INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–683/2014/16–1–153–159
http://www.fspublishers.org

Full Length Article

Over-expression of *GmWRKY111* Enhances NaCl Tolerance of Salt-Sensitive Genotype of *Glycine max*

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Abstract

WRKY transcription factors are a large family in higher plants, play significant functions in plant growth and stress response, such as plant development, metabolism, and responses to biotic and abiotic stresses. In this study, we identified one WRKY transcription factor gene *GmWRKY111* from soybean with an open reading frame of 1197 bp encoding 398 amino acids with a molecular weight of 44 kD and a pI of 5.7. Analysis of expression profiles *GmWKRY111* highly expressed in root at differential growth period, and weakly expressed in flower, stem and leaf, and significantly responded to 200 mM NaCl. In addition, the multiple alignments analysis indicated that GmWRKY111 possessed a conserved WRKYGQK domain and a zinc-finger structure; the phylogenetic tree analysis showed that GmWRKY111 belongs to group IIc clade. Meanwhile, Over-expression of the *GmWRKY111* improved seedling tolerance to salt in composite transgenic soybean plants. These results indicate that *GmWRKY111* improves the ability of plant to confer salt tolerance. Further studies are needed to explore its interaction with other proteins and explore the pathway in which it is involved. © 2014 Friends Science Publishers

Keywords: Salt tolerance; Soybean; RT-PCR; qPCR; Composite seedling; Mosaic seedling

Introduction

Soil salinity affecting large areas of cultivated land is naturally present in more than 100 countries of the world (Rengasamy, 2006), and is among the most important environmental stresses which considerably reduces plant growth and grain yield. Increased and continued salinization of cultivated land threatens to crop production globally, particularly in irrigated systems (Munns and Tester, 2008) and consequently has negative effects on food security. The consequences are damaging in both socioeconomic and environmental terms. Increasing the salinity tolerance of crop plants may provide an important contribution to the maintenance and stability of crop yields in salt-affected soils.

WRKY transcription factors (TFs) along with other TFs gene families are involved in plant responses to abiotic stresses and show vital roles in regulating several stress reactions in plants. A number of studies have shown that WRKY proteins specifically recognized W-box sequences (TTGACC/T) present in the promoter regions of various genes particularly those involved in plant defense to various stresses. Both bioinformatics and functional studies unraveled presence of groups of W boxes in stress-inducible plant promoters (Maleck *et al.*, 2000). Various studies have reported that WRKY genes are involved in several biological processes such as response to senescence (Hinderhofer and Zentgraf, 2001; Miao *et al.*, 2004), wounding (Hara *et al.*, 2000), stresses either biotic (Xu *et al.*, 2006) or abiotic (Mare` *et al.*, 2004; Jiang and Deyholos, 2009; Wu *et al.*, 2009; Gong *et al.*, 2010) etc.

The evidences of WRKY functions associated to abiotic stress responses emanated either from functional analyses or transcription profiling. For instance, microarray experiments identified WRKY genes to be inducible by various abiotic stresses like in Arabidopsis, WRKY genes were among several families of TF genes being induced by cold, drought or high-salinity stress (Fowler and Thomashow, 2002; Seki *et al.*, 2002), in tomato, WRKY TFs response to drought (Gong *et al.*, 2010), and thus provided indirect evidence. However, functional analyses based studies have provided more direct evidence like, in barley, expression analysis of HvWRKY38 showed its regulatory role in cold and drought stress tolerance (Mare` *et al.*,2004), in rice, heat shock inducible HSP101 promoter-driven over-expression of OsWRKY11 led to enhanced heat and drought tolerance (Wu *et al.*, 2009) and that of OsWRKY45 resulted in boosted drought and salt tolerance as well as improved disease resistance (Qiu and Yu, 2009), in Arabidopsis, over-expression of either AtWRKY25 or

To cite this paper: Xu, Z.L., Z. Ali, J.X. Yi, L. Xu, X.Q. Liu, X.L. He, Y.H. Huang, I. Karim, M. Rasul, H.X. Ma and D.Y. Zhang, 2014. Over-expression of *GmWRKY111* enhances NaCl tolerance of salt-sensitive genotype of *Glycine max*. *Int. J. Agric. Biol*., 16: 153–159

AtWRKY33 increased salt tolerance (Jiang and Deyholos, 2009).

In the present study, we report (1) *in silico*and digital gene expression profiling (DGEP) of GmWRKYs to identify salt responsive GmWRKYs, (2) all GmWRKYs including *GmWRKY111* mainly expressed in root, (3) *GmWRKY111* significantly responded to 200 mM NaCl, (4) that *in planta* over-expression of *GmWRKY111* enhanced salt tolerance in soybean.

Materials and Methods

Plant Materials and Treatments

The varieties of soybean include salt susceptible genotype of *Glycine max* (SSGoGM) and salt tolerant genotype of *Glycine soja* (STGoGS). Seedlings were grown in pots in green house for one week. SSGoGM seedlings were used for injecting and transgenic analysis. Two weeks old seedlings of SSGoGM and STGoGS were treatedwith200 mM NaCl over-night and collected the roots for DGEP (Ali *et al*, 2012). Root, leaf and stemtissuesat V1 (first trifoliate stage) including, flowerat R2 (Full flower stage) and podat R4 (Full pod stage) of SSGoGM were collected for tissues specific expression analysis. Three weeks old SSGoGM plants were treated with200 mM NaCl and collected the roots at 0, 2, 4, 8, 12, 24 h for NaCl inducible expression analysis. All the tissues were quick-frozen in liquid nitrogen and stored at - 80℃ till further use.

Identification of all GmWRKYs and Phylogentic Analysis

All GmWRKYs in soybean were downloaded from the database of soybean genome (www.phytozome.net/cgi-bin/gbrowse/soybean),which resulted in the identification of 214 GmWRKYs. The nomenclature of 177 GmWRKYs and 37 pseudo-GmWRKYs given at the website of The Institute of Green Energy and Clean Environment (iGECE, http://systemsbiology.usm.edu/PhytoTech/WRKY07012011/Soybean.html, last accessed on February 22, 2013) was followed. All GmWRKYs and 34AtWRKYs protein sequences(8 from group I, 3-4 from group IIa, IIb, IIc, IId, IIe, 7 from group III)were lined up together using Clustal X (http://www.ebi.ac.uk/Tools/clustalx2/index.html). The unprocessed phylogenetic tree was generated using neighbor-joining method by MEGA 5.0 software (http://mega.software.informer.com/5.0).

In silico Transcript Profilingand Identification of Salt Responsive *Gm*WRKY

The DGEP data were generated through developing a rigorous algorithm to detect differentially expressed genes (DEGs) among the control (normal) and NaCl treated samples (Audic and Claverie, 1997). The methods explained elsewhere were followed (Ali *et al.*, 2012). Twenty eight GmWRKY genes with a false discovery rate (FDR) (Benjamini and Yekutieli, 2001)$\leqslant$0.001 and the absolute value of $\log_2$ratio$\geqslant$1in the two soybean species were chosen. New WRKY TFs, displaying significant differential expression across treatments and accessions were identified from DGEP data and their expression was validated by qPCR analysis.

The young leaf, flower and root, etc expression data of every GmWRKY gene was downloaded from soybase (http://www.soybase.org), and *in silico* analysis following the procedure explained in (Zhang *et al.*, 2013), analyzed by Java Treeview1.0.4 software (http://jtreeview.sourceforge.net).

Total RNA Extraction and Reverse Transcription

The extraction of total RNA was done from roots using TRIzol® reagent (Invitrogen and Co.) following the manufacturer's instructions. The first-strand cDNA was synthesized by reverse transcription using reverse transcriptase kit (Fermentas Life Sciences) following manufacturer's instructions.

Quantitative RT-PCR

Soybean GmActin gene was used as a reference gene for data normalization and to calculate the relative mRNA levels. Total RNA from soybean roots was isolated as described above. Equal amount of cDNA prepared from roots was analyzed by quantitative real-time polymerase chain reaction(PCR) using a Roche 2.0 Real-Time PCR Detection System with the SYBR Green Supermix (TaKaRa Bio Inc, Japan). All PCRs were replicated at least three times.

Cloning and Plasmid Construction of *GmWRKY111*

The corresponding full length CDS sequence of *GmWRKY111* (Glyma17g03950) was downloaded from www.phytozome.com. The sequence specific forward (5'-ATGGAGGAGAAGAGAGA-3') and reverse (5'-CACCTACACACGATCTTCAT-3') primers were designed and got synthesized from Invitrogen™. The *GmWRKY111* gene was isolated from cDNA through PCR using a thermo-cycler (LabCycler, Senso Quest Biomedizinische Elektronik, Germany). PCR was performed with 20 µL volume containing 12.8 µL ddH_2O, 1 µL of cDNA (20 ng/µL), 0.4 µL of each primer (10µM), 2 µL Ex*Taq* buffer (10x), 1.6 µL $MgCl_2$ (25 mM), 1.6 µL dNTPs (10 mM), and 0.2 µL Ex*Taq* DNA polymerase (5 U/µL) (TaKaRa Bio Inc, Japan). Thermal cycling conditions were set with initial denaturation temperature of 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by final extension at 72°C for 3 min. The amplified product of PCR was separated on 1.0% agarose gel and purified using gel extraction kit (Axygen Biosciences) following kit's

specifications. The purified PCR product was linked to pCXSN via TA-cloning (Chen *et al.*, 2009). The linked construct was transformed into *E. coli* strain DH5-alpha. Positive and sense orientation gene's DNA clone was identified through PCR with forward 35S promoter sequence specific primer (5'-CAATCCCACTATCCTTCGCAAGACC-3') and reverse gene sequence specific primer. PCR mixture and thermal cycling parameters used are as before.

Positive clones were further grown at 37°C and used for plasmid extraction. Plasmids were extracted using plasmid extraction kit (Axygen Biosciences) following kit's specifications. Plasmids were got sequenced from GenScript (http://www.genscript.com.cn/index.html) and compared with CDS sequence of the gene using Clustal X software and right one was introduced into *A. rhizogenes* strain K599.

Plant Transformation and Function Analysis of *GmWRKY111*

In order to verify whether the salt tolerance of transgenic soybean is related to *GmWRKY111* gene, transgenic plants carrying *GmWRKY111* gene were treated with high salinity (200 mM NaCl). Transgenic soybean plants carrying empty pCXSN vector were used as control and also treated with 200 mM NaCl.

Three weeks old soybean plants injected with over-expression cassette were up-rooted from the pots. Plants having roots at the inject point were selected and their real roots were removed. The roots emerging at the inject point were immersed into ½ strength Hoagland culture solution (Hoagland and Arnon, 1950) contained in 100 mL glass tubes. The opening of the tubes was foam plugged to hold the plants. After 03 days, only one root at the inject point was kept and rest were removed. After 01 week growth of plants in solution culture NaCl was added in the culture solution to establish a 200 mM NaCl stress. The data were recorded after one week of stress. The culture solutions were changed on alternate days. The experiment was repeated for 3 times.

In order to verify whether the healthy plants have transgenic roots and contain *GmWRKY111* gene, gene's amplification, with forward 35S promoter sequence specific primer and reverse gene sequence specific primer, through PCR was adapted using genomic DNA as template. Only transgenic plants amplified the gene compared to control plants.

Statistical Analysis

Data were analyzed using SPSS ver. 13.0 (Statistical Package for Social Science for Windows, ver. 13.0) for the ANOVA test. Significant differences among means were determined by the LSD at $P < 0.05$.

Results

All WRKYs Detected in the DGEP Data and Cloning of *GmWRKY111*

In the previous study, the digital gene expression profiling (DGEP) was performed to uncover the salt response genes between salt sensitive genotype of *Glycine max* (SSGoGM) and salt tolerant genotype of *Glycine soja* (STGoGS) (Ali *et al.,* 2012). The data showed that there were 28 GmWRKY TFs exhibited up-regulated expression in the two genotypes in response to 200 mM NaCl stress for 12h (Fig.1A). Among the up-regulated WRKY transcription factors,21were in STGoGS, 31were in SSGoGM and 19were in both species (data not presented). *GmWRKY111*, one of the WRKY genes up-regulated in both genotypes, up-regulated 5.6 folds under salt stress in STGoGS and 3.7 folds in SSGoGM. To further validate the DGEP data, quantitative RT-PCR was done which showed that expression of *GmWRKY111* was significantly higher in both STGoGS and SSGoGM after 200 mM NaCl stress for 12 h compared with the control (0h), which was accorded with the DGEP result (Fig. 1B). Thereafter, the full length cDNA*GmWRKY111* was isolated via RT-PCR and cloned its open reading frame (ORF) using gene specific primers.

Sequence Analysis of *GmWRKY111*

BLASTx search in the NCBI database reveals that the predicted product of *GmWRKY111* contains an 1197 bp ORF encoding 398 amino acids with a molecular weight of 44 kD (kilo-dalton) and a pI (isoelectric point) of 5.7. The multiple alignments using Clustal X software indicated that *GmWRKY111* possessed a conserved WRKYGQK domain and a zinc-finger structure (Fig. 2B). Meanwhile, all WRKYs in soybean were downloaded for phylogenetic tree analysis. The results showed that there were 177 GmWRKYs in the soybean genome and divided into seven groups (or subgroups), *GmWRKY111* clustered into group IIc (Fig.2A).

Expression Analysis of *GmWRKY111*

In order to figure out the *GmWRKY111* expression distribution in soybean plants, *in silico* expression of gemome-wide GmWRKYs performed, showing that most of WRKY genes expressed in roots (Fig. 3), some expressed in flower and young pod, and no expression in seed, but *GmWRKY111* mainly expressed in root, flower and nodule (Fig. 3A). To validate the above mentioned results, the tissue expression of *GmWRKY111* was studied using quantitative RT-PCR. The results showed that *GmWRKY111* highly expressed in root at different growth stages, and weakly expressed in flower, stem and leaf in our soybean sample (Fig. 3B).

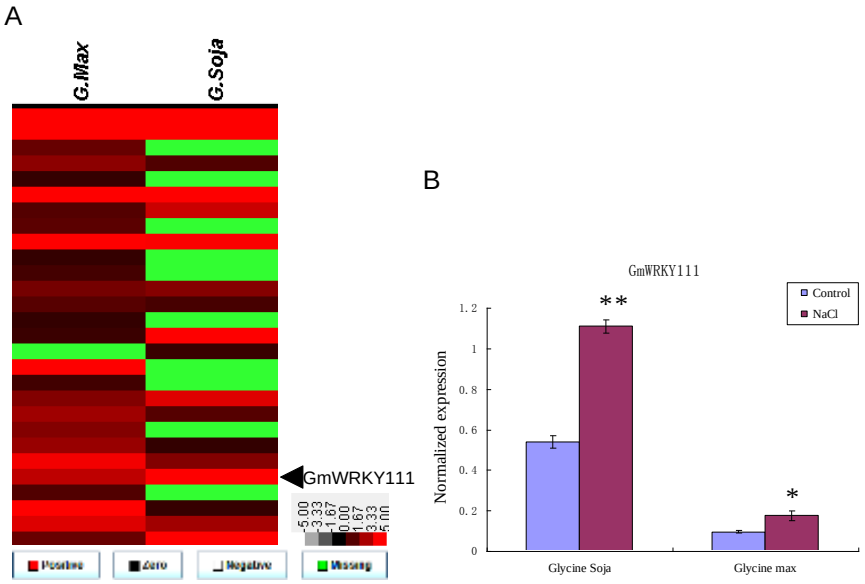

Fig. 1: Expression analysis of GmWRKYs in roots in salt tolerant genotype of *Glycine soja* (STGoGS) and a salt sensitive genotype of *Glycine max* (SSGoGM) at 12h of 200 mM NaCl stress. (A) Expression profile of 28 up-regulated Gm WRKYs using DGEP data. (B) expression pattern of *GmWRKY111* using qPCR data. *Significant at P <0.05, **significant at P<0.01, compared with control level

In addition, the salt-induced expression of *GmWRKY111* in root was done using different time points treated by 200 mM NaCl including 0, 2, 4, 8, 12 and 24 h. The results found that the expression of *GmWRKY111* first decreased at 4 h and then increased at 8 h to peak at 12 h and sustained the same level at 24 h in STGoGS, however, *GmWRKY111* was highest at 2 h of treatment in SSGoGM and declined with increasing time. Its expression showed impulsive increase at 2 h followed by immediate decrease at 4 h of NaCl stress in SSGoGM (Fig. 4).

Over-expression Analysis of *GmWRKY111*Could Increase the Tolerance to High Salt Stress

Soybean mosaic plants having transgenic roots and non-transgenic shoots were developed. The transgenic roots were tested through PCR. The mosaic plants over-expressing *GmWRKY111* were tested against 200 mM NaCl added in the nutrient solution. Mosaic plants growing in saline nutrition solution were green after one week of their growth (Fig. 5A). Average number of survived mosaic plants was 28.3, average survival rate was 68.5%, while the average survival rate of control transgenic plants with empty vector pCXSN was 10% (Fig. 5B).

Discussion

WRKY gene family is one of the largest groups of the transcription factors. It is mostly said to be plant specific, but it is not completely restricted to the plant kingdom. In higher plants WRKY gene family expanded abundantly but absent in animal kingdom and in yeast (Ülker and Somssich, 2004). WRKY conserved signature is part of domain which consists of about 60 peptides having WRKYGQK sequence at N terminal of the chain and zinc finger like pocket towards the C terminal of domain (Eulgem *et al.*, 2000). In higher plants, many WRKY transcriptional factors have been well studied and characterized. Arabidopsis WRKY family has 74 members (Ülker and Somssich, 2004), rice WRKY gene family has 109 members (Song *et al.*, 2010), papaya WRKY gene family includes 66 genes, sorghum WRKY gene family has 68 genes (Pandey and Somssich, 2009), pinus WRKY gene family has 80 members, poplar WRKY gene family has 104 members, barley WRKY gene family includes more than 45 and the present studies report 177 WRKY genes (Fig. 2A), while 37 pseudo-WRKY genes in soybean (data not presented).

WRKY proteins are classified on the basis of number of WRKY domains and feature of zinc finger like motif (Eulgem *et al.*, 2000). There are three main groups, group I is the progenitor of group II and III. Group I contains two WRKY motifs and zinc pocket. Group II and III contain single WRKY motif and zinc pocket, but Group II was further classified into 5 classes (a-e) due to the presence of additional amino acid sequences along with conserved domain (Eulgem *et al.*, 2000; Rushton *et al.*, 2010). Arabidopsis and rice WRKY gene family is consisted of three main groups (I, II, III). Group I is classified in to 2 subgroups (Ia, Ib), group II is divided in to four subgroups(IIa, IIb, IIc, IId), group III is classified into two subgroups (IIIa, IIIb) (Wu *et al.*, 2005). Now it has been reported that in higher plants like in Arabidopsis, rice,

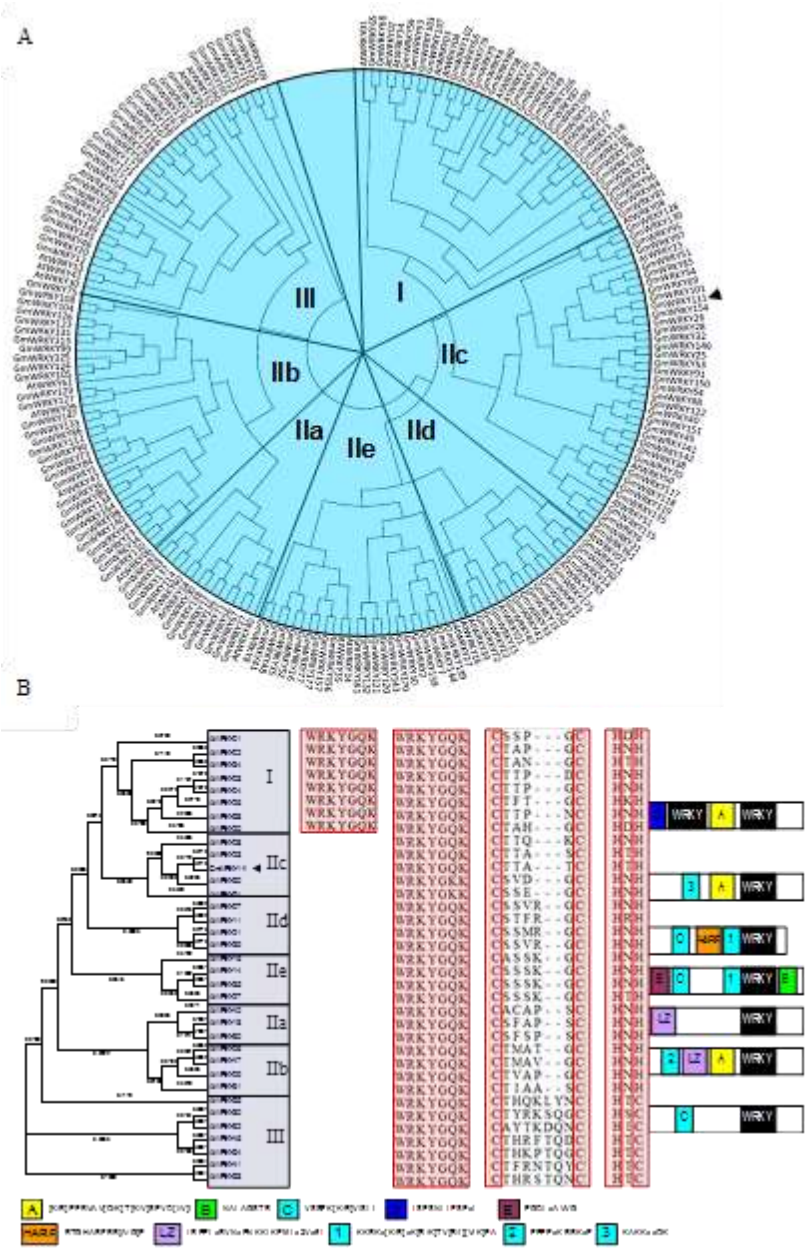

Fig. 2: Phylogenetic and structure analysis of all soybean (*Glycine max*) and some Arabidopsis WRKY proteins, the black arrow pointing GmWRKY111. (A) phyblogenetic tree of 177 WRKY TFs in soybean and 34 WRKY TFs in Arabidopsis(8 from group I, 3-4 from group IIa, IIb, IIc, IId, IIe, 7 from group III) were aligned with ClustalX and tree constructed using the neighbor-joining method in MEGA 5.0. Different groups (or subgroups) were divided into different sectors. (B) Phylogenetic analysis of 34 members of the AtWRKY and GmWRKY111, aligned with Clustal X and the grouping method was cited from Thomas E (2000), GmWRKY111 belonged group IIc, having a zinc-finger structure Cx4Cx23HxH, the WRKYGQK motif, cysteines and histidines, which form the zinc-binding pocket are in red frame

poplar, maize, barley, and soybean WRKY gene family consisted of three main groups; group I, II and III. Group II is further sub grouped into; IIa, IIb, IIc, IId and IIe. Total seven WRKY subfamilies (I, IIa, IIb, IIc, IId, IIe and III) are present in higher plants (Rushton *et al.*, 2010). Soybean WRKY transcription factors gene family is divided into total seven subfamilies (Fig. 2A). Group I and group IIc appeared into one clade, which indicated that group I have higher similarity with group IIc. Clustering of group I and group IIc in same clade is also reported in grapevine (Liu *et al.*, 2011). It suggested that they might be derived from the same ancestor. GmWRKY111 clustered in group IIc.

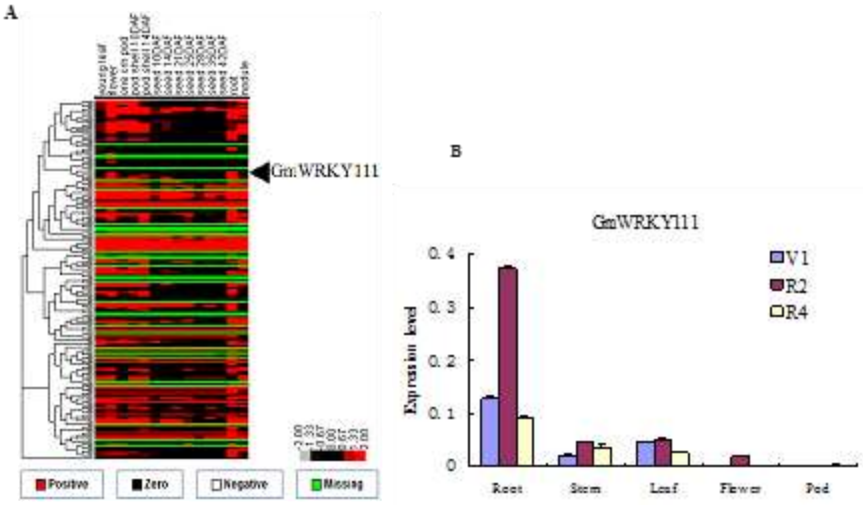

Fig. 3: Expression of WRKYs in soybean (*Glycine max*). (A)*In silico* expression of GmWRKYs in various tissues, (B) Expression of *GmWRKY111* in various tissues at different growth stages in SSGoGM. V1 First trifoliate stage, R2 Full bloom stage, R4Full pod stage.

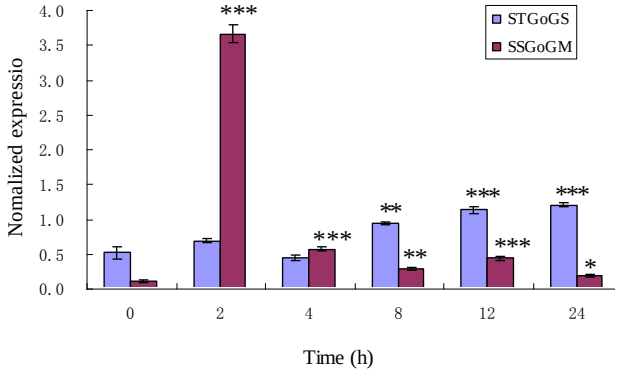

Fig. 4: Expression analysis of *GmWRKY111* inroots in salt tolerant genotype of *Glycine soja* (STGoGS) and a salt sensitive genotype of *Glycine max* (SSGoGM) at various time points using real-time PCR data. * Significant at P<0.05, ** significant at P <0.01 and *** significant at P<0.001, compared with 0 h levels

biological processes like development, metabolism, signaling and responses to biotic and abiotic stresses. However, few studies report the role of soybean WRKY genes especially under abiotic stress. For example, *GmWRKY13,GmWRKY21*and *GmWRKY54* are implicated in simulated abiotic stress tolerance in Arabidopsis plants. It was reported that there is an enhancement in the lateral root in GmWRKY13 transgenic plants. So Gm*WRKY13*play a role in both abiotic stress response and lateral root development. *GmWRKY13* of soybean showed good response against salinity but its over-expression revealed higher sensitivity to mannitol and salt stress (Zhou *et al.*, 2008). *GmWRKY111* showed enhanced tolerance of soybean mosaic plants in 200 mM NaCl stress (Fig. 5). *In silico* expression showed that *GmWRKY111* is highly expressed in root and flower (Fig. 3A), qRT-PCR expression showed that *GmWRKY111* also highly expressed in root, and a little expression in stem, leaf and flower (Fig. 3B). Salt tolerance response of WRKY transcription factors is also reported in other plant species like microarry analysis of *OsWRKY111*

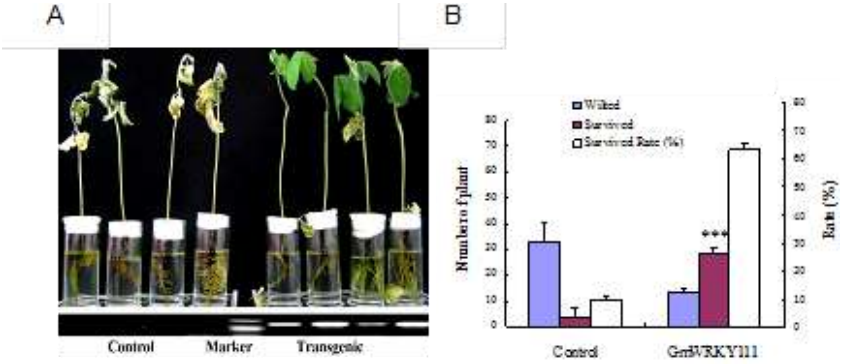

Fig. 5: Over expression analysis of *GmWRKY111* TF enhances NaCl tolerance of salt sensitive genotype of *Glycine max* (SSGoGM). A transgenic mosaic seedlings under 200 mM NaCl treated 1 week. B statistics of wilted or survived mosaic seedlings under200 mM NaCl treated 1 week.*** Significant at P <0.001, compared with control levels

exhibited its abundance in meristematic tissues of shoot, however it didn't express in shoots at 24 h post salt treatment (Berri *et al.*, 2009). *AtWRKY25* is reported to confer salinity tolerance in Arabidopsis(Rushton *et al.*, 2012).

Conclusively it was observed that *GmWRKY111* can be induced by salt stress. Over expression of the *GmWRKY111* improved seedling tolerance to salt in composite transgenic soybean plants. These results indicate that *GmWRKY111* improves the ability of plant to confer salt tolerance. Further studies are needed to explore its interaction with other genes and explore the pathway in which it is involved.

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(Received 22 May 2013; Accepted 26 August 2013)