytohormones. *GmPEPC6*, *GmPEPC8* and *GmPEPC9* were significantly induced by aluminum toxicity, cold, osmotic and salt stresses. In addition, the enzyme activities of soybean PEPCs were also up-regulated by these treatments, suggesting their potential roles in soybean response to abiotic stresses.

The enzyme phosphoenolpyruvate (PEP) carboxylase (PEPC; EC 4.1.1.31) catalyzes the irreversible β -carboxylation of PEP in the presence of bicarbonate (HCO₃⁻) and Mg²⁺ or Mn²⁺ to yield oxaloacetate (OAA) and inorganic phosphate (Pi)¹. It is widely distributed in archaea, bacteria, cyanobacteria, green algae, protozoa, and vascular plants, but is absent from animals and fungi^{1,2}. In plants, the reaction catalyzed by PEPC is the primary fixation step of photosynthetic CO₂ assimilation in C₄ photosynthesis and crassulacean acid metabolism (CAM). In most nonphotosynthetic tissues and in the leaves of C₃ plants, PEPC plays the anaplerotic role of replenishing tricarboxylic acid (TCA) cycle with intermediates that are consumed for a variety of biosynthetic pathways and nitrogen assimilation^{1,2}. Therefore, besides its fundamental role in the initial fixation of atmospheric CO₂ during C₄ and CAM photosynthesis, PEPC has a wide range of non-photosynthetic roles including supporting carbon–nitrogen interactions², seed formation and germination^{2,3}, fruit ripening², guard cell metabolism during stomatal opening⁴, and provision of malate as a respiratory substrate for symbiotic N₂-fixing bacteroids in legume root nodules⁵. Recently, accumulating evidence has confirmed that a large number of PEPC genes are induced by abiotic and biotic stresses and play important roles in regulation of plant tolerance to stress^{2,6,7}. For example, an up-regulated PEPC expression in response to salinity or drought stress has been well documented in C₃, C₄ and CAM plants^{6–10}. Meanwhile, overexpression of PEPC gene in transgenic plants enhanced their tolerance to drought or salt stress¹¹, whereas knockdown of PEPC leading to increased sensitivity to osmotic stress¹².

Plants have two types of PEPCs that belong to different evolutionary lineages, namely, plant-type PEPC (PTPC) isozyme and bacterial-type PEPC (BTPC) isozyme types^{1,3,13}. Previous studies showed PTPC genes encode highly closely related 100–110 kDa polypeptides that contain a conserved N-terminal seryl-phosphorylation site and

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Gene Name	Previously named	Gene Model Name (Wm82.a2.v1)	GenBank Protein ID	Chr	ORF (bp)	Protein			
						Size (amino acids)	Molecular Weight (Da)	pI	Subcellular localization
<i>GmPEPC1</i>		Glyma.01G091000	XP_006573282	1	3171	1056	118167.68	6.38	Cyto/Nucl
<i>GmPEPC2</i>		Glyma.02G130700	XP_014622374	2	3168	1055	118230.60	6.28	Cyto
<i>GmPEPC3</i>		Glyma.06G229900	XP_014632171	6	2904	967	110754.93	5.72	Cyto
<i>GmPEPC4</i>		Glyma.06G277500	XP_003527347	6	2901	966	110379.84	5.52	Cyto
<i>GmPEPC5</i>	<i>GmPEPC17</i> ²⁵	Glyma.10G205500	NP_001237378	10	3099	1032	115946.45	6.20	Cyto
<i>GmPEPC6</i>	<i>GmPEPC16</i> ²⁴	Glyma.12G161300	NP_001237602	12	2904	967	110790.15	5.66	Cyto
<i>GmPEPC7</i>	<i>GmPEPC4</i> ²³	Glyma.12G210600	NP_001237394	12	2901	966	110644.39	6.00	Cyto
<i>GmPEPC8</i>	<i>GmPEPC15</i> ²³ / <i>GmPPC1</i> ²²	Glyma.12G229400	XP_006591890	12	2904	967	110864.62	6.06	Cyto
<i>GmPEPC9</i>	<i>GmPEPC7</i> ²³	Glyma.13G270400	XP_006593478	13	2904	967	110779.59	6.05	Cyto
<i>GmPEPC10</i>		Glyma.13G290700	XP_003543306	13	2937	978	111982.73	5.93	Cyto

Table 1. Characteristics and nomenclature of soybean PEPC genes and their encoded proteins. Note: Chr, chromosome; ORF, open reading frame.

typically exist as homotetrameric Class-1 PEPCs^{1,2}. It is suggested that all PTPCs evolved from a common ancestral gene and display a high degree of conservation at the genetic level¹⁴. BTPC genes encode larger 116–118 kDa polypeptides that exhibit low (40%) sequence identity with PTPCs, and contain a prokaryotic-like (R/K) NTG C-terminal tetrapeptide³. Although key residues and domains involved in the catalytic reaction and binding of substrates and inhibitors are conserved in both PTPCs and BTPCs, BTPCs lack the distinctive N-terminal serine phosphorylation motif of PTPCs, and appear to only exist as catalytic and regulatory subunits of extraordinary heteromeric complexes (Class-2 PEPCs)³.

Since the release of whole-genome sequences of many plant species, numerous PEPC proteins have been successfully identified and investigated in plants including *Arabidopsis thaliana*¹³, *Hordeum vulgare*¹⁵, *Lotus japonicus*⁵, *Solanum lycopersicum*¹⁶, *Solanum tuberosum*¹⁷, and *Triticum aestivum*¹⁸. For instance, four PEPC genes (*PEPC1*–*PEPC4*) were characterized in *A. thaliana*¹³. Three dicot C₄ PEPC (*ppc-A*, *ppc-B*, *ppc-C*) genes were analyzed in *Flaveria* (Asteraceae), with the *ppc-A* gene being identified as the gene recruited for use in the C₄ photosynthetic pathway¹⁹. In grasses, there are four PEPC genes that have been predominantly characterized, and *ppc-C₄* and *ppc-B2* are often recruited for use in C₄ photosynthesis²⁰. In sedges, there are five PEPC genes that have been predominantly characterized, with the *ppc-1* gene being recruited for use in C₄ photosynthesis²¹.

Soybean (*Glycine max*) is one of the most important crops for vegetable protein and oil worldwide, and its capacity to fix atmospheric nitrogen through symbioses with soil-borne microorganisms would help sustainable agriculture development. Previously, five PEPC genes including *GmPEPC1*²² (also termed *GmPEPC15*²³), *GmPEPC4*²³, *GmPEPC7*²³, *GmPEPC16*²⁴, and *GmPEPC17*²⁵ have been identified in soybean. The release of the complete soybean genome sequences makes it possible to identify genome-wide PEPC genes in soybean²⁶. To get a more comprehensive understanding of the PEPC gene family in soybean, a genome-wide identification and characterization of soybean PEPCs was performed. Their phylogenetic relationships and protein motifs were analyzed together with the PEPCs from 12 other plant species. In addition, their expression patterns in deferent organs and in response to various abiotic stresses and hormones were also investigated, including aluminum (Al), cold, salt, and osmotic stresses as well as exogenously applied abscisic acid (ABA), aminocyclopropane carboxylic acid (ACC), gibberellic acid (GA), and jasmonic acid (JA). To our knowledge, this is the first study on genome-wide analysis of the soybean PEPC gene family, which would provide valuable information for further functional investigations of soybean PEPC.

Results

Identification and phylogenetic analysis of soybean PEPC genes. Blast searches of the soybean genome (*G. max* var. Williams 82) were performed by using all of the four *Arabidopsis* PEPC sequences as baits, and a total of 10 putative PEPC genes were identified (Table 1). Their deduced proteins were subjected to domain analysis using Pfam and SMART databases. These 10 non-redundant genes were confirmed as PEPC and denominated as *GmPEPC1*–*GmPEPC10*, according to the soybean nomenclature based on physical locations. The detailed information of *GmPEPC* gene family members, including gene name, gene model name, protein ID, chromosomal position, length of open reading frame (ORF), as well as the size (number of amino acids), molecular weight (MW), isoelectric point (pI), and proposed subcellular localization of the deduced protein, is shown in Table 1. These 10 *GmPEPC* genes locate on six chromosomes in soybean, with three *GmPEPC* genes on chromosome 12, two *GmPEPC* genes on chromosome 6 and chromosomes 13, respectively, and one *GmPEPC* on chromosome 2 and 12 each (Table 1).

To investigate the evolutionary relationship between soybean PEPC proteins and known PEPCs from other species, a neighbor-joining (NJ) phylogenetic tree was constructed using 75 PEPC proteins from 13 different plant species (Fig. 1). The dendrogram showed that these PEPCs could be classified into two subfamilies (PTPC and BTPC), based on their sequence similarity. PTPC subfamily contains 61 members and is further separated into five groups (PTPC I to PTPC V) (Fig. 1). BTPC subfamily is further separated into two groups (BTPC I and BTPC II) (Fig. 1). The 10 *GmPEPCs* were assigned to three groups with high bootstrap values: *GmPEPC4*, *GmPEPC7*, and *GmPEPC10* in PTPC IV group; *GmPEPC3*, *GmPEPC6*, *GmPEPC8*, and *GmPEPC9*

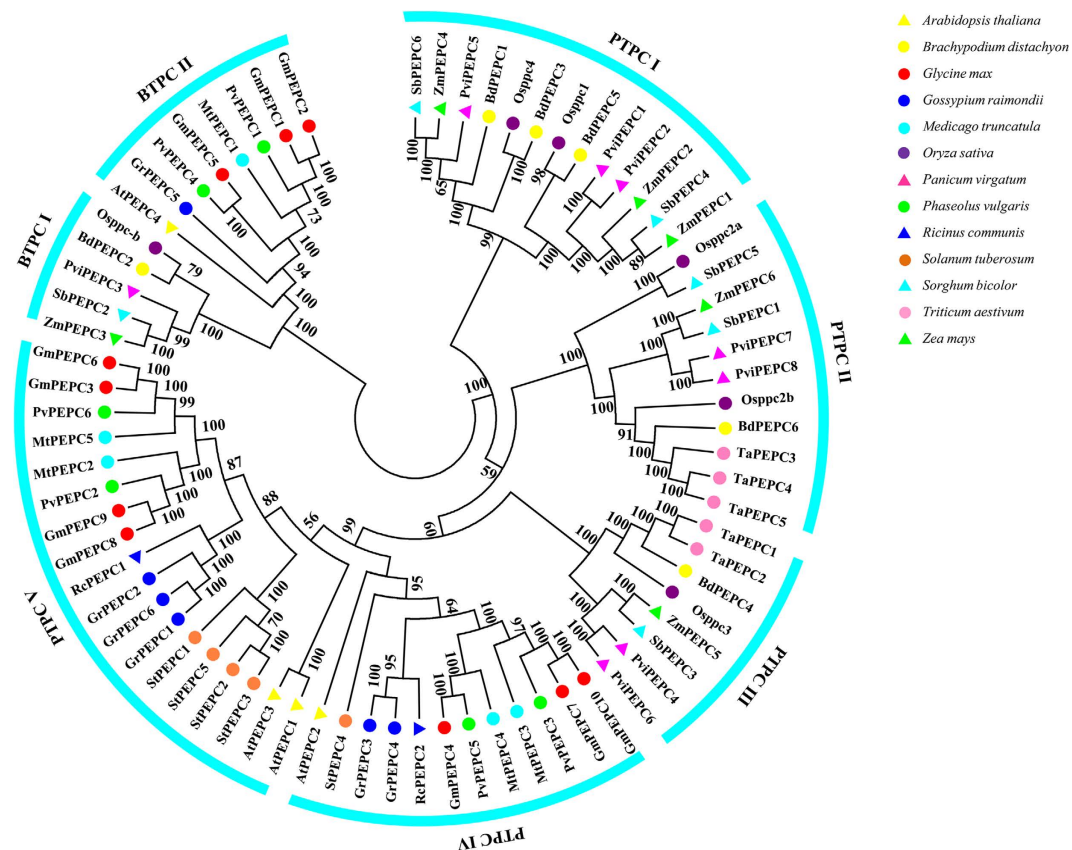


Figure 1. Phylogenetic analysis of PEPC proteins in soybean and other plant species. The full-length amino acid sequences of 75 PEPC proteins from 13 different plant species were used to construct the neighbor-joining tree using ClustalX 2.0 and MEGA 6.0 with 1000 bootstrap replicates. Branches with less than 50% bootstrap support were collapsed. The PEPCs were classified into two subfamilies (PTPC and BTPC). Subfamily PTPC is further separated into five clades from PTPC I to PTPC V, whereas subfamily BTPC is separated into two clades including BTPC I and BTPC II. Different plant species were distinguished by different colors.

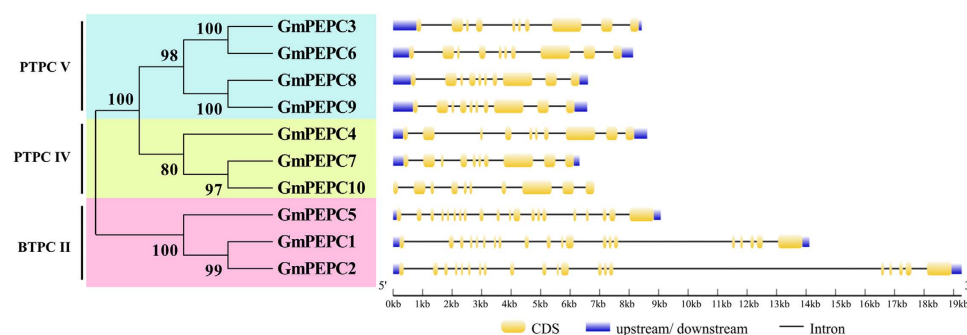


Figure 2. The phylogenetic relationship and exon-intron structures of PEPC family in soybean. Exon-intron structure was analyzed by online tool Gene Structure Display Server (GSDS). Lengths of exons and introns of *GmPEPC* genes were exhibited proportionally as indicated by the scale on the bottom. The classification of soybean PEPCs was indicated by the phylogenetic relationship on the left.

in PTPC V group; and *GmPEPC1*, *GmPEPC2*, and *GmPEPC5* in BTPC II group (Figs 1 and 2). Similar group distribution patterns of PEPCs were also found in *Gossypium raimondii*, *Medicago truncatula* and *Phaseolus vulgaris* (Table 2). The total number of PEPCs in soybean is the largest compared with the other 12 plant species (Table 2). Phylogenetic analysis also identified some closely related orthologous PEPCs between *G. max* and *P. vulgaris* (Fig. 1; Supplementary Figure S1): *GmPEPC7*, *GmPEPC10* and *PvPEPC3*, *GmPEPC4* and *PvPEPC5*, in PTPC IV group; *GmPEPC3*, *GmPEPC6* and *PvPEPC6*, *GmPEPC8*, *GmPEPC9* and *PvPEPC2*, in PTPC V group; *GmPEPC1*, *GmPEPC2* and *PvPEPC1*, *GmPEPC5* and *PvPEPC4*, in BTPC II group, suggesting that an ancestral set of PEPCs existed prior to the divergence of *G. max* and *P. vulgaris*.

Species	PTPC I	PTPC II	PTPC III	PTPC IV	PTPC V	BTPC I	BTPC II	Total No.
<i>Arabidopsis thaliana</i>	0	0	0	1	2	0	1	4
<i>Brachypodium distachyon</i>	3	1	1	0	0	1	0	6
<i>Glycine max</i>	0	0	0	3	4	0	3	10
<i>Gossypium raimondii</i>	0	0	0	2	3	0	1	6
<i>Medicago truncatula</i>	0	0	0	2	2	0	1	5
<i>Oryza sativa</i>	2	2	1	0	0	1	0	6
<i>Panicum virgatum</i>	3	2	2	0	0	0	1	8
<i>Phaseolus vulgaris</i>	0	0	0	2	2	0	2	6
<i>Ricinus communis</i>	0	0	0	1	1	0	0	2
<i>Sorghum bicolor</i>	2	3	0	0	0	1	0	6
<i>Solanum tuberosum</i>	0	0	0	1	4	0	0	5
<i>Triticum aestivum</i>	0	3	2	0	0	0	0	5
<i>Zea mays</i>	3	1	1	0	0	1	0	6
Total no.	13	12	7	12	18	4	10	75

Table 2. The distribution of PEPCs in 13 different plant species.

Gene structure and conserved motif of PEPC family. To obtain further insights into the possible structural evolution of *PEPC* genes, their exon-intron organizations were compared. As shown in Fig. 2, *GmPEPC* genes of different subfamilies exhibit different exon-intron structures. *GmPEPC* genes in PTPC V and PTPC IV contain 10 exons and 9 introns, while *GmPEPC* genes in BTPC II contain 20 exons and 19 introns. During the evolution of multigene families, diversification of gene structure is a common phenomenon, which may facilitate evolutionary cooption of genes for new functions to adapt to environmental changes. Different exon-intron structures between subfamilies were also observed for *PEPC* genes in both C_3 (such as *G. max* and *Oryza sativa*) and C_4 (such as *Zea mays* and *Sorghum bicolor*) plant species (Supplementary Figure S1), indicating conserved features of the gene structure of *PEPC* family. Meanwhile, the numbers of exons and introns are also conserved within each subfamily (Supplementary Figure S1). For example, most genes from PTPC subfamily usually contain 10 exons and 9 introns; most genes from BTPC subfamily contain 20 exons and 19 introns (Supplementary Figure S1). However, the sizes of plant *PEPC* genes vary dramatically, while the lengths of exons are similar within each subfamily, which suggests that the sizes of the genes depend largely on the sizes of the introns.

The *GmPEPC* protein sequences show highly conserved features of plant *PEPC* proteins (Fig. 3). The multiple alignments of the 10 soybean *PEPC* amino acid sequences showed 70.23% identical positions. The average identity scores of soybean PTPC proteins and soybean BTPC proteins were 92.30% and 92.44%, respectively. We predicted 10 most conserved motifs of plant *PEPCs* from all tested plant species using MEME software (Table 3; Fig. 4; Supplementary Figure S2). Results showed that PTPC proteins contain 10 motifs, and BTPC proteins contain 9 motifs (lacking motif 8, Supplementary Figure S2), which supported the phylogenetic relationship and classification of plant *PEPCs*. Notably, seven (1–5, 8 and 10) out of 10 motifs were annotated as key domains involved in the catalytic reaction and in the binding of substrates and inhibitors, which are conserved in both *PEPC* types (PTPC and BTPC). Additionally, we also analyzed *PEPC* motifs taking locations in the simulated structure into consideration. As shown in Fig. 4, five motifs contain PEP binding sites, including motif 1 to motif 5. These five *PEPC* motifs in all analyzed plant species showed the following patterns: motif 1 (SWMGGDRDGNP[RN]VT) has a highly conserved residue PEP binding site W; motif 2 (GKQEVMI[V]GYSDSGKDAGR[LF][ST]AAW) contains a PEP binding site M, also with a D774 that constitutes the binding site for Mg^{2+} and with a K777 that constitutes the binding site for HCO_3^- ; Motif 3 (GI[EG][ST]LRAIPW[IY]F[AS]WTQ) has a PEP binding site R, also with a S/A that constitutes the binding site for S/A 755 site; Motif 4 (G[TS]YGRGGGP[TC][HY]L) and Motif 5 ([RI][ML]NIGSRP[SA]) only has a PEP binding site R. Motif 8 (EMVFAKG[DN]PG) and Motif 10 ([LI][REQ][LSIN]R[DLEN][SAP][YF]) only has an aspartate binding site K or R, respectively. Whereas another three unknown functional motifs of 6, 7 and 9 were found in all 75 plant *PEPCs* (Table 3; Fig. 4; Supplementary Figure S2).

***cis*-elements in the promoter regions of *GmPEPC* genes.** It has been reported that a large number of *PEPC* genes are induced by abiotic and biotic stresses and play important roles in the regulation of plant tolerance to stresses^{2,6,7}. To investigate the possible roles of *PEPCs* identified in the soybean genome, the 1500 bp sequences upstream of the start codon of *GmPEPC* genes were used to analyze *cis*-elements in their promoter regions by PlantCARE (Fig. 5; Supplementary Table S4). Nineteen putative *cis*-elements responsive to biotic stresses [including EIRE (elicitor-responsive element²⁷), S-box (pathogen-related *cis*-element²⁸), W-box²⁹, TC-rich repeats (defense and stress-responsive element³⁰), and WUN-motif (wound-responsive element³¹)], abiotic stresses [ARE (anaerobic-responsive element), HSE (heat stress-responsive element³²), LTR (low-temperature responsive element³³), MBS (MYB binding site³⁴)], and phytohormones such as ABA [abscisic acid, ABRE (ABA responsive element³⁵)], auxin (TGA-element³⁶; AuxRR-core³⁷), ET [ethylene, ERE (ethylene-responsive element³⁸ and GCC-box³⁹)], GA (gibberellin, GARE⁴⁰), JA (jasmonic acid, CGTCA-motif⁴¹ and TGACG-motif⁴²), SA (salicylic acid, TCA-element⁴³) and endosperm development (P-box⁴⁴) were present in the promoters of *GmPEPC* genes (Fig. 5, Supplementary Table S4). There are other *cis*-elements (Supplementary Table S4) such as light responsive elements (Box I⁴⁵, GATA-motif⁴⁶ and GT1-motif⁴⁷) were also present in the promoter regions of *GmPEPC* genes. These results provide further support

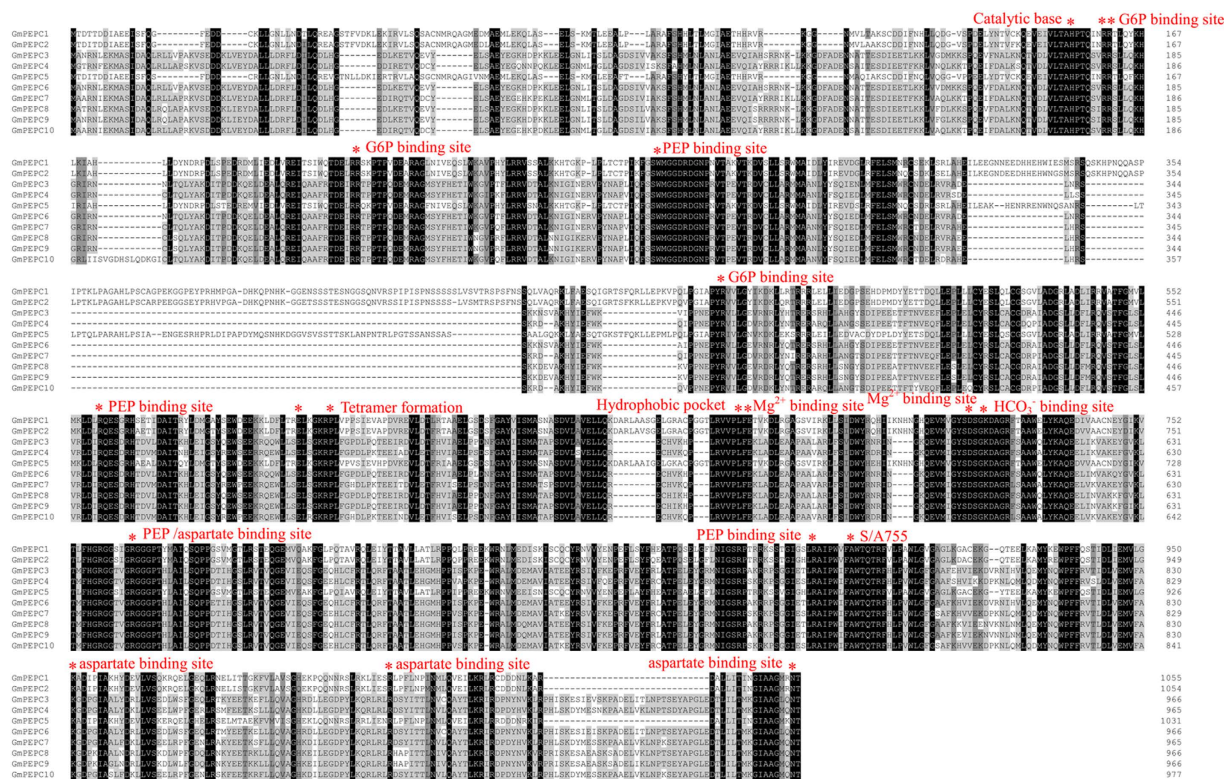


Figure 3. Multiple alignment of Soybean PEPC amino acid sequences. Black, gray, and light shading indicate 100%, 75%, and 50% similarities, respectively. Spots represent gaps. Amino acid residues of experimentally proven function are indicated by *above the alignment.

Motif ID	Amino acid residues	Function of amino acid residues	MEME regular expression
Motif 1	W	PEP binding site	SWMGGDRDGNP[RN]VT[PA][EK]VT[RK]DV[CS]LL[AS]R[MW]MA[AI][ND]
Motif 2	M D K	PEP binding site; Mg ²⁺ binding site; HCO ₃ ⁻ binding site	GKQEV[M]V]GYSDSGKDAGR[LF][ST]AAW[QF][LM]YKAQE[ED][LV][KA]
Motif 3	R [AS]	PEP binding site; S/A 755 site	GI[EG][ST]LRAIPW[IY]F[AS]WTQTRF[HV]LP[VA]WLG[FV]G[FV]G
Motif 4	R	PEP/Aspartate binding site	GG[TS]YGRGGG[TC][HY]LAI[LQ]SQPP[DH][TS][IV][HNM]G[ST]LR[VS]
Motif 5	R	PEP binding site	[RE]F[VL]ATP[EQ][LIA]E[LIA]E[YL]G[R][M]NIGSRP[SA]
Motif 6	Unknown	Unknown	PTP[QV]DE[MA]RAG[ML][SN][YI][FV][HE][EQ][TS][IL]W[KN][GA]
Motif 7	Unknown	Unknown	VL[DG][TA]F[HR]V[IL][AS]EL[PG][SA]D[SN][FL]GAY[IV]ISMA[TS]
Motif 8	K	Aspartate binding site	WPFVRV[IL]DL[VL]EMVFAKG[DN]PGIAA[YL]D[KR]LLY[SA][ER]
Motif 9	Unknown	Unknown	[FL]LEPL[EL]LCY[RK]SL[CQ][SA]CGD
Motif 10	R	Aspartate binding site	[LI][REQ][LSIN]R[DLEN][SAP][YF][IL][TN][TPA][LMI]N[VM][CLF]

Table 3. Conserved motifs of 75 PEPC proteins from 13 different plant species generated by MEME analysis.

for the diverse roles of *GmPEPC* genes in soybean developmental processes, as well as response to biotic and abiotic stresses.

Expression patterns of *GmPEPC* genes in different soybean tissues. The expression patterns of *GmPEPC* genes in soybean different tissues were analyzed by qRT-PCR. The results showed that these 10 *GmPEPC* genes were expressed with different tissue-specific patterns (Fig. 6). For example, *GmPEPC4* was expressed higher in stem, leaf, flower, and younger seed but lower in the seed of 15 days after flowering (DAF) and 50 DAF. Both *GmPEPC3* and *GmPEPC8* were expressed at lower levels in soybean leaf, but *GmPEPC3* showed relatively higher expression levels during seed development, while *GmPEPC8* expressed higher in root. Both *GmPEPC1* and *GmPEPC5* showed higher expression in soybean leaf but low in seed at 35 DAF stage. *GmPEPC6*, *GmPEPC7* and *GmPEPC9* showed high expression levels in all tested soybean tissues except seed of 35 DAF. The expression levels of *GmPEPC2* and *GmPEPC10* were low in all tested tissues (Fig. 6).



Figure 4. Conservation and diversity of the motifs in PEPC proteins. The schematic representation of ten motifs in PEPC family is elucidated by MEME. Amino acid residues of experimentally proven function are indicated by black arrows.

Expression profiles of *GmPEPC* genes in response to abiotic stresses and phytohormones. The presences of stress-responsive *cis*-elements in the promoter regions of the *GmPEPC* genes suggest their involvement in soybean response to different stresses. To further investigate the possible functions of soybean *PEPC* genes, the transcriptional expression of 10 *GmPEPC* genes in soybean plants were analyzed by qRT-PCR analysis after exogenous application of ABA, ACC, GA, and JA as well as under Al toxicity, cold stress, osmotic stress, and salt stress (Fig. 7; Supplementary Figures S3 and S4). Almost all *GmPEPC* genes responded to the exogenously applied hormones and stress treatments, with the majority being upregulated most of the time, except *GmPEPC2*, *GmPEPC4* and *GmPEPC10*, which were non-responsive or downregulated at most of the tested time points under these treatments (Fig. 7; Supplementary Figures S3 and S4). In soybean leaves (Fig. 7A), under ABA treatment, the relative expression levels of *GmPEPC1*, *GmPEPC3*, *GmPEPC6*, and *GmPEPC8* were induced at least two time points, while *GmPEPC4*, *GmPEPC5* and *GmPEPC10* showed decreased transcripts at least two time points. After ACC treatment, *GmPEPC1*, *GmPEPC5*, *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* were substantially upregulated. The increase in their levels started as early as 3 h after ACC stress and continued till 24 h of stress. On the other hand, several *GmPEPC* genes (*GmPEPC2*, *GmPEPC4*, and *GmPEPC7*) were significantly downregulated during ACC stress. With GA treatment, except *GmPEPC2* and *GmPEPC10* were significantly downregulated at 6 h, all of the rest *GmPEPC* genes were induced at least two time points. The expression profile of soybean *PEPC* genes under JA treatment is similar with GA stress, except *GmPEPC2* and *GmPEPC10*, which were down-regulated, and all of the rest genes were induced at least two time points. Under Al stress, the expression of *GmPEPC1*, *GmPEPC3*, *GmPEPC5*, *GmPEPC6* and *GmPEPC8* was induced at most of the time points, while the expression of *GmPEPC9* was only induced at 6 h. The relative expression of *GmPEPC4* and *GmPEPC7* were inhibited under Al stress. Most *GmPEPC* genes, except for *GmPEPC2*, *GmPEPC3* and *GmPEPC10*, were upregulated during cold stress treatment. Under salt stress, the transcript abundance of *GmPEPC3*, *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* increased in at least three time points, while *GmPEPC10* decreased across all time points (Fig. 7A; Supplementary Figure S3). Water-deficit conditions were imposed by transferring the soybean seedlings to 20% PEG solution and the expression levels of *GmPEPC* genes were examined during stress. *GmPEPC3*, *GmPEPC5*, *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* showed induced expression in at least two time points whereas *GmPEPC2* and *GmPEPC10* showed downregulation across all time-points. The cluster analysis showed that *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* were grouped together and showed more responsive to abiotic stresses.

We also analyzed the expression patterns of *GmPEPC* genes in soybean roots under phytohormone and abiotic treatments (Fig. 7B; Supplementary Figure S4). The transcript levels of four *GmPEPC* genes (*GmPEPC1*, *GmPEPC3*, *GmPEPC5*, and *GmPEPC6*) can be significantly induced in response to exogenous application of ABA,

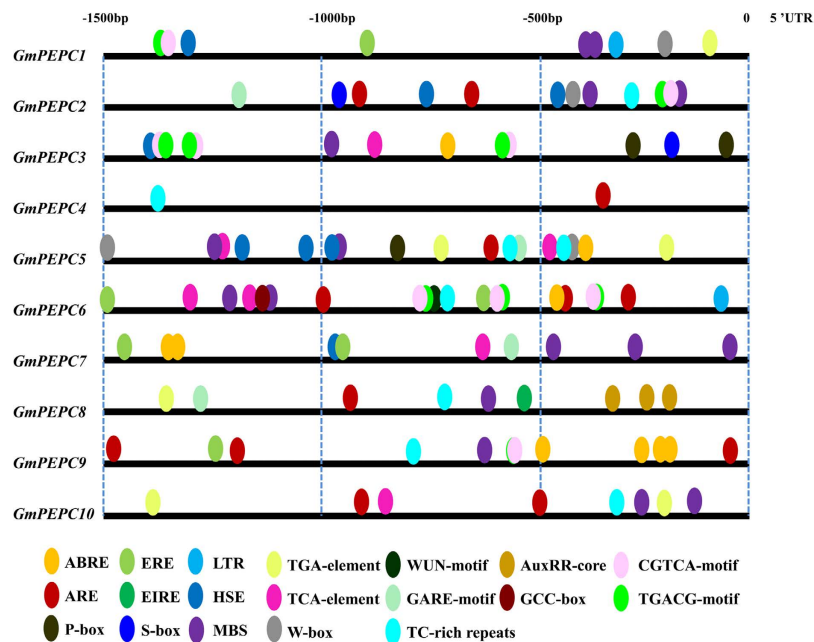


Figure 5. Predicted *cis*-elements in the promoter regions of *GmPEPC* genes. The 1500 bp promoter regions of 10 *GmPEPC* genes were analyzed to predict the *cis*-elements, which were presented as colored ellipses: ABA responsive element (ABRE), anaerobic responsive element (ARE), auxin responsive element (TGA-element, AuxRR-core), light-responsive element (G-box), gibberellin responsive element (GARE-motif), ethylene responsive element (ERE, GCC-box), heat stress responsive element (HSE), low-temperature-responsive element (LTR), MYB binding site (MBS), pathogen-related *cis*-element (S-box), defense and stress-responsive element (TC-rich repeats), salicylic acid responsive element (TCA-element), jasmonic acid responsive element (TGACG-motif, CGTCA-motif), wound-responsive element (WUN-motif), endosperm development (P-box), and WRKY binding site (W-box). The numbers on the top indicate the relative positions to the start codon.

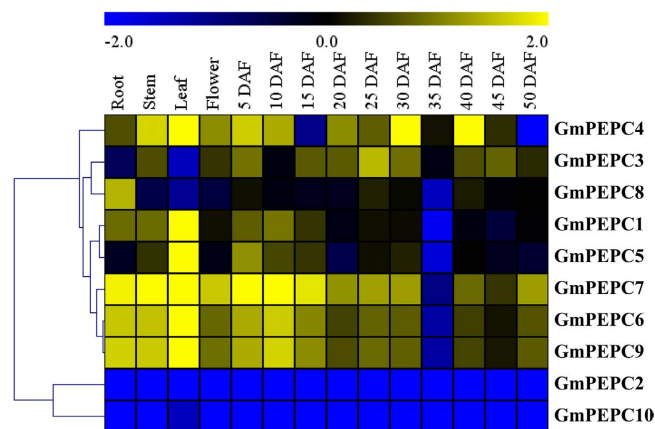


Figure 6. Expression patterns of *GmPEPC* genes in different soybean tissues. The expression patterns of 10 *GmPEPC* genes in 14 soybean tissues were investigated by qRT-PCR, using soybean *GmRP15* gene as the internal control. Root, stem, leaf, flower, and seeds from different development stages were subjected to analysis. DAF: day after flowering. The experiments were repeated three times, log₂ based value was used to create the heat map with clustering of genes. The expression levels are shown from the lowest (blue) to highest (yellow) in heat colors as indicated by the scale on the top.

while all other genes especially *GmPEPC10* are downregulated by ABA. *GmPEPC1*, *GmPEPC6* and *GmPEPC9* were highly upregulated by ACC. However, the expression levels of *GmPEPC2* and *GmPEPC3* declined in at least three time points. With GA treatment, *GmPEPC5*, *GmPEPC8*, and *GmPEPC9* were induced while *GmPEPC7* was downregulated during the whole treatment. Under JA treatment, the transcript levels of four *GmPEPC* genes (*GmPEPC3*, *GmPEPC5*, *GmPEPC8*, and *GmPEPC9*) can be significant induced, while *GmPEPC2* showed significant downregulation across all time points. Under Al stress, except for *GmPEPC4* and *GmPEPC7*, the expression

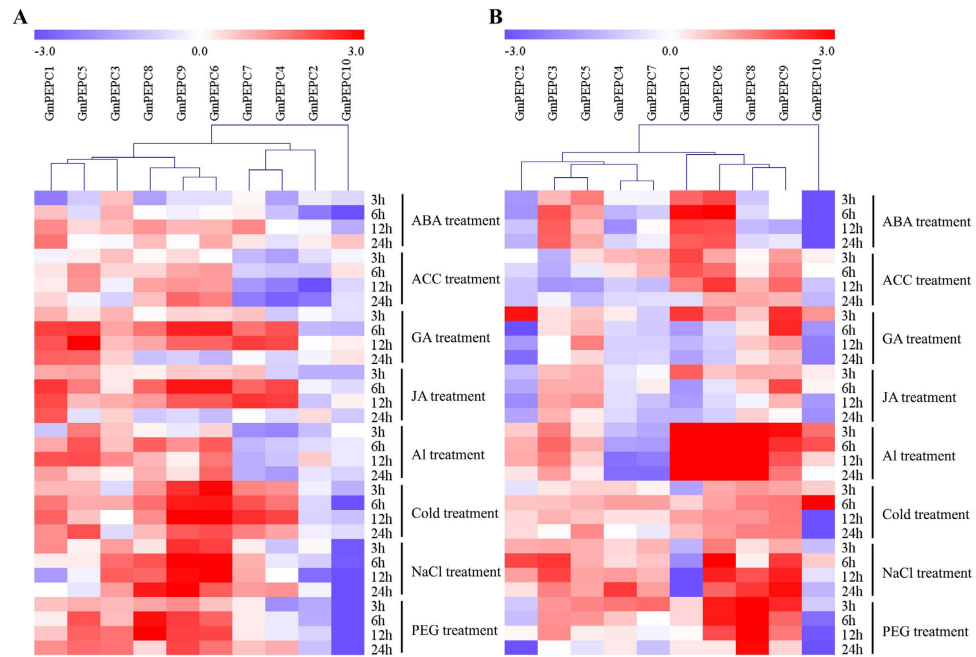


Figure 7. Expression profiles of *PEPC* genes in leaves (A) and roots (B) of soybean in response to ABA, ACC, GA, JA, Al, cold, salt (NaCl) and osmotic (PEG) treatments. The relative expression levels of the 10 *PEPC* genes were quantified by qRT-PCR, using soybean *GmRP15* gene as the internal control. Leaves and roots of 14-d soybean seedlings are used to investigate the changes in *PEPC* expression under different treatments, including 100 μM ABA (Abscisic acid), 100 μM ACC (aminocyclopropane carboxylatesynthase), 100 μM GA (Gibberellin), 100 μM JA (Jasmonic acid), 25 μM AlCl_3 (pH 4.3), 4 $^\circ\text{C}$ cold, 200 mM NaCl, and 20% PEG6000. The experiments were repeated three times, log₂ based value (fold change) was used to create the heat map with clustering of genes. All data were normalized to the expression level of control (0 h). The scale represents the relative expression levels from low (blue) to high (red).

levels of all other eight *GmPEPC* genes were increased. Under cold stress, the mRNA levels of most *GmPEPC* genes (except *GmPEPC10*) exhibited significantly upregulated in most time points. Under salt stress, the transcript levels of eight *GmPEPC* genes were upregulated except *GmPEPC1* and *GmPEPC10*. When treated with 20% PEG, the expression of *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* was induced significantly, while *GmPEPC2* and *GmPEPC10* showed down-regulation (Fig. 7B; Supplementary Figure S4). Three genes, *GmPEPC6*, *GmPEPC8*, and *GmPEPC9* showed significant induced expression in soybean roots under abiotic stresses and were cluster together, which is consistent with their expression patterns in soybean leaves (Fig. 7).

PEPC activity in response to abiotic stresses and phytohormones. To further investigate the potential functions of *GmPEPC* genes in soybean response to abiotic stresses, the enzyme activities of *GmPEPCs* in soybean leaves and roots under different phytohormones and abiotic treatments were assayed. As shown in Fig. 8, ABA and ACC treatments significantly increased the *PEPC* activity compared with control in soybean leaves. JA treatment resulted in a significant increased *PEPC* activity in both leaves and roots. Under abiotic stresses, the *PEPC* activity in leaves was significantly increased by cold, PEG, and salt stresses, especially PEG treatment. In soybean roots, the *PEPC* activity was significantly upregulated by cold and Al stresses.

Discussion

PEPC plays an important role in assimilating atmospheric CO_2 during C_4 and CAM photosynthesis, and also participates in various non-photosynthetic processes. However, a comprehensive analysis of *GmPEPC* genes in soybean has not been reported. In the present study, a total of ten *PEPCs* were identified in the soybean genome from Phytozome database⁴⁸. The numbers of *PEPC* genes in soybean were slightly more than that in other plant species, such as *A.thaliana* (4 *PEPC* genes), *Brachypodium distachyon* (6 *PEPC* genes), *G. raimondii* (6 *PEPC* genes), *M. truncatula* (5 *PEPC* genes), *O. sativa* (6 *PEPC* genes), *Panicum virgatum* (8 *PEPC* genes), *P. vulgaris* (6 *PEPC* genes), *Ricinus communis* (2 *PEPC* genes), *S. tuberosum* (5 *PEPC* genes), *S. bicolor* (6 *PEPC* genes), *T. aestivum* (5 *PEPC* genes), and *Z. mays* (6 *PEPC* genes) (Table 2). As the soybean genome is larger than the genomes of rice and Arabidopsis, it is possible that the soybean genome contains more *PEPC* genes than them. Interestingly, the genome size of soybean is smaller than maize and sorghum, but it also contains more *PEPC* genes. This might be due to the two ancient genome duplication events happened in soybean around 59 and 13 million years ago, which resulted in multiple copies of around 75% of its genes²⁶.

Phylogenetic analyses of higher plant *PEPC* genes has been previously carried out in families of Cyperaceae, Poaceae, and Molluginaceae to determine which *PEPC* gene is recruited for use in C_4 biochemistry^{20,49,50}. Plants have two types of *PEPCs* including plant-type *PEPC* (PTPC) and bacterial-type *PEPC* (BTPC)^{1,3,13}. In the core

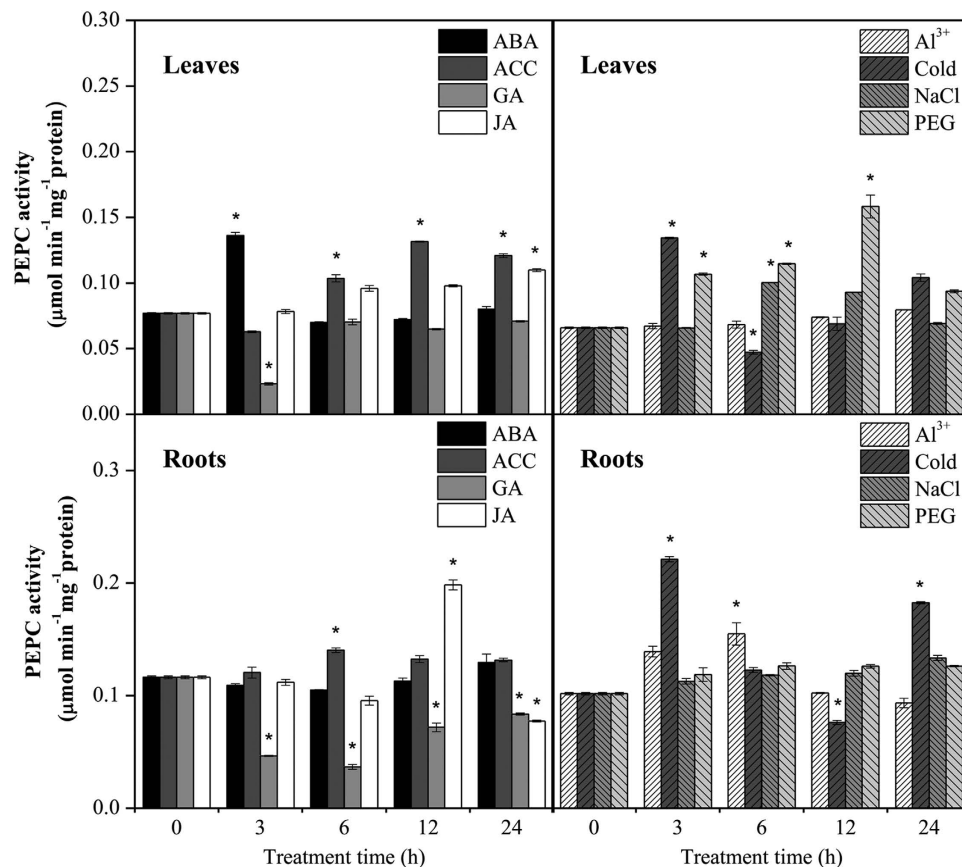


Figure 8. PEPC activities in soybean leaves and roots subjected to different abiotic and phytohormone treatments. Leaves and roots of 14-d soybean seedlings are used to investigate the changes in PEPC activities under different treatments, including 100 μM ABA (Abscisic acid), 100 μM ACC (aminocyclopropane carboxylatesynthase), 100 μM GA (Gibberellin), 100 μM JA (Jasmonic acid), 25 μM AlCl_3 (pH 4.3), 4 $^\circ\text{C}$ cold, 200 mM NaCl, and 20% PEG6000. Data shown are means \pm SD of three independent experiments. Statistical significance of differences between control and treated groups was analyzed using Student's t-test (*indicates $P < 0.05$).

edudicots, there are two primary *PEPC* gene lineages that have been studied to date: *ppc-1* (PTPC) and *ppc-2* (BTPC)⁵⁰. For example, in Arabidopsis, there are four *PEPC* genes, including three PTPC and one BTPC¹³. In rice, five rice *PEPC* genes (*Osppc1*, *2a*, *2b*, *3*, and *4*) encode the PTPCs and the other (*Osppc-b*) encodes a BTPC. In addition, all plant genomes sequenced to date, including that of ancestral green algae, contain at least one BTPC gene². In this study, soybean *PEPC* genes were also distributed into two subfamilies (PTPC and BTPC), with seven genes (*GmPEPC3*, *GmPEPC4*, *GmPEPC6*, *GmPEPC7*, *GmPEPC8*, *GmPEPC9*, and *GmPEPC10*) in PTPC subfamily, and three genes (*GmPEPC1*, *GmPEPC2*, and *GmPEPC5*) in BTPC group. Moreover, the gene structures of PTPC and BTPC genes were different: PTPC genes have a highly conserved genomic structure composed of approximately ten exons, whereas BTPC genes have a very different and more complex structure with approximately twenty exons¹. Similar results were also observed in this study, where *GmPEPCs* in PTPC subfamily contain 10 exons, and *GmPEPCs* in BTPC subfamily contain 20 exons (Fig. 2). We predicted 10 most conserved motifs in 75 *PEPCs* from 13 different species (Fig. 4; Supplementary Figure S3), and the ten *PEPCs* in soybean also possessed 10 conserved motifs (Table 3; Fig. 4). These data, together with previous studies, suggest similar origins and evolution patterns of the *PEPC* genes in different species.

PEPC genes have been reported in a wide range of tissues, including cell cultures and seedlings of *A. thaliana*⁵¹, seeds of *H. vulgare* and *R. communis*¹⁵, root nodules of *L. japonicus* and *G. max*^{5,52}, cell cultures of *S. lycopersicum*¹⁶, seedlings of *S. tuberosum*¹⁷ and leaves of *T. aestivum*¹⁸. Besides, *PEPC* has a wide range of non-photosynthetic roles including seed formation and germination, fruit ripening, guard cell metabolism during stomatal opening². Former study showed that the soybean bacterial-type gene *GmPEPC17* (termed *GmPEPC5* in this study) was expressed mainly in aboveground organs²⁵. We also noticed that a higher expression level of *GmPEPC5* in stem and leaf, but not in the flower and root. Besides, the other two *GmBTPC* genes (*GmPEPC1* and *GmPEPC2*) have no or low expression in flowers (Fig. 6), which is consistent with the previous report that Arabidopsis BTPC transcripts were detected at low levels in siliques and flowers¹³. It is noteworthy that almost no detectable expression level of *GmPEPC2* was found in the examined tissues (Fig. 6). On the other side, three *GmPTPC* genes (*GmPEPC6*, *GmPEPC7* and *GmPEPC9*) expresses at relatively higher levels in most tissues and

showed a similar expression pattern under normal condition (Fig. 6), whereas these genes expressed differently as reported before²⁵. In addition, *GmPEPC8* is expressed at low levels in the tissues tested, except a relative higher level in root (Fig. 6), whereas the broad but low-level expression pattern of this gene has been reported²⁵.

Extensive studies suggested *PEPC* genes play important roles in plant response to abiotic stresses. For example, studies have shown that *PEPC* overexpressing transgenic rice has a relatively higher photosynthetic rate under high light and temperature conditions⁵³. In addition, abiotic stresses, such as salt and chilling injury, can induce *PEPC* gene expression in wheat, *Arabidopsis* and sorghum^{8,10,54}. In this study, we showed that the promoter regions of most *GmPEPC* genes contained 19 stress-responsive *cis*-regulated elements such as ABA, cold, drought, heat, salt, and wound-related elements, indicating their potential roles in soybean response to phytohormone and stresses (Fig. 5; Supplementary Table S4). Previous reports also demonstrated the crucial roles of *PEPC* genes in various stress responses and in hormone signaling transduction^{2,7–10}, which motivated us to perform expression profile analyses of *GmPEPC* genes under various abiotic stress and hormones treatments. The results showed that Al, cold, salt or PEG stress could alter the expression level of soybean *PEPC* genes and soybean *PEPC* activity (Figs 7 and 8). *GmPEPC6*, *GmPEPC8* and *GmPEPC9* were significantly induced by Al, cold, salt or osmotic stress, suggesting that these three *GmPEPC* genes are important for soybean response to abiotic stresses (Fig. 7). It has been reported that the exudation of *PEPC*-derived organic acids functions to chelate metals in the rhizosphere to alleviate heavy metal toxicity by preventing their uptake into the cell². Overexpression of a *C₄* *PEPC* isoenzyme in rice led to increased oxalate exudation and Al tolerance⁵⁵. In our study, eight out of ten *GmPEPC* genes in soybean roots were responsive to Al stress (Fig. 7B). On the other hand, although the up-regulation of *PEPC* genes has been well documented in plants², we still found that a few *GmPEPC* genes (such as *GmPEPC2*, *GmPEPC4* and *GmPEPC10*) showed down-regulating or non-responsive tendency during the abiotic and phytohormone treatments. The down-regulation or non-responsiveness of *GmPEPC2*, *GmPEPC4* and *GmPEPC10* might be due to the low transcript abundance of *GmPEPC2* and *GmPEPC10* in all tested soybean tissues (Fig. 6) and few (only two) *cis*-elements responsive to hormones or stresses in the promoter region of *GmPEPC4* (Fig. 5), which indicates their less importance in soybean response to these hormone and stress treatments. The possible biological meaning of this phenomenon could be that different *GmPEPC* genes have different regulation mechanisms and diverged functions. In addition, the *PEPC* activity in soybean leaves increased after salt or PEG treatment, and *PEPC* activity in soybean roots was upregulated in response to Al or cold stress (Fig. 8). These results suggested that *PEPC* might be involved in soybean response to abiotic stresses.

In vascular plants, *PEPCs* are strictly regulated enzymes by a variety of mechanisms due to their irreversible nature of the enzymatic reactions. *PEPC* is activated by its positive effector, glucose 6-phosphate, and inhibited by its negative effectors, malate, aspartate, and glutamate, as well as phosphorylation catalyzed by a specific Ca^{2+} -independent serine/threonine kinase known as *PPCK* (*PEPC* protein kinase)², and dephosphorylation by a *PP2A* (protein phosphatase 2 A)^{1,2,56}. The control of reversible phosphorylation catalyzed by *PPCK* is an important mechanism that regulates the activity of *PEPC*⁵⁷. In this reaction, phosphorylation catalyzed by *PPCK* changes the sensitivity of *PEPC* to its allosteric effectors². Previously, four *PPCK* genes (*GmPPCK1*, Glyma.03G251400; *GmPPCK2*, Glyma.20G222600; *GmPPCK3*, Glyma.10G166600; *GmPPCK4*, Glyma.10G186000) have been characterized in soybean⁵⁸. In order to investigate the relationship between *GmPEPC* activity and *GmPPCK* expression, we examined the expression patterns of *GmPPCK* genes in soybean leaves and roots in response to abiotic stress and phytohormone applications (Supplementary Figure S5). The results showed that the expression patterns of four *GmPPCK* genes were not same. For example, under ABA treatment, only *GmPPCK1* was upregulated in soybean leaves, suggesting different *GmPPCK* genes have different regulation mechanisms in specific tissues and environments. And the changes in *GmPEPC* activities (Fig. 8) were not consistent with the changes in the transcript abundance of *GmPPCK* genes (Supplementary Figure S5) under these phytohormone and abiotic stress treatment, which revealed the complexity of the regulation mechanisms of *PEPC* activity. Indeed, degradation of both *PEPC* and *PPCK* by the polyubiquitin–proteasome pathway has been reported^{59,60}. Recently, monoubiquitination of *PEPC* during sorghum seed development and germination were also documented⁶¹. Additionally, the dephosphorylation of *PEPC* is catalyzed by *PP2A*^{1,2,56}. In summary, our results suggest a complex regulation mechanism of *GmPEPC* activity.

In conclusion, we conducted a genome-wide survey of the *PEPC* family in soybean. In silico analysis of the soybean genome database identified 10 *PEPC* genes, supported by conserved domain and multiple sequence alignments. Phylogenetic analyses of 75 *PEPC* genes from 13 species indicated that these *PEPCs* could be divided into two subfamilies. This classification was further supported by gene structure and motif analyses, with each group sharing common features of exon-intron and protein motifs. The *GmPEPC* genes were expressed in soybean roots, stems, leaves, flowers and developing seeds. The presence of important *cis*-regulatory elements related to various stresses in the promoter regions of *GmPEPC* genes indicates their putative functions in soybean response to stresses. Their transcript abundance and enzyme activities in soybean leaves and roots were altered by Al, cold, salt or PEG stress, as well as the exogenous application of ABA, GA, JA, and SA, implying that soybean *PEPCs* may participate in soybean response to abiotic stresses. Taken together, this work would provide a foundation for future functional investigation of the *PEPC* family in soybean.

Methods

Plant material and stress treatments. The seeds of soybean [*G. max* (L.) Merr.] variety ‘Kefeng No. 1’ (Al-tolerant and drought-tolerant cultivar)^{62,63} were obtained from the National Center for Soybean Improvement (Nanjing Agricultural University, Nanjing), and were sterilized in 3% (v/v) sodium hypochlorite for 10 min and rinsed thoroughly with deionized water. Seeds were placed on wet paper towels for 4 d in a plant growth chamber (Dongnan Equipment, Ningbo, China) with a 16-h-light/8-h-dark cycle at 28 °C/25 °C and a light intensity of 150 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The chamber had a relative humidity of 70%. The seedlings were then transferred to plastic boxes (1 L) filled with half-strength Hoagland nutrient solution for hydroponic culture⁶⁴ and incubated in the

same growth chamber. The nutrient solution was changed every 3 days. For tissue-specific expression analysis, young leaves, stems, and roots were collected from 4-week-old seedlings at V2 stage; blooming flowers were sampled from plants at R2 stage, whereas developing seeds were collected since the beginning of R3 to R7 stages, at five days intervals⁶⁵. For different abiotic stress and hormone treatment, 14-day-old seedlings were subjected to 25 μ M AlCl₃ (pH 4.3), 20% (w/v) polyethylene glycol (PEG) 6000, 200 mM NaCl, 4 °C cold treatment, 100 μ M abscisic acid (ABA), 100 μ M 1-aminocyclopropane-1-carboxylic acid (ACC), 100 μ M jasmonic acid (JA), and 100 μ M gibberellins (GA), respectively. Meanwhile, control plants were treated with half-strength Hoagland nutrient solution or 0.5 mM CaCl₂ (pH = 4.3) solution containing either 0 μ M AlCl₃ (control). Leaves and roots from all treatments were harvested separately at 0, 3, 6, 12, 24 and 48 h after treatment, then immediately frozen in liquid nitrogen and stored at -80 °C until use. Each sample was the mixture of three seedlings and each treatment was repeated three times.

Identification and sequence analysis of PEPC genes. We searched the PEPC genes from thirteen plant genomes and their sequences and corresponding annotations were downloaded from Phytozome database (<http://phytozome.jgi.doe.gov/pz/portal.html>). We used the following steps to identify the PEPC genes. First, the amino acid consensus sequences of four Arabidopsis PEPC were used as queries to conduct BLASTP searches in the soybean Proteome (<http://phytozome.jgi.doe.gov/pz/portal.html>). The protein domain and motif analysis was performed using PFAM (<http://pfam.sanger.ac.uk/>), SMART (<http://smart.emblheidelberg.de/>) and MEME (<http://meme.nbcr.net/meme/>).

Multiple alignments of the sequences were performed using ClustalW with the default options⁶⁶ in MEGA Version 6.0⁶⁷. Phylogenetic trees were constructed based on the neighbor-joining (NJ) method with a Kimura 2-parameter model using MEGA v6.0. The stability of the internal nodes was assessed with a bootstrap analysis of 1,000 replicates.

Gene structure analysis. The gene structures were predicted using the Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). Multiple expectation maximization for motif elicitation (MEME⁶⁸) was employed to identify and analyze the conserved motifs of PEPC sequences in this study. Only the motifs with *P* values < 10⁻⁶ and no overlap with each other were reported.

Analysis of cis-acting elements in GmPEPC promoter regions. The 1500 bp upstream sequences of the start codon were used to analyze the cis-elements in GmPEPC promoter regions using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and PLACE database⁶⁹.

Quantitative real-time PCR. Total RNA samples were extracted using RNeasy Plant Mini Kits with on-column DNase (RNase free DNase set) treatment (Qiagen, Dusseldorf, Germany). The RNA concentration and integrity were checked by spectrophotometry and gel electrophoresis. A total of 0.5 μ g RNA per sample was reverse transcribed into cDNA with the PrimeScriptTM II reverse transcription kit (TaKaRa, Dalian, China). The cDNAs were diluted 1:10 with nuclease-free water prior to the qRT-PCR analyses. Soybean GmRP15 gene was used as the internal standard, and all gene-specific primers were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are listed in Supplementary Table S1. Quantitative real-time PCR (qRT-PCR) analysis was carried out with the IQ5 light cycler (Bio-Rad, Hercules, USA). Each PCR mixture contained 10 μ L SYBR Premix Ex Taq II (TaKaRa), 0.5 μ M gene-specific primers and 50–100 ng cDNA in a final volume of 20 μ L. For tissue expression pattern analysis, the gene expression levels in different tissues were calculated using the 2^{- Δ Ct} method⁷⁰. The relative gene expression levels in response to various abiotic and phytohormone treatments were calculated using the 2^{- $\Delta\Delta$ Ct} method⁷¹, using the control plants (0 h) as the reference. The expression heatmaps with clustering of genes were constructed by MeV 4.9 software with Euclidean distance metric using the average linkage method⁷².

PEPC activity measurement. Approximately 0.5 g soybean leaves or roots were homogenized with 1.5 mL of extraction buffer containing 100 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, 1% (w/v) polyvinylpyrrolidone (PVP), and 1% (v/v) protease inhibitor cocktail (Sigma, USA). Then the extract was centrifuged at 12000 \times rpm at 4 °C for 15 min. PEPC activity was measured at 30 °C by an enzyme-coupled spectrophotometric method⁷³ with slight modifications. The standard assay mixture (a total volume of 1.0 ml) contained 100 mM HEPES-NaOH (pH 8.0), 0.2 mM NADH, 1 mM NaHCO₃, 6 mM PEP, 10 mM MgCl₂, and 12 units of malate dehydrogenase (MDH) from porcine heart mitochondria (Roche Diagnostics, Germany). PEPC activity was recorded by monitoring NADH oxidation at 340 nm in a spectrophotometer (Beckman DU200, Germany) at 25 °C. Assays were initiated by the addition of protein extracts. Protein amounts were determined by the method of Bradford⁷⁴.

Statistical analysis. Statistical analyses were performed with the SPSS version 17.0 software (SPSS, Chicago, IL, USA) for Windows. Data are presented as mean values \pm SD of three independent experiments. Differences between treatments were analyzed using Student's *t* test.

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Author Contributions

N.W., X.Z., Y.C., T.W., S.Y. performed the experiments, N.W. carried out the analyses and drafted the manuscript, Y.L. and J.G. conceived the study, N.W. and Y.L. revised the manuscript. All authors reviewed and approved the final manuscript.

Additional Information

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