Characterization of interactions between the soybean plasma membrane- intrinsic proteins GmPIP1s and GmPIP2s responding to salt stress

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Abstract

Background: Salt tolerance is a key trait in soybean breeding and plant responses to salt stress include physiological and biochemical changes that affect the movement of water across the plasma membrane. In this study, we report the interactions of a set of aquaporins, soybean (*Glycine max*) plasma membrane-intrinsic proteins (*Gm*PIPs), in response to salt stress.

Results: *Gm*PIP1;5 and *Gm*PIP1;6 formed hetero-tetramers with *Gm*PIP2;4, *Gm*PIP2;6, *Gm*PIP2;8, *Gm*PIP2;9, *Gm*PIP2;11, and *Gm*PIP2;13. We detected interactions between *Gm*PIP1;6 and *Gm*PIP1;7, but not between *Gm*PIP1;6 and *Gm*PIP1;5. Furthermore, *Gm*PIP2;9 formed homo-tetramers, and this interaction was strengthened under salt and osmotic stress. Expression analysis indicated complex and unique responses to salt stress depending on the duration of the stress. For example, *GmPIP2;8*, encoding one of the heteromer-forming PIP proteins, was highly up-regulated under early salt stress.

Conclusions: Our study highlights the vital role of hetero- and homo-tetramers, in salt tolerance; and improves understanding of the mechanisms by which soybean aquaporin isoforms respond to abiotic stress.

1. Background

Plasma membrane intrinsic proteins (PIPs) are aquaporins that localize to the plasma membrane and regulate the flow of water and solutes through membranes. PIPs cluster into two evolutionary subgroups, PIP1 and PIP2. The copy numbers of the *PIP1* and *PIP2* isoforms vary among species: there are five *PIP1*s and four *PIP2*s in *Arabidopsis thaliana*[1], eight *PIP1*s and fourteen *PIP2*s in soybean (*Glycine max*; Zhang et al. 2013)[2] and *Brassica rapa* [3], and four *PIP1*s and five *PIP2*s in chickpea (*Cicer arietinum* L.) [4].

Plant PIPs contain two conserved domains: transmembrane domains (TMDs) and NPA motifs (asparagine–proline–alanine, NPA). Moreover, plant PIPs vary in the lengths of their N- and C-terminal ends (Yaneff et al. 2015)[5]. Previous biochemical and crystallography-based 3D structural analyses reported that PIPs assemble as hetero- or homo-tetramers (Murata et al. 2000; Gonen et al. 2004; Beesesims et al. 2011)[6-8]. Moreover, assays in *Xenopus* oocytes suggested that *PIP1* and *PIP2* co-expression could help PIP1 reach the plasma membrane (PM) and increase the permeability coefficient (P_f) in comparison with expressing *PIP2* alone (Fetter et al. 2004; Bienert et al. 2012). These results imply that PIP1 and PIP2 regulate several physiological processes jointly and may form functional units that aid solute transport (Yaneff et al. 2015)[5].

PIP1 and PIP2 form a complex that regulates water transport [9-11]. Previously, Harvengt *et al.* [12] isolated two isoforms of aquaporin from the protein-storage vacuoles of *Lens culinaris* Med. seeds. Using chemical cross-linking, they found that both isoforms belonged to the same oligomer in the membrane. Co-expression of maize (*Zea mays*) *ZmPIP1;2* with *ZmPIP2;1*, *ZmPIP2;4*, or *ZmPIP2;5* led to an increase in P_f [13]. Moreover, the interaction between PIP proteins enhanced the delivery of PIP2 from the Golgi to

the plasma membrane [14]. Additionally, in the extremophile *Thellungiella halophila*, the interaction between PIP1 and PIP2 triggered multiple physiological responses when the plant was exposed to salt stress [15]. When co-expressed in *Xenopus* oocytes, *Beta vulgaris BvPIP1;1* and *BvPIP2;2* enhanced PM water permeability [16], but *Bv*PIP2;1 did not bind *Bv*PIP1;1 or transport it to the oocyte PM[17]. Thus, the phenomenon of PIP interaction is specific, and not conserved in all isoforms.

Protein–protein and protein–macromolecule interactions play crucial roles in maintaining various physiological activities and molecular functions in plants. Salt stress is an important abiotic stressor of soybean that disrupts many physiological processes and reduces potential yield. We hypothesized that physical interactions between PIPs are a key component of the soybean response to salt stress. To detect these interactions, we carried out yeast two-hybrid assays. Our results show hetero- and homo-tetramerization of PIPs, and changes in PIP gene expression, which might affect water channel activity and plant salt tolerance.

2. Results

2.1 Sequence analysis of soybean *Gm*PIPs

Soybean *Gm*PIPs contain six TMDs and two conserved NPA motifs. Even though the transmembrane regions of both types of PIPs share high similarity, we found several differences in the amino acid sequences of TMD2 and TMD4 (Supplementary Fig. 1). The structural differences between PIP1 and PIP2 are limited to their N and C terminal ends (Yaneff et al., 2015)[5]. In soybean, the N-terminal ends of *Gm*PIP1s are longer than those of *Gm*PIP2s by about 15 amino acids. However, the C-terminal ends of GmPIP1s are shorter than those of GmPIP2s by about 8 amino acids (Supplementary Fig. 1). Phylogenetic analysis indicated that the *Gm*PIP1s and the *Gm*PIP2s formed two separate clusters (Supplementary Fig. 2). The clusters remained even after the addition of PIPs from other species, such as rice (*Os*PIPs) and *Arabidopsis* (*At*PIPs), to the phylogenetic analysis (Supplementary Fig. 3).

We downloaded and analyzed whole genome duplication data for soybean from PGDD (http://chibba.agtec.uga.edu/duplication/). We found variation in segmental-duplication between some of the isoforms (Reference to Table or Figure): among *GmPIP1;1, GmPIP1;2, GmPIP1;7* and *GmPIP1;8; GmPIP1;3, GmPIP1;4, GmPIP1;5* and *GmPIP1;6; GmPIP2;1* and *GmPIP2;2; GmPIP2;3, GmPIP2;4, GmPIP2;5, GmPIP2;6* and *GmPIP2;8; GmPIP2;7, GmPIP2;4, GmPIP2;6, GmPIP2;8, GmPIP2;13, GmPIP2;14,* and *GmPIP1;8, GmPIP2;13, GmPIP2;9, GmPIP2;10, GmPIP2;11* respectively, however, tandem-duplication variation happened only in *GmPIP2;10* and *GmPIP2;11*.

2.2 Hetero- and homo-tetramerization in *Gm*PIP1s and *Gm*PIP2s

Based on our phylogenetic analysis, we selected some (Supplementary Fig. 3) of the *Gm*PIPs for yeast two-hybrid assays. A total of 255 PIP1–PIP1, PIP1–PIP2, and PIP2–PIP2 combinations were tested. We found that *Gm*PIP1;5 and *Gm*PIP1;6 could each interact with *Gm*PIP2;2, *Gm*PIP2;4, *Gm*PIP2;6, *Gm*PIP2;8, *Gm*PIP2;9, *Gm*PIP2;11, and *Gm*PIP2;13 (Fig. 1). Among the PIP1–PIP1 pairs tested, we found an

interaction only between *Gm*PIP1;6 and *Gm*PIP1;7 (Fig. 1). In addition, we observed that salt treatment strengthened most of the interactions, but osmotic stress induced by mannitol treatment did not (Fig. 2). Furthermore, we found that only GmPIP2;9 could form homo-tetramers, and that this interaction was enhanced by salt stress, but weakened by osmotic stress (Fig. 3).

2.3 The transcriptional profiles of *GmPIP*s under salt stress

Two-week old plants were subjected to salt stress (200mM NaCl) for 0 h, 2 h, and 12 h, and the transcriptional profile of *GmPIP* genes was examined. The expression levels of three *GmPIP* genes (*GmPIP1;3*, *GmPIP1;4*, and *GmPIP2;8*) were significantly elevated in response to salt stress (Fig. 4). Of particular note was *GmPIP2;8*, whose transcript levels were about 6 times higher after 12 h of salt stress. Most of the other *PIP* genes were down-regulated after treatment for 12 h, except for *GmPIP2;7* (not shown in the figure). While the expression levels of five *GmPIP* genes (*GmPIP1;1*, *GmPIP1;7*, *GmPIP2;3*, *GmPIP2;5*, and *GmPIP2;8*) were lowered immediately in response to salt stress, the levels of others (such as *GmPIP1;6*, *GmPIP2;13*, and *GmPIP2;1*) decreased gradually. Furthermore, the transcript levels of *GmPIP1;5*, *GmPIP1;8*, and *GmPIP2;2* did not change even after 12 h of salt stress. The transcript levels of *GmPIP1;2* and *GmPIP2;4* decreased significantly at 2 h and then recovered at 12 h to the same level as at 0 h. By contrast, the transcript levels of four *GmPIP* genes (*GmPIP2;1*, *GmPIP2;9*, *GmPIP2;10*, and *GmPIP2;11*) increased at 2 h, and then sharply decreased afterwards (Fig. 4). Therefore, the *GmPIP* genes show different transcript profiles in response to salt stress.

3. Discussion

Numerous studies have reported the identification of functional genes that are relevant to plant stress tolerance and that can be used for crop improvement[18-21]. To aid efforts to breed salinity tolerance in soybean, we analyzed the orthologs of plasma membrane intrinsic proteins (PIPs) in soybean and tested their interactions using a yeast two-hybrid system. The interaction between PIP1 and PIP2 proteins functions as the main signal for cell membrane water and salt exchange and PIPs have been considered as functional units that perform their physiological roles under different environmental stresses, such as salt and drought stress. Aquaporins play an important role in growth regulation of plants by influencing root water uptake and leaf gas exchange. The soybean PIP1, *GmPIP1;6*, has previously been well characterized. Its function in growth regulation and salt tolerance was analysed by constitutive overexpression [22]. A separate study highlighted the involvement of several aquaporin homologs in response to a variety of environmental stressors that interrupt plant cell osmotic balance [23].

PIP1 and PIP2 have highly conserved peptide sequences, and the main differences between them are the lengths of their N and C terminal ends [5]. In soybean, the N terminal ends of *Gm*PIP1 are longer than that of *Gm*PIP2 (approximately 15 amino acids), however, the C terminal ends of *Gm*PIP1 type are shorter than that of *Gm*PIP2 type (approximately 8 amino acids). Interestingly, the transmembrane region of both *Gm*PIP1 and *Gm*PIP2 nearly share the same section (Supplementary Figure 1).

Ispolatov *et al.* [24] proposed that duplicated proteins were more likely to interact among themselves than with other proteins, and that paralogous interactions were inherited from ancient homo-dimeric proteins, rather than established *de novo* after gene duplication. In evolution progress, gene duplication events increase gene number by tandem- and segmental-duplication [25]. Soybean (*Glycine max* (L.) Merr.) is an important crop and well-studied. Previous research reported that soybean is a paleopolyploid, and at least two rounds of large-scale duplication occurred in its ancestral genome at approximately 14- to 42- million ago [26]. In this study, we searched the database PGDD (http://chibba.agtec.uga.edu/duplication/), and found many duplication events in the *GmPIP* family. Expansion of aquaporin gene families via genome duplication events have been reported in other plants [27].

The transcriptional profiles of *PIP*s may provide evidence for PIP1–PIP2 interactions. For example, a joint increase (or decrease) in the expression of specific *PIP1–PIP2* pairs in plants under stress may indicate shared functionality. Thus, the formation of hetero-tetramers composed of specific PIP1s and PIP2s could be affected by their mRNA abundance [5]. Transcriptional profiles in rice, maize, and *Arabidopsis* indicate interactions between PIP1–PIP2 pairs in these species [5]. Additionally, Zargar *et al.* [28] developed a gene co-expression network of rice aquaporin genes (*OsPIPs*) and tonoplast intrinsic proteins (*OsTIPs*) using Rice Friend server (http://ricefrend.dna.affrc.go.jp). They found co-expression of *PIP1–PIP2* pairs, indicating likely physical interaction between these proteins. In this study, we found physical interactions among *Gm*PIPs using yeast two-hybrid assays. We detected both homotetramers and heterotetramers among these proteins. Salt, but not mannitol, enhanced these interactions. Only *Gm*PIP2;9 could form homotetramers, and this interaction was enhanced by salt stress, but weakened by osmotic stress. Our results corroborate those of Bienert *et al.* [29], who reported PIP heterotetramerization under salt stress in *Selaginella moellendorffii*.

The TMDs of *Gm*PIPs were predicted by SMART software. All *Gm*PIPs contained six TMDs. *Gm*PIP1;5 and *Gm*PIP1;6 differed by two amino acids in TMD2 (D/Y) and TMD6 (H/Q). However, there were many differences in TMD2, TMD4, and TMD6 among *Gm*PIP2;4, *Gm*PIP2;6, *Gm*PIP2;8, *Gm*PIP2;9, *Gm*PIP2;10, and *Gm*PIP2;11. This indicates that the highly conserved sequences in TMD1, TMD3, and TMD5 may play a crucial role in the formation of PIP1–PIP2 pairs between *Gm*PIP1;5 and *Gm*PIP1;6, and among *Gm*PIP2;4– *Gm*PIP2;11. Using extensive amino acid substitution mutagenesis, Yoo et al. (2016) studied tetramer formation in *ArabidopsisAt*PIP2;1. They demonstrated that TMD1, TMD2, and TMD5 contained essential amino acid residues key to tetramer formation.

In addition, the expression profiles of *GmPIPs* under salt stress also showed similar expression patterns in *GmPIP1;5* and *GmPIP1;6*, and in *GmPIP2;3*, *GmPIP2;4*, *GmPIP2;5*, *GmPIP2;6*; *GmPIP2;9*, *GmPIP2;10*, *GmPIP2;11*; *GmPIP2;13* and *GmPIP2;14* respectively, which is partly consistent with the sequence similarities and interaction patterns among *Gm*PIPs. The expression of *GmPIP1;3*, *GmPIP1;4*, *GmPIP2;1*, *GmPIP2;8*, *GmPIP2;9, GmPIP2;10*, *GmPIP2;11* was significantly up regulated by salt stress. However, the other *GmPIPs* were significantly downregulated by salt stress, except *GmPIP1;9, 1;10, 2;7 and 2;12*.

Aquaporins are implicated in a variety of stress responses that disturb plant cell osmotic balance and nutrient homeostasis (Yaneff et al. 2015)[5]. They are involved in the *Arabidopsis* response to drought stress (Afzal et al., 2016)[23], in leaves and roots of sugar beet under salt stress (Lv et al., 2018)[30], and in rice tolerance to salt stress and cold stress (Qiang et al. 2015)[15]. We have discovered that some *GmPIPs* are significantly up- or down-regulated in response to salt stress. *GmPIP1;6* is the closest ortholog to *ArabidopsisAtPIP1;2* (with an amino acid sequence identity of 84.8%), which localizes to the Golgi apparatus and the membrane system. *At*PIP1;2 was considered as a functional water channel when it was expressed alone in *Xenopus* oocytes [31]. A previous study also suggested that many *Gm*PIPs have high sequence similarity but diverse functions (Zhang et al. 2013)[2]. *Gm*PIP1;6 could interact with other *Gm*PIP2 type aquaporins, which implied they may play crucial roles in aquaporin trafficking from the Golgi apparatus to the membrane system in plants. The functions and molecular mechanisms of the diverse families of plant PIPs still need further study.

4. Conclusions

In this study we identified the *PIP* gene families in the soybean genome (*GmPIP*) and analyzed the expression patterns of *GmPIPs* under salt stress and interactions among the encoded proteins. We found that *Gm*PIP1;5 and *Gm*PIP1;6 each formed hetero-tetramers with six *Gm*PIP2-type aquaporins. *Gm*PIP1;6 interacted with *Gm*PIP1;7. Furthermore, *Gm*PIP2;9 formed homo-tetramers. These interactions were strengthened by salt stress, but not by osmotic stress. Most of the genes encoding interacting GmPIPs exhibited a similar expression pattern under salt stress. Our results improve our understanding of the interactions among soybean PIP isoforms and the role of homo- and hetero-tetramers in the response to salt stress.

5. Abbreviations

Glycine max – Gm; plasma membrane-intrinsic protein – PIP, transmembrane domains -TMD; plasma membrane - PM; permeability coefficient - P_f; *Zea mays* - *Zm*; stand derivation – SD.

6. Methods

6.1 Soybean plant materials and treatments

The seeds of soybean variety Willimas82 (*Glycine max*) were from the seed stock of our lab, and they were grown in pots for the salt treatment. Two-week-old seedlings were placed into 1/2Hoagland solution for two days as an adjustment process, then subjected to 200 mM NaCl solution made with 1/2 Hoagland solution for 12 hours and the treatment with 1/2Hoagland solution as a control respectively, all the treated seedlings were kept in a growth chamber with a temperature of 24℃.

6.2 Bioinformatics analysis of soybean PIPs

To analyze the duplication events among soybean *Gm*PIPs, the data were downloaded from http://chibba.agtec.uga.edu/duplication/ and the tandem and segmental duplication of *Gm*PIPs were found out respectively. The transmembrane region for *Gm*PIPs was predicted using http://smart.embl-heidelberg.de/ online software. The evolutional trees for *Gm*PIPs and other plant species PIPs proteins, downloaded from http://ricefrend.dna.affrc.go.jp, were constructed using MEGA5.0 software [32]. The multiple alignment was performed using Cluxtal X and GeneDoc.

6.3 RNA extraction and cDNA synthesis

Total RNA was extracted from the root tissue as well as other samples of the seedlings subjected to 200 mM NaCl for 0 (CK), 2 and 12 h, respectively, with Promega RNA extraction kit. After the DNA removal through DNase I digestion, RNA quality and integrity was detected using 1.2% agarose gel electrophoresis. The cDNA synthesis was performed according to the procedure of the reverse transcription kit (HaoJia Technology Development Co., Ltd. Shanghai, China).

6.4 *GmPIPs* gene cloning and constructs preparation

Full-length ORF of *GmPIPs* was amplified from the root or leaf tissues of soybean (variety *Willimas* 82) seedlings using primers containing enzyme digestion sites (Supplementary Table 1). PCR products were transferred to the pGEM-T Easy Vector and the resulting vector pGEM-T– *Gm*PIPs were sequenced by Nanjing TSINGKE Biological Technology Co., Ltd to validate the sequence, every gene repeated three times. The pGADT7, pGBKT7 vectors and pGEM-T-*Gm*PIPs were digested using *Nco* I and *EcoR* I, then the digested vector and gene fragments were ligased using T4 DNA ligase enzyme. After transformation, positive clones detection and sequencing validation for pGADT7-*Gm*PIPs and pGBKT7-*Gm*PIPs, and then they were prepared for the following yeast two hybrid assay.

6.5 Yeast two hybrid assay

The yeast two-hybridization assay was performed according to the protocol of The Matchmaker ^{TM} Gold Yeast Two-Hybrid System (Clontech).

After the co-transformation of pGADT7-GmPIPs and pGBKT7-GmPIPs, the equal amounts of yeast clones were transferred and plated on selective mediums of SD-Leu-Trp and SD-Leu-Trp-His-Ade+Aba+X-a-gal respectively. They were incubated at 30°C until the appearance of distinguishable colonies. Yeast cells having pGBKT7–53 and pGADT7-SV40 plasmids were as positive controls, and those having pGBKT7-Lam and pGADT7-SV40 were the negative. Meanwhile, the yeast clones were also plated on SD-Leu-Trp-His-Ade+Aba+X-a-gal with 100 mM NaCl or 200 mM Mannitol respectively. The ddH_2O was used for the yeast dilution and then 1 ml of OD and 1 ml of the solution was poured in a cuvette to confirm the OD.

6.6 Gene expression analysis

Quantitative real-time PCR was performed to assay the expression level of *GmPIP* genes in response to salt stress. Primers for each gene were listed in Supplementary Table 2. The soybean *GmTUBB3* (NM_001252709.2) and *GmActin* (NM_001289231.1) were used as internal references. All of reactions were carried out with the SYBR® Premix Ex Taq™ (Takara, China) reaction mixture in a Bio-Rad CFX connect Real-Time system (Bio-Rad Laboratories, California, USA). The experimental conditions were set as: 10 s at 95°C, followed by 40 cycles of 5 s at 95°C for denaturing and 30 s at 60°C for annealing and extension. The results were obtained from the relative gene expression levels calculated by the $2^{-\Delta\Delta Ct}$ method and the plants of 0h treatment were as control.

6.7 Statistical analysis

The data are the average of two replicates ± stand derivation (SD) for each treatment. The data was analyzed by ANOVA using SPSS 20.0. Values of significantly difference ($P<0.05$) are marked by lowercase letters (a-d) in the figures.

7. Declarations

7.1 Ethics approval and consent to participate

There is no experimental material of animal and human used in present research. Here we declare the experiments and researches have no conflict against ethics at all.

7.2 Consent to publish

All the participators involved in present research have thoroughly read the manuscript and approved the submission.

7.3 Availability of data and materials

The data and results present in the manuscript are all available and free to be accessed by the readers.

7.4 Competing interests

The authors declare that they have no conflict of interest.

7.5 Funding

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Authors' Contributions

WQ, JL and DZ conceived, designed, and conducted the experiments. YH, QW and W Q analysed the data and results. DZ wrote the manuscript. HS and HL monitored the experiments and critically. All authors have read and approved the manuscript.

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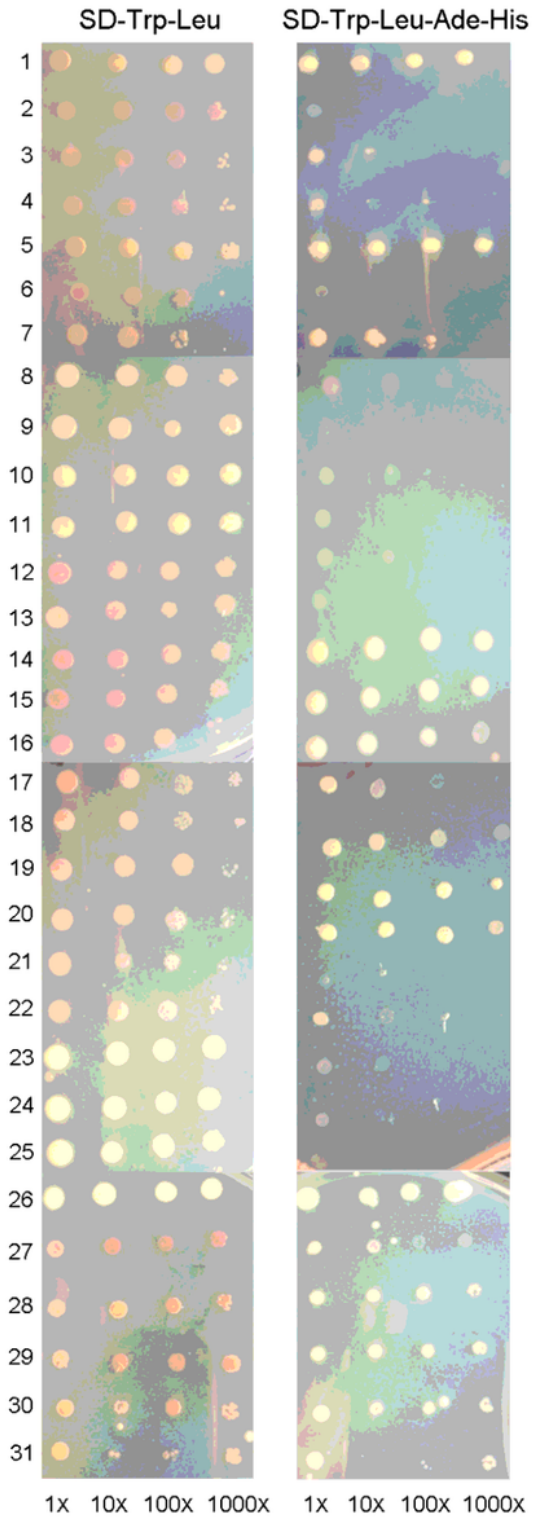
Figures



No.	AD	BD
1	pGADT7-SV40	pGBKT7–53
2	pGADT7-SV40	pGBKT7-Lam
3	pGADT7-*Gm*PIP1;3	pGBKT7–*Gm*PIP2;4
4	pGADT7-*Gm*PIP1;4	pGBKT7–*Gm*PIP2;6
5	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;2
6	pGADT7-*Gm*PIP1;7	pGBKT7–*Gm*PIP1;8
7	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;2
8	pGADT7-*Gm*PIP1;2	pGBKT7–*Gm*PIP2;4
9	pGADT7-*Gm*PIP1;3	pGBKT7–*Gm*PIP2;6
10	pGADT7-*Gm*PIP1;4	pGBKT7–*Gm*PIP2;8
11	pGADT7-*Gm*PIP1;7	pGBKT7–*Gm*PIP2;9
12	pGADT7-*Gm*PIP1;8	pGBKT7–*Gm*PIP2;11
13	pGADT7-*Gm*PIP2;2	pGBKT7–*Gm*PIP1;3
14	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;4
15	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;6
16	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;8
17	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;9
18	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;11
19	pGADT7-*Gm*PIP1;5	pGBKT7–*Gm*PIP2;13
20	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP1;7
21	pGADT7-*Gm*PIP1;7	pGBKT7–*Gm*PIP2;2
22	pGADT7-*Gm*PIP1;7	pGBKT7–*Gm*PIP2;4
23	pGADT7-*Gm*PIP1;7	pGBKT7–*Gm*PIP2;6
24	pGADT7-*Gm*PIP1;8	pGBKT7–*Gm*PIP2;2
25	pGADT7-*Gm*PIP1;8	pGBKT7–*Gm*PIP2;4
26	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;4
27	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;6
28	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;8
29	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;9
30	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;11
31	pGADT7-*Gm*PIP1;6	pGBKT7–*Gm*PIP2;13

Figure 1

Yeast two-hybrid (Y2H) assays show GmPIP1s and GmPIP2s interact to form hetero-tetramers. Full-length GmPIP genes were cloned into pGADT7 (with the activation domain, AD) and pGBKT7 (with the DNA-binding domain, BD). Varied combinations of pGADT7 and pGBKT7 constructs were co-transformed into the Y187 and Y2H gold yeast strains. Yeast cells were grown in liquid selective medium until OD600 = 1.0, and then spotted at 1-, 10-, 100-, and 1000-fold dilutions on the SD-Leu-Trp and SD-Leu-Trp-Ade-His

selective solid-medium plates. They were put into the incubator with temperature at 30 °C, until the colonies emerged. Yeast cells harboring pGBKT7–53/pGADT7-SV40 vectors were set as the positive control, and those with pGBKT7-Lam/pGADT7-SV40 the negative. The numbers 1–31 represent the AD and BD vectors listed in the table on the right.

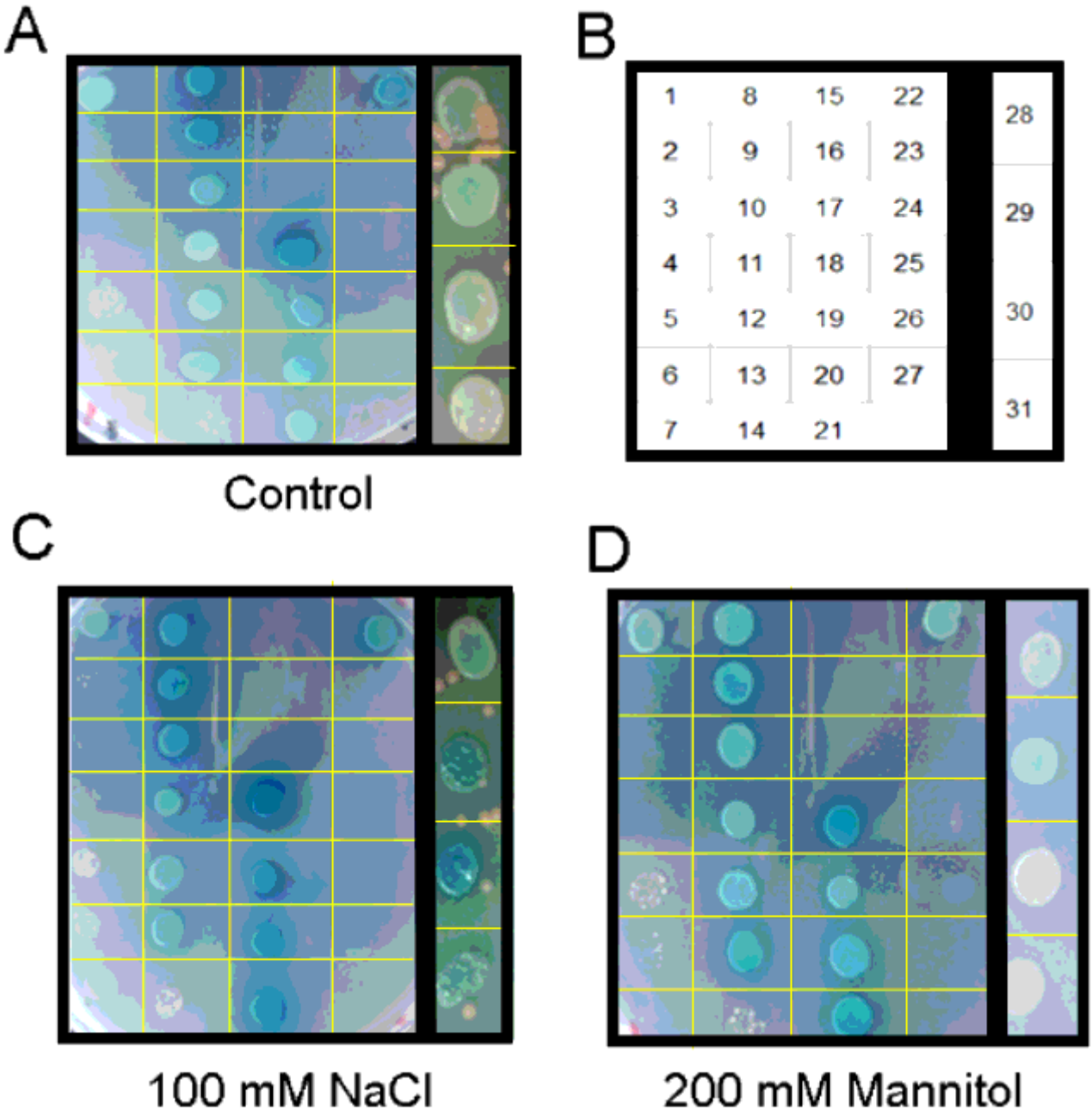


Figure 2

The effect of salt and osmotic stress on the interaction of GmPIP1s and GmPIP2s. (A) All the yeast clones mentioned in Figure 2 were plated on selective plates (SD-Leu-Trp-His-Ade) with Aba (100 ng/ml) and X-a-gal. (B) The position of the yeast clones on the plates. (C) The clones were spotted on the selective plates SD-Leu-Trp-His-Ade with Aba (100 ng/ml), X-a-gal and 100 mM NaCl. (D) The clones were spotted on the selective plates SD-Leu-Trp-His-Ade with Aba (100 ng/ml), X-a-gal, and 200 mM mannitol.

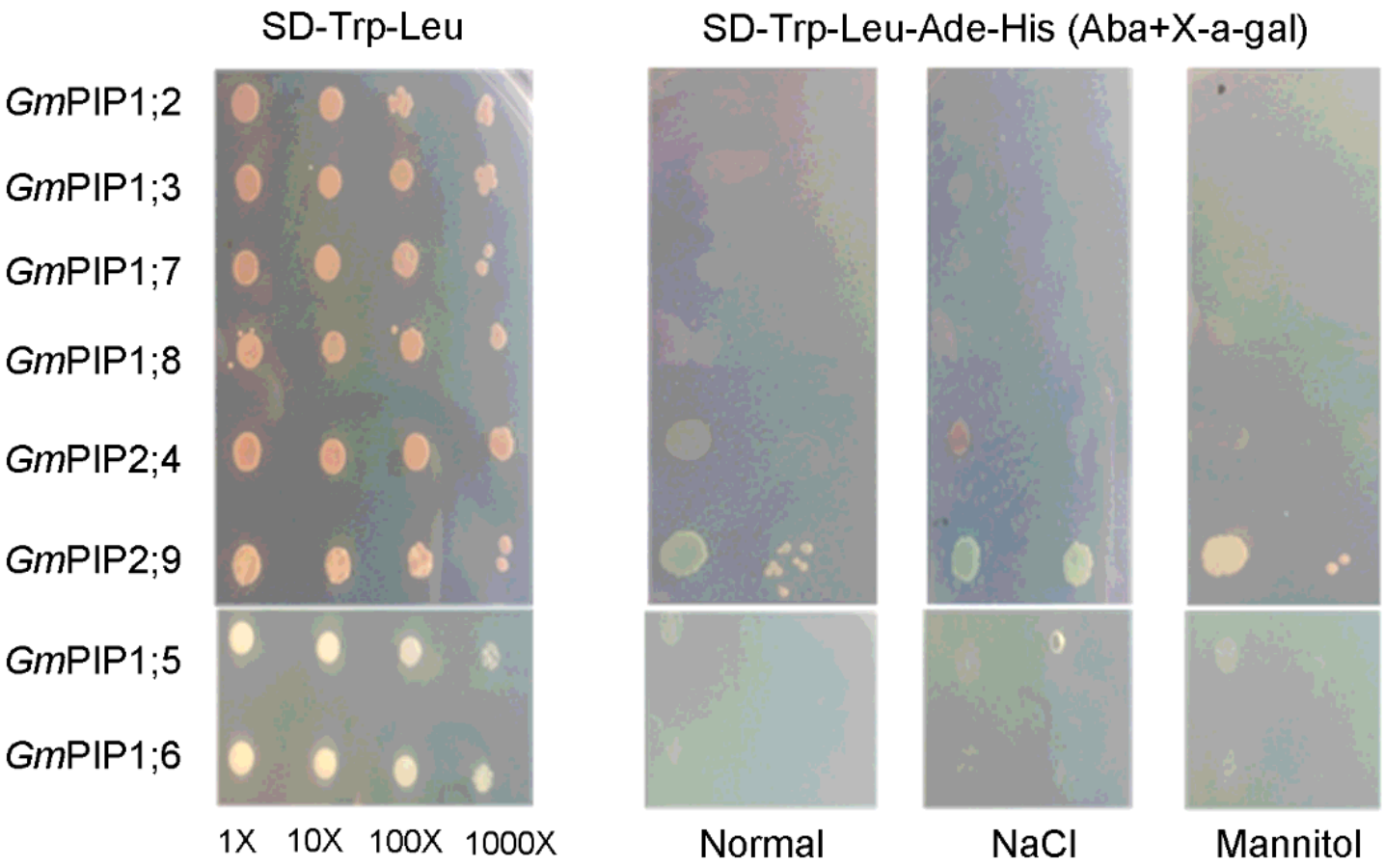


Figure 3

Y2H to test homo-tetramerization of GmPIPs. Six GmPIP1 and two GmPIP2 genes were selected to construct the pGADT7 and pGBKT7 vectors. The same genes expressed in AD and BD were co-transformed into the Y187 and Y2H gold yeast strain. The fresh yeast cells (OD600 = 1.0) were spotted at 1, 10, 100 and 1000-fold dilutions media on the SD-Leu-Trp plates or SD-Leu-Trp-His-Ade+Aba (100 ng/ml)+X-a-gal with 100 mM NaCl or 200 mM mannitol

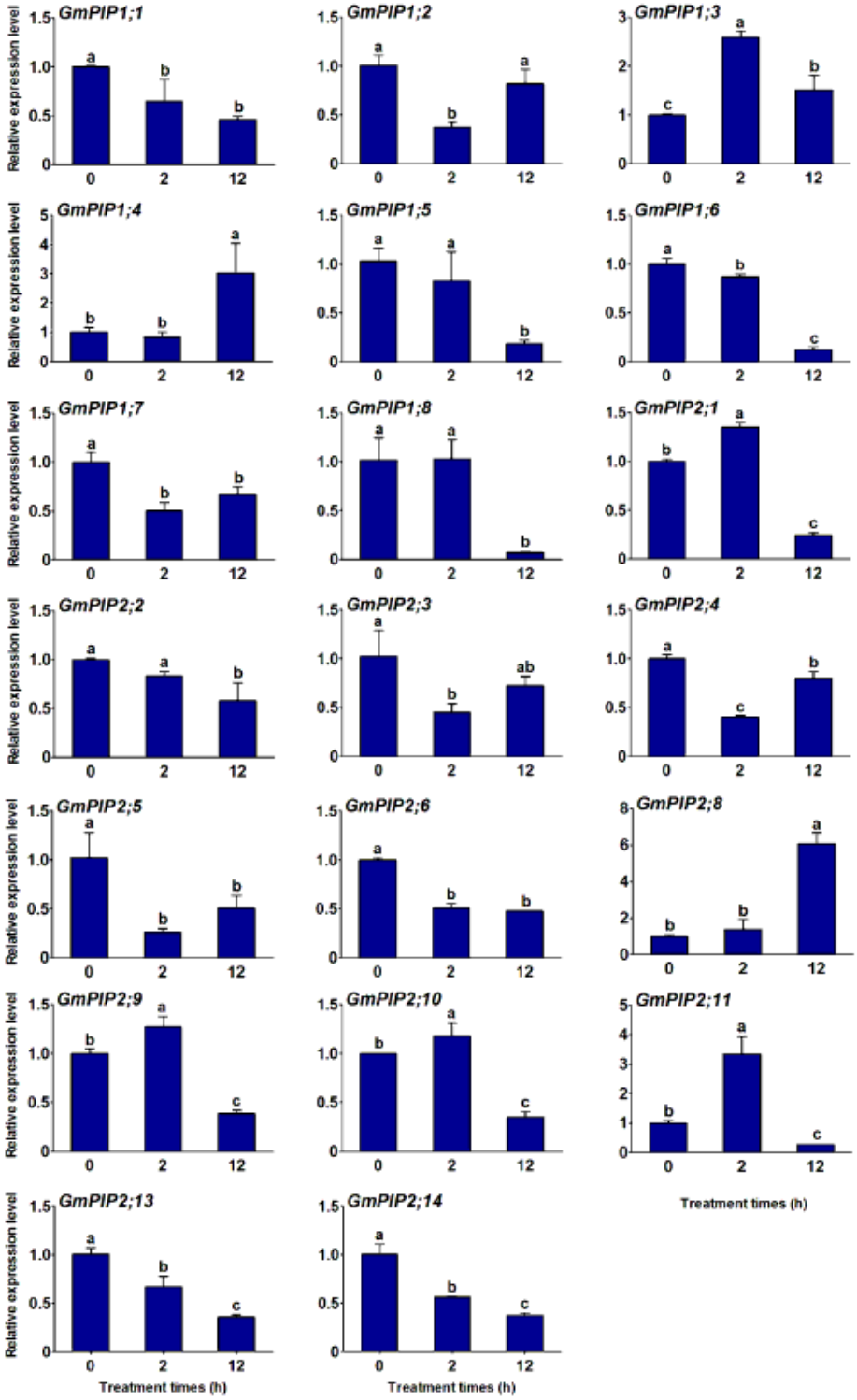


Figure 4

Expression profile of GmPIPs in response to salt stress. The relative transcript levels of GmPIP genes were assayed by qRT-PCR, with soybean GmTubulin set as internal control. Roots of 2-week-old soybean seedlings were used to evaluate the changes of gene expression in response to salt stress (200 μM NaCl for 0, 2, and 12 h). The results are presented as means ± SD of which three independent experiments were

performed. Different alphabets indicate significant differences between controls and treated plants ($P<0.05$, one-way ANOVA).

Supplementary Files

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