Mutation of GmAITR Genes by CRISPR/Cas9 Genome Editing Results in Enhanced Salinity Stress Tolerance in Soybean

Tianya Wang1†, Hongwei Xun1,2†, Wei Wang3, Xiaoyang Ding2, Hainan Tian1, Saddam Hussain1, Qianli Dong1, Yingying Li1, Yuxin Cheng1, Chen Wang1, Rao Lin1, Guimin Li1, Xueyan Qian2, Jinsong Pang1, Xianzhong Feng2, Yingshan Dong2, Bao Liu1 and Shucai Wang3*

1 Key Laboratory of Molecular Epigenetics of MOE, Northeast Normal University, Changchun, China, 2 National Engineering Research Center for Soybean, Soybean Research Institute, Jilin Academy of Agricultural Sciences, Changchun, China, 3 Laboratory of Plant Molecular Genetics and Crop Gene Editing, School of Life Sciences, Linyi University, Linyi, China, 4 Key Laboratory of Soybean Molecular Design Breeding, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, China

Breeding of stress-tolerant plants is able to improve crop yield under stress conditions, whereas CRISPR/Cas9 genome editing has been shown to be an efficient way for molecular breeding to improve agronomic traits including stress tolerance in crops. However, genes can be targeted for genome editing to enhance crop abiotic stress tolerance remained largely unidentified. We have previously identified abscisic acid (ABA)-induced transcription repressors (AITRs) as a novel family of transcription factors that are involved in the regulation of ABA signaling, and we found that knockout of the entire family of AITR genes in Arabidopsis enhanced drought and salinity tolerance without fitness costs. Considering that AITRs are conserved in angiosperms, AITRs in crops may be targeted for genome editing to improve abiotic stress tolerance. We report here that mutation of GmAITR genes by CRISPR/Cas9 genome editing leads to enhanced salinity tolerance in soybean. By using quantitative RT-PCR analysis, we found that the expression levels of GmAITRs were increased in response to ABA and salt treatments. Transfection assays in soybean protoplasts show that GmAITRs are nucleus proteins, and have transcriptional repression activities. By using CRISPR/Cas9 to target the six GmAITRs simultaneously, we successfully generated Cas9-free gmaitr36 double and gmaitr23456 quintuple mutants. We found that ABA sensitivity in these mutants was increased. Consistent with this, ABA responses of some ABA signaling key regulator genes in the gmaitr mutants were altered. In both seed germination and seedling growth assays, the gmaitr mutants showed enhanced salt tolerance. Most importantly, enhanced salinity tolerance in the mutant plants was also observed in the field experiments. These results suggest that mutation of GmAITR genes by CRISPR/Cas9 is an efficient way to improve salinity tolerance in soybean.

Keywords: GmAITRs, salinity tolerance, ABA, CRISPR/Cas9, genome editing, soybean
INTRODUCTION

As the fourth major crop and a nitrogen-fixing plant, soybean (*Glycine max*) is one of the most important protein- and oil-rich seed crops worldwide (Zhang et al., 2015; Vanliyodan et al., 2017), and it plays an important role in maintaining the cycling of nitrogen in ecosystems (Deshmukh et al., 2014). However, similar to other crops, growth and yield of soybean is largely affected by abiotic stresses including drought, salinity and extreme temperatures. As an example, drought alone can cause up to 40% yield loss of soybean globally (Wang et al., 2003; Fujita et al., 2006; Manavalan et al., 2009; Ray et al., 2013). In addition, drought and salinity are common in many different regions, and long-term drought caused by accelerated climate changes and global warming usually led to salinity. As a result, more than 50% of all arable lands on the earth may get seriously salinized by the year 2050, a dramatically increase from a currently ∼50% of all arable lands on the earth may get seriously salinized (Deshmukh et al., 2014). As the fourth major crop and a nitrogen-fixing plant, soybean is induced by both ABA and salt, and GmAITRs may serve as targets for CRISPR/Cas9 genome editing to improve abiotic stress tolerance in crops.

We report here the characterization of soybean AITRs (GmAITRs). We found that expression of *GmAITRs* is induced by both ABA and salt, and GmAITRs function as transcription repressors in transgenic soybean protoplasts. We generated transgene-free *gmaitr* mutants by using CRISPR/Cas9 genome editing to target *GmAITR* genes, and found that the *gmaitr* mutants showed enhanced tolerance to salt in both laboratory and field assessments.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Williams 82 (Wm82) wild type soybean (*Glycine max*) was used for plant transformation, protoplasts isolation and as control for the experiments. The transgene-free *gmaitr*36 double and *gmaitr*23456 quintuple mutants were generated by using CRISPR/Cas9 gene editing in the Wm82 wild type background.

For generation assays, ABA and salt tolerance assays, and gene expression in response to ABA and salt, seeds of the Wm82 wild type and the *gmaitr* mutants were generated on the surface of two
and one saline-alkali soil field, i.e., normal soil field 1 (E124° fields in Jilin province, including two fields with normal soil and root mean square deviation (RMSD) values were analyzed by PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). The protein structure of GmAITR2 was drawn by BIOVIA Discovery Studio Visualizer 2020.3

Sequence Alignment, Conserved Motif Analysis, and Three-Dimensional Protein Structure Prediction of GmAITRs

The full-length amino acid sequences of the six GmAITRs identified previously (Tian et al., 2017), were subjected to amino acid sequence alignment by using BioEdit with default settings, to motif analysis by using MEME with default settings (Bailey et al., 2009). The GmAITR sequences in the Wm82 wild type and the gna14t mutants were used for three-dimensional protein structures prediction by using Alphafold v2.0 with default settings (Jumper et al., 2021). The protein structural alignment and root mean square deviation (RMSD) values were analyzed by PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). The protein structure of GmAITR2 was drawn by BIOVIA Discovery Studio Visualizer 2020.3

Phylogenetic Analysis

The full-length amino acid sequences of GmAITRs, or GmAITRs and AITRs from Arabidopsis, Medicago, and rice were used for alignment on MAFFT (Katoh and Standley, 2013). Phylogenetic tree was generated based on the sequence alignment result, by using MEGA7 (Kumar et al., 2016). The cross-species analysis of AITRs was performed by using the Neighbor-Joining method based on the Poisson correction substitution model. All ambiguous positions were removed for each sequence pair. The sequences used in phylogenetic analysis have been listed by Tian et al. (2017).

Abscisic Acid and NaCl Treatment

To examine the expression of GmAITRs in response to ABA and NaCl, healthy and uniform-sized seeds of the Wm82 wild type were selected and grown hydroponically in distilled water for 14 days. The seedlings were then transferred to 100 μM ABA, 200 mM NaCl or distilled water as a control, and treated for 6 h. Roots and leaves were dissected from the seedlings immediately after the treatments, frozen in liquid nitrogen and stored in −80°C for RNA extraction.

To examine ABA response of the ABA signaling key regulator genes, seeds of the Wm82 wild type and the gna14t mutants grown in the plastic bags with distilled water for 14 days, then transferred to 100 μM ABA and distilled water as a control, and treated for 6 h. After the treatments, roots were collected and frozen in liquid nitrogen and stored in -80°C for RNA extraction.

RNA Isolation, cDNA Synthesis and qRT-PCR

For ABA response of GmAITR genes and ABA signaling key regulator genes, the above mentioned samples collected were used for RNA isolation. For tissue expression analysis, roots, stems and leaves were collected from 28-day-old soil pot-grown Wm82 wild type plants when the trifoliate leaf fully opened, frozen in liquid nitrogen and stored in -80°C for RNA extraction.

Total RNA was isolated from the samples collected by using an OminiPlant RNA kit (CWBio) according to the manufacturer’s instructions. During the isolation, RNA was treated with RNase-Free DNase (CWBio) to avoid the contamination of DNA. After the DNase treatment, 1 or 2 μg total RNA was used to synthesize cdNA by oligo(dt)20-primed reverse transcription using the EasyScript One-Step gDNA Removal and cdNA Synthesis SuperMix (TransGen Biotech). The synthesized cdNA was used as the template for gene expression analysis. For qRT-PCR, each sample was amplified in three parallel reactions as technical replicates, and the GmEF-1a (Glyma.17G186600) was amplified as a reference gene. The primers used for genes GmPYL9, GmPYL10, GmPYL12, and GmPP2C1 have been described previously (Bai et al., 2013), and the primers used for expression analysis of other genes are listed in Supplementary Table 1.

Constructs

The reporter construct Lexa-Gal4:GUS, and the effector constructs GD, GD-GmAITRs, GFP-GmAITRs, and LD-VP have been described previously (Tiwari et al., 2004; Wang et al., 2005; Tian et al., 2017). To generate CRISPR/Cas9 constructs for GmAITRs gene editing, the potential target sequences within the exons of GmAITRs were selected by using targetDesign on CRISPR-GE.5 Target specificity was then evaluated by using offTarget on CRISPR-GE. A total of six target sequences were selected. Due to the high CDS sequence similarity (>85%) between GmAITR gene pairs, i.e., GmAITR1 and GmAITR4, GmAITR2 and GmAITR5, and GmAITR3 and GmAITR6, each of the six target sequences was able to target one pair of genes. The six targets were divided into two groups with each group contains three target sequences that can target all the six genes. The target sequences were inserted into the pYL-CRISPR/Cas9Pubi-B vector to generate CRISPR/Cas9 genome editing constructs using the method described previously (Ma et al., 2015). The target sequences in construct one are

3http://skl.scau.edu.cn/home/
FIGURE 1 | Abscisic acid (ABA) induced transcription repressors (AITRs) in Soybean. (A) Phylogenetic relationship and gene structures of GmAITRs. Coding sequence (CDS) and untranslated region (UTR) sequences were indicated in black and gray boxes, respectively, and the lengths were drawn to scale. The phylogenetic analysis was performed by using MEGA 7.0. (B) Amino acid sequence alignment of GmAITRs. Full-length amino acid sequences of GmAITRs were obtained from phytozome (https://phytozome-next.jgi.doe.gov/), and used for alignment on BioEdit. Identical and similar amino acids are shaded in black and gray, respectively. Conserved motifs are predicted by MEME analysis (http://meme-suite.org), and indicated by underlines in different colors. Red box indicates the LxLxL transcriptional repression motif. (C) Three-dimensional structure of GmAITR2 predicted by AlphaFold v2.0 (https://www.alphafold.ebi.ac.uk/). The LxLxL repression motif was highlighted in scaled-atom form and the atom charge was added as the surface of the protein.

5’-GGATGCACC GG GTACATAC TACATACC(TGG)-3’ targets GmAITR2 and GmAITR5, 5’-GGA GGGTTTGGGCGATA(GGG)-3’ targets GmAITR1 and GmAITR4, and 5’-GG GTGACAGGCAC CG TGCAT(GGG)-3’ targets GmAITR3 and GmAITR6. The target sequences in construct two are 5’-GTGGTGTTCGT GTGTGACGG(TGG)-3’, 5’-G AGGGTTTCACGTG CAGGGTG(AAG)-3’ targets GmAITR1 and GmAITR4, and 5’-GTGAAAGCTGCGCTCAGTTT(GGG)-3’ targets GmAITR3 and GmAITR6. The primers used for making the constructs are listed in Supplementary Table 2.

Plant Transformation, Transgenic Plant Selection, and Transgene-Free Mutant Isolation

pYL-CRISPR/Cas9Pubi-B constructs for GmAITRs were transformed into the Agrobacterium tumefaciens strain of EHA105, and then used to transform soybean by using Agrobacterium-mediated cotyledinary node transformation method as previously described (Paz et al., 2004).

Transgenic plants generated were initially examined by using GMO DETECT kit (bar/pat) (Artron Laboratory Inc., Beijing) flowing the manufacturer’s instructions, and then examined by PCR amplification of Cas9 gene fragment. Gene editing status in the confirmed T1 transgenic plants was examined by amplifying and sequencing the genomic sequence of GmAITR genes. Transgene-free homozygous mutants were isolated from T2 progeny of gene edited T1 plants by PCR amplification of Cas9 gene fragment, and sequencing of GmAITR genes.

DNA Isolation and PCR

DNA was isolated from leaves of the T1 transgenic plants and T2 progeny of gene edited T1 plants by using a method described previously (Edwards et al., 1991).

To confirm the transgenic status of the T1 plants and to isolate transgene-free mutants in T2 progeny of gene edited T1 plants, DNA isolated was used as templates to amplify Cas9 gene fragment by PCR. The primers used were 5’-CGCTCAGATTGGAGATCAGT-3’ and 5’-CGAAGGTGATA-3’.

To examine genome editing status of GmAITR genes, DNA isolated was used as templates to amplify genome sequence of GmAITR genes by PCR, and the PCR products was isolated and sequenced. The sequencing results were aligned with wild type sequences of the Corresponding GmAITR gene. The primers
FIGURE 2 | Expression of GmAITRs in different tissues and organs, and in response to ABA and salt treatments. (A) Expression of GmAITRs in different tissues and organs. Roots, stems and leaves were collected from 28-day-old soil-grown plants with the trifoliate leaf fully opened, total RNA was isolated and qRT-PCR was used to examine the expression of GmAITRs. The expression of GmEF-1α was used as an inner control. The expression levels of GmAITRs in roots were set as 1. Data represent the mean ± SD of three replicates. (B) Expression of GmAITRs in response to ABA and salt treatments in roots (up panel) and leaves (low panel). Fourteen-day-old seedlings grown in plastic growth bags were exposed to distilled water, 100 µM ABA or 200 mM NaCl for 6 h, then the roots and leaves were dissected, total RNA were isolated and qRT-PCR was used to examine the expression of GmAITRs. The expression of GmEF-1α was used as an inner control. The expression levels of GmAITRs in distilled water control were set as 1. Data represent the mean ± SD of three replicates. The experiments were repeated three times with similar results. The asterisks in the figure indicate significant different from the control (*P < 0.05; **P < 0.01).

used for PCR amplification of GmAITR genes are listed in Supplementary Table 3.

Plasmid Isolation, Protoplast Isolation, and Transient Transfection

Plasmids of the reporter and effector constructs were extracted using a GoldHi EndoFree Plasmid Maxi Kit (CWBIO) according to the manufacturer’s instructions. Protoplasts were isolated and transfected by following a procedure previously described (Xiong et al., 2019). Briefly, protoplasts were isolated from trifoliate leaves of 2-week-old soil pot-grown Wm82 wild-type plants, plasmids were transfected or co-transfected into the protoplasts isolated, and transfected protoplasts were incubated under darkness at room temperature. For subcellular localization assays, the transfected protoplasts were incubated for 16–18 h, and then GFP fluorescence was examined under an Olympus BX61 fluorescence microscope. For transcription activity assays,
the transfected protoplasts were incubated for 22–24 h, and then GUS activities were measured by using a Synergy™ HT fluorescence microplate reader (BioTEK).

**Seed Germination Assays**

Healthy and uniform-sized seeds of the Wm82 wild type and the gmaitr mutant plants were placed in Petri plates on the surface of two layers of filter papers soaked with 100 μM ABA 200 mM NaCl, or distilled water as a control. The plates were kept in a growth room, and germinated seeds were counted at indicated time points. Each plate contains ten seeds and seeds with radicles longer than 0.5 cm were calculated as germinated seeds at the eleven time points (Kan et al., 2015).

**Seedling Growth Assays**

Healthy and uniform-sized seeds of the Wm82 wild type and the gmaitr mutant plants were germinated and grown with distilled water in plastic growth bags (PhytoTC, Beijing) (Li et al., 2019) for 3 days, and then initiated the salt treatment by adding the 200 mM NaCl solution or fresh distilled water as a control. Two parallel bags were used for each treatment, and two plants for each genotype were included in one bag, and different genotypes in different growth bags were placed in different order to minimize the position effects. After grown in a growth room for 2 weeks, seedlings were taken out from the growth bags for measurement of the shoot and root length.

**Field Production Assays**

For the agronomic traits comparison, seeds of the Wm82 wild type and the gmaitr mutant plants were sown in the experimental fields in plots by genotypes. Each plot in the two normal soil fields includes four rows, and each plot in the saline-alkali field includes three rows. The plot length was 2 m, the space between rows was 0.5 m, and the space between plants in the rows was 10 cm. The seeds were sown in May and the plants were harvested in October in the year 2020.

**Statistical Analysis**

A statistical analysis of the phenotypic data and expression levels was performed using two-tailed Student’s t-test in Excel (*P < 0.05, **P < 0.01).

**RESULTS**

**Abscisic Acid Induced Transcription Repressors in Soybean**

We have previously identified that there are six genes in soybean encoding AITRs, a number identical to that in Arabidopsis (Tian et al., 2017). Similar to the Arabidopsis AITR genes (Tian et al., 2017), all the 6 GmAITRs are genes with a single exon (Figure 1A). Phylogenetic analysis shows that GmAITR2 is closely related to GmAITR5, whereas GmAITR1 is closely related to GmAITR4, and together, these four GmAITRs formed one clade. On the other hand, GmAITR3 is closely related to GmAITR6, and they formed another clade (Figure 1A). Expanded phylogenetic analysis with AITRs from the dicot plant Arabidopsis, soybean and Medicago and the monocot plant rice (Oryza sativa) shows that the two OsAITRs formed a distinct clade, whereas two other clades were formed by AITRs from the three dicot plants, and both of the clades contain AITRs from all the three dicot plants (Supplementary Figure 1).

Sequence alignment shows that GmAITRs shared high amino acid identity and similarity, and contain a conserved LxLxL motif at their C-terminal (Figure 1B). Protein domain assays indicates that these three conserved domains in all the GmAITRs, one at the N-terminal, one in the middle region and the third is the LxLxL motif containing domain at the C-terminal (Figure 1B and Supplementary Figure 2). In addition, GmAITRs are hydrophilic and non-transmembrane.

\[\text{http://www.detaibio.com/sms2/protein_gravy.html}\]
proteins\textsuperscript{7}, and protein structure prediction with AlphaFold v2.0 (Jumper et al., 2021) indicate that all the GmAITRs have similar three-dimensional structures (Figure 1C and Supplementary Figure 3). These results suggest that GmAITRs may have similar functions.

\textsuperscript{7}http://www.cbs.dtu.dk/services/TMHMM/

Expression of GmAITRs Is Induced by Abscisic Acid and Salt, and GmAITRs Function as Transcription Repressors

To examine the functions of GmAITRs in ABA signaling and abiotic stress tolerance, we first examined the expression pattern of GmAITR genes. We found that GmAITR genes showed diverse expression patterns in the tissues and organs examined. In
general, relative higher expression levels for all the 6 \textit{GmAITR} genes were observed in stems, and all but \textit{GmAITR2} also have relative higher expression levels in leaves (Figure 2A). However, difference in expression levels in different tissues and organs were observed for different \textit{GmAITR} genes, for example, the highest expression level of \textit{GmAITR1} was observed in stems, but it was only about 2.5-fold of that in root, whereas that of \textit{GmAITR4} in leaves was nearly 50-fold of that in root (Figure 2A).

We have previously shown that the expression of \textit{GmAITR} genes is induced by treating excised soybean roots with ABA (Tian et al., 2017). Having shown that \textit{GmAITRs} showed different expression patterns in the tissues and organs, we then compared ABA response of \textit{GmAITR} genes in roots and leaves. We found that the expression levels of all the \textit{GmAITR} genes were increased in response to ABA treatment in both root and leaves, but to different levels. For instance, an ~80- 110-fold increase for \textit{GmAITR1} and \textit{GmAITR3}, respectively in roots, and an ~27-fold increase for \textit{GmAITR2} in leaves (Figure 2B).

We also examined the expression of \textit{GmAITRs} in response to salt stress, and found that salt treatment induced the expression of different \textit{GmAITR} genes at least in roots or leaves, although to a relative lower levels when compared to ABA treatment (Figure 2B).

We further examined subcellular localization and transcriptional activity of \textit{GmAITRs} in soybean protoplasts. Similar to the results observed in transfected Arabidopsis protoplasts (Tian et al., 2017), \textit{GmAITRs} were localized in nucleus (Figure 3A), and they repressed the expressed \textit{Gal4:GUS} reporter gene when recruited to the \textit{Gal4} promoter by the fused \textit{Gal4} DNA binding domain (Figure 3B). These results suggest that \textit{GmAITRs} function as transcription repressors in soybean.

\textbf{Generation of Genome Edited Transgene-Free Mutants for \textit{GmAITR} Genes}

Our previously studies have shown that \textit{AITR}s are conserved in angiosperms, and \textit{AITR} genes may be good targets for CRISPR/Cas9 genome editing to improve abiotic stress tolerance in crops (Tian et al., 2017; Chen et al., 2021; Wang et al., 2021).
Our results described above indicate that GmAITRs and Arabidopsis AITRs shared similar features, we therefore decided to generate transgene-free mutants of GmAITR genes by using CRISPR/Cas9 genome editing, and examine their response to ABA and abiotic stresses.

Two different CRISPR/Cas9 constructs were generated using the pYL-CRISPR/Cas9\textsubscript{ubi}-B vector (Ma et al., 2015), and each construct contains three target sequences with each is aimed to target a pair of GmAITR genes. The Wm82 wild type soybean was used for plant transformation, and gene edited status were examined in T1 plants, and transgene-free homozygous mutants were isolated from progeny of gene edited T1 plants. Editing of GmAITR3 and GmAITR6 were observed in T1 plants generated with one construct, and editing of GmAITR2-GmAITR6 were observed in T1 plants generated with another construct. Finally, transgene-free gmair3 gmair6 (gmair36) double and gmair2 gmair3 gmair4 gmair5 gmair6 (gmair23456) quintuple homozygous mutants were obtained from construct one and two transformed plants, respectively.

In all the mutants obtained, either a single nucleotide insertion or one to up to 60 nucleotides deletion was occurred at the target sites for the GmAITR genes (Figure 4A), resulting in changes of amino acid sequence of the corresponding GmAITR proteins. In both gmair36 double mutants, amino acid substitutions and premature stop occurred in GmAITR3, whereas amino acid substitutions and premature stop occurred in GmAITR6 in the gmair36-c1 double mutant, and immediately premature stop occurred in GmAITR6 in the gmair36-c2 double mutant, respectively (Figure 4B). In the gmair23456 quintuple mutants, 20 amino acids deletion occurred in GmAITR2, an amino acid substitution and premature stop occurred in GmAITR4, and amino acid substitutions and premature stop occurred in GmAITR5 (Figure 4B). However, nucleotides deletions in GmAITR3 and GmAITR6 in the gmair23456 quintuple mutants led to amino acid substitution and addition of extra amino acids in corresponding GmAITR proteins (Figure 4B). The positions of amino acids changes in the GmAITR proteins for the gmair36 double and gmair23456 quintuple mutants were diagrammed in Figure 4C. Moreover, protein structures of genome edited GmAITRs were predicted by AlphaFold v2.0 (Supplementary Figure 3), and obvious differences can be found in gmair36 double mutants for protein GmAITR3 and GmAITR6. In gmair23456 quintuple mutants, protein structures of GmAITR4 and GmAITR5 were severely damaged compared with wild type, while GmAITR2, GmAITR3, and GmAITR6 preserved similar structures as wild type.

The gmair Mutants Are Hypersensitivity to Abscisic Acid

By using seed germination assays, we examined ABA response of the gmair mutants generated. Different from the results observed in the Arabidopsis aitr mutants, which showed a decreased ABA sensitivity (Tian et al., 2017; Chen et al., 2021), we found...
that seeds of all the gmait mutants were more sensitivity to ABA treatment when compared to the Wm82 wild type seeds (Figure 5A). Quantitative assays show that no difference was observed for the Wm82 wild type and the mutant seeds on control plates (plates soaked with distilled water, which is the dissolvent of ABA and salt solution), seeds of all the plants reached a maximum germinate rate, i.e., ∼100% 48 h after treatment. On the other hand, when compared to the Wm82 wild type seeds, a reduced germination rate was observed for seeds of all the mutants on the ABA treated plates (Figure 5B), indicating that ABA sensitivity in the mutants was increased. However, we found that germination rate of the gmaitr36 double mutant seeds is largely indistinguishable from that of the gmaitr23456 quintuple mutant seeds (Figure 5B). Our previously results indicated that AITRs in Arabidopsis function as feedback regulators in ABA signaling by inhibiting ABA responses of some ABA signaling regulators genes (Tian et al., 2017; Chen et al., 2021). Having shown that ABA response in the gmaitr mutants was affected, we further examined if expression levels of the core ABA signaling regulator genes may be changed in the gmaitr mutants. We treated the gmaitr mutants and Wm82 seedlings with different concentration of ABA solution, and ABA key regulator genes were significantly induced in soybean seedlings treated with 100 μM ABA, thus 100 μM ABA was used for expression analysis. We found that the basal expression levels of some ABA signaling key regulator genes identified previously (Bai et al., 2013), including the GmPYL receptor genes GmPYL9, GmPYL10, GmPYL12, and the PP2C phosphatase gene GmPP2C1 remained largely unchanged in the gmaitr mutants (Figure 6A). However, ABA induced responses of these genes were reduced in the gmaitr mutants, even though little, if any difference was observed between the gmaitr36 double and the gmaitr23456 quintuple mutants (Figure 6B).

The gmaitr Mutant Plants Are Tolerant to Salt Stress

Changes in the expression levels of the ABA signaling regulator genes including Arabidopsis AITR genes have been shown to affect plant abiotic stress tolerance (Fujita et al., 2009; Park et al., 2015; Yoshida et al., 2015; Zhao et al., 2016; Tian et al., 2017; Chen et al., 2021), but so far only aitr mutants showed enhanced tolerance to drought and salt, make AITRs good candidate genes for CRISPR/Cas9 genome editing to improve abiotic stress tolerance in plants (Tian et al., 2017; Chen et al., 2021).

To examine if mutation of GmAITR genes may indeed improve abiotic stress tolerance in soybean, we first examined the effects of salt treatment on seed germination of the gmaitr mutants. We found that the gmaitr mutant seeds showed enhanced tolerance to salt treatment (Figure 5A), and quantitative assays showed that an increased germination rate were observed for seeds of gmaitr mutants at all the time points examined (Figure 5B). But similar to the results observed with ABA treatment, little, if any difference was observed between
FIGURE 8 | Field production of the Wm82 wild type and the gmaitr mutant plants in normal and saline-alkali soil lands. (A) Plants of the Wm82 wild type and the gmaitr mutants in normal and saline-alkali soil lands. The Wm82 wild-type and the gmaitr mutants were grown in two normal soil and one saline-alkali land (pH 8.1–9.8; soluble saline 0.1–0.7%) for field production analysis in the year 2020. Seeds were planted in plots by genotypes. Each plot in normal fields includes four rows, and plots in saline-alkali field include three rows. Numbers of seeds planted in a row for each plot were the same. Upper panel, field images of 4-month-old plants from one of the normal soil land the saline-alkali soil land. The white frames were used to indicate the edges of the plots. Low panel, images of five bundled representative mature plants for each genotype from one of the normal soil land the saline-alkali soil land. (B) Yield indexes of the Wm82 wild type and the gmaitr mutant plants in normal and saline-alkali soil lands. The Wm82 wild-type and the gmaitr mutant plants were harvested and plants randomly selected were used for yield indexes measurement, including plant height, pods produced per plant, and hundred-seed weight. For each field, the measurement was repeated four times with four different set of plants. Each set of plants contain five randomly selected plants from each plot. Data represent the mean ± SD of at least four replicates. The asterisks indicate significant differences (*P < 0.05; **P < 0.01).
the gmaitr36 double and the gmaitr23456 quintuple mutant seeds (Figure 5B).

We then examined the effects of salt treatment on seedling growth of the gmair mutants. As shown in Figure 7A, the gmair mutant seedlings showed enhanced tolerance to salt treatment, as they produced longer roots and shoots when compared with the Wm82 wild type seedlings (Figure 7B).

At last, we compared growth and yield of the Wm82 wild type and the gmair mutant plants in both normal soil field and saline-alkali soil field. We found that the gmair mutant plants are morphological similar to the Wm82 wild type plants in the normal soil field, but growth better in the saline soil field (Figure 8A). Both the Wm82 wild type and the gmair mutant plants reached a height of ∼110 cm at mature stage, with ∼45 pods per plant, and produced seed with hundred-seed weight of ∼18 g (Figure 8B). Plants height, number of pods per plants and hundred-seed weight were all dramatically decreased in the saline-alkali soil field, however, the gmair mutant plants were less affected (Figure 8B).

**DISCUSSION**

Even though CRISPR/Cas9 genome editing has been successfully used to improve important agronomic traits in several different crops (Ma et al., 2015; Gao et al., 2016; Li X. et al., 2017; Lu et al., 2017; Shimatani et al., 2017; He et al., 2018; Zsögön et al., 2018; Chen et al., 2019), identification of suitable candidate genes in ABA signaling pathway for genome editing to improve abiotic stress tolerance in crops is a big challenge. In soybean, several different types of transcription factors involved in abiotic stress tolerance have been reported to be related to ABA signaling pathway, such as the AP2/ERF transcription factor GmERF3 (Zhang et al., 2009), the bZIP transcription factor GmbZIP1, GmbZIP15 and GmFDL19 (Gao et al., 2011; Li Y. et al., 2017; Zhang et al., 2020), the R2R3 MYB transcription factor GmMYB84 (Wang et al., 2017), the WRKY transcription factor GmWRKY12 and GmWRKY54 (Shi et al., 2018; Wei et al., 2019), and the NAC transcription factor GmNAC1, GmNAC06 and GmNAC8 (Li et al., 2019, 2021; Yang et al., 2020). However, among all these transcription factors, only GmbZIP15 functioned as a negative regulator of abiotic stress tolerance in soybean, yet no enhanced tolerance was observed in the transgenic soybean plants expressing a repressor form of GmbZIP15 (Zhang et al., 2020). These results suggest that none of these transcription factor genes can serve as targets for CRISPR/Cas9 gene editing to improve abiotic stress tolerance in soybean.

We have previously identified AITRs as a novel family of transcription factors conserved in angiosperms, and loss-of-function of AITR genes enhanced abiotic stress tolerance in Arabidopsis without fitness costs, indicating that AITRs may be good candidates for gene editing to improve abiotic stress tolerance in crops (Tian et al., 2017; Chen et al., 2021). By using a combination of different assays including gene expression assays, transcriptional activity assays, generation of transgene-free gene edited mutants, and physiological and field yield analysis, we show that GmAITRs are ABA and salt inducible transcription repressors, and GmAITRs can be targeted to improve salinity stress tolerance in soybean.

First, we show that the expression of GmAITRs was induced by both ABA and salt treatments, eventhough these genes have different expression pattern, and there are difference among these genes in responses to ABA and salt (Figure 2). Second, we found that, similar to the results observed in Arabidopsis protoplasts (Tian et al., 2017), GmAITRs proteins localized in nucleus and they repressed reporter gene expression in soybean protoplasts (Figure 3). Third, ABA inhibited seed germination was affected in the gmair mutants (Figure 5), and ABA response of some ABA signaling key regulator genes was altered in the gmair mutants (Figure 6). These results suggest that GmAITRs are ABA responsive transcription repressors and they regulate ABA response in soybean via affecting ABA signaling. Forth, the gmair mutants showed enhanced tolerance to salt in both seed germination and seedling growth assays (Figures 5, 7). Last but not least, field experiments suggest that the gmair mutants performed better in the saline-alkali soils when compared to the Wm82 wild type plants (Figure 8). These results suggest that genome editing of GmAITR genes is able to enhance salt tolerance in soybean.

It should be noted that in ABA inhibited seed germination assays, the gmair mutants showed increased sensitivity to ABA (Figure 5), a result different from that of the Arabidopsis aitr mutants, which showed decreased sensitivity to ABA (Tian et al., 2017; Chen et al., 2021), suggest that there is some difference between GmAITRs and Arabidopsis AITRs in regulating ABA responses. However, the gmair mutants also showed enhanced tolerance to salt (Figures 5, 7), similar to that observed in the Arabidopsis aitr mutants (Tian et al., 2017; Chen et al., 2021), making them good targets for genome editing to improve abiotic stress tolerance in soybean.

We also noted that the gmair23456 quintuple mutants are largely indistinguishable to the gmair36 double mutants in both ABA and salt tolerance assays, and in field growth conditions (Figures 5, 7, 8). Even though we cannot rule out the possibility that some of the GmAITRs may have a dominate roles in regulation ABA response and salt tolerance, as we previously observed for the Arabidopsis AITRs (Chen et al., 2021). Based on the conserved motif analysis (Figure 4) and protein structure prediction results (Supplementary Figure 3), a possible explanation is that the editing to GmAITR2, GmAITR3, and GmAITR6 in the gmair23456 quintuple mutants may not led to loss-of-function of these genes. First, as the genome editing of GmAITR2 in the gmair23456 quintuple mutants only resulted in a deletion of 20 amino acids outside the conserved motifs (Figure 4), whereas genome editing of both GmAITR3 and GmAITR6 in the gmair23456 quintuple mutants only disrupted the LxxLxxL motif at the C-terminal of GmAITR3 and GmAITR6, respectively. Our previous results with Arabidopsis AITRs have already shown that the deletion of LxxLxxL motif affected AITRs’ transcriptional repression activities, but they are still able to function as transcription repressors (Tian et al., 2017). Second, according to the three-dimensional protein structure prediction, the protein binding pockets structure, which is important for protein functionality (Stank et al., 2016), were
barely not damaged for GmAITR2, GmAITR3 and GmAITR6 in the gmaitr23456 quintuple mutants compared with wild type (Supplementary Figure 3). Therefore, it will be of great interest to generate high-order loss-of-function mutants of GmAITR genes and to examine if increased tolerance to abiotic stresses can be achieved, and if there are any fitness costs. It will be of great interest to compare physiological/biochemical index in the Wm82 and the gmaitr mutants, and use more negative controls for the ABA and salt related response analysis, therefore to understand the subtle changes and physiological mechanism of GmAITR in abiotic stress tolerance. It will be also of great interest to edit GmAITR genes in soybean cultivars with other good agronomic traits to see if enhanced abiotic stress tolerance can be obtained without affecting these agronomic traits, thereby accelerating the molecular breeding process of soybean with different benefit agronomic traits.

On the other hand, considering that in all the major crops, AITRs are encoded by multiple genes (Tian et al., 2017), loss-of-function of a few AITR genes can already led to enhanced abiotic stress tolerance making it more practicable for editing AITR genes to improve abiotic stress tolerance in crops. After all, it is not easy to edit all the AITR genes simultaneously in a crop.

Nevertheless, our results show that GmAITRs are involved in the regulation of ABA response and abiotic stress tolerance in soybean, and CRISPR/Cas9 genome editing of GmAITR genes is able to enhance salt tolerance in soybean.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

REFERENCES

Bai, G., Yang, D., Zhao, Y., Ha, S., Yang, F., Ma, J., et al. (2013). Interactions between soybean ABA receptors and type 2C protein phosphatases. Plant Mol. Biol. 83, 651–664. doi: 10.1007/s11103-011-0114-4

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., et al. (2009). MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208.

Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu. Rev. Plant Biol. 70, 667–697.

Chen, S., Zhang, N., Zhou, G., Hussain, S., Ahmed, S., Tian, H., et al. (2021). Knockout of the entire family of AITR genes in Arabidopsis leads to enhanced drought and salinity tolerance without fitness costs. BMC Plant Biol. 21:137. doi: 10.1186/s12870-021-02907-9

Deshmukh, R., Sonah, H., Patil, G., Chen, W., Prince, S., Mutava, R., et al. (2014). Integrating omic approaches for abiotic stress tolerance in soybean. Front. Plant Sci. 5:244. doi: 10.3389/fpls.2014.00244

Dong, T., Park, Y., and Hwang, I. (2015). Abscisic acid: biosynthesis, inactivation, homoeostasis and signalling. Essays Biochem. 58, 29–48. doi: 10.1042/bse0580029

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.

AUTHOR CONTRIBUTIONS

SW, BL, YD, XF, and JP conceived the study. TW and SW designed the experiments, analyzed the data, and drafted the manuscript. HT made the CRISPR/Cas9 constructs. HX and XQ generated the mutants. TW, WW, and GL examined the gene editing status in the mutants. TW, XD, SH, YL, YC, CW, and RL did the experiments. QD performed the bioinformatics analysis. All authors participated in the revision of the manuscript, read and approved the final manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (32071938), the National Key R&D Program of China (2016YFD0101900), and a startup funding from Linyi University (LYDX2019BS039). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Yaoguang Liu (South China Agricultural University) for sharing the pYL-CRISPR/Cas9_pubi-B vector, and all the lab members for helpful discussion.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.779598/full#supplementary-material

Fujita, H., Verslues, P. E., and Zhu, J. K. (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. Plant Cell 19, 485–494. doi: 10.1105/tpc.106.048538

Fujita, H., and Zhu, J. K. (2009). Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc. Natl. Acad. Sci. U. S. A. 106, 8380–8385. doi: 10.1073/pnas.0903144106

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., et al. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. 9, 436–442. doi: 10.1016/j.pbi.2006.05.014

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., et al. (2009). Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. Plant Cell Physiol. 50, 2123–2132. doi: 10.1093/pcp/pcp147

Gao, S. Q., Chen, M., Xu, Z. S., Zhao, C. P., Li, L., Xu, H. J., et al. (2011). An effective strategy for reliably isolating heritable and Cas9-free Arabidopsis mutants generated by RISPR/Cas9-mediated genome editing. Plant Physiol. 171, 1794–1800. doi: 10.1104/pp.110.160663
Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013). Matres, J. M., Hilscher, J., Datta, A., Armario-Nájera, V., Baysal, C., He, W., Ray, D. K., Mueller, N. D., West, P. C., and Foley, J. A. (2013). Programmed self-elmination of the CRISPR/Cas9 construct greatly accelerates the isolation of edited and transgene-free rice plants. Mol Plant 11, 1210–1213. doi: 10.1016/j.molp.2015.08.005

Jumper, J., Evans, R., Pritzel, A., Figurnov, M., Ronneberger, O., et al. (2018). Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589.

Kan, G., Zhang, W., Yang, W., Ma, D., Zhang, D., Hao, D., et al. (2015). Association mapping of soybean seed germination under salt stress. Mol Genet. Genomics 290, 2147–2162. doi: 10.1007/s00438-015-1066-y

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. doi: 10.1093/molbev/mst010

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874. doi: 10.1093/molbev/msw054

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D., Bush, J., et al. (2015). Protein binding pocket dynamics. Acc. Chem. Res. 49, 809–815.

Tian, H., Chen, S., Yang, W., Wang, T., Zheng, K., Wang, Y., et al. (2017). A novel family of transcription factors conserved in angiosperms is required for ABA signalling. Plant Cell Environ. 40, 2958–2971. doi: 10.1111/pce.13058

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol. 31, 686–688. doi: 10.1038/nbt.2650

Shi, W. Y., Du, Y. T., Ma, J., Min, D. H., Jin, L. G., Chen, J., et al. (2018). The WRTKY transcription factor GmWRKY12 confers drought and salt tolerance in soybean. Int. J. Mol. Sci. 19:4087. doi: 10.3390/ijms1924087

Shimatzu, Z., Kasahoji, S., Takayama, M., Terada, R., Arazoe, T., Ishii, H., et al. (2017). Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat. Biotechnol. 35, 441–443. doi: 10.1038/nbt.3833

Song, L., Huang, S. C., Wise, A., Castanon, R., Nery, J. R., Chen, H., et al. (2016). A transcription factor hierarchy defines an environmental stress response network. Science 354:aaag1550. doi: 10.1126/science.aaa5350

Stank, A., Kokh, D. B., Fuller, J. C., and Wade, R. C. (2016). Protein binding pocket dynamics. Acc. Chem. Res. 49, 809–815.

Tiwari, S. B., Hagen, G., and Guilfoyle, T. J. (2004). AuxI/IAA proteins contain a potent transcriptional repression domain. Plant Cell 16, 533–543. doi: 10.1105/pcp.107384

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., et al. (2010). Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. Plant Cell Physiol. 51, 1821–1839. doi: 10.1093/pcp/pcq156

Vanlijnendon, B., Ye, H., Song, L., Murphy, M., Shannon, J. G., and Nguyen, H. T. (2017). Genetic diversity and genomic strategies for improving drought and waterlogging tolerance in soybeans. J. Exp. Bot. 68, 1835–1849. doi: 10.1093/jxb/erw433

Wang, N., Zhang, W., Qin, M., Li, S., Qiao, M., Liu, Z., et al. (2017). Drought tolerance conferred in soybean (Glycine max (L) by GmMYB84, a Novel R2R3-MYB transcription factor. Plant Cell Physiol. 58, 1764–1776. doi: 10.1038/pcc.201711

Wang, S., Tiwari, S. B., Hagen, G., and Guilfoyle, T. J. (2005). AUXIN RESPONSE FACTOR5 restores the expression of auxin-responsive genes in mutant Arabidopsis leaf mesophyll protoplasts. Plant Cell 17, 1979–1993. doi: 10.1105/tpc.105.031096

Wang, T., Dong, Q., Wang, W., Chen, S., Cheng, Y., Tian, H., et al. (2021). Evolution of AITR family genes in cotton and their functions in abiotic stress tolerance. Plant Biol. 23, 58–68. doi: 10.1111/plb.13218

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Plant Sci. 198, 1–14. doi: 10.1016/s0168-9452(01)00211-2

Wei, W., Liang, D. W., Bian, X. H., Shen, M., Xiao, J. H., Zhang, W. K., et al. (2019). GmWRKY54 improves drought tolerance through activating genes in abscisic acid and Ca++ signaling pathways in transgenic soybean. Plant J. 100, 384–398. doi: 10.1111/tpj.14449

Xiong, L., Li, C., Li, H., Luy, X., Zhao, T., Liu, J., et al. (2019). A transient expression system in soybean mesophyll protoplasts reveals the formation of cytoplasmic GmCR1R1 photobody-like structures. Sci. China Life Sci. 62, 1070–1077. doi: 10.1007/s11427-018-9496-5

Xu, Y., Lu, Y., Xie, C., Gao, S., Wan, J., and Prasanna, B. M. (2012). Whole-genome association mapping of soybean seed germination under salt stress. Mol Genet. Genomics 287, 391–404. doi: 10.1007/s00438-012-1064-x

Yang, C., Huang, Y., Lv, W., Zhang, Y., Bhat, J. A., Kong, J., et al. (2020). GmNAC8 acts as a positive regulator in soybean drought stress. Plant Sci. 293:110442. doi: 10.1016/j.plantsci.2020.110442
Yoshida, T., Mogami, J., and Yamaguchi-Shinozaki, K. (2014). ABA-dependent and ABA-independent signaling in response to osmotic stress. *Curr. Opin. Plant Biol.* 21, 133–139.

Zhang, G., Chen, M., Li, L., Xu, Z., Chen, X., Guo, J., et al. (2009). Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. *J. Exp. Bot.* 60, 3781–3796. doi: 10.1093/jxb/erp214

Zhang, M., Liu, Y., Cai, H., Guo, M., Chai, M., She, Z., et al. (2020). The bZIP transcription factor GmbZIP15 negatively regulates salt- and drought-stress responses in soybean. *Int. J. Mol. Sci.* 21:7778. doi: 10.3390/ijms21207778

Zhang, Y., He, J., Wang, Y., Xing, G., Zhao, J., Li, Y., et al. (2015). Establishment of a 100-seed weight quantitative trait locus-allele matrix of the germplasm population for optimal recombination design in soybean breeding programmes. *J. Exp. Bot.* 66, 6311–6325. doi: 10.1093/jxb/erv342

Zhao, Y., Chan, Z., Gao, J., Xing, L., Cao, M., Yu, C., et al. (2016). ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proc. Natl. Acad. Sci. U. S. A.* 113, 1949–1954. doi: 10.1073/pnas.1522840113

Zsögön, A., Čermák, T., Naves, E. R., Notini, M. M., Edel, K. H., Weinl, S., et al. (2018). *De novo* domestication of wild tomato using genome editing. *Nat. Biotechnol.* 36, 1211–1216. doi: 10.1038/nbt.4272

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Wang, Xun, Wang, Ding, Tian, Hussain, Dong, Li, Cheng, Wang, Lin, Li, Qian, Pang, Feng, Dong, Liu and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.