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Over-expression of a novel JAZ family gene from *Glycine soja*, increases salt and alkali stress tolerance

Dan Zhu [a,1], Hua Cai [a,1], Xiao Luo [a], Xi Bai [a], Michael K. Deyholos [b], Qin Chen [c], Chao Chen [a], Wei Ji [a], Yanming Zhu [a,*]

[a] Plant Bioengineering Laboratory, Northeast Agricultural University, Harbin 150030, China
[b] Department of Biological Sciences, University of Alberta, Edmonton, Canada T6G 2E9
[c] Lethbridge Research Centre, Agriculture and Agri-Food Canada, 5403-1 Ave., South P.O. Box 3000, Lethbridge, AB, Canada T1J 4B1

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ABSTRACT

Salt and alkali stress are two of the main environmental factors limiting crop production. Recent discoveries show that the JAZ family encodes plant-specific genes involved in jasmonate signaling. However, there is only limited information about this gene family in abiotic stress response, and in wild soybean (*Glycine soja*), which is a species noted for its tolerance to alkali and salinity. Here, we isolated and characterized a novel JAZ family gene, *GsJAZ2*, from *G. soja*. Transcript abundance of *GsJAZ2* increased following exposure to salt, alkali, cold and drought. Over-expression of *GsJAZ2* in *Arabidopsis* resulted in enhanced plant tolerance to salt and alkali stress. The expression levels of some alkali stress response and stress-inducible marker genes were significantly higher in the *GsJAZ2* overexpression lines as compared to wild-type plants. Subcellular localization studies using a GFP fusion protein showed that GsJAZ2 was localized to the nucleus. These results suggest that the newly isolated wild soybean *GsJAZ2* is a positive regulator of plant salt and alkali stress tolerance.

1. Introduction

Saline and alkaline environments, including Na^+, high pH, CO_3^{2-} and HCO_3^-, elicit adverse effects on the productivity of crops. To cope with the limitations of a sessile lifestyle, plants have developed combinatorial mechanisms to sense and respond to salt and alkali stress [1–3]. Tremendous progress has been made in understanding salt and alkali stress tolerance in plants, and many salt and alkali stress related genes have been isolated and characterized in the past years. However, salt and alkali tolerance are complex traits controlled by multiple genes involved in different biological processes [4,5], including selective ion uptake and exclusion [6,7]; accumulation of osmo-protectants and antioxidant [8,9]; regulation by transcription factors (TF) [10] and signal perception and transduction [11].

The JAZ (JASMONATE ZIM-DOMAIN) family is a novel, plant-specific, gene family encoding proteins characterized by a conserved Jas domain [12]. Recently, many JAZ family genes have been extensively investigated, focusing on the proteins' roles in jasmonic acid signaling. There are 12 members of the JAZ family proteins in *Arabidopsis*. A microarray analysis of early responses to jasmonate (30 min) showed that the expression of 10 members of the JAZ family was quickly and specifically induced by jasmonate treatment [13]. Over-expression of wild-type *JAZ* genes does not show any apparent JA-related defect [13–15]. By contrast, over-expression of the mutant of *AtJAI3/JAZ3* gene (At3g17860), which encodes an aberrant protein lacking the Jas domain, showed a dominant jasmonate-insensitive phenotype [13,14]. Yan et al. (2007) identified another JAZ gene (At5g13220) named *AtJAS1* (JASMONATE-ASSOCIATED1). Over-expression of *AtJAS1* in *Arabidopsis* resulted in a decreased sensitivity to methyl jasmonate (MeJA) and reduced wound-induced growth inhibition. Plants bearing an RNAi construct of targeting *AtJAS1* showed increased MeJA sensitivity [15]. In addition, AtJAZ1 protein has been revealed that it can repress transcription of jasmonate-responsive genes. Jasmonate treatment causes AtJAZ1 degradation and this degradation is dependent on the SCFCOI1-dependent 26S proteasome pathway [14]. Together, these data confirmed the importance of the Jas motif in the regulation of JAZ activity and suggesting that JAZ proteins are repressors of JA signaling. Knowledge of JAZ family genes in species other than *Arabidopsis* and *JAZs* function other than JA signaling repressors, is very limited.

Glycine soja L.G07256 is an ideal plant candidate for isolating salt and alkali tolerance related genes, as the seeds of this wild soybean can germinate in sodic soils of pH9.0 and continue to survive in nutrient solutions containing 50 mM $NaHCO_3$ [16]. In

* Corresponding author. Fax: +86 451 86668757.
E-mail address: ymzhu@neau.edu.cn (Y. Zhu).
[1] These authors contributed equally to this work.

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this study, we isolated a novel JAZ family gene from *G. soja* L.G07256 and named it *GsJAZ2*. Here, we investigated this gene's expression patterns in response to several abiotic stresses, using real-time quantitative PCR. Furthermore, we found that overexpression of *GsJAZ2* in *Arabidopsis* enhanced plant tolerance to salt and alkali stresses. Moreover, *GsJAZ2* was located in the nucleus suggesting it might play a role as a transcriptional regulator. Together, these data illustrate an important role of *GsJAZ2* as a regulator of salt and alkali stress responses in plants.

2. Materials and methods

2.1. Plant materials, growth conditions and stress treatments

Seeds of *G. soja* L.G07256 were obtained from the Jilin Academy of Agricultural Sciences (Changchun, China). For gene expression analysis, seedlings of *G. soja* L. were grown in a culture room with the following settings: 60% relative humidity, 24 °C and a light regime of 16 h light/8 h dark. The light source SON-T ARGO 400 W generated constant illumination of 30,000 lx. Before sowing, seeds of *G. soja* L. were shaken for 10 min in 98% sulfuric acid. Subsequently, seeds were washed five times with sterile water. Nineteen days after sowing, seedlings in the stress treatment group were transferred into 1/4 strength Hoagland's solution with 200 mM NaCl for salt stress, 50 mM $NaHCO_3$ for alkali stress, 4 °C for cold stress, 30% PEG for drought stress, respectively. Equal amounts of leaves and roots were sampled at 0, 0.5, 1, 3 and 6 h time points respectively.

Arabidopsis thaliana ecotype Columbia (Col-0), used for transformation, was grown in a greenhouse under controlled environmental conditions (21–23 °C, 100 μmol photons m^{-2} s^{-1}, 60% relative humidity, 16 h light/8 h dark cycles). For the expression analysis of salt and alkali stress-response marker genes, seeds from WT and *GsJAZ2* over-expression lines were sown on filter paper (Whatman 3MM) saturated with 0.5× MS solution. After 21 d of growth, seedlings were saturated with water (control), NaCl (200 mM) or $NaHCO_3$ (50 mM) respectively. Rosette leaf samples were taken as three biological replicates at 0, 1, 3, and 6 h after treatment.

2.2. Isolation and sequence analysis of GsJAZ2

The full-length cDNA of *GsJAZ2* was cloned based on microarray hybridization data and homology to Affymetrix® Soybean GeneChip® microarray probe Gma.10342.1.S1_at and the full cDNA of its matching gene Glyma01g41290.1. Total RNA was isolated from whole seedlings of *G. soja* L.G07256 using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and the cDNA was prepared using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The full-length cDNA of *GsJAZ2* was obtained using gene-specific primers: 5′-AATACCTCAGTGGAGAACAAGAT-3′ and 5′-TTACTAAAACTATGCTAACCAAACT-3′. The PCR products were cloned into the pGEM-T cloning vector (Promega, Madison, WI) and subjected to sequencing. Protein sequences were aligned using ClustalX [17].

2.3. Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), The isolated RNA was subjected to reverse transcription using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) with the oligo d(T)18 reverse primer. Prior to the qRT-PCR assays, the quality of the cDNA was assessed by PCR using *GADPH*-specific primers to test for genomic DNA contamination. One microliter of synthesized cDNA (diluted 1:5) was used as template for quantitative real-time PCR. qRT-PCR was performed on each cDNA template using the SYBR Green Master Mix on an ABI 7500 sequence detection system, according to the manufacturer's protocol (Applied Biosystems, USA).

GADPH (AFFX-r2-Gma-gapdh-M_at, accession # DQ355800) expression was used to normalize all values in the qRT-PCR assays in wild soybean (*G. soja*), and *ACTIN2* (At3g18780) was used as an internal control for salt and alkali stress response marker genes in *A. thaliana*. Primers for qRT-PCR were designed using Primer3 software [18]. Primer sequences are listed in Table 1. To enable statistical analysis, three fully independent biological replicates were obtained and subjected to real-time PCR runs in triplicate. Expression levels for all candidate genes were calculated using the $2^{-\Delta\Delta CT}$ method [19]. Relative amounts were calculated and normalized as described previously [20].

2.4. Transformation of Arabidopsis

The coding regions of *GsJAZ2* were amplified from pGEM-T-GsJAZ2 (described above) with the primer pair (5′-AATACCTCAGTG GAGAACAAGAT-3′) and (5′-GGTTTAAUTTACTAAAACTATGCTAAC-CA AACT-3′). The sequence was inserted into the pCAMBIA230035Su vector with the USER™ cloning technique [21], placed under the control of a CaMV35S promoter, with nptII as the selectable marker. The construct was introduced into *Agrobacterium tumefaciens* strain LBA4404, and transgenic *Arabidopsis* plants were generated by floral-dip [22]. Transformants were selected for on 0.5× MS medium containing 50 mg/L kanamycin. Seeds from each T_1 plant were individually collected. Selected T_2 plants were propagated, and homozygous overexpression lines were confirmed by RT-PCR analysis.

2.5. Phenotypic analysis of transgenic Arabidopsis plants

For salt and alkali-tolerance testing, seeds from WT and homozygous transgenic *Arabidopsis* were surfaced-sterilized [23], then were germinated on 0.5× MS agar medium for 7 d, followed by a transfer to fresh medium (in the absence or presence of 140 mM NaCl or 8.5 mM $NaHCO_3$) for 14 d of vertical growth before being photographed and measured for plant bolting rates.

All experiments were repeated at least three times, and the results from one representative experiment are shown. The numerical data was subjected to statistical analyses using Excel 2007 (Microsoft, http://www.microsoft.com) and SPSS statistical software 13.0 (SPSS Inc, http://www.spss.com).

Table 1
Gene-specific primers used for quantitative real-time PCR assays.

Gene name	Primer Sequence (5′ to 3′)
GsJAZ2	Forward: CACTGCCAATAATTCGGTTCA
	Reverse: GCTGCCATGTGATTGCTTGT
GAPDH	Forward: GACTGGTATGGCATTCCGTGT
	Reverse: GCCCTCTGATTCCTCCTTGA
ACTIN2	Forward: GAAGATGGCAGACGCTGAGGAT
	Reverse: ACGACCTACAATGCTGGGTAACAC
NXH1 (At5g527150)	Forward: CCACTCGAACCGTGCATTACT
	Reverse: CTCAAGCCTTACTAAGATCAGGAGG
SOS1 (At2g501980)	Forward: CGGCAGCATGGTTAATGTGTAC
	Reverse: TTGGCTGAAACGAGACCTTGA
NADP-ME (At5g11670.1)	Forward: TGGTCTGATCTACCCGCCATT
	Reverse: CGCCAATCCGAGGTCATAGG
H^+-Ppase (At1g15690.1)	Forward: ATGACGATGATGAAGAAGAAGAAGAT
	Reverse: TTTTTTAACCACCTACGGTAAACG
RD29B (At5g52300.1)	Forward: TGAAGGAGACGCAACAAGGG
	Reverse: CAACGGTGGTGCCAAGTGAT
KIN1 (At5g15960)	Forward: AACAAGAATGCCTTCCAAGC
	Reverse: CGCATCCGATACACTCTTTCC

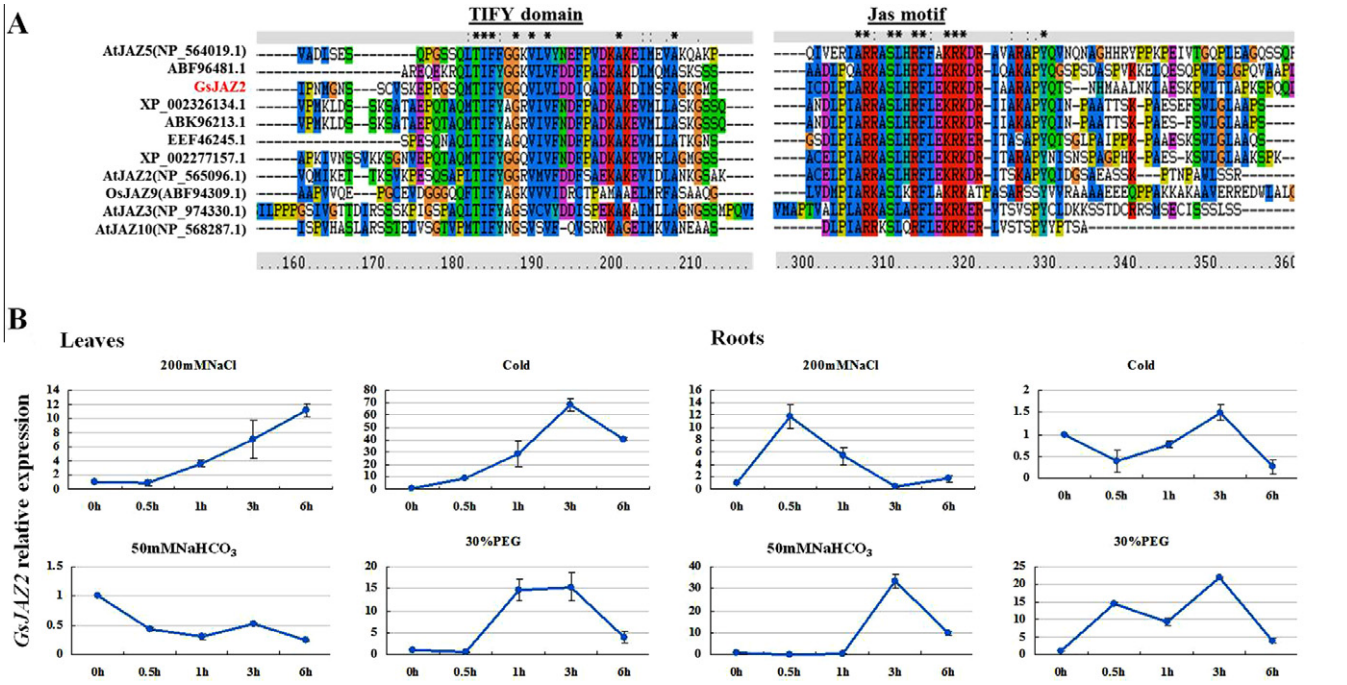

Fig. 1. Amino acid sequence analysis and expression patterns of the *GsJAZ2* under abiotic stresses. (A) Sequence alignment between GsJAZ2 and other JAZs. (B) *GsJAZ2* relative expression levels under the treatment of 200 mM NaCl, 50 mM $NaHCO_3$, 4 °C and 30% PEG at the indicated time points, respectively.

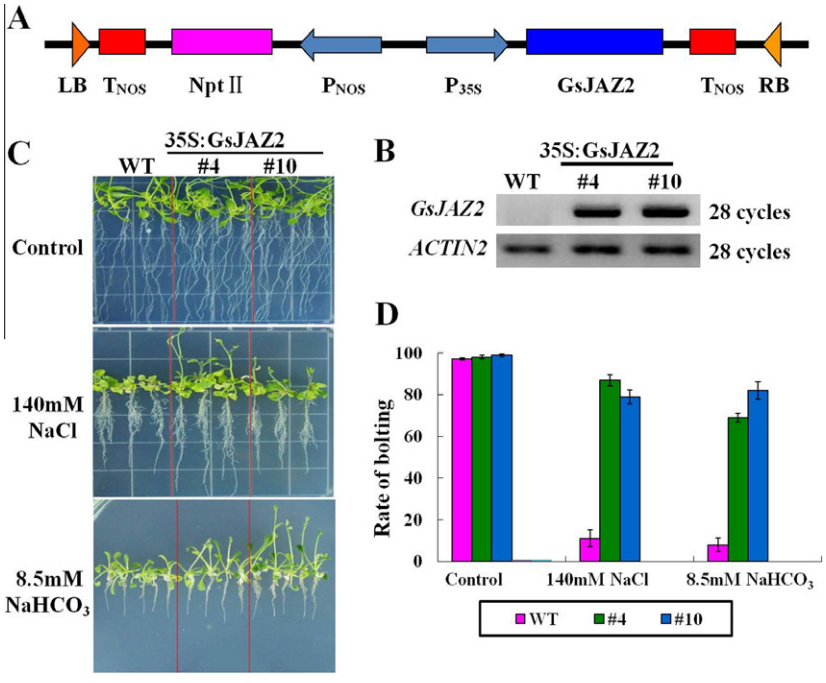

Fig. 2. Improvement of salt and alkali stress tolerance from the overexpression of *GsJAZ2 Arabidopsis*. (A) Schematic representation of a construct used for *Agrobacterium tumefaciens*-mediated transformation of the *GsJAZ2* gene. (B) Expression of *GsJAZ2* gene in WT and two homozygous transgenic lines (line4, line10). (C) Phenotypes of WT and transgenic seedlings (line4, line10) grown in either 0.5× MS medium or 0.5× MS medium containing 140 mM NaCl or 8.5 mM $NaHCO_3$. (D) Plant bolting rates are shown in (C). All values are means (±S.E.) from three independent experiments (30 seedlings per experiment).

2.6. Protein subcellular localization assay

For subcellular localization assays, the *GsJAZ2* gene was amplified with primer pair: 5′-CTTGTCGACGGAGAACAAGATG-3′ and 5′-GTCAGATCTCGTAACACAAAGCTGG-3′. The product was then cloned into *SalI*/*BglII*-digested pCAMBIA1302 to generate pCAMBIA1302-GsJAZ2:GFP, in which the *GsJAZ2* coding sequence was fused in-frame to the 5'end of the green fluorescent protein (GFP). The plasmids pCAMBIA1302 and pCAMBIA1302-GsJAZ2:GFP were then precipitated onto gold beads and transformed into onion (*Allium cepa*) epidermis as described [24]. Localization of fluorescent proteins in the epidermis was observed

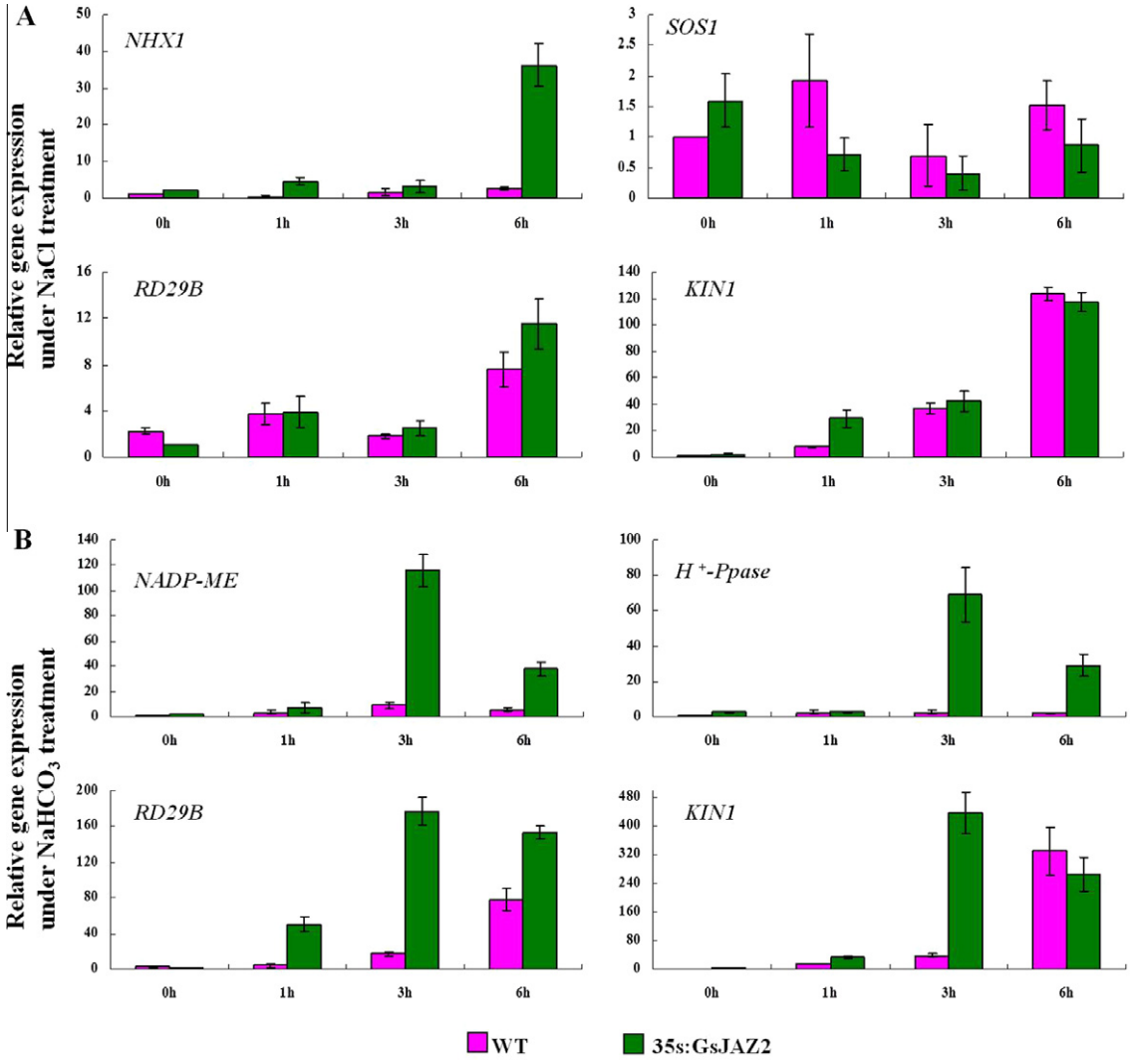

Fig. 3. Expression patterns of salt and alkali stress induced marker genes in WT and *GsJAZ2* transgenic *Arabidopsis* seedlings in response to salt or alkali stress. (A) The relative transcript levels of salt-responsive genes (*NHX1*, *SOS1*, *RD29B* and *KIN1*) under NaCl treatment. (B) The relative transcript levels of alkali-responsive genes (*NADP-ME*, *H*$^+$*-Ppase*, *RD29B* and *KIN1*) under $NaCO_3$ treatment.

at 488 nm using a confocal laser-scanning microscope (SP5, Leica, Germany).

3. Results

3.1. Isolation and sequence analysis of GsJAZ2

A novel saline-alkaline stress response JAZ family gene was previously identified by analyzing global transcriptome profiles of *G. soja* under $NaHCO_3$ treatment [25]. The full-length cDNA of this JAZ family gene was obtained by homologous cloning with gene-specific primers designed according to *Glycine max* cDNA sequences. We designated this gene *GsJAZ2*.

Sequence alignments showed that, like other JAZ family members, GsJAZ2 protein contained a Jas domain and a TIFY domain (Fig. 1A). The C-terminal conserved domain designated as Jas, with a characteristic motif of SLX2FX2KRX2RX5PY [26]. The Jas domain acts as a key regulator of jasmonate responses in *Arabidopsis* [13,14]. Besides the Jas domain, GsJAZ2 contained a TIFY domain, which normally contains 36 amino acids and the conserved motif of different proteins in the family has variant forms [12].

3.2. Expression of GsJAZ2 transcripts are induced by multiple abiotic stresses

Real-time PCR analysis was carried out to investigate the expression patterns of *GsJAZ2* under abiotic stresses in the leaves and roots of *G. soja*. Results showed in Fig. 1B. Under salt treatment, relative transcript abundance of *GsJAZ2* increased continuously in leaves starting after 0.5 h treatment. In salt-treated roots, transcript abundance also peaked at 0.5 h. During alkali treatment, *GsJAZ2* abundance decreased about a quarter of the normal level in leaves, but increased in roots with a peak at 3 h (32-fold). During the cold treatment, the *GsJAZ2* transcript in leaves greatly increased after 0.5 h and peaked at 3 h with 68-fold induction. In contrast, transcript abundance in cold-treated roots, showed relatively little change. For PEG-treated tissues, transcript abundance increased and peaked at 3 h in both leaves and roots. These results suggest that *GsJAZ2* is involved in responses to a wide variety of abiotic stresses, including salt, alkali, cold, dehydration.

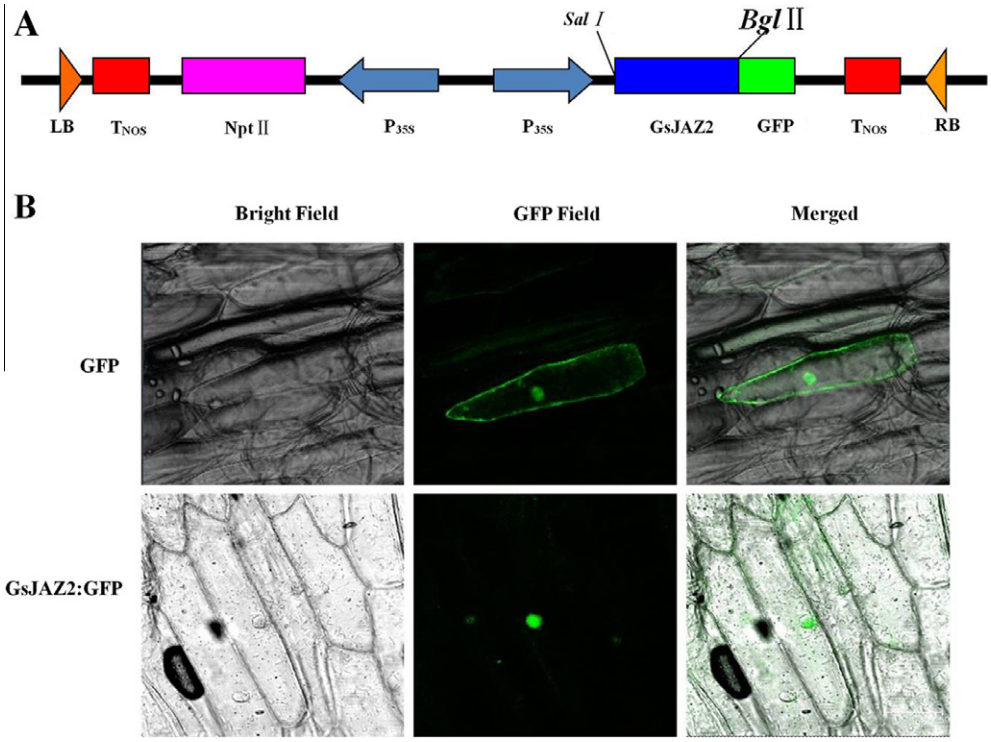

Fig. 4. Subcellular localization of GsJAZ2-GFP. (A) Schematic representation of constructs used for transformation and subcellular localization of the GsJAZ2 protein. (B) Subcellular localization assay of the GsJAZ2 protein.

The differences in the kinetics of transcript abundance changes observed in different tissues and treatments may indicate that *GsJAZ2* regulates more than one response pathway.

3.3. Overexpression of GsJAZ2 in Arabidopsis enhances plant salt and alkali stress tolerance

To further characterize the function of *GsJAZ2*, we generated *Arabidopsis* transgenic plants, in which *GsJAZ2* was overexpressed under the control of the strong constitutive CaMV35S promoter (Fig. 2A). Two independent homozygous transgenic lines (line4, line10) were obtained and the transcript abundance of *GsJAZ2* in each was estimated by semi-quantitative RT-PCR (Fig. 2B). Results showed that *GsJAZ2* transcript expression was strongly increased in both overexpression lines. No obvious effects on growth or development were observed in *GsJAZ2* transgenic plants under normal growth conditions.

Under standard culture conditions, we did not observe a noticeable difference on the growth of two transgenic lines (#4 and #10) over-expressing *GsJAZ2* compared with WT. However, when seven-day-old seedlings were subjected to NaCl or $NaHCO_3$ treatments, growth and development were inhibited in the wild-type plants compared to *GsJAZ2* overexpression lines, The overexpressing lines also exhibited significantly faster bolting rate than wild-type (Fig. 2C), statistical analysis of the measurements confirmed this result (Fig. 2D).

3.4. Salt and alkali stress response and stress-inducible marker genes are affected by GsJAZ2

To evaluate the role of *GsJAZ2* in salinity and alkalinity responses, the expression of some known salt or alkali stress-related marker genes was analyzed in *GsJAZ2* overexpression and wild-type plants following NaCl exposure or $NaHCO_3$ application. The expression of RD29B [27] and *KIN1* [28], vacuolar Na^+/H^+ antiporter *NHX1* and plasma membrane Na^+/H^+ antiporter *SOS1* have long been proposed to play roles in various stress responses [7,29]. We found that with the exception of a single time point, the expression of these genes did not differ significantly between wild-type and *GsJAZ2* overexpressing lines following NaCl treatment (Fig. 3A). However, *NHX1* was induced substantially at 6 h, in the *GsJAZ2* overexpression lines only. *NADP-ME* and *H^+-Ppase* have been experimentally shown to adjust the pHc in the cytoplasm by balancing the levels of malic acid or by excluding H^+ ions with H^+ pumps located in the plasmalemma and tonoplast of plant cells [30–32]. Therefore *NADP-ME* and *H^+-Ppase* were selected as marker genes of pH rebalancing activity under alkali stress. We found that the expression of *NADP-ME* and *H^+-Ppase* were significantly induced by $NaHCO_3$ stress at 3 h and 6 h in transgenic plants (Fig. 3B), however, neither gene was induced in wild-type. Therefore, it may be that *GsJAZ2* is positively regulates the expression of *NADP-ME* and *H^+-Ppase*, although it is not yet clear whether this effect is direct or indirect. Furthermore, expression of *RD29B* and *KIN1* were both more highly induced by alkali stress in *GsJAZ2* lines than in wild-type lines (Fig. 3B).

3.5. GsJAZ2 protein is targeted to the nucleus

To investigate the subcellular localization of the GsJAZ2, its coding sequence was fused in-frame to the N-terminus of GFP (Fig. 4A). This was expressed transiently, after biolistic delivery to onion epidermal cells. An examination of the fluorescent protein by confocal laser scanning microscopy showed that GFP fluorescence was localized to the nucleus of the onion cells when tagged to GsJAZ2, whereas untagged GFP exhibited fluorescence in both the cytosol and the nucleus (Fig. 4B).

4. Discussion

Salt and alkali are two major abiotic stresses that limit crop growth and productivity, with nearly half of the irrigated lands worldwide being affected by these conditions [33]. High salinity

and alkalinity cause both hyperionic and hyperosmotic stresses, which inhibit metabolism and can lead to plant death. Wild soybean (*G. soja* L.G07256) exhibits a relatively high adaptability to soil salt and alkali stress [16], the functional characterization of *GsJAZ2*, which can be strongly induced by abiotic stresses, will provide insight into plant abiotic stress adaptation and signal transduction.

In a recent study in our laboratory, we identified and characterized 34 *JAZ* genes from *G. soja* genome. Using microarrays, we found that most *GsJAZs* were induced under $NaHCO_3$ treatment, though with different spatial and temporal patterns (Results unpublished). In the current study, we showed that transcript expression of *GsJAZ2* was induced by salt, alkali, cold and drought (Fig. 1B). This suggests that the JAZ gene family may play important roles in abiotic stress tolerance.

We demonstrated that overexpressing *GsJAZ2* in *Arabidopsis* resulted in an increased tolerance to both salt and alkali stresses. The examination of the effects of *GsJAZ2* on the transcript levels of some abiotic stress-inducible marker genes (*NHX1*, *SOS1*, *NADP-ME*, *H^+-Ppase*, *RD29B* and *KIN1*), showed that, under alkali stress, *NADP-ME*, *H^+-Ppase*, *RD29B* and *KIN1* all displayed a higher induced expression level in transgenic plants than wild type plants (Fig. 3B). Previous reports have shown that *NADP-ME* and *H^+-Ppase* can be induced by alkali stress [34]. These enzymes play major roles in intracellular pH regulation, aiding plant cells in coping with the potential acidification of the cytoplasm under environmental stress [35,36]. *GsJAZ2* may regulate plant alkali stress tolerance by inducing the high expression of the two defense genes directly. The accumulation of RD29B and KIN1 proteins plays an important role in adjusting physiological conditions in plant cells [37]. The ability of *GsJAZ2* to upregulate *RD29B* and *KIN1* under alkali stress suggests that it may be an important regulator of alkali stress response. Under salt stress, *SOS1*, *RD29B* and *KIN1* showed no significant difference in expression levels between *GsJAZ2* overexpression lines and wild-type plants, except *NHX1*, was induced substantially at 6 h, in the *GsJAZ2* overexpression lines only. *NHX1* gene encodes a vacuolar Na^+/H^+ antiporter, included in sodium ion transmembrane transporter activity, which is important in salt tolerance. So the role of *GsJAZ2* in plant salt stress regulation may have relation with *NHX1*, and may play as a regulator in sodium ion transport or sodium ion export.

Subcellular localization assays indicate that *GsJAZ2* is a nuclear protein (Fig. 4), therefore it is likely that the GsJAZ2 protein is functional in this compartment, acting as a transcriptional regulator. Recently, yeast two-hybrid and in vitro pull-down assays demonstrated a direct physical interaction between the C-terminus of AtJAZ3 (containing the Jas motif) and the N-terminus of AtMYC2 (containing the activation domain), which is a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor and can regulate *AtJAZ3* gene expression via feedback [38,39]. Those further suggest a transcriptional regulator role of JAZ family genes.

In conclusion, we describe here a novel gene, *GsJAZ2*, identified in wild soybean, which can modify plant salt and alkali stress tolerance. However, the molecular mechanisms of plant salt and alkali stress tolerance are far from clear. Further study at the protein level of *GsJAZ2* may provide insight into the gene function and shed light on the molecular mechanisms of plant salt and alkali stress tolerance.

Acknowledgments

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