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Genome-wide analysis and expression profiling of PP2C clade D under saline and alkali stresses in wild soybean and *Arabidopsis*

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Abstract Protein phosphatase 2Cs (PP2Cs) belong to the largest protein phosphatase family in plants. Some members have been described as being negative modulators of plant growth and development, as well as responses to hormones and environmental stimuli. However, little is known about the members of PP2C clade D, which may be involved in the regulation of signaling pathways, especially in response to saline and alkali stresses. Here, we identified 13 PP2C orthologs from the wild soybean (*Glycine soja*) genome. We examined the sequence characteristics, chromosome locations and duplications, gene structures, and promoter *cis*-elements of the PP2C clade D genes in *Arabidopsis* and wild soybean. Our results showed that GsPP2C clade D (*GsAPD*) genes exhibit more gene duplications than AtPP2C clade D genes. Plant hormone and abiotic stress-responsive elements were identified in the promoter regions of most PP2C genes. Moreover, we investigated their expression patterns in roots, stems, and leaves. Quantitative real-time PCR analyses revealed that the expression levels of representative GsPP2C and AtPP2C clade D genes were significantly influenced by alkali and salt stresses, suggesting that these genes might be associated with or directly involved in the relevant stress signaling pathways. Our results established a foundation for further functional characterization of PP2C clade D genes in the future.

Keywords PP2C clade D · Alkali · Salt · *Arabidopsis* · Wild soybean

Introduction

Saline and alkali stresses are considered major threats to plant growth and development. Numerous studies have focused on the mechanisms of salt stress (Zhu 2001; Phang et al. 2008; Huang et al. 2012; Sanchita et al. 2014). However, only a few studies on alkali stress have been reported so far. Salt stress affects plant growth mainly by ionic poisoning and osmotic stress (Tuteja 2007). Alkaline stress, which is characterized by HCO_3^-, CO_3^{2-}, Na^+, and high pH, imposes detrimental effects on plant cells (Alhendawi et al. 1997; Wang et al. 2007; Zhang et al. 2013). We previously identified some alkaline stress-responsive genes, such as *GsTIFY10*, using a high throughput RNA sequencing technique, and described their functions under alkaline stress (Zhu et al. 2011). Some PP2C genes were also found to be significantly upregulated under alkaline stress and may function as putative alkaline stress-responsive genes.

Protein phosphatase 2C proteins are Mg^{2+}/Mn^{2+}-dependent enzymes (MacKintosh et al. 1991). In the genomes of *Arabidopsis thaliana* and *Oryza sativa*, a total of 80 and 78 putative PP2C genes were identified, respectively (Singh et al. 2010). Based on multiple sequences alignments, the AtPP2C family can be divided into 10 or 13 subfamilies

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(Schweighofer et al. 2004; Xue et al. 2008). Some members have been described as modulators in response to developmental signaling cascades or environmental stimuli. For example, PP2Cs of clade A function as co-receptors of ABA signaling and play significant roles in ABA-dependent stress responses, including drought, cold, and salt (Christmann et al. 2006; Santiago et al. 2009; Park et al. 2009; Banerjee and Roychoudhury 2017). Some members of clade B have been demonstrated to be negative regulators of MAPK activities (Schweighofer et al. 2007). Members of clade C, including *POLTERGEIST* and *POLTERGEIST-LIKE*, participate in stem-cell identity and shoot and root meristem (Song and Clark 2005; Song et al. 2006). Whereas in clade E, AtPP2C52 can interact with UMP1 (proteasome maturation factor) and RD21a (cysteine proteinase), and AtPP2C6-6 can interact with a histone acetyltransferase GCN5 to regulate stress-responsive genes (Benhamed et al. 2006; Servet et al. 2008; Liu et al. 2013). However, besides a single report (Tovar-Mendez et al. 2014), little is known about PP2C clade D in *Arabidopsis* or other plants, especially in respond to stress responses until now.

Wild soybean (*Glycine soja*) exhibits very high adaptability to saline-alkali soil compared to cultivated soybean (*Glycine max*) (Ying et al. 2009). It can germinate and grow well in the saline-alkali soil (pH 9.02) and can survive in the Hoagland nutrient solution containing 50 mmol/L $NaHCO_3$ at pH 8.5 (Ge et al. 2010). Previously, we identified some PP2C clade D genes in wild soybean as bicarbonate stress-responsive genes based on transcriptome sequencing data (DuanMu et al. 2015). In this study, we highlighted the important features of PP2C clade D genes in wild soybean and *Arabidopsis*, as well as the differences in evolution and divergence. In addition, we also examine the tissue-specific and alkali-salt stress-inducible expression patterns of the PP2C clade D genes in wild soybean and *Arabidopsis*.

Materials and methods

Sequence retrieval and identification

To assemble all members of the PP2C gene family in soybean, we performed a BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/) and PHYTOZOME (http://phytozome.Jgi.doe.gov/pz/portal.html) using the *G. max* database. All putative non-redundant PP2C gene sequences were assessed with Pfam database and SMART (http://smart.embl-heidelberg.de/) analyses (Sonnhammer et al. 1997; Schultz et al. 2000). The sequences of *Arabidopsis* PP2C family genes were also obtained from PHYTOZOME.

Information regarding amino acid sequences and chromosome locations was obtained from PHYTOZOME. The isoelectric point (pI) and molecular weight (MW) were predicted by online softwares (http://au.expasy.org).

Phylogenetic analysis of PP2C family in wild soybean and *Arabidopsis*

Multiple sequence alignments of the full-length protein sequences were performed using MEGA 5.0. Phylogenetic trees were constructed by the neighbor-joining (NJ) method, with 1000 replicates of bootstrap analysis to support the values for each branch (Tamura et al. 2011).

Chromosomal location and gene duplication

Information about the chromosome locations of PP2C clade D genes was extracted from the PHYTOZOME database and NCBI. Gene locations were mapped on chromosomes using the MapInspect tool. Gene duplications were investigated according to two criteria: (1) the shorter sequence covers at least 80% of the longer sequence, and (2) the similarity of two genes was at least 70% (Gu et al. 2002; Yang et al. 2008).

Analyses of genetic structures and *cis*-acting elements

Genomic sequences and CDS sequences were obtained from the PHYTOZOME database and NCBI. Genetic structure maps were produced using Gene Structure Display Server (GSDS) online tools (Hu et al. 2015). The 3000-bp promoter sequences of GsPP2C clade D genes and 1500-bp promoter sequences of AtPP2C clade D genes were obtained from the *G. soja* sequence database (http://www.ncbi.nlm.nih.gov/assembly/ GCA) and the *A. thaliana* database (http://phytozome.Jgi. doe.gov/pz/portal.html) (Sun et al. 2014), respectively. *Cis*-acting elements were analyzed using PLANT CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Plant materials, growth conditions, and stress treatments

Wild soybean (07256) seeds were obtained from Jilin Academy of Sciences (Changchun, China). To promote germination, the seeds were treated with 98% sulfuric acid and shaken for 12–15 min, and then, were washed with distilled water for three times. The seeds were incubated in the dark for 1 day. Subsequently, the seeds were transferred to Hoagland's nutrient solution at 23–28 °C and germinated under 16-h light/8-h dark environmental conditions. To explore the gene expression patterns, the roots, stems, and leaves of 21-day-old seedlings were harvested, respectively. For the alkali and salt treatments, the 21-day-old seedlings were transferred into Hoagland's nutrient solution containing 50 mM $NaHCO_3$ or 200 mM NaCl. Equal amounts of seedling roots were harvested at six time

points (0, 1, 3, 6, 12, and 24 h), and the samples were snap-frozen in liquid nitrogen and stored at −80 °C.

The soybean (DN50) seeds were incubated and germinated in the dark for 2 days. Subsequently, the seeds were transferred to Hoagland's nutrient solution at 23–28 °C with 16-h light/8-h dark environmental conditions. For the alkali and salt stress treatments, 15-day-old seedlings were transferred into Hoagland's nutrient solution containing 50 mM $NaHCO_3$ or 200 mM NaCl. Equal amounts of seedling roots were harvested at six time points (0, 1, 3, 6, 12, and 24 h), and the samples were snap-frozen in liquid nitrogen and stored at −80 °C.

Arabidopsis thaliana (Col-0) seedlings were grown in a greenhouse at 21–23 °C and with a 16-h light/8-h dark cycle. To investigate gene expression patterns, the roots, stems, and leaves of 18-day-old seedlings were harvested, respectively. For the gene expression analyses under alkali and salt stresses, the 12-day-old seedlings were treated with 10 mM $NaHCO_3$ or 150 mM NaCl. Equal amounts of seedlings were harvested at six time points (0, 1, 3, 6, 12, and 24 h), and the samples were frozen in liquid nitrogen and stored at −80 °C.

Quantitative real-time PCR

Total RNA was isolated from wild soybean, soybean, and *Arabidopsis* materials using the EasyPure™ Plant Kit (TransGen, Beijing, China), and cDNAs was synthesized using the GoldScript cDNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR assays were performed with SYBR Green Master Mix on an ABI 7500 sequencer according to the manufacturer's protocols. *ACTIN2* in *Arabidopsis* and *GAPDH* in wild soybean and soybean were used as internal controls (Czechowski et al. 2005; Huis et al. 2010). All experiments were performed with three independent biological replicates for statistical analyses. Statistical analyses were performed by one-way ANOVA with Bonferroni post-test or one-way ANOVA with Tukey's pairwise comparison tests. The primers used for quantitative RT-PCR are listed in Supplemental Table S1.

Results

Identification of PP2C genes in cultivated soybean and PP2C clade D genes in wild soybean

Although the genome sequence data for wild soybean are available now, the genome annotation is still in progress. Because the wild and cultivated soybean species have a close genetic relationship, and their genomes share a high level of sequence similarity (Zeng et al. 2012; Chung et al. 2014), we employed the *G. max* database from NCBI and PHYTOZOME databases to identify the soybean PP2C genes.

As a result, a total of 131 putative PP2C genes were obtained. Subsequently, 103 non-redundant soybean PP2C genes were extracted using the Pfam database and SMART online tool analysis. Simultaneously, 80 PP2C genes were obtained from *Arabidopsis*.

Using 103 GmPP2C and 80 AtPP2C full-length amino acid sequences, we performed phylogenetic analysis to construct a neighbor-joining phylogenetic tree. According to a previous study, AtPP2Cs were divided into 13 subfamilies. In this study, 10 soybean PP2C subfamilies (A, B, C, D, E, F1, F2, G, H, I) were classified, which is consistent with the previous study (Fig. 1) (Schweighofer et al. 2004). The soybean and *Arabidopsis* PP2Cs genes are closely related to each other within each subfamily. The GmPP2C clade D subfamily contains 14 genes according to the PP2C clade D nomenclature in *Arabidopsis*. Moreover, PP2C clade D in soybean and *Arabidopsis* has high bootstrap support, indicating that the genes in the same subfamily have a close evolutionary relationship. However, eight genes could not be clustered according to the nomenclature of the AtPP2C subfamily.

We also obtained PP2C clade D gene sequences of wild soybean from www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi and http://www.ncbi.nlm.nih.gov/assembly/ GCA (Kim et al. 2010; Qi et al. 2014), by comparing to the sequences of soybean PP2C clade D genes. We found GsPP2C and GmPP2C clade D proteins share high sequence similarity (Fig. S1). Additionally, phylogenetic and gene structure analyses showed that these genes have reasonably high bootstrap support and share analogous exon/intron structures (Fig. S2). These results indicated that GsPP2C and GmPP2C clade D genes share a high level of sequence similarity.

Identification and analysis of the physical locations and duplications of PP2C clade D genes in wild soybean and *Arabidopsis*

To explore the basic properties of PP2C clade D proteins, the polypeptide lengths, theoretical MW, and pI were predicted (Table 1). The polypeptide lengths of GsPP2C clade D proteins range from 344 to 397aa, shorter than those of AtPP2C clade D proteins, which range from 370 to 400aa. Moreover, the theoretical MWs of GsPP2C clade D proteins are in the range of 38,198.54 to 44,137.56 Da, also smaller than those of AtPP2C clade D proteins, which range from 41,334.32 to 44,140.21 Da. The average pI of GsPP2C clade D proteins is 7.37, which is lower than that of AtPP2C clade D with an average pI at 7.91.

To determine the genomic distribution and duplication of GsPP2C clade D genes, their physical locations on the wild soybean chromosomes were determined using the soybean genome database (http://phytozome.jgi.doe.gov/pz/portal.html). The results demonstrated that 14 PP2C clade D genes
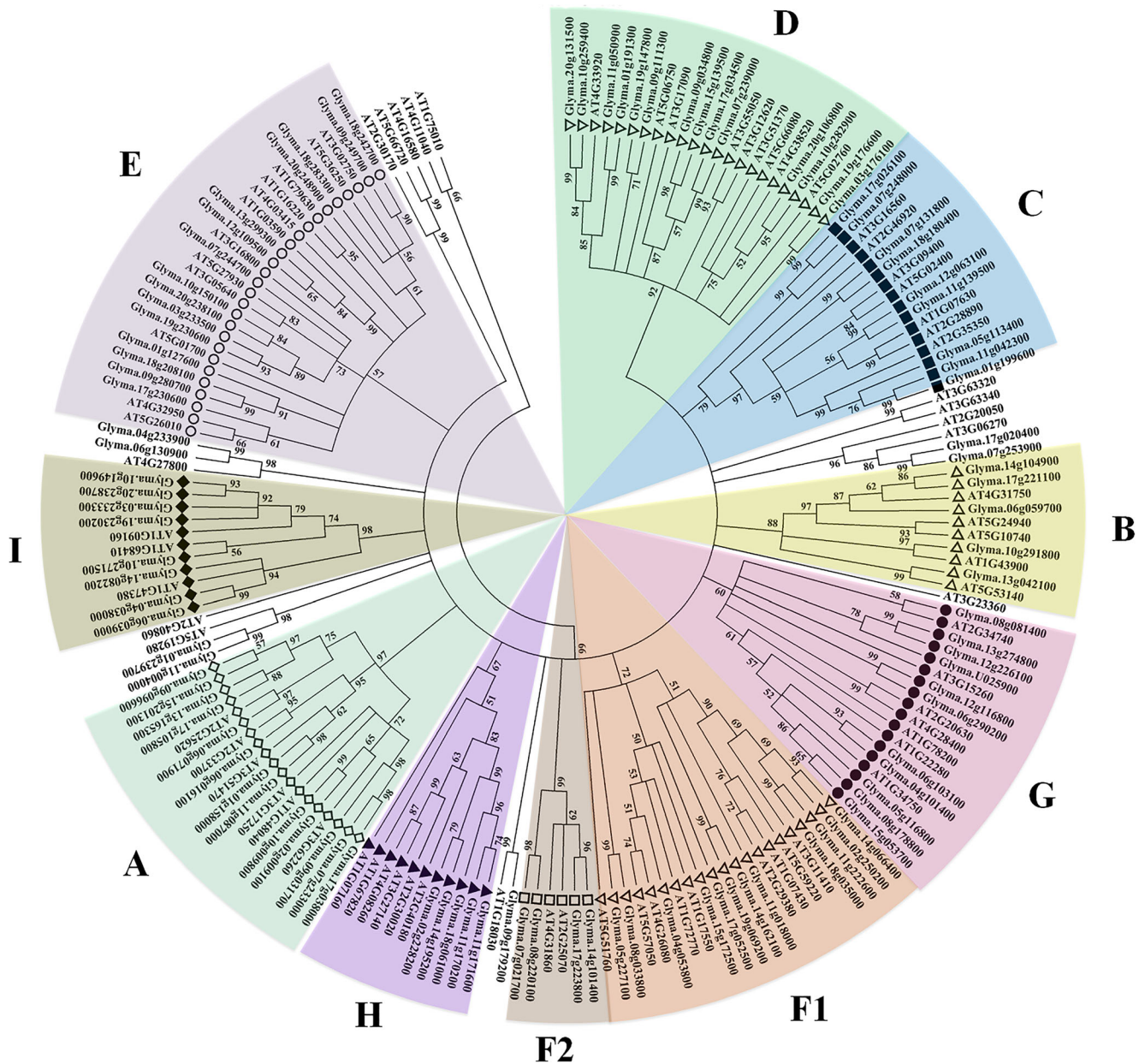
Fig. 1 Phylogenetic relationship of PP2C families of soybean and *Arabidopsis*. Multiple sequence alignments were generated by using MEGA 5.0 with full-length protein sequences. The phylogenetic tree was constructed by the neighbor-joining (NJ) method, and the bootstrap values were based on 1000 replications for each branch. The frequency values > 50 (%) are shown. GsPP2Cs were divided into 10 subfamilies (A, B, C, D, E, F1, F2, G, H, and I) and marked by different shapes

were mapped to 10 chromosomes, and each chromosome was assigned to one or two genes (Fig. 2a). Moreover, 12 genes were mainly located on the edges of the chromosomes except for *GsAPD4*. Additionally, nine AtPP2C clade D genes were unevenly distributed among three chromosomes, with the exception of chromosomes 1 and 2. All genes were found to be located near the edges of the chromosomes, which is similar to the locations of GsPP2C clade D genes (Fig. 2b). In addition, we investigated gene duplication events. The results of this analysis showed that all GsPP2C clade D

genes were linked to each other, whereas four paralogous genes were identified in AtPP2C clade D.

## Phylogenetic and structure analyses of PP2C clade D genes in wild soybean and *Arabidopsis*

To gain insights into the evolutionary relationships and structural diversity of GsPP2C and AtPP2C clade D genes, we constructed a neighbor-joining phylogenetic tree and compared the gene structure between these two clades

Table 1 Protein information of PP2C clade D genes in wild soybean and *Arabidopsis*

| Gene name	Gene ID	Amino acid residues	MW (Da)	pI	Chromosome
GsAPD1	*Glysoja.01g191300*	377	41,879.77	6.43	1
GsAPD2	*Glysoja.03g176100*	357	39,901.23	6.84	3
GsAPD3	*Glysoja.07g239000*	374	41,517.38	7.81	7
GsAPD4	*Glysoja.09g111300*	385	43,080.32	8.71	9
GsAPD5	*Glysoja.10g259400*	378	42,452.47	8.13	10
GsAPD6	*Glysoja.10g282900*	397	43,516.33	7.28	10
GsAPD7	*Glysoja.11g050900*	344	38,198.54	5.72	11
GsAPD8	*Glysoja.15g139500*	372	41,084.92	7.69	15
GsAPD9	*Glysoja.17g034500*	375	41,437.23	7.80	17
GsAPD10	*Glysoja.19g147800*	391	44,137.56	7.69	19
GsAPD11	*Glysoja.19g176600*	369	40,923.39	7.16	19
GsAPD12	*Glysoja.20g106800*	397	43,657.47	7.73	20
GsAPD13	*Glysoja.20g131500*	373	41,357.11	6.93	20
AtAPD1	*AT3G12620*	385	42,851.24	9.39	3
AtAPD2	*AT3G17090*	384	42,489.10	6.44	3
AtAPD3	*AT3G51370*	379	42,179.43	8.51	3
AtAPD4	*AT3G55050*	384	42,850.84	6.71	3
AtAPD5	*AT4G33920*	380	42,318.44	9.25	4
AtAPD6	*AT4G38520*	400	44,122.99	7.74	4
AtAPD7	*AT5G02760*	370	41,334.32	8.99	5
AtAPD8	*AT5G06750*	393	44,140.21	6.41	5
AtAPD9	*AT5G66080*	385	42,962.92	7.77	5

(Fig. 3a–b). The results suggested that these two clades have a close evolutionary relationship. For example, GsPP2C and AtPP2C clade D genes have a high bootstrap support, and they shared 48 to 76% sequence similarity. *GsAPD6* and *AtAPD3*, *AtAPD6* and *AtAPD9*, *GsAPD12* and *AtAPD1* and *AtAPD4*, *GsAPD5* and *AtAPD5*, and *GsAPD4* and *AtAPD8* share high sequence similarities. Furthermore, almost all *Arabidopsis* clade D genes contain four exons and three introns, except for *AtAPD4*. However, GsPP2C clade D genes have longer sequences than AtPP2C clade D genes, although they share similar protein sequence lengths. Moreover, more paralogous pairs of GsPP2C clade D genes have high bootstrap support and share analogous exon/intron structures than AtPP2C clade D genes, which is consistent with the characteristics of genomic distribution and duplication.
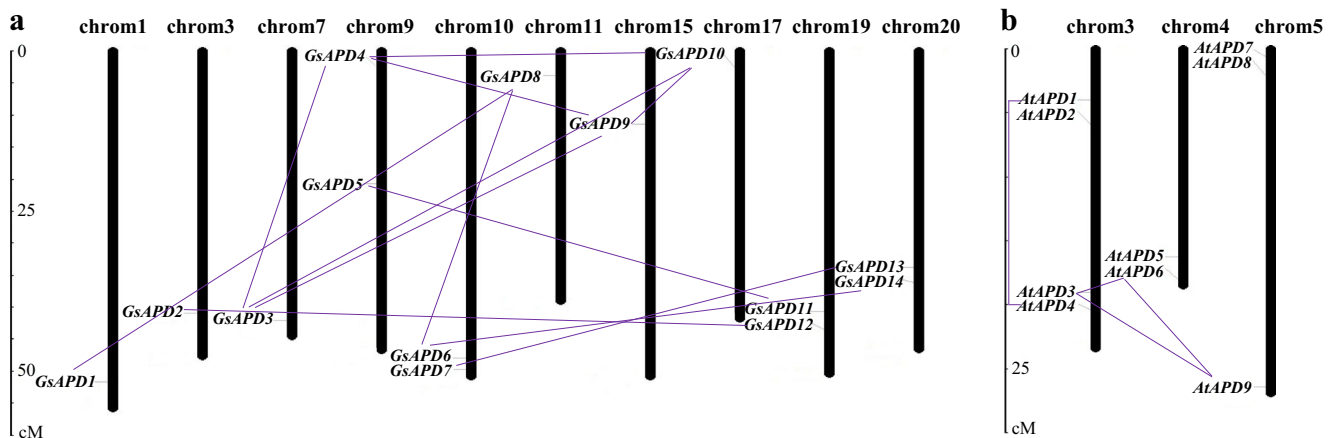
Fig. 2 Physical locations and duplications of PP2C clade D genes in wild soybean (**a**) and *Arabidopsis* (**b**). The black bars represent the chromosomes and the scales represent megabases (Mb). The chromosome numbers are shown on the top of each chromosome representation. GsPP2C (**a**) and AtPP2C (**b**) genes are distributed on 10 and 3 chromosomes, respectively. The paralogous genes are identified and connected by purple lines
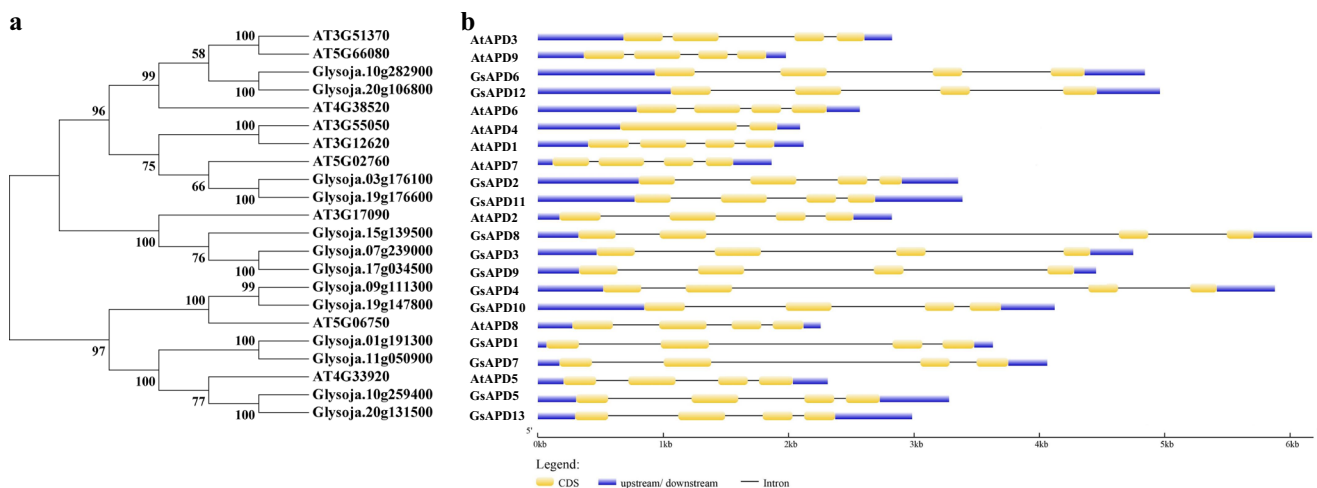
Fig. 3 Phylogenetic relationships and exon-intron structure analysis of PP2C clade D genes in wild soybean and *Arabidopsis*. **a** The phylogenetic tree was produced using MEGA 5.0 by the N-J method. Bootstrap values are based on 1000 replications for each branch. Pair of genes with bootstrap values higher than 98% were considered to be paralogues. **b** Structure analysis of PP2C clade D genes was performed using GSDS online tools. The untranslated regions (UTRs), CDSs, and introns are indicated by light blue boxes, yellow boxes, and black lines, respectively

Analyses of *cis*-acting elements of PP2C clade D gene promoters in wild soybean and *Arabidopsis*

Previous studies have suggested that hormone and abiotic stress-related *cis*-acting elements play active roles in response to stresses (Narusaka et al. 2003; Hernandez-Garcia and Finer 2014; Passricha et al. 2017). To elucidate whether PP2C clade D genes contain such *cis*-acting elements, we analyzed the PP2C clade D gene promoters in wild soybean (3 kb) and *Arabidopsis* (1.5 kb) using the PLANTCARE database. Our analyses demonstrated that almost all members carried putative stress-related *cis*-acting elements in their promoters, including ABA (ABRE), MeJA, ERE (ethylene), SA (Salicylic acid), GA (Gibberellin), Defense and Stress, HSE (heat), and MBS (drought)-responsive elements (Table 2). For example, all GsPP2C clade D genes contain HSE-responsive elements. All GsPP2C clade D genes contain defense and stress- and GA-responsive elements except for *GsAPD12*. In contrast, only 61.53% of GsPP2C clade D and 33.33% of AtPP2C clade D members have ERE-responsive elements. In all, these results suggested that both PP2C clade D genes may be responsive to abiotic stresses and hormone stimuli.

Expression patterns of PP2C clade D genes in wild soybean and *Arabidopsis*

Expression patterns are useful to assess the potential functions of a gene in certain developmental stages or under certain environmental conditions. To further understand the tissue-specific expression patterns of PP2C clade D genes, we

examined the spatial expression levels of these genes in roots, stems, and leaves of *Arabidopsis* and wild soybean.

Table 2 Distribution of *cis*-acting elements on the promoters of PP2C clade D genes in wild soybean and *Arabidopsis*

Name	ABRE	MeJA	ERE	SA	GA	MBS	HSE	Defense and stress
GsAPD1	1	0	1	3	2	2	3	2
GsAPD2	1	0	0	2	4	1	1	1
GsAPD3	1	4	1	1	2	0	5	6
GsAPD4	0	2	2	1	2	2	3	3
GsAPD5	3	2	2	2	3	1	2	2
GsAPD6	2	0	3	5	1	1	4	3
GsAPD7	2	2	0	2	3	1	1	5
GsAPD8	1	2	0	3	2	1	3	4
GsAPD9	1	2	2	4	2	3	3	1
GsAPD10	1	2	0	1	1	2	5	2
GsAPD11	2	2	0	2	3	2	1	1
GsAPD12	2	2	2	0	0	5	3	0
GsAPD13	1	2	1	0	1	1	4	3
AtAPD1	0	8	0	1	1	1	2	4
AtAPD2	1	6	0	2	0	1	3	1
AtAPD3	2	4	1	2	1	2	1	1
AtAPD4	0	0	2	2	2	1	1	3
AtAPD5	0	4	3	4	1	2	2	1
AtAPD6	0	0	0	2	4	1	2	4
AtAPD7	1	4	0	4	3	1	0	2
AtAPD8	0	4	0	1	6	1	0	2
AtAPD9	2	6	0	1	1	1	0	0

Transcripts of all the clade D genes were detectable in these three organs of *Arabidopsis* and wild soybean (Fig. 4). Five GsPP2C clade D genes, including *GsAPD5, -6, -11, -12,* and *-13,* displayed high expression levels in roots. Eight AtPP2C clade D genes, *AtAPD1, -2, -3, -4, -5, -6, -8,* and *-9,* showed higher expression levels in roots than the other organs. The high expression of these genes in roots or in all three organs suggested that these genes may significantly play functional roles in developmental processes or under specific conditions. In addition, we identified some PP2C clade D genes shared highly correlated expression pattern in wild soybean or *Arabidopsis* using correlation analysis. We found 15 pairs of GsPP2C clade D genes showed a highly correlated expression pattern ($r > 8$), such as *GsAPD2* and *GsAPD8*, *GsAPD5* and *GsAPD13*, and *GsAPD6* and *GsAPD12* (Fig. S3a). Sixteen pairs of PP2C clade D genes showed a highly correlated expression pattern ($r > 8$) in *Arabidopsis*, such as *AtAPD1* and

AtAPD2, AtAPD1 and *AtAPD3,* and *AtAPD2* and *AtAPD8* (Fig. S3b). These results showed that these genes with highly correlated expression patterns may play similar roles in plant developmental processes.
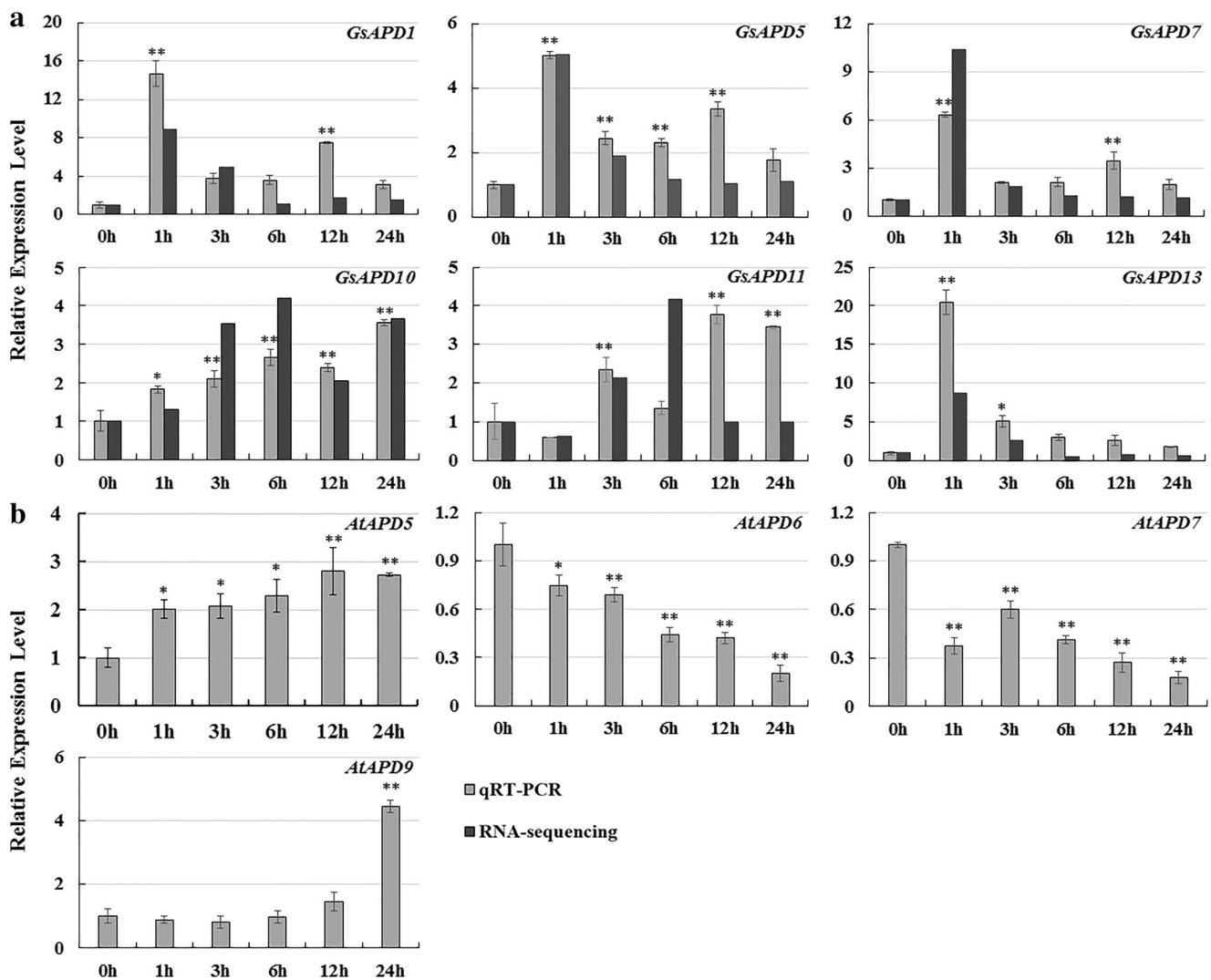
Expression analysis of PP2C clade D genes in wild soybean and *Arabidopsis* under alkaline treatment

Previously, we identified six GsPP2C clade D genes (*GsAPD1, -5, -7, -10, -11,* and *-13*) by transcriptome sequencing data and revealed that these PP2C genes were rapidly and significantly upregulated under alkaline treatment. To verify the data from the transcriptome sequencing analysis, we examined the expression patterns of these genes in wild soybean under alkaline treatment using qRT-PCR analysis (Fig. 5a). The gene expression patterns were roughly consistent with the transcriptome sequencing data, except that *GsAPD7* had
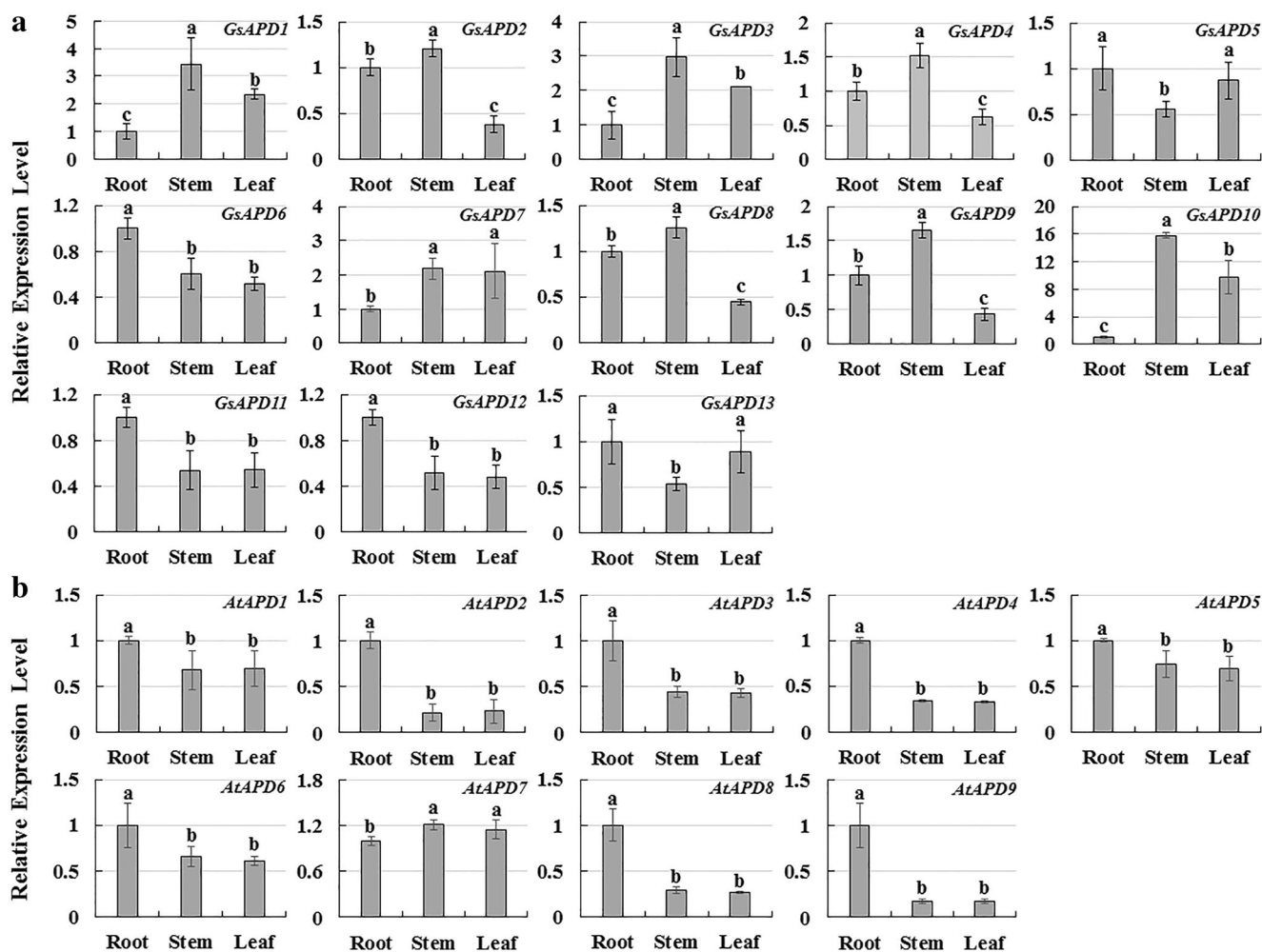
Fig. 4 Tissue-specific expression of PP2C clade D genes in wild soybean (**a**) and *Arabidopsis* (**b**). Total RNA samples were extracted from root, stems, and leaves of 21-day-old wild soybean and 18-day-old *Arabidopsis* plants. The relative expression levels were detected by qRT-PCR. *GAPDH* and *ACTIN2* were used as internal controls in wild soybean and *Arabidopsis*, respectively. Statistical analyses were performed by one-way ANOVA followed by Tukey's test. $*P < 0.05$, $**P < 0.01$

Fig. 5 Expression analysis of PP2C clade D genes in wild soybean (**a**) and *Arabidopsis* (**b**) under alkaline treatment. Total RNA samples were extracted from 21-day-old wild soybean seedlings under 50 mM NaHCO₃ treatment and 12-day-old *Arabidopsis* seedlings under 10 mM NaHCO₃ treatment, respectively. The relative expression levels were detected using qRT-PCR. *GAPDH* and *ACTIN2* were used as internal controls in wild soybean and *Arabidopsis*, respectively. All experiments were performed in three independent biological replicates to enable statistical analyses. Statistical analyses were performed by one-way ANOVA with Bonferroni post-test. *$P < 0.05$, **$P < 0.01$, as compared with the control before treatment (0 h)

contrary results at 12 and 24 h. In addition, the expression of *GsAPD1*, -5, -10, and -13 increased and reached a maximum point at 1 h. *GsAPD7* and *GsAPD11* were obviously induced at 12 and 24 h, respectively.

Similarly, the expression profiles of all AtPP2C clade D genes in *Arabidopsis* under alkali stress were also examined using qRT-PCR (Fig. 5b). Four genes (*AtAPD5*, -6, -7, and -9) showed differential expression. Interestingly, *AtAPD6* and *AtAPD7* were downregulated in response to alkali stress across all time points, and *AtAPD5* and *AtAPD9* were upregulated at different time points. In conclusion, our results showed that most of GsPP2C and AtPP2C clade D genes showed significant responses to alkali stress, providing the novel clues for us to better understand and to further dissect

the mechanisms of alkali-related stresses at the cellular and molecular levels in plant.

Expression analyses of PP2C clade D in wild soybean and *Arabidopsis* under salt treatment

Plants growing on saline-alkali soils suffer from not only sodium toxicity but also from HCO₃⁻ and CO₃²⁻ toxicity. To determine whether PP2C clade D genes are responsive to salt stress, we assessed the expression levels of all clade D genes under salt stress (Fig. 6). All eight genes (*GsAPD1*, -2, -5, -7, -8, -10, -11, and -13) were upregulated at different time points under salt stress in wild soybean. However, only two genes (*AtAPD4* and *AtAPD7*) appeared to be upregulated in
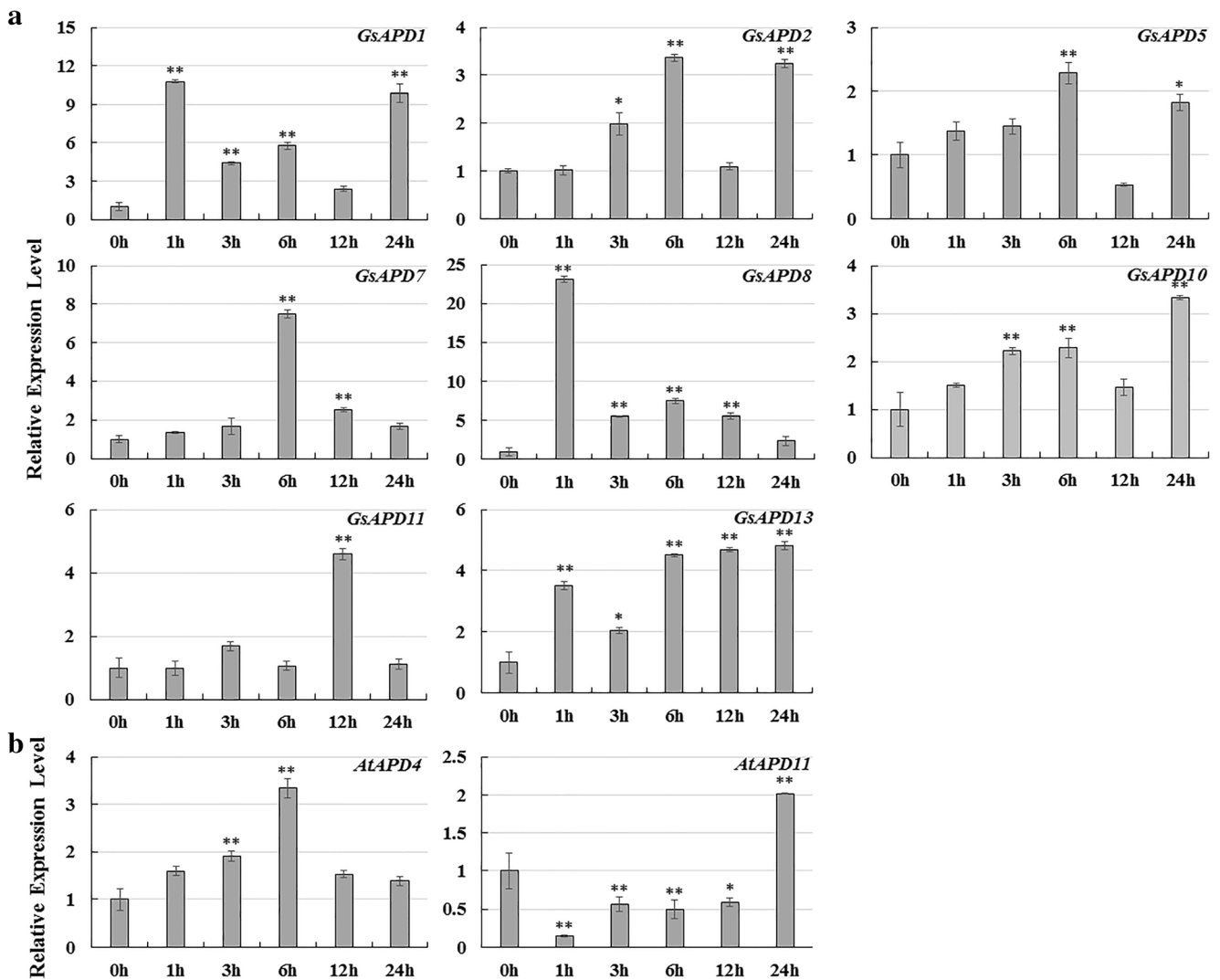
Fig. 6 Expression analysis of PP2C clade D genes in wild soybean (**a**) and *Arabidopsis* (**b**) under salt treatment. Total RNA samples were extracted from 21-day-old wild soybean seedlings and 12-day-old *Arabidopsis* seedlings under 200 mM NaCl and 150 mM NaCl treatment. qRT-PCR was performed to detect the relative expression levels. All experiments were performed in three independent biological replicates to enable statistical analysis. Statistical analyses were performed by one-way ANOVA with Bonferroni post-test. *$P < 0.05$, **$P < 0.01$, as compared with the control before treatment (0 h)

Arabidopsis, and *AtAPD7* was downregulated at 1, 3, 6, and 12 h, and then was upregulated at 24 h. These results indicate that more GsPP2C genes than AtPP2C genes in the clade D subfamily may participate in salt resistance and play important roles in response to salt stress.

Discussion

Phosphatase 2C family proteins play key regulatory roles in multiple signaling transduction pathways related to diverse biological processes, especially abiotic stress responses (Fuchs et al. 2013). Previously, we found that six GsPP2C clade D genes were significantly upregulated under alkaline treatment by transcriptome sequencing data (DuanMu et al.

2015). However, little is known about members of PP2C clade D, except for their localization and possible roles in regulating PM H$^+$-ATPase activity (Tovar-Mendez et al. 2014). In this study, we identified soybean PP2C clade D genes by investigating the relationship between the soybean and *Arabidopsis* PP2C protein families by phylogenetic analysis (Fig. 1). Then, we compared the sequences of GsPP2C clade D genes using GmPP2C clade D genes as references in the absence of wild soybean genome annotation.

In this study, to explore a genome-wide overview of the PP2C clade D subfamily, we compared the chromosome locations and duplications and gene structures in wild soybean and *Arabidopsis* and found that AtPP2C clade D genes underwent fewer gene duplications than GsPP2C clade D (Fig. 2), although a number of studies have showed that the

Arabidopsis genome has undergone large-scale gene duplications and contains numerous large duplicated chromosomal segments (Blanc et al. 2003). First, all GsPP2C clade D genes had gene paralogs, compared with only four pairs of AtPP2C clade D gene paralogs. Second, more paralogous gene pairs of GsPP2C clade D genes have relatively high bootstrap support and share similar exon/intron structures compared to AtPP2C clade D (Fig. 3a–b). In plants, the gene duplication is an important evolutionary mechanism, resulting in novel gene generation, gene loss, and gene rearrangement, which helps plants to adapt to different and diverse environments (Bowers et al. 2003; Shang et al. 2013). Thus, our results indicated that the PP2C clade D genes expanded differently in wild soybean and *Arabidopsis*, and suggested that the wild soybean genome is a result of large-scale gene duplications to adapt to different environmental conditions.

There are 15 and 16 pairs of PP2C clade D genes which exhibited similar expression patterns in wild soybean and *Arabidopsis*, respectively (Fig. S3a–b). Some paralogous pairs of PP2C clade D genes also exhibited similar expression patterns in wild soybean and *Arabidopsis*, such as *GsAPD5* and *GsAPD13*, *GsAPD6* and *GsAPD12*, and *AtAPD3* and *AtAPD9*, indicating that they may play similar roles in plant developmental processes. However, eight of the AtPP2C clade D genes displayed higher expression in roots than the other organs, whereas only five GsPP2C clade D genes showed observable expression in roots. In particular, *GsAPD10* displayed high expression in stem, indicating that it may play special roles in stem. In total, these results clearly suggested that AtPP2C clade D genes may mainly play functional roles in roots under specific environmental conditions. Additionally, GsPP2C and AtPP2C clade D genes may play different functional roles at certain developmental processes or under different stresses based on their different expression patterns.

Previous reports have illustrated that SAUR proteins can interact with some AtPP2C clade D proteins to regulate PM H⁺-ATPase activity, which plays regulatory roles in cell expansion, intracellular pH, salinity, and alkalinity (Rober-Kleber et al. 2003; Gevaudant et al. 2007; Spartz et al. 2014; Li et al. 2016). We screened putative *cis*-acting elements in the promoter regions of wild soybean and *Arabidopsis* PP2C clade D genes and found that most of them contain plant hormone-responsive elements and stress-responsive elements (Table 2 and Table S2). Subsequently, we determined the expression patterns PP2C clade D genes under salt and alkali stresses in wild soybean and *Arabidopsis*. The results demonstrated that six (*GsAPD1*, *-5*, *-7*, *-10*, *-11*, and *-13*) and four (*AtAPD5*, *-6*, *-7*, and *-9*) genes responded to alkali stress in wild soybean and *Arabidopsis*, respectively (Fig. 5). And eight (*GsAPD1*, *-2*, *-5*, *-7*, *-8*, *-10*, *-11*, and *-13*) and two (*AtAPD4* and *AtAPD7*) genes responded to salt stress in wild soybean and *Arabidopsis*, respectively (Fig. 6). These results indicate

that both GsPP2C and AtPP2C clade D subfamily genes may play important roles in response to alkali and salt stresses.

In addition, the data show that much more PP2C clade D genes from wild soybean than *Arabidopsis* were significantly regulated under salt and alkali stresses, suggesting that the wild soybean genome has undergone more large-scale gene duplications than the *Arabidopsis* genome, which might result in better adaptability of wild soybean to environmental stresses. Moreover, AtPP2C clade D genes displayed more variable expression patterns compared to GsPP2C clade genes, which were all upregulated. For example, *AtAPD5* and *AtAPD9* showed significant upregulation, whereas, *AtAPD6* and *AtAPD7* were downregulated under alkali stress, indicating AtPP2C clade D genes may participate in different signaling pathways compared with GsPP2C clade D genes. In our previous studies, we found that *GsCML27* plays opposite roles in plant responses to alkali and salt stresses, even though it is upregulated under both alkali and salt treatments (Chen et al. 2015). *GsACA1* overexpression in alfalfa increased plant tolerance to both alkaline and salt stresses (Sun et al. 2016). In this study, we also found that some genes were induced by both alkali and salt stresses, such as *GsAPD1*, *GsAPD5*, *GsAPD7*, *GsAPD10*, and *AtAPD7*, and these genes may have important functions under alkali and salt stresses. However, further functional characterization of PP2C clade D genes is required to understand the molecular mechanisms of their responses to saline and alkali stresses.

Wild soybean is the ancestor of the cultivated soybean, and wild soybean exhibits high adaptability to saline-alkali soil compared to the cultivated species (Ying et al. 2009). In this study, we extracted the sequences of GsPP2C clade D genes using GmPP2C clade D genes as references because they have high sequence homology. Therefore, it is worthy to discuss the PP2C clade D genes in soybean and wild soybean. We found that GsPP2C and GmPP2C clade D genes not only shared high protein sequence similarity but also had high bootstrap support and shared analogous exon/intron structures (Figs. S1-S2). These results are consistent with the previous studies, indicating that there is only 0.31% genomic difference between wild soybean and domesticated soybean (Kim et al. 2010).

We also assessed the expression levels of four GmPP2C clade D genes (*GmAPD1*, *5*, *7*, and *10*) under alkali and salt stresses (Fig. S4), and found that all were upregulated in wild soybean. Although these genes were upregulated under alkali and salt stresses, they have some different expression patterns compared with GsPP2C clade D genes (*GsAPD1*, *5*, *7*, and *10*). For example, *GmAPD1* showed no response to alkali stress, whereas *GsAPD1* was obviously induced at 1, 3, 6, and 24 h under alkali stress. *GsAPD7* was induced and reached a maximum point at 6 h, whereas *GmAPD7* was induced and reached a maximum point at 12 h. We demonstrated that all GmPP2C clade D genes harbored putative stress-related *cis*-

acting elements in their promoters, but there were some different stress-related *cis*-acting elements in GsPP2C clade D genes (Table S3). Overall, these results indicated that some GsPP2C and GmPP2C clade D genes may participate in different signaling pathways. This may be consistent with the differences in adaptability to stress tolerance mechanisms between wild soybean and cultivated soybean (Zhang et al. 2016).

Author contribution statements Chao Chen, Yang Yu, Huizi Duanmu, and Lei Cao performed the experiment, analyzed data; Chao Chen, Yu Yang, and Dan Zhu wrote the manuscript; Beidong Liu, Xiaoli Sun, and Zaib-un-Nisa edited the manuscript; and Qiang Li, Yanming Zhu, and Xiaodong Ding provided ideas, designed the research; all authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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