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# Cloning and function analysis of a plasma membrane intrinsic protein gene, *GmPIP* in soybean (*Glycine max* L. Merr)

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**Abstract**

A full-length mRNA that encoded a plasma membrane intrinsic protein (PIP) was isolated by electronic elongation and RT-PCR, containing an open reading frame of 861bp (GenBank HM245628), which encodes a protein of 281 amino acid residues whit a termination codon. The GmPIP included an Asn-Pro-Ala (NPA) domain and major intrinsic protein (MIP) superfamily domain, protein sequence alignment studies and phylogenetic analysis strongly suggested that GmPIP belongs to the group of plant PIPs. The semi-quantitative RT-PCR results showed that *GmPIP* mRNA abundance was increased in roots under treatments with salt. Sense and antisense expression vectors of *GmPIP* gene were constructed. Transgenic soybean hair roots overexpressing sense *GmPIP* gene showed stronger viability than the hair roots overexpressing antisense *GmPIP* gene and control upon salt stress. The transgenic *Lotus corniculatus* line was transferred to MS medium (pH6.8) containing 150 mM NaCl. About 15 days later, the transgenic *L. Corniculatus* plants had better growth than the wild plants. Therefore *GmPIP* may be involved into salt tolerance, and the enhanced expression of *GmPIP* gene may have caused the increased salt tolerance of soybean.

**Keywords:** *GmPIP*, salt tolerance, soybean, suppression subtractive hybridization (SSH).
**Abbreviations:** PIP_plasma membrane intrinsic protein; NPA_Asparagine-Proline-Alanine; MIP_major intrinsic protein; AQPs_ plant aquaporins; LEA_late embryogenesis abundant proteins; EST_expressed sequence tag; SSH_suppression subtractive hybridization; RT-PCR_reverse transcription polymerase chain reaction; HPTII_hygromycin phosphotransferase; PEG_polyethylene glycol; ABA_abscisic acid; GFP_green fluorescent protein; ORF_open reading frame.

## Introduction

Plants aquaporins are ubiquitous membrane channel proteins that facilitate and regulate the permeation of water across biological membranes, which play an important role in seed germination, cell elongation, stomatal movement, fertilization and so on. In plants, aquaporins (AQPs) are an ancient family of channel proteins. AQPs are present in the tonoplast, the plasma membrane, and possibly in other internal membranes (Barkla et al., 1999). According to sub-cellular localization combining with the homologies analysis, the aquaporins are classified into four types: plasma membrane intinsic proteins (PIPs), tonoplast intrinsic proteins, Nod26-like intrinsic proteins, and small and basic intrinsic proteins. But all sequences share structural motifs and conserved amino acids, six transmembrane helices connected by five loops (loops A-E). Loops A, C and E are extracellular and loops B and D are intracellular (Schäffner, 1998). The protein comprises two internal tandem repeats, covering roughly the amino- and carboxy-terminal halves of the protein. Each repeat consists of three transmembrane helices and a highly conserved loop following the second transmembrane helix (loops B and E, respectively). This loop includes a conserved signature motif, asparagine-proline-alanine (NPA). Aquaporins are members of the MIP family. Plasmamembrane intrinsic proteins (PIPs) belong to a subgroups in the plant MIP family (Schäffner, 1998; Johanson et al. 2001). The PIP group can be further divided into two subfamilies named PIP1 and PIP2, which are each highly conserved and share a high degree of homology (Schäffner, 1998). These water channel proteins aquaporins (AQPs) can transport water and neutral solutes across cell membranes, which is the major pathway of water uptake for plants. Whereas, many kinds of abiotic stress such as drought, salt stress, low temperatures can reduced or blocked water uptake (Munns, 2002). Some reports have showed differential gene expression of aquaporins in response to abiotic stresses, and that the aquaporins play important role in the response of plants to conditions that affect water availability. The rice PIP gene *OsPIP1; 3* (RWC3) was found to be up regulated in only the drought tolerant rice cultivar under water insufficiency (Lian et al., 2004). The abundance of total PIP1 proteins of both chilling-tolerant and -sensitive phenotypes was increased by chilling, and that of phosphorylated form of PIP2 increased dramatically in both phenotypes (Aroca et al., 2005), suggesting that activation of the protein by phosphorylation plays some role in the response process. Overexpression of a rapeseed (*Brassica napus*) PIP (*BnPIP1*) in transgenic tobacco plants has been shown to result in increased tolerance to water stress, while an antisense plant shows reduction in growth and germination and tolerance to water stress (Yu et al., 2005). In salt stress conditions, the rate of *AtPIP2; 1* cycling was enhanced and endocytosis was cooperated by a membrane raft-associated salt-induced pathway and a clathrin-dependent pathway (Alexandre et al., 2012). Two aquaporin genes (*PIP* and *TIP*) preferentially expressed in the salt gland cells were rapidly induced in response to increasing salt concentration,

which suggests that aquaporins are involved and contribute to the re-absorption of water during salt removal in Avicennia officinalis salt glands (Tan et al., 2013). *TdPIP1; 1* and *TdPIP2; 1* are differentially regulated in roots and leaves in the salt-tolerant wheat variety when challenged with salt stress and abscisic acid (Ayadi et al., 2011). *ZmPIP1; 1* protein amount under salt-stressed conditions was higher in inoculated leaves than in non-inoculated ones (Marulanda et al., 2010).

Soybean is an important cash crop and a major cause of its productivity loss is abiotic stress. Salinity is one of the major abiotic stresses. Although soybean is classified as a moderately salt-tolerant crop, the final yield of soybean will be reduced when soil salinity exceeds 5 dS $m^{-1}$ (Ashraf and Wu, 1994). To understand better the molecular basics of the salt tolerance complicated mechanism, people have identified and cloned some salt tolerance-related genes from soybean, and research the relation of some genes to salt tolerance in transgenic plants. Such as *GmNHX1* and *GmNHX2* (Li et al., 2006; Sun et al., 2006), LEA proteins (Phang et al., 2008; He et al., 2002; Cai et al., 2006), DREB type transcription factor family (Chen et al., 2007; Li et al., 2005), WRKY-type transcription factor family(Zhou et al., 2008), bZIP-like transcription factor family(Liao et al., 2008) and so on. However, the reports on plasma membrane intinsic proteins (PIPs) in soybean are less. By far, there is only one report that two PIP genes from soybean were cloned, *GmPIP1* and *GmPIP2*, their expression pattern was studied in arbuscular mycorrhisal (AM) and non-AM plants cultivated under well-watered or drought stressed conditions. But the function of the GmPIPs was unknown (Porcel et al., 2006).

In this study, an EST sequence was found out from the SSH library of a salt-tolerant soybean roots upon salt stress. The homologous comparing analysis showed that putative function involved into PIPs. The full-length cDNA sequence was forecasted by electronic elongation and cloned by RT-PCR. The protein owes the same structure as the AQPs family with six transmembrane helices connected by five loops, the loop B and E were conserved signature motifs, asparagine-proline-alanine (NPA). The function of the gene was analysis by soybean roots transformation system and the *Superroot* regeneration transformation system. The results showed that the sense overexpression of the gene can enhance the salt tolerance of the transgenic soybean hair roots and the transgenic *Lotus corniculatus* plants to some extent.

## Results

### *Cloning and sequence analysis of GmPIP cDNA*

One of the subtractive fragments (*GmPIP*) was identified from the SSH library for the root tissue of *Wenfeng7*, a salt-tolerant cultivar upon salt treatment. The gene might be involved into the plasma membrane intrinsic protein by BlastX in the GenBank non-redundant protein database. The full-length was forecasted by electronic elongation. There were 14 ESTs found out to joint and elongate the segment of *GmPIP*. An open reading frame (ORF) containing 861bp bases (GenBank HM245628) was got from the predicted sequence owed 1076bp bases. The primers were designed according to the end sequences of the ORF. RT-PCR was carried out to amplify the full-length of *GmPIP* in the roots of *Wenfeng7*, the products of expected size for the genes were verified by 1% agarose electrophoresis, and recall the target bands to sequence. The result of sequencing agreed with the forecast of *GmPIP*. The gene could was translated into 286 continuous amino acids containing a termination codon. Sequence comparison showed that the putative soybean *GmPIP* protein was homologous to plant aquaporin family proteins from other organisms. Its deduced amino acid sequence displayed 80.9%, 87.11%, 78.82% and 81.03% similarity with *GmPIP1* (aaa69490), *GmPIP2;2* (aax86046), *AtPIP1A* (np_190910) and *ZmPIP2-1* (np_001105024), respectively. The proteins were all of similar lengths and owned the similar Asn-Pro-Ala (NPA) and MIP conserved domains (Fig.2).

To understand the relationships between the GmPIP and the other plant aquaporin family protein, the phylogenetic tree was built using the DNAMAN5.0 program (Fig.3). The result showed that GmPIP was closer to GmPIP2; 2, GmPIP1, AtPIP1A and ZmPIP2; 1 than the others, which indicted that GmPIP belonged to plasma membrane intrinsic proteins in plant aquaporin families.

### *GmPIP transcript abundance was increased by salt in soybean roots*

The abundance of *GmPIP* transcript was examined under 0.9% (W/V) NaCl treatment at 48h by semiquantitative RT-PCR. The *GmPIP* mRNA was present examined in roots and leaves of *Wenfeng7*, *Union* (Fig. 4). The mRNA abundance of *GmPIP* was increased upon salt treatment in roots of *Wenfeng7*, but in leave almost unaltered. However, the mRNA abundance of *GmPIP* in the roots of *Union* under salt treatment was similar with that under non-salt treatment. In the leaves of *Union* the abundance of *GmPIP* was increased under salt treatment. The increased abundance of *GmPIP* in the roots of a salt-tolerant cultivator, *Wenfeng7* appeared to be expected for a gene related to salt tolerance of soybean.

### *Validation of the gene function by the transgenic hairy roots of soybean*

The soybean hairy roots survived in solid MS medium with 14mg $L^{-1}$ HPTII were identified as being transgenic by GFP detection (Fig. 5A) and PCR analysis. The PCR was performed with primers designed to *GFP* fragments, and the results showed the presence of *GFP* (Fig. 5B) bands of the expected sizes (641 bp) in the corresponding transgenic samples and their absence in the negative controls, indicating that all the positively transgenic hairy roots contained the *GFP* genes.

Six soybean transgenic hairy roots induced by K599 with pGFP*GmPIP* (S) and pGFP*GmPIP* (A) identified as being transgenic by GFP detection and PCR analysis were respectively transferred to solid MS mediums, which contained 0, 50, 100, 150 and 200mM NaCl, the hairy roots induced by K599 were used as control (Fig.6). The experiment was repeated five times. After salt treatment for 15 days, statisical comparison of the survival quantity of the hairy roots was carried out. Upon the 100 mM NaC1 treatment, the transgenic *GmPIP* (A) hairy roots and the control started to die, but the transgenic *GmPIP* (S) hairy roots all survived. Upon the 150 mM NaC1 treatment, about 78% of the transgenic *GmPIP* (S) hairy roots and 28% of the control survived, proportion of the survival transgenic *GmPIP* (A) hairy roots was only about 6%. About 40% of the transgenic *GmPIP* (S) hairy roots and 11% of the control survived, but all of the transgenic *GmPIP* (A) hairy roots were dead upon the 200 mM NaCl treatment (Table 1). The results indicated that the salt tolerance of the transgenic *GmPIP* (S) hairy roots was increased, but the *GmPIP* (A) decreased the salt tolerance of the transgenic hairy roots comparing with the control upon the 150 mM NaCl treatment.

**Table1.** Survival proportation of transgenic hairy roots and control under various NaCl concentrations.

| The transgenic hairy roots | NaCl (mM) treatment | | | | |
|---|---|---|---|---|---|
| | 0 | 50 | 100 | 150 | 200 |
| GmPIP(S) | 1.0000 | 1.0000 | 1.0000 | 0.7778a | 0.3889a |
| CK | 1.0000 | 1.0000 | 0.7666 | 0.3888b | 0.1111b |
| GmPIP(A) | 1.0000 | 0.8889 | 0.7778 | 0.0556c | 0.0000c |

(Note: a, b, c indicated that the probability of a significant level of 0.05; GmPIP (S), the transgenic hairy roots with sense GmPIP; GmPIP (A), the transgenic hairy roots with sense GmPIP; CK, the hairy roots induced by A. rhizogenes harboring without binary vector)

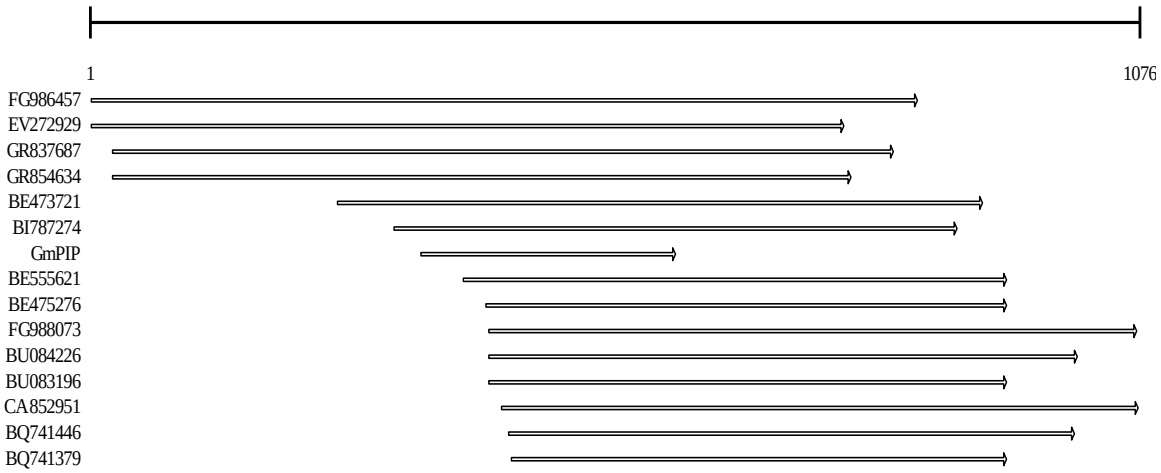


**Fig 1.** Electronic elongation of GmPIP in soybean. Fourteen the high homology ESTs with GmPIP EST from the soybean EST data was found out by the Blastn program, and used to elongate the GmPIP EST. A full-length sequence (1076bp) was predicted. GenBank ID: FG986457, EV272929, GR837687, GR854634, BE473721, BI787274, BE555621, BE475276, FG988073, BU084226, BU083196, CA852951, BQ741446, BQ741379.

***Validation of the gene function by the transgenic Superroot-derived L. corniculatus plants***

The hairy roots identified as being transgenic by GFP detection (Fig. 7A) were transferred to in solid MS medium with 4mg $L^{-1}$ HPTII for shoot induction. PCR analysis of the regenerated plants was performed with primers designed to amplify *GmPIP*. The PCR results showed the presence of *GmPIP* bands of the expected sizes (861bp) in the corresponding transgenic samples and their absence in the negative controls (Fig. 7C). To further verify gene transfer, GFP expression was monitored in leaves of the whole plantlets regenerated from the hairy roots (Fig. 7B). Southern blot analysis was also carried out to identify the transgenic events. Genomic DNA of regenerated plants and the pGFEGUS*plus* vector DNA as positive control were digested with *Hind* III which cuts at a single site within the T-DNA. Restriction-digested DNA was then blotted and hybridized with digoxigenin (DIG)-labelled *GmPIP* full-length sequence as a probe. The five randomly selected regenerated plants showed a different single integration event of the T-DNA, thereby confirming their independent transgenic nature. Nohybridization signal was observed in the control plant (Fig. 7D). In order to rapidly obtain a large number of transgenic *GmPIP* plants for salt tolerance assays. Six plantlets regenerated from individual hairy root randomly selected were cut into stem segments with one or two nodes and then inserted into MS medium for rooting. After 10–13 days, 90% segments produced roots. To test the salt tolerance of transgenic *Superroot* plants overexpressing *GmPIP*, ten plantlets of each independent transgenic *L. corniculatus* line and the negative control were used. An example is shown in Figure 7E, most of the control plants grown for 15 days on MS medium (pH 5.8) containing 150 mM NaCl bleached, roots were stunted and plants were arrested in their growth. In contrast, the transgenic *Superroot* plants over-expressing *GmPIP* survived and exhibited healthy growth (Fig. 7E).

**Discussion**

In a search for salt-inducible genes in soybean using suppression subtractive hybridization techniques, we cloned a novel PIP-like gene. We named this gene *GmPIP* to distinguish it from the two other PIP-like genes reported previously in soybean (Porcel et al., 2006). Three major characteristics of this PIP suggest that it is indeed a member of the PIP family: (1) it exhibits high homology to classical PAPs such as *GmPIP1* (80.9%), *GmPIP2; 2* (87.11%), *AtPIP1A* (78.82%) and *ZmPIP2-1* (81.03%), (2) it possesses all amino acid residues (Fig. 2) that are conserved in all PAPs, (3) phylogenetic analysis showed that GmPIP was closer with the PIP proteins than the other proteins (Fig.3). Another unique feature of *GmPIP* is that it is strongly induced by NaCl stress. Our results showed that NaCl stress causes a general induction of *GmPIP* expression in roots of *Wenfeng7*, but down-regulation of the expression of the *GmPIP* gene in roots of Union (Fig. 3). The expression pattern of *GmPIP1* and *GmPIP2* found in soybean is decreased by drought stress(Porcel et al., 2006), and PIP2 are more active in water flow across plasma membranes than PIP1(Chaumont et al., 2000; Fetter et al., 2004; Bots et al., 2005). In the other plants, there are many reports that the expression of some PIPs genes was regulated up or down under all kinds of abiotic stresses. The treatment such as low temperature 4℃, 150mM NaCl stress and drought could decreased the gene *OsPIP1; 1* expression (Sakurai et al., 2005; Li et al., 2000), but the expression of *OsPIP1; 1* was regulated up under the treatment with 10% PEG and low temperature 7°C (Guo et al., 2006; Yu et al., 2006). The gene *OsPIP2; 2* expression was increased by the 150 mM NaCl stress and 10%


**Fig 2.** Alignment of the deduced amino acid sequence of *GmPIP* with that of plant homologues. Sequences and their accession numbers are listed as follows: *GmPIP1* (AAA69490), *GmPIP2; 2* (AAX86046), *AtPIP1A* (NP_190910) and *ZmPIP2-1* (NP_001105024). Identical amino acid residues are shadowed in black, and similar amino acid residues are shadowed in purple.

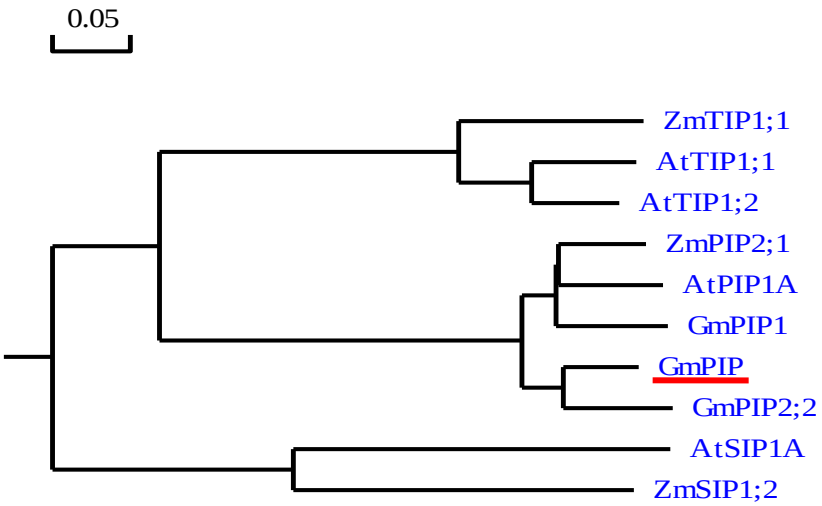


**Fig 3.** Phylogenetic relationships between the *GmPIP* and some other plant AQP families. The tree was generated by the software DNAMAN5.0, including GmPIP and its homologues: ZmTIP1; 1 (np_001104896), AtTIP1; 1 (P25818), AtTIP1; 2 (Q41963), GmPIP1 (AAA69490), GmPIP2; 2 (AAX86046), AtPIP1A (NP_190910), ZmPIP2-1 (NP_001105024), AtSIP 1A (NP_187059) and ZmSIP1; 2 (Q9ATM2). The analyzed polypeptides were divided into three major groups, plant TIP proteins (Group I), plant PIP proteins, plant SIP proteins (Group III).

PEG (Guo et al., 2006; Kawasaki et al., 2001). The expression of *ZmPIP2; 4* and *ZmPIP1; 1* was regulated up by 100mMNaCl stress. The reports indicated that the structure and physical composition of the water channel proteins might be changed by the abnormal expression of the PIPs genes to adapt the abiotic stresses. In this study, the mRNA abundance of *GmPIP* was increased upon salt treatment in roots of *Wenfeng7*, which suggested that the physiological role of *GmPIP* might be related to the adaptation to NaCl stress. To validate the function of *GmPIP*, sense and antisense expression vectors of *GmPIP* were constructed, *GmPIP*(S) and *GmPIP*(A) were transferred into the soybean hairy roots. Statisical comparison of the survival quantity of the transgenic hairy roots showed that the percent of the survived transgenic *GmPIP* (S) hairy roots was higher than that of the control, and the percent of the control was higher than that of survived transgenic *GmPIP* (A) hairy roots was higher than upon the 150 mM NaC1 treatment, which indicated that overexpression of *GmPIP* could increase the tolerance of the soybean hairy roots. Bcause of that the activity of *GmPIP* proteins enhanced by overexpression of *GmPIP* may facilitate the water absorption of roots, which maintains the osmotic pressure of the cells to avoid the salt injure, but the activiy of PIP proteins was decresed when the *GmPIP* (A) was transferred into the hairy roots to make the intrinsic GmPIP silence. The test of the salt tolerance of transgenic *Superroot* plants overexpressing *GmPIP* showed that most of transgenic plants survived and exhibited healthy growth comparing with the control plants under the treatment with 150mM NaCl for 15days. The result agreed with that of the transgenic soybean hairy roots research, the gene *GmPIP* was a plasma membrane intinsic protein gene related to the salt tolerance of plants, the overexpression of the gene could increased the tolerance of the transgenic soybean hairy roots and *Superroot* plants to some extent.

## Materials and Methods

### *Plant materials, growth conditions and treatments*

Two soybean cultivars (*Glycine max* L. Merr.), *Wenfeng7*

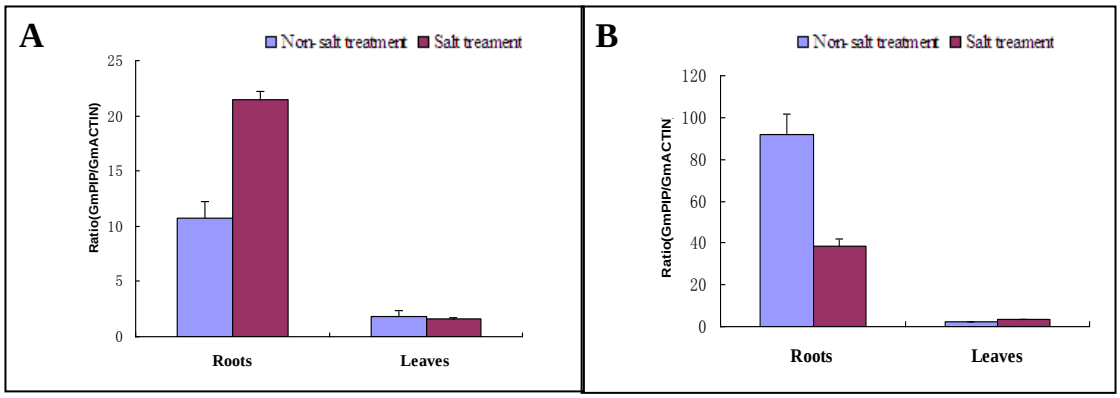


**Fig 4.** The expression of GmPIP under salt treatment(■) and non-salt treatment (■). A. The expression of GmPIP in Wenfeng7; B. The expression of GmPIP in Union. Values are the means of three replicates ± S.E.

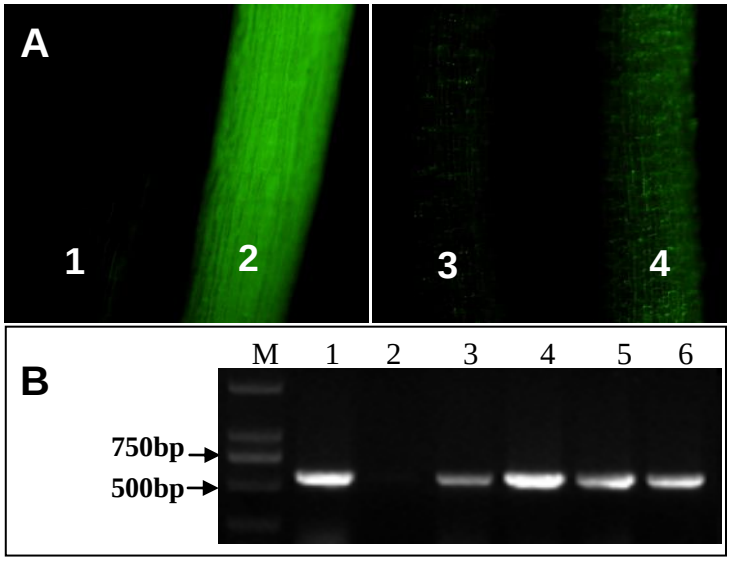


**Fig 5.** GFP detection of hairy roots. (A) GFP-derived fluorescence detected by confocal laser scanning microscopy in a transgenic hairy root (1 and 3, negative hairy roots; 2 and 4, transgenic hairy roots); (B) PCR-amplification of GFP (M, DNA marker; 1, plasmid DNA; 2, negative control; 3-6, transgenic hairy roots).

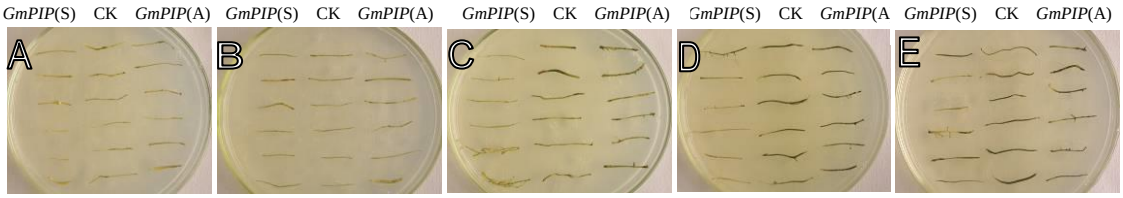


**Fig 6.** Salt tolerance of transgenic hairy root on solid culture medium. GmPIP(S): transgenic hairy roots with sense GmPIP; GmPIP(A): transgenic hairy roots with antisense GmPIP; A: 0 mM NaCl; B: 50 mM NaCl; C: 100 mM NaCl; D: 150 mM NaCl; E: 200 mM NaCl

(salt-tolerant) and *Union* (salt-sensitive), were grown with the temperature ranging from 24 to 27 °C, the humidity ranging from 60 to 80% and a short-day photoperiod (12h light/12h dark) in the greenhouse. The seeds were germinated in vermiculite and irrigated with deionized water. The seedlings with the first pair of fully expanded leaves were used in the following treatments. For NaCl treatment, the roots of the seedlings were immersed in half strength Hoagland's solution containing 0.9% (W/V) NaCl or nothing for control.

***Total RNA and mRNA isolation***

The roots and leaves were then collected for total RNA extraction at 48 h after the treatment, frozen in liquid nitrogen, and then kept at -70℃. Frozen samples were ground in liquid nitrogen. Total RNA was isolated using TriZOL reagent (GIBCOL/BRL) according to the manufacturer's instructions. For suppression subtracted hybridization, equal amounts of total RNA for each sample from treatment or control were mixed and the mRNA was purified from the mixed total RNA using the Oligotex mRNA Min Kit (QIAGEN) according to the manufacturer's protocol.

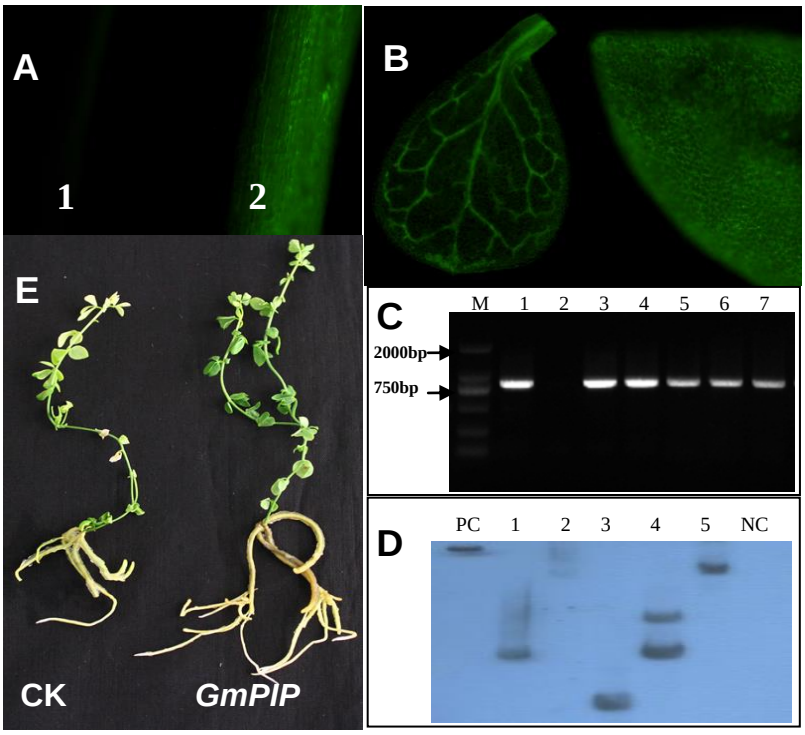


**Fig 7.** Analysis of transgenic GmPIP events. (A): GFP-derived fluorescence detected in hairy root by laser scanning confocal microscopy. 1, a negative control hairy root; 2, a transgenic hairy root; (B): GFP-derived fluorescence detected in leaves by laser scanning confocal microscopy; (C): PCR-amplification of GmPIP from regenerated plants. M, DNA marker; 1, plasmid DNA; 2, negative control; 3–7, transgenic regenerated plants; (D): Southern blot of the L. corniculatus cv. Superroot plants transgenic lines. PC, Hind III-digested pGFPGUSPlus plasmid DNA; NC, negative control plant; 1-5, transgenetic regenerated plants; (E): Phenotypes of representative GmPIP transgenic and control (CK) L. corniculatus plants after treatment with 150 mM NaCl for 15 days. All negative control plants were regenerated from hairy roots developed by A. rhizogenes without binary vector.

The quantity and quality of isolated total RNA were examined by spectrophotometry and gel electrophoresis, respectively.

***Molecular cloning, phylogenetic analysis***

The CLONTECH PCR-Selectk cDNA Subtraction Kit (Clontech K1804-1) was employed to construct the WF subtractive library. All procedures were performed according to the manufacturer's recommendations. The tester and driver cDNAs were originated from the roots of NaCl-treated and control WF plants, respectively. Selected subtractive fragments were cloned into pMD18-T (TAKARA), and the ligation products were then transformed into DH5a (TAKARA) by thermal stimulation method. Three-hundred and eighty four colony PCR products from SSH1 were dotted onto two Hybond-$N^+$ nylon membranes (Amersham) by Biomek2000 Laboratory Automation Workstation (Becman Coulter), one for forward and the other for reverse SSH library, were prepared to hybridize with the digoxygenin (DIG)-labeled subtracted cDNA probes. Some differential expression subtractive fragments were sequenced, all the sequences were analyzed by Blast program in the website of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). One of the subtractive fragments (*GmPIP*) was identified as a PIP-like gene based on its high homology to other known PIP genes. Blast2.2.16 software package was downloaded from the website (ftp://ftp. Ncbi. Nih. gov/blast/executables/release/ 2.2.16/), the soybean EST data were download from the website (center for Biotechnology Information, NCBI), and the soybean genome data were download from the website (ftp.://ftp.jgi-psf.org/pub/JGI_ data/Glycine_max). The Blastn program was used to find out the high homology ESTs with *GmPIP* EST from the soybean EST data, 14 ESTs were got and joined to elongate the *GmPIP* EST. A full-length sequence (1076bp) was predicted, which contain an open reading frame (861bp) with an initiation codon (ATG) and a termination codon (TGA) (Fig.1). The primers were designed according to the predicted 5' and 3' end sequence, Forward prime: 5'-ATGGCTAAAGATGTTGA- GG-3', Reverse prime: 5'-TCAAGCGTTGCTCCTGAA-3'. The RT-PCR was carried out to clone the *GmPIP* gene, the program was 35 cycles: 94 ºC for 30S, 56 ºC for 30S, 72 ºC for 50S. Moreover, phylogenetic relationships of GmPIP with other PIPs were analyzed, and multiple sequence alignment was performed using DNAMAN program (version 2.1). In addition, the bootstrap value was calculated, and the phylogenetic tree was built using DNAMAN program.

***Real-Time PCR analysis***

SYBR Green (Takara, Japan) RT-PCR were conducted to analyze the expression regulation of it in roots and leaves of a salt-tolerant cultivator, Wenfeng7 and a salt-sensitive cultivator, Union under salt and without salt treatment, *GmACTIN* was used for internal standard gene. Primers of the candidate genes were designed by Primer5 program and synthesized by Tsingke, China as forward prime: 5'-GCTGAGTTCATAGCAAC-CCTT-3', and reverse prime: 5'- CGCCCTTATCAAC-GACAC-3'. PCR conditions are 40 cycles: 95 ºC for 5s, 52 ºC

for 15s, 72 ºC for 25s. Real-Time PCR experiments were repeated three times in the Bio-Rad iCycler PCR.

***Bacterial strain and Construction of the binary vectors***

This binary vector pGFPGUS*Plus* has two reporter genes: β-glucuronidase (uidA) and *GFP*, both of which are controlled by the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The hygromycin phosphotransferase (HPTII) gene was also located between T-DNA borders, allowing the hygromycin selection of positive transformants. The restriction enzyme cutting site *Spe* Ⅰ was jointed to the 5'-end, and *BsTE* Ⅱ was jointed to the 3'-end of *GmPIP* by PCR. The following sets of primer; 5'- GCGACTAGTATGGCTAAAGATGTTGAGG-3'(forward), 5'-CCGGGTNACCTCAAGCGTTGCTCCTGAA-3'(reverse) pairs were used for amplification. The sense expression vector of *GmPIP*, pGFP*GmPIP* (S) was constructed by that the *GUS* report gene in the vector pGFPGUS*Plus* was replaced by GmPIP with the new restriction enzyme cutting sites. *BsTE* Ⅱ was jointed to the 5'-end, *Spe* Ⅰ was jointed to the 3'-end of *GmPIP* by PCR. The following sets of primer pairs; 5'- GGGCGGTNACCATGGCTAAAGATGTTGAGG -3'(forward), 5'-CGCACTAGTTCAAGCGTTGCTCCTGAA-3' (reverse) were used for amplification. The anti-sense expression vector of *GmPIP*, pGFP*GmPIP* (A) was constructed by the same way. Cucumopine-type *A. rhizogenes* strain K599 with pGFP*GmPIP* (S) and pGFP*GmPIP* (A) was used to transform. *A. rhizogenes* K599 which lacks the binary vector was used as negative control for the transformation.

***Soybean hairy roots induction***

*A. rhizogenes* K599 with pGFP*GmPIP* (S) and pGFP*GmPIP* (A) was grown in dark at 28°C were inoculated in liquid LB medium (50mL) containing 50 mg $L^{-1}$ kanamycin, and stopped culturing when $OD_{600}$ was about 0.8. The undamaged seeds of *Zigongdongdou* were surface-sterilized for 16h using chlorine gas produced by mixing 3.5 mL of 12N HCl and 100 mL commercial bleach in a tightly sealed desiccator. The sterilized seeds were germinated in *Gm* medium. After 4 days cotyledons were immerged into the *A. rhizogenes* culture at 25°C for 30min. The explants were dried on sterile filter paper and then transferred to CCM which was covered with a sterile filter paper. The CCM consisted of 1/10 MS, 3.9 g $L^{-1}$ morpholino ethanesulfonic acid, 150 mg $L^{-1}$ cysteine and 150mg $L^{-1}$ dithiothreitol. After co-cultivation 5 days, the explants were washed three times with sterile water and immerged in liquid 1/2 MS (pH5.8) medium containing 250 mg $L^{-1}$ cephalexin and 250 mg $L^{-1}$ carbenicillin. The blot-dried explants were transferred to solid MS medium (pH5.8) with 250 mg $L^{-1}$ cephalexin, 250 mg $L^{-1}$ carbenicillin and 14mg $L^{-1}$ HPTII for hairy root induction.

***Hairy root induction and plant regeneration of L. corniculatus***

*A. rhizogenes* K599 with pGFP*GmPIP* (S) was grown in dark at 28°C were inoculated in liquid LB medium (20mL) containing 50 mg $L^{-1}$ kanamycin, and stopped culturing when $OD_{600}$ was about 0.6-0.8. After a 2-day preculture, segments of *L. corniculatus* cv. *Superroot* plants were immerged into the *A. rhizogenes* culture and shook (50 rpm) at 25°C for 30min. The explants were dried on sterile filter paper and then transferred to CCM which was covered with a sterile filter paper at 22°C for 2 days. After co-cultivation, the explants were washed three times with sterile water and immerged in liquid ½ MS medium containing 250 mg $L^{-1}$ cephalexin and 250 mg $L^{-1}$ carbenicillin. The blot-dried explants were transferred to solid 1/2 MS medium (PH 6.8) with 250 mg $L^{-1}$ cephalexin, 250 mg $L^{-1}$ carbenicillin and 4mg $L^{-1}$ HPTII for hairy root induction (Jian et al., 2009). Two-week old hairy roots, which were produced at the wounding sites of explants after they were transferred to solid ½ MS medium, were cut off and placed onto RM for shoot induction. The shoots were transferred to hormone free solid MS medium for shoot elongation and rooting.

***PCR Detection of GFP and GmPIP***

Genomic DNA was extracted from the soybean hair roots and *GFP* fragment was amplified by PCR to detect the transgenic events. The following set of primer pair; 5'-GTAAACGGCCACAAGTTCAGCG-3' (forward), 5'-TCGTCCATGCCGAGAGTGATCC-3' (reverse) was used for amplification. Plasmid DNA was used as template for the amplification of positive control. Genomic DNA of soybean hair roots induced by *A.rhizogenes* K599 harbouring without binary vector was used as negative control. The PCR conditions were as follows: 10min at 94°C and 35 cycles of 30 s at 94°C, 50s at 62°C and 45 s at 72°C, followed by a final extension at 72°C for 10 min. Genomic DNA was extracted from the regenerated *L. corniculatus* plants and *GmPIP* was amplified by PCR. The following set of primer pair was used for amplification. Forward primer: 5'-ATGGCTAAAGATGT-TGAGG-3' (forward), reverse primer: 5'-TCAAGCGTTGC-TCCTGAA-3'(reverse). The pGFP*GmPIP* DNA was used as template for the amplification of positive control. Genomic DNA of regenerated plants derived from hairy roots induced by *A. rhizogenes* K599 harbouring no binary vector was used as negative control. The PCR conditions were as follows: 10min at 94°C and 35 cycles of 30 s at 94°C, 50s at 56°C and 45 s at 72°C, followed by a final extension at 72°C for 10 min. The products of expected size for the genes were verified by 1% agarose electrophoresis and SYBR Green staining.

***Southern blot analysis***

Genomic DNA was extracted from regenerated *L. corniculatus* plants. Approximately 30μg of DNA from each sample was digested overnight with *Hind* III. 200 ng *Hind* III digested pGFPGUS*Plus* plasmid DNA was used as positive control. DNA fragments were separated by electrophoresis in 0.7% agarose gel, transferred to a Hybond-$N^+$ (Amersham Biosciences) nylon membrane, and cross-linked by baking at 120°C for 30min. The DIG-dUTP (Roche) labelled *GmPIP* gene was used as a probe and hybridization was carried out according to the manufacture's instruction of DIG high prime DNA labeling and detection starter kit II (Roche).

***Histochemical localization of GFP expression***

GFP expression of the soybean hairy roots and regenerated *L. corniculatus* plants was detected by confocal laser scanning microscope (Leica, TCS SP2). The soybean hairy roots and a regenerated plant induced by *A. rhizogenes* harboring without binary vector were used as negative control.

***Assess the salt tolerance of the soybean transgenic hair roots***

Six positive soybean transgenic hair roots were selected respectively from that induced by *A. rhizogenes* K599 with pGFP*GmPIP* (S) and pGFP*GmPIP* (A) to be transferred to MS mediums containing 0mM, 50mM, 100mM, 150mM and 200mM NaCl, and six hair roots induced by *rhizogenes* harboring no binary vector were selected to be negative control.

After the 15 days observe the development and statistical analyze the survival proportation of the hair roots by Data Processing System (DPS).

***Assess the salt tolerance of transgenic Superroot-derived L. corniculatus plants***

Individual positive transgenic *GmPIP* plants identified by *GFP* detection and also southern blot detection were cut into stem segments with one or two nodes and then inserted into MS medium for rooting. After about ten days, 90% segments produced roots. A regenerated plant from hairy root developed by *A. rhizogenes* harboring no binary vector was used as negative control. Six plantlets of each independent transgenic *L. corniculatus* line and the negative control were transferred to MS medium (pH6.8) containing 150 mM NaCl. About 15 days later, the distinguishing phenotypes between the transgenic *GmPIP* plants and negative control plants were assessed.

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**References**

Alexandre M, Xiaojuan L, John R, Jinxing L, Christophe M, Doan-Trung L (2012) Salt stress triggers enhanced cycling of Arabidopsis root plasma-membrane aquaporins. Plant Signal Behave 7:4, 529–532

Aroca R, Amodeo G, Fernandez-Illescas S, Herman EM, Chaumont F, Chrispeels MJ (2005) The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. Plant Physiol 137(1):341-353

Ashraf M, Wu L (1994) Breeding for salinity tolerance in plants. Crit Rev Plant Sci 13(1):17-42

Ayadi M, Cavez D, Miled N, Chaumont F, Masmoudi K (2011) Identification and characterization of two plasma membrane aquaporins in durum wheat (Triticum turgidum L. subsp. durum) and their role in abiotic stress tolerance. Plant Physiol Biochem 49(9):1029-1039

Barkla BJ, Vera-Estrella R, Pantoja O, Kirch HH, Bohnert HJ (1999) Aquaporin localization–how valid are the TIP and PIP labels. Trends Plant Sci 4(3):86-88

Bots M, Vergeldt F, Wolters-Arts M, Weterings K, Van As H, Mariani C (2005) Aquaporins of the PIP2 class are required for efficient anther dehiscence in tobacco. Plant Physiol 137(3):1049-1056

Cai D, Zheng Y, Lan Y (2006) Expression of Em gene (LEA1) from soybean immature seeds confers salt tolerance to Escherichia coli and tobacco plants. J Shenzhen Univ (Sci. Eng.) **23**, 230–236

Chaumont F, Barrieu F, Jung R, Chrispeels MJ (2000) Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol 122(4):1025-1034

Chen M, Wang QY, Cheng XG, Xu ZS, Li LC, Ye XG, Xia LQ, Ma YZ (2007) GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. Biochem Biophys Res Comm 353(2):299-305

Fetter K, Van Wilder V, Moshelion M, Chaumont F (2004) Interactions between plasma membrane aquaporins modulate their water channel activity. Plant Cell Online 16(1):215-228

Guo L, Wang ZY, Lin H, Cui WE, Chen J, Liu M, Chen ZL, Qu LJ, Gu H (2006) Expression and functional analysis of the rice plasma-membrane intrinsic protein gene family. Cell Res 16(3):277-286

Jian B, Hou W, Wu C, Liu B, Liu W, Song S, Bi Y, Han T (2009) Agrobacterium rhizogenes-mediated transformation of Superroot-derived *Lotus corniculatus* plants: a valuable tool for functional genomics. BMC Plant Biol 9:78

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjovall S, Fraysse L, Weig AR, Kjellbom P (2001) The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 126(4):1358-1369

Katsuhara M, Hanba YT, Shiratake K, Maeshima M (2008) Expanding roles of plant aquaporins in plasma membranes and cell organelles. Funct Plant Biol 35(1):1-14

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13(4):889-905

Li LG, Li SF, Tao Y, Kitagawa Y (2000) Molecular cloning of a novel water channel from rice: its products expression in Xenopus oocytes and involvement in chilling tolerance. Plant Sci 154:43-51

Li WYF, Wong FL, Tsai SN, Phang TH, Shao G, Lam HM (2006) Tonoplast-located *GmCLC1* and *GmNHX1* from soybean enhance NaCl tolerance in transgenic bright yellow (BY)-2 cells. Plant, Cell & Environment 29(6):1122-1137

Li XP, Tian AG, Luo GZ, Gong ZZ, Zhang JS, Chen SY (2005) Soybean DRE-binding transcription factors that are responsive to abiotic stresses. Theor Appl Genet 110(8):1355-1362

Lian HL, Yu X, Ye Q, Ding XS, Kitagawa Y, Kwak SS, Su WA, Tang ZC (2004) The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol 45 (4):481-489

Liao Y, Zhang JS, Chen SY, Zhang WK (2008) Role of soybean GmbZIP132 under abscisic acid and salt stresses. J Integr Plant Biol 50(2):221-230

Marulanda A, Azcón R, Chaumont F, Ruiz-Lozano JM, Aroca R (2010) Regulation of plasma membrane aquaporins by inoculation with a Bacillus megaterium strain in maize (Zea mays L.) plants under unstressed and salt-stressed conditions. Planta 232(2):533-543

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25(2):239-250

Phang TH, Shao G, Lam HM (2008) Salt tolerance in soybean. J Integr Plant Biol 50(10):1196-1212

Porcel R, Aroca R, Azcón R, Ruiz-Lozano JM (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. Plant Mol Biol 60(3):389-404

Sakurai J, Ishikawa F, Yamaguchi T, Uemura M, Maeshima M (2005) Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant Cell Physiol 46(9):1568-1577

Schäffner AR (1998) Aquaporin function, structure, and expression: are there more surprises to surface in water relations? Planta 204(2):131-139

Sun Y, Wang D, Bai Y, Wang N, Wang Y (2006) Studies on the overexpression of the soybean *GmNHX1* in *Lotus corniculatus*: the reduced $Na^+$ level is the basis of the increased salt tolerance. Chinese Sci Bull 51(11):1306-1315

Tan WK, Lin Q, Lim TM, Kumar P, Loh CS ( 2013) Dynamic secretion changes in the salt glands of the mangrove tree species Avicennia officinalis in response to a changing saline environment. Plant Cell Environ 36(8):1410-1422

Yu Q, Hu Y, Li J, Wu Q, Lin Z (2005) Sense and antisense expression of plasma membrane aquaporin *BnPIP1* from *Brassica napus* in tobacco and its effects on plant drought resistance. Plant Sci 169(4):647-656

Yu X, Peng YH, Zhang MH, Shao YJ, Su WA, Tang ZC (2006) Water relations and an expression analysis of plasma membrane intrinsic proteins in sensitive and tolerant rice during chilling and recovery. Cell Res 16(6):599-608

Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, Wang CM, Wang HW, Zhang JS, Chen SY (2008) Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J 6(5):486-503