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Functional Analysis of *Arabidopsis thaliana* Galactinol Synthase *AtGolS2* in Response to Abiotic Stress

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Abstract Soil salt-alkalization is one of the adverse factors limiting crop yields. Identification of key salt-alkaline tolerant genes is of great significance for molecular breeding of stress-resistant crops. In this study, a T-DNA insertion Arabidopsis mutant *atgols2* showing higher sensitivity to bicarbonate salt-alkaline stress was screened out against $NaHCO_3$ treatment. Further bioinformatic analysis revealed that the *AtGolS2* gene encoded a galactinol synthase, which is a member of the glycosyltransferase family A superfamily. We predicted the protein interaction network of AtGolS2 via SMART online analysis, and found that these AtGolS2 interacting proteins were related to lipid metabolism, galactose biosynthesis and raffinose biosynthesis, and participated in abiotic stress responses. By using the online expression data, we showed that *AtGolS2* expression responded to salt, osmotic, drought and ABA stress. PCR amplification by using the three primers method verified the homozygous T-DNA insertion in atgols2. Phenotypic assays further uncovered that *atgols2* mutant was more sensitive to high salt, osmotic and ABA stresses than the wild type Arabidopsis. Taken together, results in this study revealed the positive function of *AtGolS2* in bicarbonate salt-alkaline, high salt, osmotic and ABA stresses, which will facilitate further research regarding the function and molecular mechanism of the GolS family genes in stress responses.
Keywords Arabidopsis; Galactinol synthase; *AtGolS2*; Abiotic stress; Functional analysis

Plant is inevitably affected by abiotic stress such as drought, low temperature and soil salinization during growth and development. Saline-alkaline stress is one of the main adverse factors that affect the growth and development of crops and ultimately lead to yield reduction (Ismail and Horie, 2017). The common characteristics of drought, low temperature and saline-alkaline stress are the lack of water in cells, imbalance of cell water, denaturation of protein macromolecules, and destruction of cell membrane structure, which affect growth and development (Wu et al., 2017). When plants encounter low temperature, drought, and high salt stresses, the content of intracellular soluble sugars such as glucose, sucrose, and raffinose family oligosaccharides (RFOs) increases, which will thereby help to maintain osmotic balance and enhance plant stress tolerance (Salvi et al., 2018). Therefore, identification of key sugar metabolism genes is of great significance for the cultivation of stress-resistant crops.

Galactinol synthase (GolS) was initially identified in pea seeds and can catalyze the formation of galactinol by UDP-galactose and inositol, provide activated galactosyl groups for RFOs and regulate the accumulation of RFOs in plants (Bachmann and Keller, 1995). GolS only exists in flowering plants, with 7 in Arabidopsis (Selvaraj et al., 2017), 2 in rice (*Oryza sativa* L) (Shimosaka and Ozawa, 2015), and 10 in corn (*Zea mays* L) (Zhou et al., 2012). In recent years, studies have found that overexpression of *ZmGolS2* in Arabidopsis significantly increased the content of galactinol and raffinose in leaves, and enhanced tolerance to oxidative stress (Gu et al., 2019). GolS expression was induced by low temperature in *Solanum lycopersicum* and *Ajuga reptans* (Dos Santos et al., 2011, Downie et al., 2003). At the same time, GolS also played a role in the response to heavy metal stress. *TaGolS3* was induced by $ZnCl_2$ and $CuCl_2$. Overexpression of *TaGolS3* in Arabidopsis significantly promoted ROS scavenging, improved antioxidant enzyme activity and proline content, and reduced MDA accumulation (Wang et al., 2016).

In summary, GolS is involved in plant response to oxidative, low temperature and heavy metal stresses, but little information has been reported for GolS involvement in salt-alkaline stress.

In this study, we screened out a T-DNA insertion mutant Arabidopsis *atgols2* showing increased sensitivity to bicarbonate salt-alkaline stress. Further analysis found that *AtGolS2* gene expression also responded to high salt, osmotic and ABA stress, and *atgols2* was also more sensitive to high salt, hyperosmotic and ABA stress. This study revealed the function of *AtGolS2* gene under abiotic stress, which laid the foundation for analyzing the function and mechanism of *GolS* gene in stress response.

1 Results and Analysis

1.1 Screening of bicarbonate salt-alkaline sensitive Arabidopsis Mutants

In order to explore potential genes conferring bicarbonate salt-alkaline tolerance, we purchased a set of Arabidopsis T-DNA insertion mutants from the Arabidopsis Biological Resource Center to screen bicarbonate salt-alkaline sensitive lines. Figure 1 showed the growth performance of five of the bicarbonate salt-alkaline sensitive mutants (#6, #7, #9, #11, #15) on 1/2 MS medium supplemented with 0 mM or 10 mM $NaHCO_3$. Under normal conditions, each line grew well without difference; however, seed germination was inhibited on 1/2MS medium supplemented with 10 mM NaHCO3, especially #11 showing the least number of germinated seeds (Figure 1A). The statistics of germination rates also showed that seed germination of wild type (WT), #6, #7, #9, #11 and #15 mutants under 10 mM NaHCO3 treatment slowed down, and the germination rates were 52.9%, 40.2%, 42.7%, 42.1%, 35.8%, 39.8% on the 7th day, respectively (Figure 1B).

In this study, the #11 mutant (SALK_101144) with the lowest germination rate was selected as the research object. The T-DNA insertion flanking sequence of mutant #11 was downloaded from TAIR, and it was found that the T-DNA was inserted in the *AT1G56600* gene. According to NCBI and TAIR annotation, *AT1G56600* encoded a galactinol synthase, named as *AtGolS2* (Nishizawa et al., 2008).

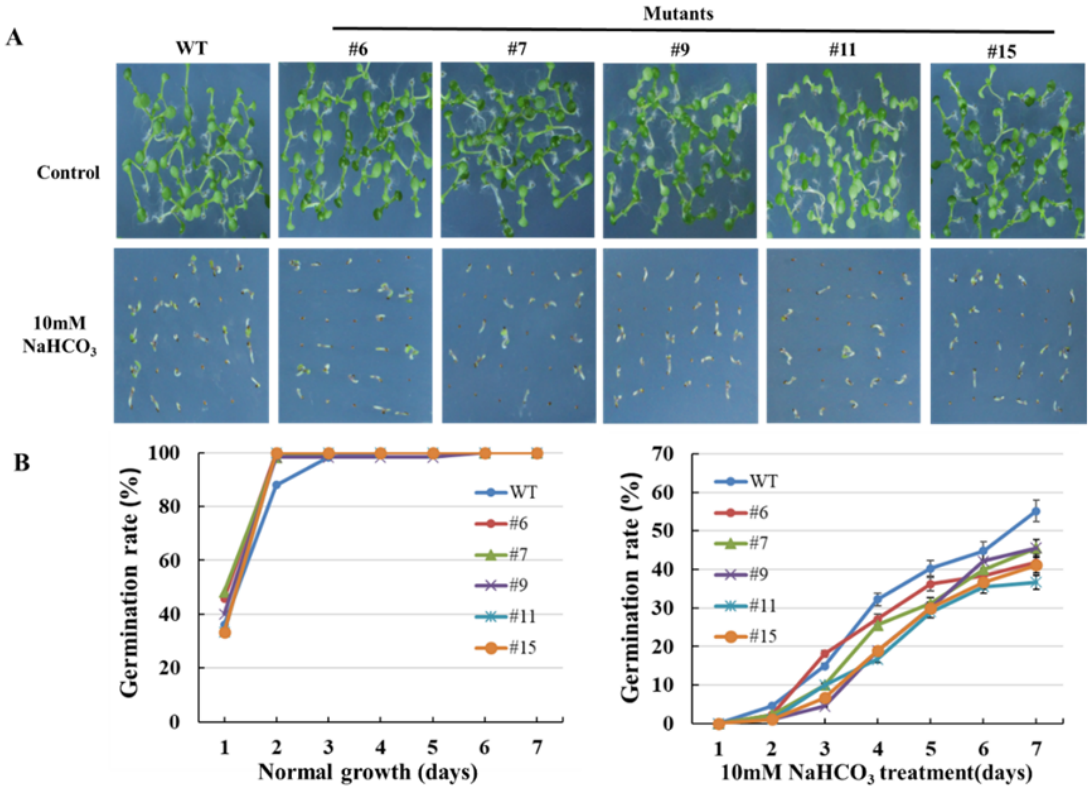

Figure 1 Phenotype and germination rates analysis of Arabidopsis mutants under bicarbonate saline-alkaline stress

Note: A: Phenotype of Arabidopsis mutants under bicarbonate saline-alkaline stress; B: Germination rates of Arabidopsis mutants under bicarbonate saline-alkaline stress

1.2 Analysis of AtGolS2 conserved domain

The conserved domain of AtGolS2 protein was analyzed by using SMART online program. AtGolS2 protein had a Glyco_transf_8 domain, and was a member of the glycosyltransferase family A superfamily (Figure 2A). In Arabidopsis, this family contained seven genes AtGolS1-7 (Nishizawa et al., 2008). Protein sequence alignment revealed that Arabidopsis galactinol synthase family proteins were highly conserved and all had a conserved Glyco_transf_8 domain (Figure 2B). This domain transfers sugar groups from activated nucleotide-sugar donor to the acceptor molecules to synthesize oligosaccharides, polysaccharides and glycoconjugates, which can increase the polysaccharide content in plants. Recent studies have shown that *AtGolS1* improved oxidative stress tolerance by increasing raffinose content (Song et al., 2016). Overexpression of *AtGolS3* in *Populus* improved the accumulation of galactitol and raffinose and enhanced oxidative tolerance (La Mantia et al., 2018). It is speculated that *AtGolS2* with the same domain may participate in abiotic stress response by regulating lipopolysaccharide biosynthesis or glycogen synthesis and increasing the content of galactitol and raffinose.

1.3 Analysis of AtGolS2 protein interaction network

By using STRING, we analyzed the signal transduction pathways that *AtGolS2* may participate in. The predicted model indicated that RD20, USP, AT5G18200, UGE2, UGE5, DIN10, SIP2, STS, RFS1 and RFS5 may interact with AtGolS2 (Figure 3). According to the NCBI protein database, these interacting proteins (Table 1) could be divided into three categories: lipid metabolism-related proteins, galactose biosynthesis-related proteins, and raffinose biosynthesis-related proteins.

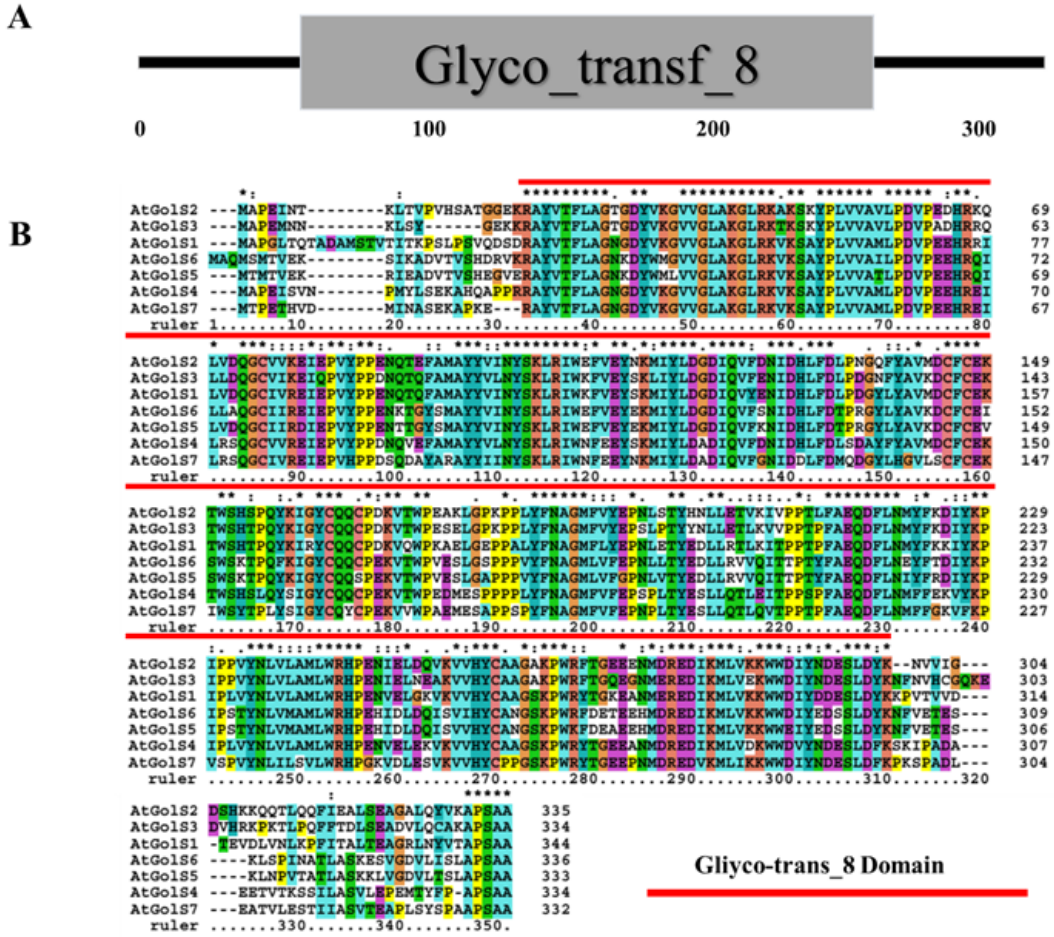

Figure 2 Analysis of conserved domains in AtGolS2 protein

Note: A: SMART prediction of the conserved domain in AtGolS2; B: Multiple alignment of AtGolS2 and homologous GolS proteins in Arabidopsis, the red line marked the position of Glyco_transf_8 domain

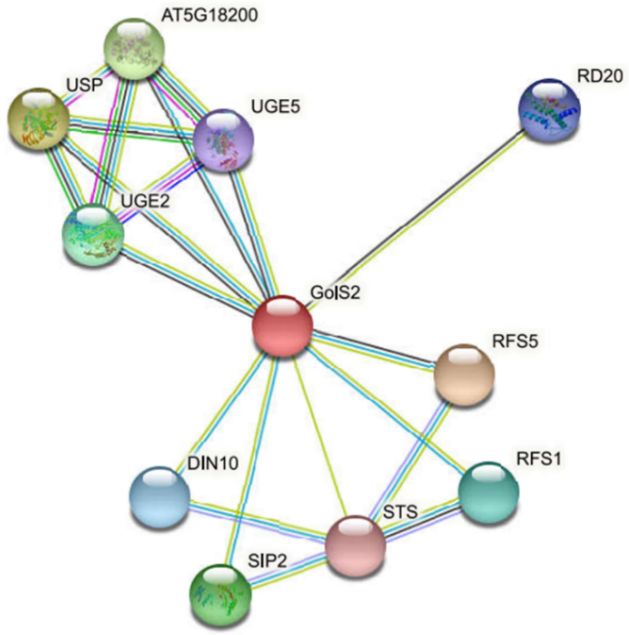

Figure 3 Schematic diagram of AtGolS2 protein interaction network

Table 1 Annotation of the function of AtGolS2 interacting protein

Gene name	Accession number	Encoding protein type	Functional annotation	Expression pattern under abiotic stress	References
RD20	AT2G33380	Caleosin	Lipid metabolism	Induced by salt, drought and osmotic stress	Aubert et al., 2011, Aubert et al., 2010, Park et al., 2018, Sham et al., 2015
UGE2	AT4G23920	Galactose epimerase	Galactose biosynthesis	Induced by low temperature and osmotic stress	Aznar et al., 2018
UGE5	AT4G10960	Galactose epimerase	Galactose biosynthesis	Induced by low temperature, osmotic and salt stress	Aznar et al., 2018
USP	AT5G52560	Galactose pyrophosphorylase	Galactose biosynthesis	Inhibited by low temperature stress	Decker and Kleczkowski, 2017
AT5G18200	AT5G18200	Galactose-1-phosphate urate transferase	Galactose biosynthesis	Induced by salt stress	Kotake et al., 2007
DIN10	AT5G20250	Glycosyl hydrolase	Glucose metabolism	Induced by high temperature, reactive oxygen stress	Lee et al., 2017, Maruyama et al., 2009
SIP2	AT3G57520	Raffinose-specific α-galactosidase	Galactose biosynthesis	Induced by osmotic stress	Fujita et al., 2005
STS	AT4G01970	Raffinose synthase	Galactose biosynthesis	Inhibited by salt stress	Nishizawa et al., 2008
RFS1	AT1G55740	Raffinose synthase	Galactose biosynthesis	Induced by low temperature and osmotic stress	Nishizawa et al., 2008
RFS5	AT5G40390	Raffinose synthase	Galactose biosynthesis	Induced by low temperature, osmotic, salt and drought stress	Nishizawa et al., 2008

Lipid metabolism-related proteins: RD20 is a member of the Caleosin family, which facilitates seeds to store lipids during germination and participates in lipid metabolism. RD20 was involved in response to salt, drought, and osmotic stress (Aubert et al., 2011, Aubert et al., 2010, Park et al., 2018, Sham et al., 2015).

Galactose biosynthesis-related proteins: *UGE2*, *UGE5*, *USP* and *AT5G18200* encoded two UDP-galactose epimerases, a UDP-galactose pyrophosphorylase and a UTP galactose-1-phosphate urate transferase, respectively. They play a vital role in the biosynthesis and decomposition of galactose. *UGE2* was induced by low temperature and osmotic stress, while *UGE5* was induced by low temperature, osmotic and salt stress (Aznar et al., 2018). *USP* was suppressed by low temperature stress (Decker and Kleczkowski, 2017), and *AT5G18200* was induced by salt stress (Kotake et al., 2007).

Raffinose biosynthesis-related proteins: *DIN10*, *SIP2*, *STS*, *RFS1* and *RFS5* encoded a raffinose hydrolase, a raffinose-specific α-galactosidase, and three raffinose synthases, respectively. These proteins play a vital role in the process of biosynthesis and decomposition, and participate in abiotic stress responses. *DIN10* was induced by low temperature and oxidative stress (Lee et al., 2017, Maruyama et al., 2009); *SIP2* was induced by osmotic stress (Fujita et al., 2005); *STS* was suppressed by salt stress; *RFS5* was induced by low temperature, osmotic, salt and drought stress (Nishizawa et al., 2008).

Based on functional annotation, we revealed that AtGolS2 interaction proteins were related to galactose and raffinose biosynthesis, and most of them responded to abiotic stress.

1.4 The expression pattern of AtGolS2 under abiotic stress

The expression pattern of *AtGolS2* gene under abiotic stress (cold, osmotic, salt, drought) and hormone treatment (ABA, GA, ETH) was analyzed based on the Arabidopsis eFP Browser. Expression of *AtGolS2* in shoot and root was basically unchanged under low temperature, GA and ETH treatments. However, expression of *AtGolS2* increased significantly after osmotic, salt, drought and ABA stress. Especially after salt and osmotic stress treatment for 3h, *AtGolS2* expression increased by 400 and 500 folds, suggesting that *AtGolS2* possibly participates in salt, osmotic and drought stress responses through ABA-dependent pathways (Figure 4).

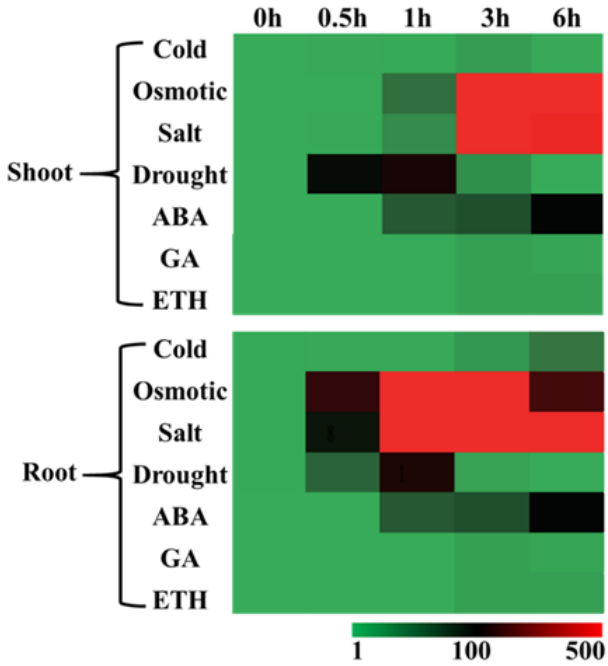

Figure 4 The expression pattern of AtGolS2 under abiotic stress and hormone treatment

1.5 PCR identification of atgols2 T-DNA insertion mutant

According to the *atgols2* T-DNA insertion flanking sequence, a diagram was generated to show the T-DNA insertion structure. As shown in Figure 5A, T-DNA was inserted in the promoter region of the *AtGolS2*. We designed gene-specific primers (P1, P2) according to the insertion site, and used the triple primers PCR method to identify whether *atgols2* is a T-DNA insertion homozygous mutant. When using LB (T-DNA sequence-specific primer) + P2 (gene-specific reverse primer), no band was amplified in WT, however *atgols2* mutant showed a 500 bp target band, indicating the existence of T-DNA insertion in the mutant (Figure 5B). When using the P1+P2 primer combination, a target band of 626 bp was observed for WT, but not for *atgols2*, indicating that the mutant was homozygous (Figure 5C). The results indicated that six individual plants were T-DNA homozygous insertion mutants of *AtGolS2*.

1.6 Phenotype analysis of *atgols2* mutant under salt, osmotic stress and ABA treatment

To validate the function of *AtGolS2* under salt, osmotic stress and ABA treatment, the growth of WT and *atgols2* lines on 1/2MS medium with or without 125 mM NaCl, 250 mM Mannitol, 0.6 μm ABA was compared. Under normal conditions, WT and *atgols2* showed the same growth performance and germination rates; germination and growth of WT and *atgols2* on NaCl, Mannitol and ABA medium were both retarded. The statistical results showed that the germination rates of *atgols2* on NaCl, Mannitol and ABA medium were significantly lower than that of WT after 2 days (Figure 6B). After 7 days of sowing, all seeds germinated, indicating the absence of *AtGolS2* only slowed down seed germination, but did not finally inhibit the germination of seeds. On the 7th day, the number of seedlings with open leaves was much lower of *atgols2* than WT under NaCl and Mannitol treatment, and seedling growth of *atgols2* was obviously repressed compared with WT (Figure 6A). The above results indicated that the absence of *AtGolS2* reduced the tolerance to high salt, hyperosmotic and ABA.

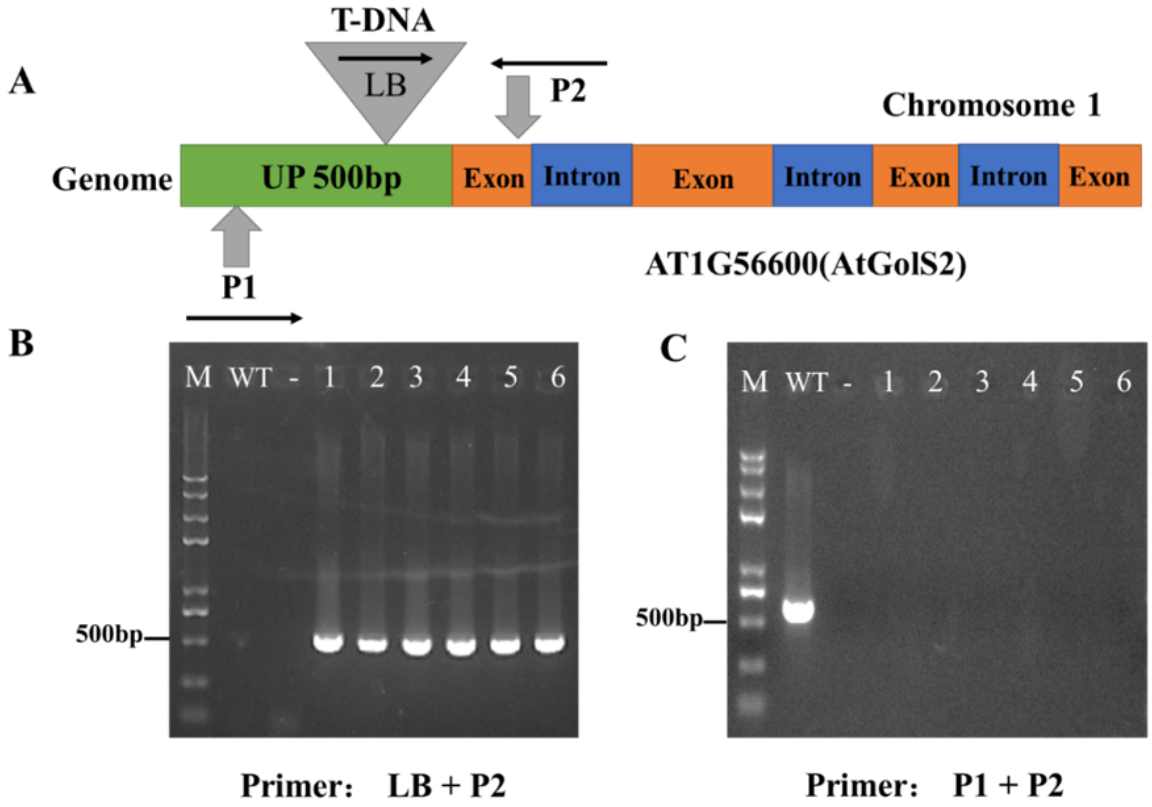

Figure 5 Detection of the T-DNA homozygous insertion in atgols2 mutant

Note: A: atgols2 mutant T-DNA insertion pattern diagram;B: T-DNA insertion identification of atgols2 mutant Arabidopsis M: DNA marker; -: Negative control; WT: wild-type control; 1-6: Mutant plant;C: Homozygous identification of atgols2 mutant Arabidopsis M: DNA marker; -: Negative control; WT: wild-type control; 1-6: Mutant plant

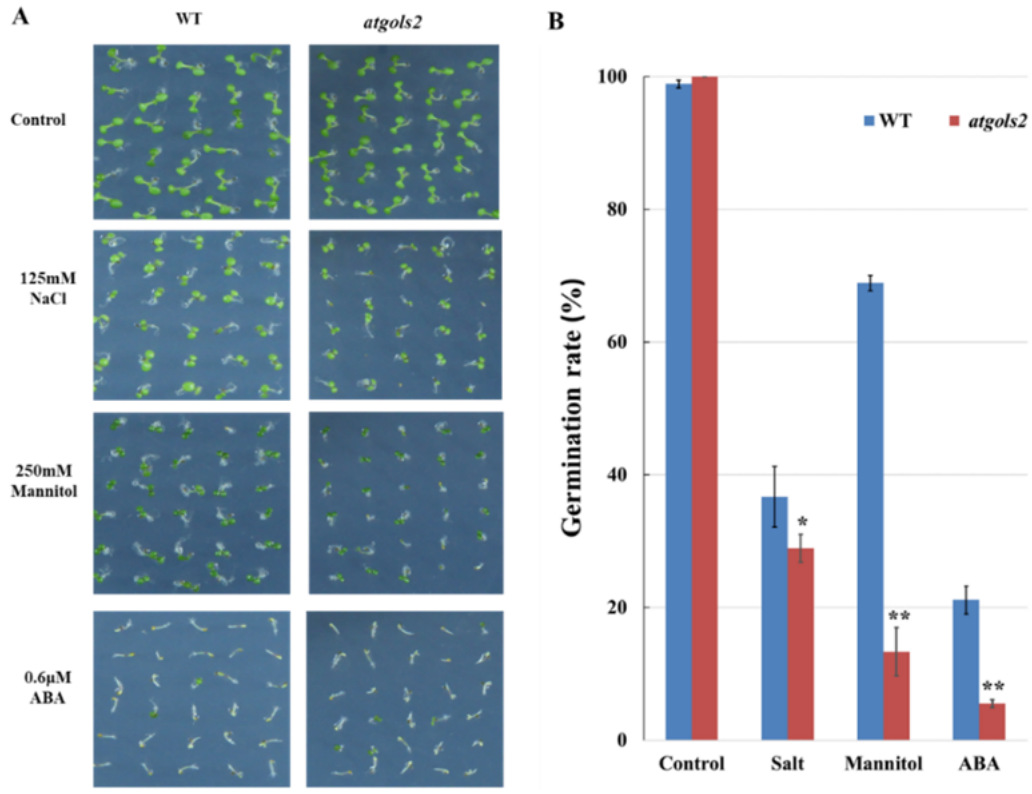

Figure 6 Phenotype and germination rates analysis of atgols2 mutant under salt, osmotic and ABA treatment

Note: A: Phenotype of atgols2 mutant under salt, osmotic and ABA treatment;B: Germination rates of atgols2 mutant under salt, osmotic and ABA treatment for 2 day;* significantly different at $p< 0.05$ level (n = 30); ** significantly different at $p< 0.01$ level (n = 30)

2 Discussion

Soil salinity-alkalinity is a global problem with dramatically negative impacts on crop yield and quality. There are 831 million hm2 of soil in the world that cannot be effectively used due to high salinity (Jin et al., 2008). Salt stress is mainly caused by neutral salts such as NaCl and Na_2SO_4 (Lv et al., 2019), while saline-alkaline stress is mainly caused by alkaline salts such as bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) (Wu et al., 2018). The damage of salt stress to plants mainly includes ion stress, osmotic stress and oxidative stress. However, saline-alkaline stress also imposes the ion stress and high pH stress caused by HCO_3^- or CO_3^{2-} on plants, and is more harmful than salt stress (Liu et al., 2018). Therefore, identification of key saline-alkaline tolerant genes is of great significance for improving crop saline-alkaline tolerance and utilizing saline-alkaline soil.

In this study, an Arabidopsis T-DNA insertion mutant, *atgols2*, is identified to be more sensitive to bicarbonate saline-alkaline stress (Figure 1). Existing studies have confirmed that GolS is involved in the response to drought, oxidative, and low temperature stress, but little is reported for bicarbonate saline-alkaline stress. This study found that atgols2 displayed increased sensitivity to bicarbonate saline-alkaline stress, which provides evidence for the participation of GolS in bicarbonate saline-alkaline stress. In addition, this study also found that *AtGolS2* expression was significantly induced by salt, osmotic, drought and ABA treatment (Figure 4), and *atgols2* was more sensitive to high salt, osmotic and ABA stress (Figure 6B), indicating that *AtGolS2* positively regulates Arabidopsis tolerance to abiotic stress.

Consistent with the finding of this study, overexpression of *AtGolS3*/*AtGolS2* in *poplar* resulted in increased expression of antioxidant enzyme synthesis genes, enhanced antioxidant capacity, reduced stomatal conductance, and improved drought tolerance (La Mantia et al., 2018, Yu et al., 2017). The *AtGolS2* ectopic expression in *Brachypodium distachyon* increased the chlorophyll content and drought tolerance (Himuro et al., 2014). Overexpression of *CaGolS1*/*CaGolS2* in Arabidopsis enhanced high temperature tolerance by reducing ROS

accumulation (Salvi et al., 2018). Transformation of *AmGolS* into *Photinia* × *fraseri* Dress increased the cold tolerance (Dos Santos et al., 2011, Downie et al., 2003). This study found that *AtGolS2* was involved in bicarbonate saline-alkaline and ABA responses. In the future, the application potential of GolS in crop saline-alkaline improvement will be further evaluated.

The AtGolS2 protein belongs to the glycosyltransferase family A superfamily, which is responsible for the synthesis of galactosidase synthase, and catalyzes the first step of RFOs biosynthesis. RFOs use the galactosyl group provided by inositol galactosides to synthesize a series of different oligosaccharides (Saravitz et al., 1987). Previous studies found that overexpression of *AtGolS2* in Arabidopsis increased the content of galactitol and raffinose, reduced leaf stomatal conductance, reduced transpiration rate, and improved drought tolerance (Nishizawa et al., 2008). Compared with WT, *AtGolS2* transgenic soybean (*Glycine max*) and rice showed increased galactitol accumulation and improved yields under drought stress (Honna et al., 2016, Selvaraj et al., 2017). *TsGolS2* is a homolog of *AtGolS2* in *Thellungiella salsuginea*, and overexpression of *TsGolS2* in Arabidopsis significantly increased the content of galactitol, raffinose and α-ketoglutarate, and improved the tolerance to high salt and hyperosmotic stress (Sun et al., 2013). In addition, *AtGolS3*, *CaGolS1* and *CaGolS2* expression all promoted raffinose accumulation (Dos Santos et al., 2011, Downie et al., 2003). However, whether the GolS participates in the ABA response by promoting the accumulation of galactitol and raffinose and other oligosaccharides needs further validation.

Proteins are the executor of various physiological activities in plants, which cannot be achieved by a single protein independently, but is mediated by protein-protein interaction (Cheng et al., 2018). The AtGolS2 protein interaction network was predicted via STRING, and they could be divided into three categories: lipid metabolism-related proteins, galactose biosynthesis-related proteins, and raffinose biosynthesis-related proteins (Figure 3, Table 1). These proteins play important roles in regulating lipid metabolism, galactose, and raffinose biosynthesis and decomposition. In addition, their expression also responded to abiotic stress (Table 1). RD20 encodes a Caleosin (oil body calcium) protein, which is induced by drought, salt and ABA. Compared with WT, *rd20* knockout mutants increased stomatal opening and showed higher transpiration rate. *RD20* improved the drought tolerance by controlling the stomata opening under water deficit conditions. At the same time, *rd20* knockout mutant also showed a salt-sensitive phenotype (Aubert et al., 2011, Aubert et al., 2010 , Park et al., 2018, Sham et al., 2015). *DIN10* encoded a glycosyl hydrolase, which responded to cold stress and reactive oxygen stress. Overexpression of *DIN10* in Arabidopsis could improve the cold tolerance (Lee et al., 2017, Maruyama et al., 2009). *SIP2* encoded a raffinose-specific α-galactosidase, and overexpression of SIP2 in Arabidopsis could improve drought tolerance (Fujita et al., 2005). *STS*, *RFS1* and *RFS5* encoded a raffinose synthase, *sts* and *rfs5* mutants displayed reduced raffinose content and cold tolerance (Nishizawa et al., 2008). In the future, the interaction between AtGolS2 and the above proteins need be validated by yeast two-hybrid, pull down, co-immunoprecipitation and other methods, which will provide a theoretical basis for further revealing the *AtGolS2*-mediated saline-alkaline signal transduction pathway.

3 Materials and Methods

3.1 Plant materials

The *atgols2* T-DNA insertion mutant (SALK_101144) was purchased from the ABRC (Arabidopsis Biological Resource Center). The wild type *Arabidopsis thaliana* (Columbia) was kept by the Crop Stress Molecular Biology Laboratory.

3.2 Germination and growth assays of *atgols2* mutants

In the plate germination experiments, seeds of WT and *atgols2* mutants were sterilized with 5% NaClO for 10 min, then washed with ddH_2O for 6-10 times, and placed in dark at 4℃ for 3 days. Seeds were sown on half-strength MS medium supplemented with none or 10 mM $NaHCO_3$ (bicarbonate saline-alkali stress), or 125 mM NaCl (high salt stress), or 250 mM Mannitol (osmotic stress), or 0.6 μM ABA (hormone stress), and grown in a plant

growth chamber for 7 days (22°C, 60% relative humidity, and a 16 h day/8 h night photoperiod). Each experiment was performed with at least 30 seeds per line. All experiments were replicated at least three times.

3.3 PCR identification of *atgols2* T-DNA insertion mutant

According to the Arabidopsis database TAIR, the flanking sequence of the *atgols2* T-DNA insertion mutant was obtained. Gene-specific primers were designed across the T-DNA insertion site, labeled P1 (5'-CGTGTCCACATAATAACCAATCAGA-3') and P2 (5'-CCCCTTTCACGTAGTCTCCAGTT-3'). The triple primers PCR method was used to identify whether atgols2 was a homozygous T-DNA insertion mutant with primers of LB (5'-ATTTTGCCGATTTCGGAAC-3'), P1 and P2. The gDNA was extracted using the Easy Pure genomic DNA extraction kit (Transgen). The reaction conditions were as follows: 95°C for 5 min; 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s, 30 cycles, 72°C for 10 min. PCR reaction mixture: 2 μL of gDNA, 2×EasyTaq® PCR Super Mix 7.5 μL, Sense primer 0.3 μL, Anti-sense primer 0.3 μL, ddH2O 4.9 μL. PCR products were analyzed by agarose gel electrophoresis.

3.4 Bioinformatic analysis of *AtGolS2*

AtGolS2 genomic sequence, CDS sequence, amino acid sequence, and protein function annotation were obtained through Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), and SMART (http://smart. embl.de/) was used to predict the conserved domains. Arabidopsis *AtGolS2* homologous genes were identified via BlastP search on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of Arabidopsis GolS genes (*AtGolS1-7*) were performed by using Clustal X1.83. STRING (https://version11.string-db.org/cgi/network) was used to predict the AtGolS2-mediated interaction network, and functional annotation of the interaction proteins was acquired through the Uniprot (https://www.uniprot.org/) database.

3.5 Analysis of *AtGolS2* expression pattern under abiotic stress

The expression data of *AtGolS2* under cold, osmotic, salt, drought, ABA, GA, and ETH treatment was obtained from the Arabidopsis eFP Browser (http://bar.utoronto.ca/eplant/) (Kilian et al., 2007). The expression data under 0 h, 0.5 h, 1 h, 3 h, 6 h after stress treatments were downloaded and subjected to generate a heat map by TBtools.

Authors' contributions

SY and JBW were the executors of this research; WJY, CXX and SMZ were responsible for *atgols2* phenotype observation and data processing; HBS, CY and WY were responsible for *AtGolS2* bioinformatic analysis; SXL acquired the funding and supervised the project; SMZ and SY originally drafted the work; SXL and JBW edited and revised the manuscript critically. All authors reviewed the final version of this manuscript.

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