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Cloning and expression analysis of a stress-induced *GmIMT1* gene in soybean (*Glycine max*)

H.T. Wang, N. Guo, J.M. Zhao, A. Karthikeyan, D. Xue, C.C. Xue, J.Y. Xu, Z.H. Xu, J.Y. Gai and H. Xing

Soybean Research Institute/National Center for Soybean Improvement/ National Key Laboratory for Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing, Jiangsu, China

Corresponding author: X. Han
E-mail: hanx@njau.edu.cn

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ABSTRACT. Here, we aimed to clone and identify the *GmIMT1* gene related to the salt stress response in soybean. The full-length cDNA sequence of the *GmIMT1* gene was amplified in soybean using degenerate primers of *Mesembrythmum crystallium*. To understand the stress response, the *GmIMT1* gene was cloned and sequenced. Then, the expression vectors of the gene were constructed, and introduced into the model plant *Arabidopsis thaliana* through *Agrobacterium* mediated transformation, and the salt tolerance was analyzed in the transgenic plants. In addition, the expression patterns of *GmIMT1* gene in soybean were analyzed. The expression was examined in different organs (roots, leaves, flower seeds, and stem) and under different stress conditions (drought, high salt, low temperature, salicylic acid, ethane, abscisic acid, and methyl jasmonate) by real-time fluorescent quantitative polymerase chain reaction analysis. The results showed that the root,

leaves, and stems exhibited high level of *GmIMT1* gene expression, whereas there was no expression in the seeds. In addition, the *GmIMT1* gene expression was upregulated under all stress conditions. Overall, the results clearly indicate that *GmIMT1* might be involved in multiple plant response pathways to the different environmental conditions. Furthermore transgenic plants exhibited higher salt-tolerance compared to wild type plants.

Key words: Abiotic stress; Genetic engineering; *GmIMT1* gene; Soybean

INTRODUCTION

Abiotic stress caused by salinity is one of the major threats to crop yields worldwide, with about one third of the world's irrigated land being affected by salt. The development of salt tolerant crops presents an ongoing challenge, because this trait is complex both genetically and physiologically (Liu et al., 1998; Yamaguchi and Blumwald, 2005; Tuberosa and Salvi, 2006). For soybean [*Glycine max* (L.) Merrill], abiotic stress is an inescapable and persistent condition. Water stress caused by high salinity is the most widespread abiotic stress, and constitutes the most stringent factor limiting soybean productivity. Soybean cultivars exhibit differential tolerance to salinity during seed germination and plant growth (Hao et al., 2011). It has been reported that the differential effects of salinity on growth rates and photosynthesis might be associated with the specific ion toxicity of plants. Therefore, salinity management has always been one of the main objectives of soybean improvement programs (Valliyodan and Nguyen, 2008).

To reduce the plant losses caused by salinity, plant biologists have adopted numerous methods to engineer salt-tolerant plants. In recent years, efforts have been directed toward the development of plants tolerant to high salinity stress through genetic engineering. Different approaches, based on genetic and molecular studies, have shown that many genes, proteins, and metabolites are modulated in a very complex network of plant adaptations to salt stresses (Qifeng and Guanyi, 2000). The *Imt1* gene encodes myo-inositol-*O*-methyltransferase (Imt1), which uses *S*-adenosyl-*L*-methionine to methylate myo-inositol to form *D*-ononitol. This gene was identified from the facultative CAM (Crassulacean acid metabolism) plant (*Mesembrythmum crystallium*), and is induced by salt stress, drought, and low temperature (Vernon and Bohnert, 1992). The molecular and physiological functions of this gene were first studied in *M. crystallium* and *Escherichia coli* (Vernon and Bohnert, 1992; Rammesmayer et al., 1995). Previous studies showed that, under salt stress conditions, the *Imt1* gene induces inositol substrate to generate a polyhydroxy sugar alcohol compound, ononitol. This compound reduces physiological drought caused by the loss of water, and improves the salt tolerance of plants (Zhu et al., 2012). Transgenic tobacco plants carrying a cDNA encoding *Imt1* gene accumulated *D*-ononitol and, as a result, acquired enhanced photosynthesis protection and increased plant recovery under drought and salt stress conditions (Sheveleva et al., 1997). Subsequently, Yunzhou (1999) and Yunzhou and Xueyan (2000) also reported that *Imt1* gene expression improves salt tolerance in tobacco plants. Based on the results of these studies, the present investigation focused on the cloning and expression of the *GmIMT1* gene related to the salt stress response in soybean.

MATERIAL AND METHODS

Plant materials, growth stages, and treatments

The soybean cultivar Qi Huang 22 and *Arabidopsis thaliana* (Col-0) seeds were used in this study. First, the soybean cultivar Qi Huang 22 was grown in the Jiangsu experimental station. The leaves, stem, root, flower, and seeds were sampled at 15 and 25 days after flowering (DAF) to analyze *GmIMT1* gene expression by real-time fluorescent quantitative PCR. All fresh organ samples were immediately frozen in liquid nitrogen, and stored at -80°C. For the salt stress treatments, soybean seeds were washed with distilled water, and sown in pots. Then, after growing 2 leaves, the plants were transferred to Hoagland nutrient solution, and maintained in a growth chamber. After a further 2 weeks of growth, various drought stresses [200 mM salt (NaCl), 10% PEG 6000, 200 μM abscisic acid (ABA), 40 μM ethane (ET), 2 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), and 4°C treatment] were given to the plants two weeks. Then, leaf samples were collected at 0, 0.5, 1, 3, 6, 12, 24, and 36 h after treatment, immediately frozen in liquid nitrogen, and stored at -80°C.

RNA isolation and cDNA first strand synthesis

Total RNA was extracted by the TRIZOL method using a total RNA isolation kit (Tiangen, Beijing, China). The quality of the RNA was checked on 1.5% agarose gel, by visualizing the extent to which RNA bands were intact electrophoresis on agarose gel. Then, RNA was quantified by a Nanodrop Spectrophotometer (Thermo Fisher China Ltd., Shanghai, China). The RNA extracted was converted into cDNA using RevertAid™ H minus first strand cDNA synthesis kit (Nanjing Shengxing Company, China), following manufacturer protocols, and was used as a template for gene cloning and real-time fluorescent quantitative PCR analysis.

Cloning and sequence analysis of the *GmIMT1* gene

To obtain the full length cDNAs of the *Imt1* gene, similar sequences of the *Imt1* gene were identified from the soybean genome database using a homologous sequence search. Subsequently, primers GmIMT1F1 and GmIMT1R1 (Table 1) were designed for PCR amplification. The PCR was carried out in a DNA thermal cycler (BIO OVEN III) that was programmed to run the following specific temperature profile: 95°C for 5 min, initial denaturation for 1 min, 32 cycles each consisting of a denaturation step for 30 s at 94°C, an annealing step for 30 s at 56°C, an extension step for 2 min at 72°C, and a final extension for 8 min at 72°C. The amplified fragments were cloned with pMD19-T simple vector, and sequenced by Shanghai Pvt. Ltd., China. Sequence data were assembled and analyzed using the BioXM2.6 software program. The BLASTX program was used to identify similar sequences available from the GenBank databases. The Genscan software (http://genes.mit.edu/GENSCAN.html) was used in combination with ORF finder to identify the integrity of the gene coding region. Protein properties were analyzed with the support of the DNAMAN software. Conserved protein domains were identified using Conserved Domain Databases (NCBI CDD). Multiple sequence alignments were made using CLUSTAL X (1.8). Sequence phylograms were constructed using the MEGA software version 4.1, and uprooted trees were constructed using the TREEVIEW software.

Table 1. Primers used to isolate *GmIMT1* in soybean.

Primer	Sequences
GmIMT1F1	5'-CAATACGGAATCTAAGGCAAAG-3'
GmIMT1R1	5'-CACAGCAAACAACTCGGAAA-3'
GmIMT1F2	5'-GGCACTACCAGACAATGGGAAG-3'
GmIMT1R2	5'-ACAGCAAACAACTCGGAAACC-3'
GmIMT1F3	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTTCAACAGGTGAGACT-3'
GmIMT1R3	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAATCTTTTTGAGAAATTCCATGA-3'

Real-time quantitative PCR fluorescent analysis for *GmIMT1* gene expression in soybean

GmIMT1 gene expression in the plant organs (15 and 25 DAF) and under various stress treatments was analyzed by the real-time quantitative PCR fluorescent technique. The primers used for real-time PCR were GmIMT1F2 and GmIMT1R2 (Table 1). qRT-PCRs were conducted using the ReverTra Ace® qPCR RT Kit (TOYOBO, Japan) and a Bio-Rad iQ5 system following manufacturer protocols. The qRT-PCR mixture contained 8 mL diluted cDNA, 10 mL 2X iQ SYBR Green Supermix (Bio-Rad, Singapore), and 200 nM of each gene-specific primer in a final volume of 20 mL. Control PCR without cDNA templates was also performed for each primer pair. qRT-PCR was performed using a Bio-Rad iQ5 system under the following conditions: 2 min at 50°C, followed by 2 min at 95°C, 32 cycles for 15 s at 95°C, and for 30 s at 60°C on a 96-well optical reaction plate (Bio-Rad iQ5).

Gateway recombinant expression vector construction

For Gateway cloning, Multisite Gateway Three-Fragment Vector Construction kits were used following manufacturer protocols (Invitrogen). P Early Gate 103 was used as the destination vector. P Early Gate 103-35 S - *GmIMT1*plasmids were constructed by amplifying the coding region of *GmIMT1* by PCR with the primer sequences, GmIMT1F3 and GmIMT1R3 (Table 1). The donor vectors used in this experiment were constructed from pDONR™ 201 (Invitrogen Corp). pDONR™ 201 was used to clone DNA fragments by exchanging their assette flanked by a pair of attB GmIMT1F3 and GmIMT1R3 sequences by a BP reaction. The cloned sequences were assembled by Shanghai.pvt.ltd, China, and named as pEntry clone. Plant expression vector (P Early Gate 103) was constructed via an LR reaction between the pEntry clone and plasmid P Early Gate 103 (Invitrogen). The LR reaction system consisted of: 4 µL TE Butter, pH 8.0, 2 µL Entry clone, 2 µL pEarlygate103, 2 µL LR clonase TMII enzyme mix. The gun head was gently sucked up after mixing via short centrifugation. The mixture was then incubated at 25°C incubation for >1 h. Then, 1 µL protease K was added at 37°C for 10 min in the termination reaction.

Agrobacterium-mediated transformation

GmIMT1 was transferred into *A. thaliana* plants via *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent, 1998). The plants were then grown under continuous white light at 22°C for 16-48 h (Qifeng and Guanyi, 2000). To confirm the transgenic lines, T1 seeds were collected and selected on MS medium mgVL basta. Then, PCR and RT-PCR

analysis were performed with the support of actin primers. Plasmid and water were used as the negative and positive controls, respectively. In addition, T2 and T3 homozygous lines were developed, and used in the subsequent experiments.

Transgenic *A. thaliana* germination rate and phenotypic analysis under different stress conditions

Transgenic lines of T3 and wild-type plant (non transgenic) seeds were sown on 1/2 MS medium [1/2 MS + ABA (0.5 μM ABA), 1/2 MS + NaCl (concentration of 150 mM)]. The medium was then maintained at 23°C in a growth chamber under stress conditions. The percentage germination and root length were calculated at 7 and 10 day intervals, respectively.

Proline content

Free proline content was determined by the ninhydrin method of Bates et al. (1973). *Arabidopsis* plants were homogenized in 4 mL sulfosalicylic acid (3%), and the homogeneous mixture was placed in boiling water for 10 min. Then, the mixture was passed through filter paper. The 1-mL extract was added to a new tube, and mixed with 1 mL sulfosalicylic acid (3%), 1 mL acetic acid, and 2 mL ninhydrin acid. The reaction mixture was boiled for 1 h, cooled in an ice bath, and then 2 mL toluene was added to the extract and thoroughly mixed. Finally, 150 μL toluene extract was removed to measure absorbance at 520 nm in a TECAN M200 Microplate reader. A standard curve was drawn using proline (Sigma, USA).

RESULTS

Cloning, sequencing, and phylogenetic analysis

The full-length cDNA of sequence *GmIMT1* was 1208 bp in length, and contained a 1098-bp ORF encoding a polypeptide with 365 amino acids, a 5'-UTR 84-bp upstream from the start codon ATG, and 1179 bp 3'-UTR incidental 30-bp poly (A) tail from the stop codon TAA, as shown in Figure 1. Protein properties were analyzed with the support of DNAMAN-software. The proteins had a molecular weight of 106.3 kDa, and isoelectric point of 4.82. To further understand the *GmIMT1*gene evolutionary position, a relationship phylogenetic tree was constructed, and the results were analyzed. The phylogenetic tree results showed that the *GmIMT1* protein is more similar to Aaimt and Llimt proteins compared to other proteins. In addition, amino acid sequence alignment showed that *GmIMT1* had 99% homology with Aaimt and Llimt (Figure 2).

GmIMT1 gene expression in different plant organs

Soybean plant organs (leaves, stem, root, pod, and seeds) were sampled at 2 different plant developmental stages, 15 and 25 DAF. The expression of the *GmIMT1* gene was analyszd in different organs using the fluorescence quantitative PCR technology, with actin being used as the internal reference gene. *GmIMT1* gene mRNA expression was high in the root, leaves, and stem; however, it was not expressed in the seeds (Figure 3).

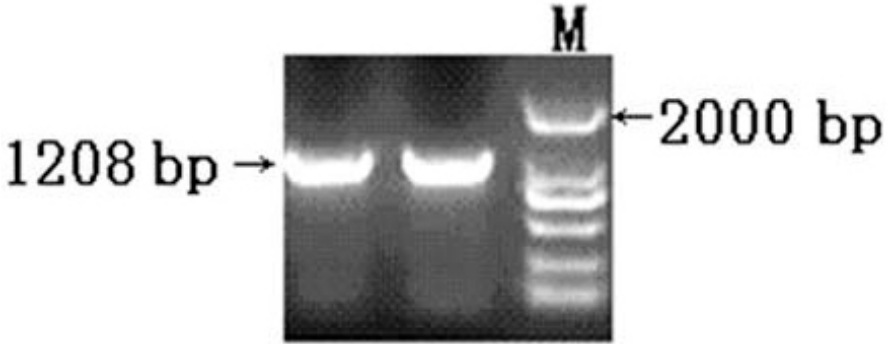

Figure 1. PCR amplification of ORF.

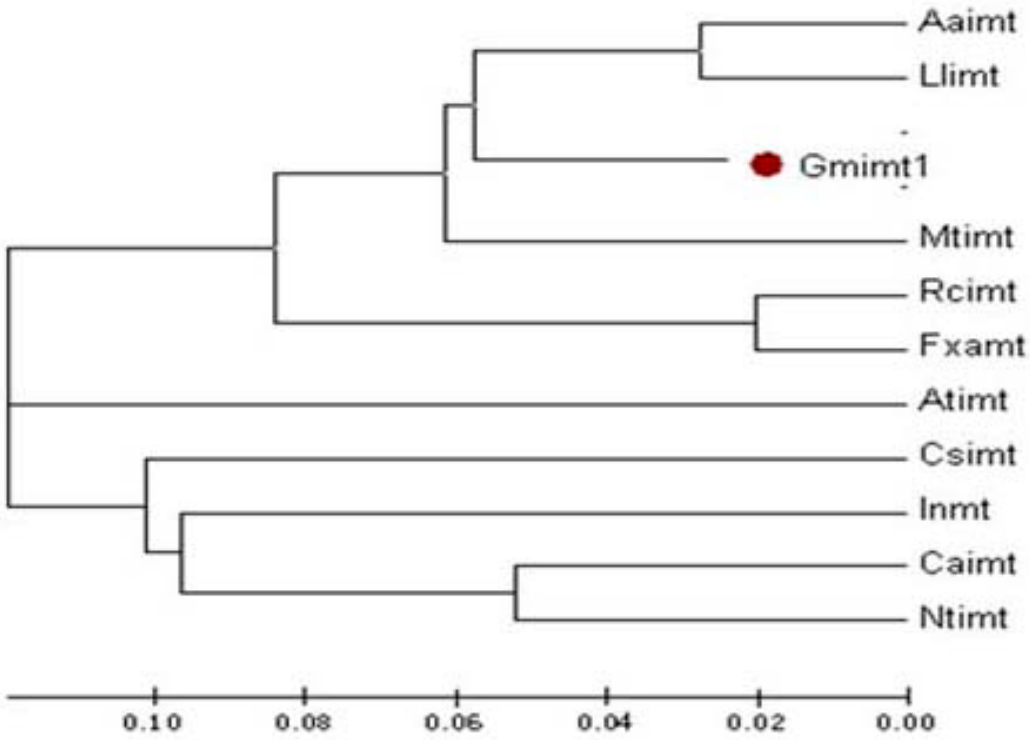

Figure 2. Phylogenetic tree of GmIMT1 protein from soybean and other species. Phylogenetic tree: using the MEGA program (v. 4.1) neighbor-joining software method to construct a phylogenetic tree. Occur in the protein GenBank registration number as follows: Aaimt (*Acacia* genus, *Acacia auriculiformis* x *Acacia mangium*, AAY86361.1), Llimt (*Leucaena*, *Leucaena leucocephala*, ABS57468.1), Mtimt (*Medicago*, *Medicago truncatula*, XP_003602396), Rcimt (*Rosa chinensis* var. *rose*, *spontanea*, BAC78827.1), Fxamt (*Fragaria* x *ananassa*, strawberry, AAF28353.1), Atimt (*Arabidopsis thaliana*, *Arabidopsis thaliana*, NP_200227.1 Csimt (*Camellia*, *Camellia sinensis*, ADN27527.1), Inmt (*Ipomoea*, *Ipomoea nil*, BAE94400.1), Caimt (*Capsicum annuum*, pepper, AAG43822.1), Ntimt (tobacco, *Nicotiana tabacum*, CAA52462.1).

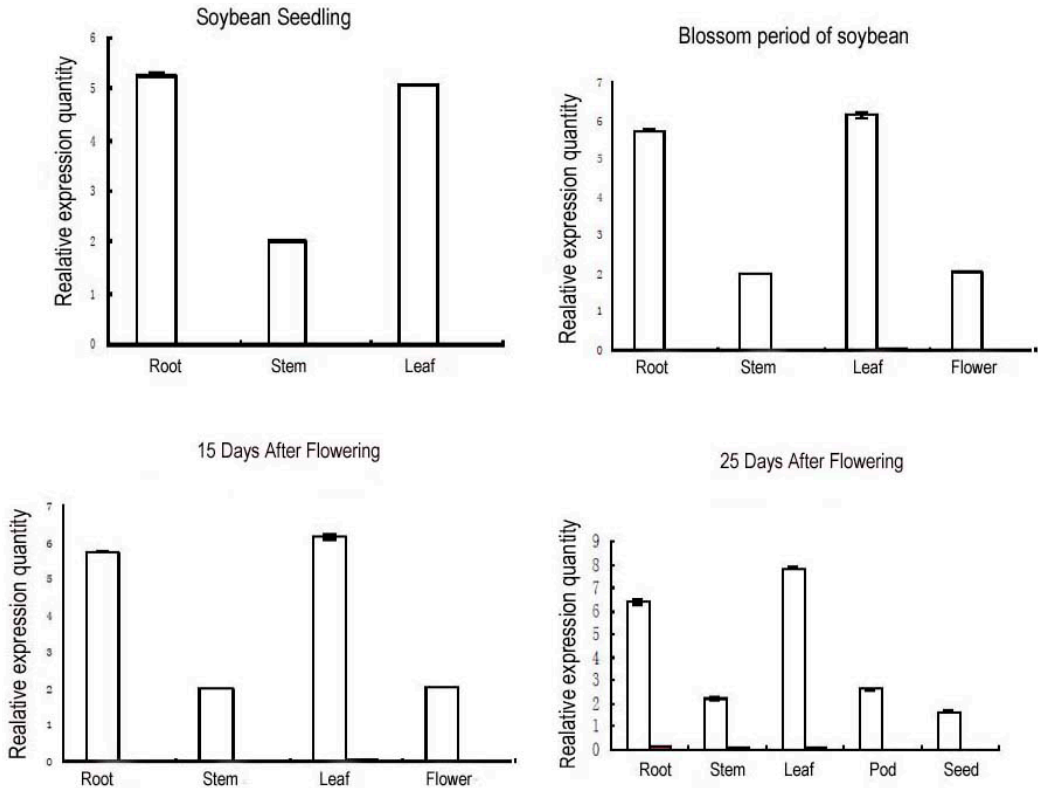

Figure 3. Expression pattern of *GmIMT1* in various soybean organs. Note: 15 DAF = 15 days after flowering; 25 DAF = 25 days after flowering.

Expression patterns of the *GmIMT1* gene under different stress conditions

The *GmIMT1* gene was significantly affected by the stress induced expression of NaCl. Initially, the gene was inhibited, but had the highest expression level at 24 h. *GmIMT1* expression in response to PEG showed a similar pattern to the NaCl stress response. Under low temperature stress (4°C), *GmIMT1* expression was inhibited, and then gradually increased until it peaked at 24 hs. *GmIMT1* gene expression was high at 1 after MeJA stress, and then declined, with no uniform expression. mRNA accumulation rapidly increased at 0.5 h after ABA stress, with *GmIMT1* gene transcription peaked at 36 h. The stresses SA and ET were induced by *GmIMT1* gene. *GmIMT1* gene transcription peaked at 3 h after SA stress, declined at 6 h, and then slowly increased at 12, 24, and 36 h (Figure 4).

Transgenic *A. thaliana*

To elucidate the function and stress response of the *GmIMT1* gene, it was cloned and sequenced. The expression vector of the gene was constructed (Figure 5), and was then introduced into the model plant *A. thaliana* through *Agrobacterium* mediated transformation. To confirm the transgenic lines, T1 seeds were collected and placed on MS medium mgVL basta, and confirmed

by PCR and RT-PCR analysis. The transgenic lines were amplified (expected amplicon size 1208 bp). The negative control was not amplified in the targeted fragment, while the positive control fragment size was consistent with the objective fragment (Figure 6). It was further confirmed in T2. Out of 31 transgenic lines, 23 lines were confirmed as positive seedlings.

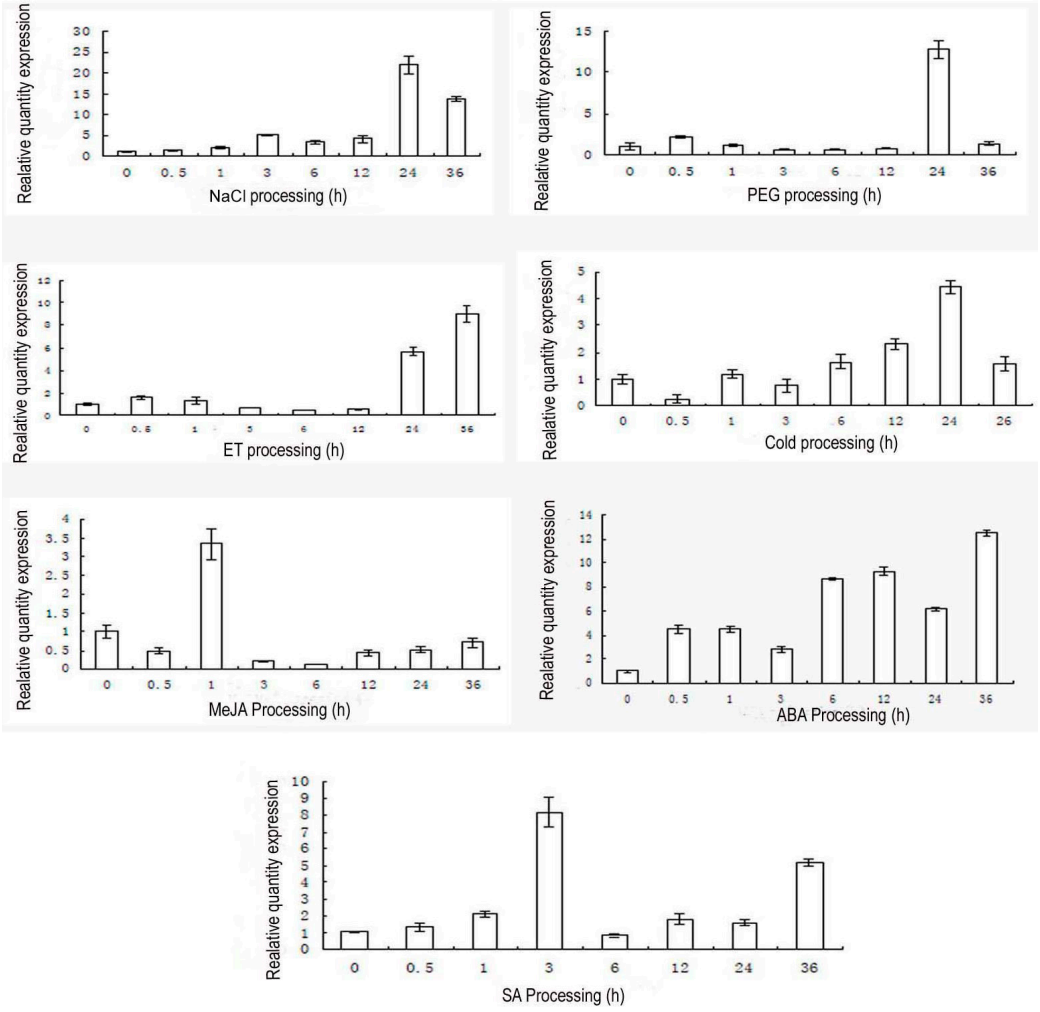

Figure 4. Induction of the *GmIMT1* gene expression. 1. Soybean seedlings were treated, respectively, under 2 mM SA, 200 mM NaCl, 100 μM ET, 200 μM ABA, 4°C, 100 μM MeJA JA and 10% PEG condition. *GmIMT1* expression in the soybean seeding detected by RT-PCR and actin used as internal reference gene.

Constitutive expression of *GmIMT1* in transgenic *Arabidopsis* enhances salt and ABA tolerance

The germination rate and root growth of transgenic and wild type *Arabidopsis* plants

was tested in 1/2 MS, 1/2 MS + NaCl and 1/2 MS + ABA. In 1/2 MS medium, the germination rate and root growth of wild type and transgenic plants was identical. In the 1/2 MS + NaCl culture medium, the germination rate of the wild type plants was 40%, while that of the 2 transgenic lines was 91 and 88%. In 1/2 MS +ABA stress, the germination rate of the wild type was <20%, while that of the 2 transgenic lines was 74 and 78% (Figure 7). The root length of wild type *Arabidopsis* plants was just half that of the w transgenic lines (Figure 8).

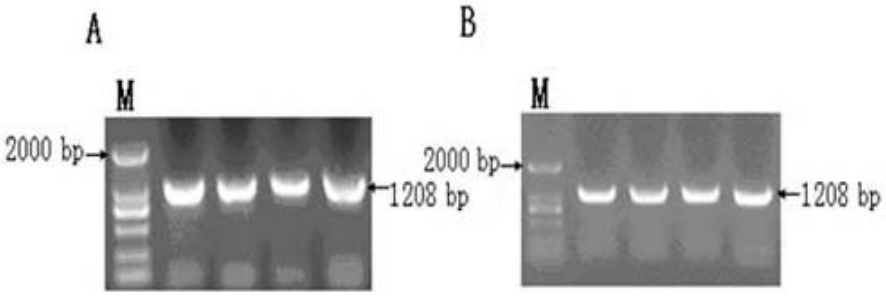

Figure 5. Construction of expression vector pEarlygate-GmIMT1. **A.** BP reaction of liquid PCR verification; **B.** LR responses after expression vector PCR verification.

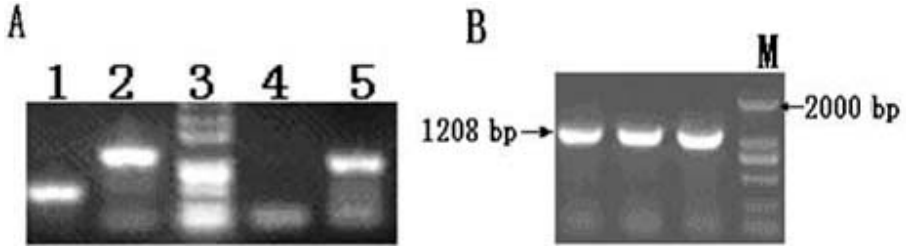

Figure 6. PCR identification of T_1 transgenic *Arabidopsis thaliana*. **A.** T1 generation transgenic *A. thaliana* results of PCR **B.** T1 generation transgenic *Arabidopsis* RT-PCR test results. *Lane 1* = Actin; *lane 2* = positive control; *lane 3* = marker; *lane 4* = negative control; *lane 5* = transgenic *Arabidopsis* DNA.

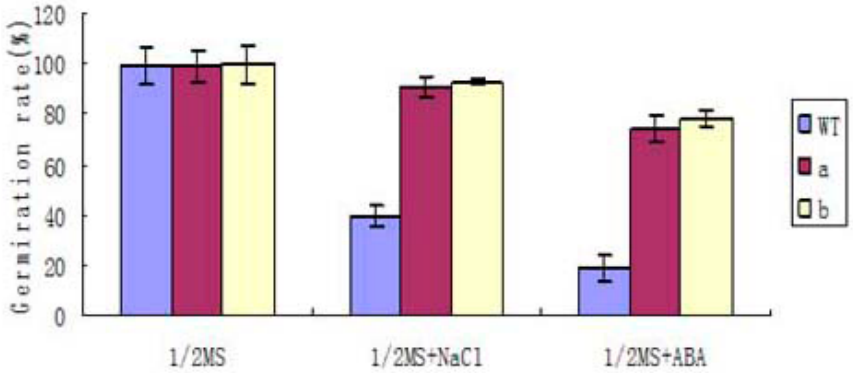

Figure 7. Germination rate of wild *Arabidopsis* and transgenic *Arabidopsis* under NaCl, ABA, stress. WT = wild *Arabidopsis;* a, b = transgenic *Arabidopsis*.

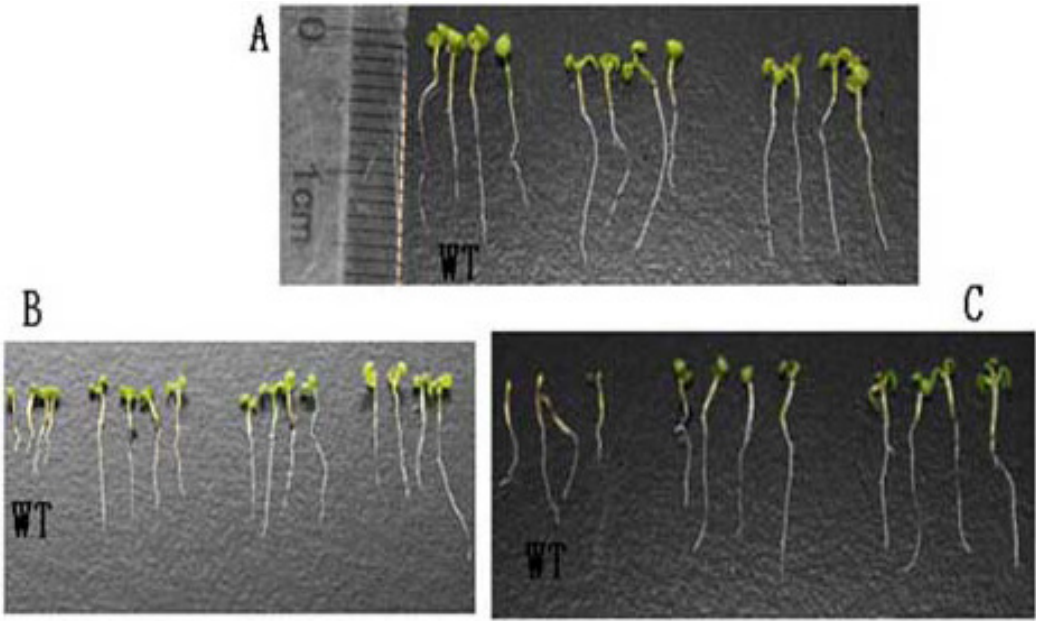

Figure 8. Root growth inhibition to NaCl, ABA stress of wild and transgenic *Arabidopsis*. **A.** 1/2 MS; **B.** 1/2 MS + NaCl; **C.** 1/2 MS + ABA.

Overexpression of *GmIMT1* in *A. thaliana* increases proline accumulation

The proline content of transgenic and wild-type *Arabidopsis* plants was calculated by the indinavir three ketone method. The results showed that transgenic *Arabidopsis* plants had higher proline content compared to wild type plants (Figure 9).

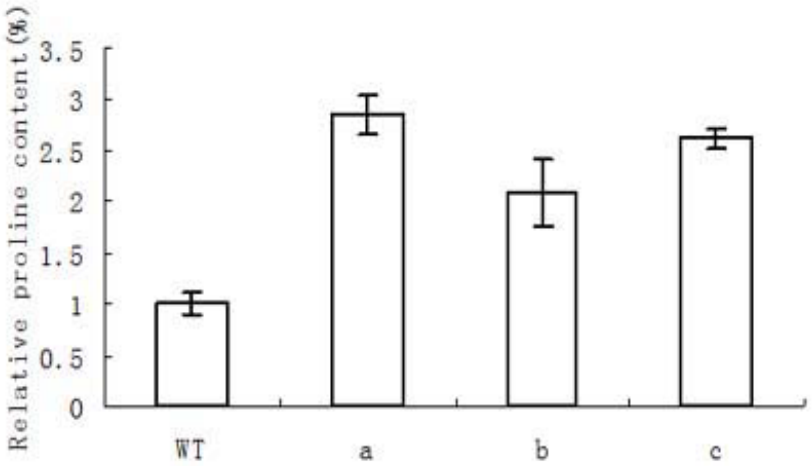

Figure 9. Overexpression of *GmIMT1* enhances proline content in transgenic *Arabidopsis thaliana*.

DISCUSSION

Abiotic stresses (such as drought, salinity, cold, freezing, high temperature, and water logging) have become an integral part of soybean production. Among the different abiotic stresses, salinity is a major threat to production in soybean producing areas. Plant breeders have attempted to develop varieties tolerant to specific abiotic stress. Recently, noticeable improvements have been made by molecular biologists regarding perturbations in genes expression and proteins during stress. In particular, advances have been made towards elucidating the mechanism of resistance and the cloning of related resistance genes through genetic engineering approaches, leading to improvements in salinity tolerance (Vannini et al., 2004; Zhao and Zhang, 2006; Park et al., 2011). Vernon and Bohnert (1992) studied the molecular mechanisms of osmotic stress tolerance in *M. crystalline* (the ice plant). The stress-initiated transcriptional induction of *Imt1* expression in this facultative halophyte provided strong support for the importance of sugar alcohols in establishing tolerance to osmotic stress in higher plants. Furthermore, *Imt1* gene expression in tobacco facilitates the intracellular accumulation of proline, glycine, mannitol, sorbitol, and trehalose, in addition to improving salt tolerance of various plants (Bohnert et al., 1995; Liu et al., 1995; Holmström et al., 1996). In this study, we cloned and characterized the full-length of cDNAs on the *GmIMT1* gene of soybean using degenerate primers of *M. crystallium* (*Imt1* gene). The full length of the *GmIMT1* gene was 1208 bp. It contained a 1098-bp open reading frame, encoding a polypeptide of 365 amino acids. Protein properties were analyzed by the DNAMAN software, and conserved protein domains were identified. The phylogenetic relationship showed that the *GmIMT1* protein was more similar to Aaimt and Llimt proteins compared to other proteins, with amino acid sequence alignment revealing that *GmIMT1* had 99% homology with Aaimt and Llimt.

In addition, we studied the pattern of *GmIMT1* gene expression in the plant organs of soybean. *GmIMT1* gene expression was high in the root, leaves, and stem; however, it was minimally expressed in the seed. *GmIMT1* gene expression was higher in mature plants compared to plants in the early stages of growth. This result provides evidence for the development process of *GmIMT1* gene in plants. In addition, *GmIMT1* gene expression was observed under different stress conditions, including drought, high salt, low temperature, SA, ET, ABA, and MeJA. These treatments were selected to help to elucidate the abiotic and biotic stress mechanisms of the *GmIMT1* gene. For instance, ABA is abiotic stress signal substance, while SA, ET, and MeJA tend to be biological stress signals (Kieber, 1997). However, the results of this study showed that *GmIMT1* gene expression was upregulated under all of these stress conditions. The *GmIMT1* gene was significantly affected by the stress induced expression of NaCl, and PEG. At 4°C stress, *GmIMT1* expression was initially inhibited, and then gradually increased. *GmIMT1* gene expression was high at 1 h after MeJA stress, but then became downregulated. *GmIMT1* gene expression showed a similar response to both ABA and NaCl stress. The SA and ET stresses were induced by the *GmIMT1* gene. The induced expression of this gene was divided into two categories, depending on the timing of expression. JA and SA stresses were grouped into the first category, because they exhibited rapid and transient expression. These stressors required a few hours to peak, before gradually degrading. NaCl, PEG, temperature, ABA, and ET were grouped into the second category. The expression of these stressors was initially slow (0.5, 1, 3, and 6 h), and started to gradually increase at 10 h. These results clearly indicate that *GmIMT1* gene expression is induced by NaCl, JA, PEG, SA, temperature, ABA, and ET, but to different levels.

To further study the soybean *GmIMT1* gene function, the Gateway technology was used to construct the *GmIMT1* gene plant expression vector and the *GmIMT1* gene was transferred into the model plant *A. thaliana*. The analysis of various stressors on *GmIMT1* gene expression in soybean showed that high salt (NaCl) and ABA stress induced high *GmIMT1* expression. To further elucidate the stress response, we compared the germination rate and root growth of wild type and *GmIMT1* transgenic seeds subject to NaCl and ABA stress. In 1/2 MS medium, germination rates were similar for both the transgenic lines and wild type plants. However, in 1/2 MS + NaCl medium and 1/2 MS + ABA medium, the 2 transgenic lines had higher germination rates compared to wild type plants. Similar results were also obtained for proline content. These results might be attributed to the direct production of Imt1 gene expression. The direct production is awn handle alcohol, which contains multiple hydrophilic hydroxyl groups. The hydrophilic ability is strong, and is able to reduce the physiological drought caused by the loss of moisture. This phenomenon enables plants to become salt tolerant (Yunzhou and Xueyan, 2000).

In conclusion, this study cloned and identified the *GmIMT1* gene related to salt stress response in soybean. Overall, the results of this study clearly indicate that *GmIMT1* might be involved in multiple plant response pathways to the different environmental conditions, with transgenic plants exhibiting higher salt-tolerance compared to wild type plants. The findings of this study provide a foundation for further studies investigating the functions and biochemical characteristics of the *GmIMT1* gene.

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