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Overexpression of a novel transcriptional repressor *GmMYB3a* negatively regulates salt–alkali tolerance and stress-related genes in soybean

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ABSTRACT

Myeloblastosis (MYB) transcription factor (TF) plays a positive role in the growth and stress response of plants; however, information on the functions of MYB repressors in soybean is limited. In the present study, the gene *GmMYB3a* was identified and characterized as a member of the R2R3 MYB repressor family, which is induced by various abiotic stresses. To understand the functions of *GmMYB3a*, a transgenic soybean over-expressing *GmMYB3a* was obtained and the photosynthetic index under salt–alkali treatments was evaluated. The transgenic line exhibited a series of negative regulation relative to the wild-type control. Quantitative real time polymerase chain reaction revealed that the physiological parameters, including soluble sugar, free proline, and chlorophyll contents; and photosynthetic rate decreased in the transgenic plants. Furthermore, *GmMYB3a* overexpression down-regulated a set of key genes associated with plant defense signal pathways. These finding suggest that *GmMYB3a* negatively affects the response of plants to salt stress.

1. Introduction

Soil salinity severely affects plant development and agricultural productivity [1]. The detrimental effects of natural salt–alkalinized soils on plants are mainly through osmotic stress caused by water loss and salt stress caused by excess sodium ions on critical biochemical processes [2]. The alkaline salts ($NaHCO_3$ and Na_2CO_3) are more destructive to crops than the neutral salts (NaCl and Na_2SO_4) [3]. During the evolutionary process against stress damage, plants have developed complex molecular signal mechanisms for survival. These include an array of function proteins and key enzymes for osmolyte biosynthesis, such as soluble sugar, proline, detoxification enzymes, and membrane transporters, that directly protect the plants from abiotic stress.

Soybean (*Glycine* max (L.)) is an important economic crop worldwide. The soybean crop is severely affected by salinity stress throughout the developmental stage [4]. Furthermore, salinity stress not only inhibits normal growth rate and seed germination [5], but also decreases biomass accumulation and seed yield [6]. The increase in salt content concomitantly decreases soybean yield by up to 40% [7].

Characterizing and employing stress-related molecular mechanism are effective approaches to minimize the loss of yield during soybean breeding. As the responses of plants to salt stress are closely related to mechanism overlap, the key enzymes underlying salt stress tolerance and adaptation have long been the focus of research. Earlier studies have confirmed that the ABA-dependent pathway plays an important role in enhancing salt tolerance [6,8]. Furthermore, ABI5 has been reported to be a positive regulator of ABA-mediated signal pathway, which is cross-regulated by other ABI genes [9]. The gene Glyma10g08370 (*GmABI5*), whose identity with ABI5 is 81.3% and has similar functions, has been isolated from soybean [10]. *DREB2* is one of the important DREB homologs of the ABA-independent pathway that regulates salt tolerance [11,12], which can transactivate downstream genes by binding to the DRE *cis*-element. Jasmonates [jasmonic acid (JA) and methyl jasmonate (MeJA)] are another group of important molecules that play crucial

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roles in abiotic and biotic stress responses, growth, and development in plants [13]. In the pathway that converts linolenic acid (LA) to JAs, the key step is catalyzed by allene oxide synthase (AOS) [14], The genes that act as inhibitors or activators under stress conditions are referred to as osmotic stress-responsive (OR) genes. The MYB protein is known to have pleiotropic functions, including resistance and development in plants [15,16]. Its function has been of great interest because as a positive effector, it is involved in stress tolerance by regulating stress-related genes, such as *GmMYB76*, *GmMYB92*, and *GmMYB177* [17]. However, most MYB repressors are uncharacterized, and their biological functions are unknown.

During the last decades, extensive studies have focused on TFs as transcriptional activators; however, information on their role as negative regulators during stress responses is limited. *Arabidopsis* gene belonging to subgroup 4 has been confirmed as a transcriptional repressor [18–20]. It alters physical traits of a plant indirectly by affecting defense-related genes. However, the function of homologous genes in soybean is not clear. To investigate the effect of transcriptional repressor on the defense mechanism of plants, a new transcriptional repressor gene, designated *GmMYB3a*, has been identified and characterized in soybean. In the present study, the expression pattern of *GmMYB3a* induced by different stresses, and its effect on osmolyte biosynthesis, photosynthesis, and expression of defense-related genes in *GmMYB3a* over-expressing plants under salt–alkali stress were also investigated. The results suggest that the R2R3 MYB gene *GmMYB3a* negatively regulates salt-stress response in soybean and provide information for plant molecular breeding.

2. Materials and methods

2.1. Plant growth condition and treatments

Homozygous seeds were germinated at 23 °C in a regime of 16:8 h light/dark cycle. Four-leaf stage seedlings were subjected to abiotic stresses. In high salt, drought, cold, and ABA treatments, the seedlings were treated with 200 mM NaCl, 20% polyethylene glycol, 4 °C, and 200 μM ABA, respectively [21]. To determine the effects of mechanical damage, a sterilized scalpel was used to create several wounds on the leaves of soybean seedlings. The samples were collected from the treatment groups 0, 1, 2, 5, 10, and 24 h after treatment.

2.2. Isolation and conservative analysis of GmMYB3a

The full-length sequence of *GmMYB3a* was obtained using NCBI Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Total RNA was extracted from soybean Jilin32 using the EasyPure Plant RNA Kit (Transgen Biotech, Beijing, China) according to the manufacturer's instruction. Furthermore, reverse transcripts were obtained using the *TransScript*®RT Enzyme Mix (One-Step gDNA Removal, Transgen, Beijing). The protein secondary structure of the gene *GmMYB3a* was predicted using Smart (http://smart.embl-heidelberg.de/). Phylogenetic analysis was conducted using ClustalX and MEGA software version 5.1.

2.3. Plasmid construction

The open reading frame (ORF) of *GmMYB3a* was inserted into the transformed vector *pTF101-35s*. The final vector was transformed into the soybean plants using the *A. tumefaciens* strain *EHA101*. The gene *GmMYB3a* was cloned into the GAL4DNA-BD binding domain in the *pGBKT7* vector. The plasmids of *GmMYB3a* and vector alone were transformed separately into the yeast strain *AH109* and grown on synthetic dropout medium lacking tryptophan (SD/-Trp). Imidazoleglycerol-phosphate dehydratase (HIS3) activity was assessed by a viability test on a medium lacking tryptophan, adenine, and histidine (SD/-Trp-Ade-His).

2.4. Quantitative real-time PCR

Total RNA was extracted from T_2 transgenic and wild-type control plants on day three of each abiotic treatment using the EasyPure Plant RNA Kit (Transgen Biotech, Beijing, China). The transcription level of *GmMYB3a* was examined using specific primers *GmMYB3a* (qRT)-F and *GmMYB3a* (qRT)-R. *ACTII* was used as a reference gene. All the primers used are shown in Supplemental Table S4. The PCR conditions were as follows: 94 °C for 30 s, followed by 42 cycles at 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s. The relative quantification $2^{-\triangle\triangle CT}$ method [22] was used to evaluate relative gene expression, and data were compared with the internal control.

2.5. Regeneration and screening of transgenic plants

The regenerated plantlets were transplanted to a greenhouse. The T_0 transgenic plants were screened using PCR and LibertyLink® strip to confirm bar expression according to the manufacturer's instruction. The positivity of the transgenic plants was examined by PCR analysis using plant DNA as template. The T_2 generation was further screened by smearing glufosinate (1‰ m/v) when the second trifoliate leaves were fully expanded. Southern blot hybridization was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany).

2.6. Determination of photosynthetic indexes and chlorophyll content

The photosynthetic indexes were evaluated using the LI-6400 portable photosynthetic system. The light intensity was maintained at 1200 μmol m^{-2} s^{-1} using fixed red and blue light sources. The measurements were taken from 10:00 to 12:00 am on the tenth day of treatment. The detection position was the small leaf in the middle of the second leaf of a compound leaf. The net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were directly read by the instrument.

Fresh leaves (0.1–0.15 g) were placed in a centrifuge tube after shearing, and immersed in 80% acetone and anhydrous ethanol for more than 48 h. The optical density (OD) was read using an ultraviolet spectrophotometer. The total amount of chlorophyll was calculated as follows: $C_T = 20.29D_{645} + 8.05D_{663}$, where, D_{645} and D_{663} are the ODs at the corresponding wavelengths.

2.7. Measurement of free proline and soluble sugar contents

Ten days after treatment, 1–2 g of fresh leaves was used for biochemical analysis. The proline content in transgenic and wild-type soybean plants was determined by the sulfosalicylic acid method [23]. The content of total soluble sugar in the leaves was determined by the modified phenol-sulfuric acid method [24].

3. Results

3.1. GmMYB3a induced by various abiotic stresses in soybean plant

Based on the cDNA sequence analysis of soybean immature embryo gene expression profile, we isolated a cDNA clone whose predicted translation product has a MYB-binding domain. This novel gene was designated as *GmMYB3a* (GeneBank ID:

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