Overexpression of Galactinol Synthase 1 From *Solanum Commersonii* (*ScGolS1*) Confers Freezing Tolerance In Transgenic Potato

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Abstract

Potato (*Solanum tuberosum* L.) is the fourth largest food crop in the world. Low temperature causes serious damage to potato plants every year, and freezing tolerance has become a hot spot in potato research. Galactinol synthase (GolS) is a key enzyme in the synthesis of raffinose family oligosaccharides (RFOs), and plays an important role in the response of plants to abiotic stress. In this study, the *ScGolS1* gene from *S. commersonii* was cloned and introduced into the *S. tuberosum* cultivars 'Atlantic' and 'Desiree' via *Agrobacterium*-mediated transformation. Phenotyping assay showed that overexpression of the *ScGolS1* could significantly improve freezing tolerance in transgenic potato plants. Further physiological and biochemical results showed that the relative conductivity, malondialdehyde (MDA) content, and 3,3'-Diaminobenzidine (DAB) staining of the transgenic lines decreased, and the plant survival rate increased compared with wild type (WT). Moreover, *CBF1*, *CBF2*, *CBF3*, *CBF* downstream cold responsive genes *COR413*, *COR47* and *ERF* transcription factor genes *ERF3*, *ERF4*, *ERF6* in the ethylene signaling pathway were all induced by freezing treatment, while higher levels were observed in *ScGolS1* overexpression lines compared with WT. In addition, other genes such as *MIPS*, *STS* and *RS* genes from RFO metabolic pathway and some sugars content were altered in response to freezing treatment. This indicates that overexpression of the *ScGolS1* gene induced both the regulation of the ethylene signaling pathway and the metabolism of raffinose series oligosaccharides, regulating the balance of sugar composition and improved anti-peroxidation capacity, and thereby improved freezing tolerance in potato. These results provide theoretical support and genetic resources for freezing tolerance breeding in potato.

Key Message

***ScGolS1* overexpression altered sugar composition and enhanced freezing tolerance in transgenic potato via inducing ET signaling and *CBF* pathways.**

Introduction

The growth and development of plants can be affected by various adverse environmental conditions such as biotic and abiotic stresses (Zhu 2016). The biosynthesis of soluble carbohydrates in plants is of great significance to plant growth, development, and environmental adaptability, especially when plants face adverse conditions such as low temperature, drought, and high salt levels. The cellular biosynthesis and content of soluble sugars such as glucose, sucrose, and raffinose family oligosaccharides (RFOs) are increased, which enhances the osmotic adjustment ability of plant cells, thereby improving the tolerance of plants to abiotic stress (Sun et al. 2013; Salvi et al. 2018). Soluble carbohydrates can enhance osmotic pressure and energy metabolism in cells and act as signal molecules to improve freezing tolerance in plants (Theocharis et al. 2012). Generally, sucrose accumulation plays an important role in stabilizing the cellular membrane and maintaining turgor under low temperature stress (Guy et al. 1992; Strand et al. 2003). The concentration of RFOs is second only to sucrose, and mainly includes three types: raffinose, stachyose, and verbascose. Galactinol synthase (GolS, EC 2.4.1.123) catalyzes the reaction of UDP-

galactose and inositol to produce galactosyl inositol, which provides galactosyl groups that undergo transfer catalysis to synthesize RFOs. This is one of the most important steps in the RFOs metabolic pathway. GolS, the key regulatory enzyme in this step, was first discovered in pea seeds. It regulates the accumulation of RFOs in plants, and plays an important role in plant growth and environmental adaptability (Bachmann et al. 1994; Sengupta et al. 2012; Sonali et al. 2015).

The *GolS* gene family is classified as a subfamily of the glycosyltransferase (GTs) *GT8* gene family. In addition to the classic structural motifs DxD and HxxGxxKPW of GT8 family proteins, several other unique conserved motifs are present in GolS proteins: YNL, FAEQDFLNMF, YFNAGMFVFEP, and KLRIWEFVEY (Yin et al. 2010). GolS is a key enzyme in the synthesis of RFOs, and it participates in the abiotic stress response in plants (Sengupta et al. 2012). The Arabidopsis *AtGolS* gene family has seven members, of which three (*AtGolS1-3*) are responsive to stress. *AtGolS1* and *AtGolS2* expression is up-regulated by drought and salinity, but not in response to low temperature stress, while expression of *AtGolS3* is induced by low temperature stress but not by drought and high salinity (Panikulangara et al. 2004). *MdGolS2* is the main *GolS* gene expressed during dormancy in apple, and it helps to protect the dormant buds from the effects of lack of water (Falavigna et al. 2018). In winter wheat, overexpression of *TaGolS1* and *TaGolS2* can significantly improve the resistance to low temperature (Shimosaka et al. 2015). In chickpeas, although the overexpression of *CaGolS1* and *CaGolS2* is beneficial to improve seed vigor and longevity, the two genes respond to different stresses (Prafull et al. 2018; Salvi et al. 2018). Overexpression of *Ammopiptanthus nanus AnGolS1* in tomato causes the plants to accumulate galactosyl inositol, which acts as a sugar signal in the ethylene signaling pathway and plays an important role in adaptation to low temperature stress (Liu et al. 2019).

The ethylene (ET) signaling pathway is known to be involved in the response to low temperature stress (Eremina et al. 2016). Ethylene-responsive factors (ERFs), which are downstream regulatory factors of the ET signaling pathway, modulate the expression of downstream stress-response genes. ERF proteins play a key role in directing changes in gene expression in response to different forms of environmental stress (Nakano et al. 2006). Overexpression of the EREBP/ AP2 DNA-binding protein *DREB1A* not only induces strong expression of the target genes under unstressed conditions, but also enhances the drought and freezing tolerance of transgenic Arabidopsis plants (Liu et al. 1998). Ectopic expression of a pepper ERF/AP2-type transcription factor, *CaPF1*, also improves the freezing tolerance of Arabidopsis (Yi et al. 2004). In addition, ERF proteins also positively promote ET biosynthesis via feedback regulation; for instance, overexpression of tomato *TERF2/LeERF2* can enhance the ET production and freezing tolerance of tobacco and tomato plants (Zhang and Huang 2010).

Potato (*Solanum tuberosum* L.) is the fourth largest food crop in the world. Low temperature can cause serious damage to potato plants, resulting in reduced or even no harvest (Chen et al. 1999). Freezing tolerance has become a major focus in potato research. The commonly-grown potato cultivars have poor freezing tolerance and no obvious cold acclimation ability (Chen et al. 1980). It is difficult to breed varieties with freezing tolerance by intraspecific hybridization of common cultivars, although there are abundant freezing-tolerant resources in wild potato species such as *S. commersonii*, *S. malmeanum*, and

S. acaule (Vega et al. 1995). A previous study reported that the semi-lethal temperature (LT₅₀) for *S. commersonii* before cold acclimation is -4.5°C, and the LT₅₀ after cold acclimation can reach -11.5°C. *S. commersonii* is one of the most freezing-tolerant of the wild potato species (Aversano et al. 2015). Because the endosperm balance number (EBN) in *S. commersonii* is 1, the difference in EBN makes it sexually incompatible with tetraploid potato cultivars (EBN is 4 in *S. tuberosum*). Cloning the freezing tolerance genes from *S. commersonii* and introducing them into cultivated varieties is an effective method to prevent and control the harmful effects of low temperature and frost. Although *Go/S* genes have been identified and isolated in many plants such as *Arabidopsis thaliana* (Falavigna et al. 2018), tomato (Liu et al. 2019), corn (Gu et al. 2013), tea tree (Zhou et al. 2017), and salvia (Wang et al. 2012), there are few studies of *Go/S* genes in potato. The goal of our study is to clone *ScGo/S1* gene from the wild diploid freezing-tolerant potato species *S. commersonii*, verify the function of the gene through genetic transformation, and then to explore the important role of *ScGo/S1* in the freezing tolerance pathway in plants. Our results will increase our understanding of freezing tolerance in plants and provide genetic resources for breeding of freezing tolerance in potato.

Materials And Methods

Plant materials and growth conditions

The tetraploid *S. tuberosum* potato cultivars 'Atlantic' (B44) and 'Desiree' (B7) are freezing-sensitive, and the diploid wild potato species *S. commersonii* (W3) is freezing-tolerant. All materials were preserved in the form of tissue culture seedlings or seed potatoes in the potato laboratory of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. Tissue culture conditions for potato propagation: 30 mL Murashige-Skoog (MS) solid medium (pH = 5.8 - 6.0) containing 3% sucrose, 2500 Lx light intensity, 22 ± 1°C for 16 h in light and 8 h in dark, 80% humidity. Transplanting tissue culture seedlings: tissue cultured seedlings at 3-4 weeks of age were transplanted into 10 × 10 cm plastic flower pots containing seedling substrate and grown in a solar greenhouse (14 h light/10 h dark) for 30 days. All samples collected were immediately frozen in liquid nitrogen and then kept in a refrigerator at -80°C before use.

ScGo/S1 gene cloning and bioinformatics analysis

Total RNA was extracted from seedlings of the wild potato species *S. commersonii* using the Plant Polysaccharide Polyphenol RNA Extraction Kit (GeneBetter, China). First-strand cDNA was synthesized with the Script III RT Kit With gDNA Eraser (GeneBetter, China), and then the primer pair ScGo/S-CDS-F/R with added restriction sites was used to amplify the *ScGo/S* CDS (Supplemental Table S1). The pBI121 plasmid was digested with *Xba* I and *Xma* I (NEB, USA) overnight at 37°C, examined by 0.8% agarose gel electrophoresis, and the linearized vector was purified from the gel. The *Go/S* CDS was cloned using the Seamless Cloning Reaction System (Vazyme, China), and individual positive clones were selected and sequenced (BGI, China).

The databases used in this study were the Potato Genome Sequencing Consortium (PGSC) database (<http://solanaceae.plantbiology.msu.edu/>) and the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>). DNAMAN software was used to perform sequence alignments; MEGA5.0 software was used to construct a phylogenetic tree by NJ (neighbor-joining) method; ProtParam (<https://web.expasy.org/protparam/>) was used to predict the basic physical and chemical properties of proteins; TMHMM (<https://www.cbs.dtu.dk/services>) was used for transmembrane domain prediction; ProtScale (<https://web.expasy.org/protscale/>) was used for protein hydrophobicity analysis; PSORT Prediction (<https://psort.hgc.jp/form.html>) was used to predict the subcellular localization of proteins; Smart (<http://smart.embl-heidelberg.de/>) was used to predict the conserved domains in amino acid sequences; SOPMA (<https://npsa-prabi.ibcp.fr>) was used to predict the secondary structure of proteins.

ScGolS1 gene transformation and plant phenotype identification

The recombinant vector plasmid CaMV35S-pBI121-ScGolS1 was transformed into *Agrobacterium tumefaciens* GV3101, and the bacteria liquid was used to infect potato stems. Callus buds were induced on callus induction medium and bud induction medium, and the callus buds were further induced to root and form shoots. A combination of the vector primer pair 73F/211R and gene-specific primers was used in PCR amplification to identify positive seedlings (Supplemental Table S1), the initial identification was performed on tissue culture seedlings and the second identification was performed on the potted seedlings after transplanting.

One month old seedlings were transferred to a low-temperature incubator or outdoor with three replicates for freezing identification, and the temperature and humidity were monitored in real time. Low-temperature incubator appraisal: plants were subjected to freezing treatment at $-2 \pm 0.5^{\circ}\text{C}$ for 24 h, and then allowed to recover at 23°C during the day/ 20°C at night for 1-2 days, during which the temperature would rise/fall slowly.

T-DNA insertion sites analysis

Three nested specific primers SP1, SP2, and SP3 were designed based on the known sequence of the recombinant vector CaMV35S-pBI121-ScGolS1. These nested specific primers were combined with four sets of degenerate primers, LAD1, LAD2, LAD3, and LAD4 (Supplemental Table S1), and used in hiTAIL-PCR for three consecutive rounds of amplification using the method of Liu and Chen with slight modifications (Liu et al. 2007). The sequences were used as queries to search the PGSC database to identify the T-DNA insertion sites, and the T-DNA insertion site sequences were then analyzed.

Real-time quantitative PCR analysis

30 days old seedlings were subjected to different freezing stress: (1) 20°C , 12°C , 4°C , 2°C and 0°C for 24h ; (2) -1°C for 0, 12, 24 and 48 h. The total RNA from root, stem, leaf of transgenic lines and WT plant was extracted by the Plant Polysaccharide Polyphenol RNA Extraction Kit (GeneBetter, China) according to the manufacturer's instructions, and cDNA was synthesized via the Script III RT Kit with gDNA Eraser

(GeneBetter, China) as directed by the manufacturer. The *EF-1 α* gene was used as the internal control for normalization of gene expression in real-time quantitative PCR (qRT-PCR) (Supplemental Table S1). qRT-PCR were performed with SYBR Green Master Mix (Mei5bio, China) on a Roche LightCycler PCR instrument (Roche, USA), using the following amplification procedure: a 95°C pre-denaturation for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s. The $2^{-\Delta\Delta C_t}$ method was used to analyze the expression data (Livak et al. 2001).

Physiological and biochemical determination

Transgenic and WT plants grown in a low-temperature incubator were treated with -1°C for 24 h, and plants grown under normal conditions for 24 h were used as control. The relative conductivity was determined according to the method of Cao et al. (Cao et al. 2007), with a slight modification. MDA content was determined using a malondialdehyde detection kit (Solarbio, China) (Wang et al. 2019). DAB staining was performed according to the method of Jambunathan with minor modifications (Jambunathan et al. 2010).

Sugar content detection and analysis

Soluble sugars were extracted from leaves under freezing treatment. The mixed sample was ground in liquid nitrogen, and then 1g of ground powder was distributed in 100 μ L water, then 1 mL saccharide internal standard (0.4 mg/mL arabinose water) was added to the water-soluble potato leaves extracts. After sonication and centrifugation, the samples were filtered through a 0.22 μ m polyethersulfone ultrafiltration membrane and mixed with an equal volume of acetonitrile for analysis. Saccharide was measured by UPLC-MS/MS (ACQUITY UPLC I-Class-Xevo TQ-S Micro, Waters). For saccharide analysis, an ACQUITY UPLC BEH Amide 1.7 μ m column was used as the analytical column (2.1 $\times$ 100 mm; Waters). The mobile phase was composed of acetonitrile as solvent A and 1 mg/mL ammonium hydroxide as solvent B. The temperatures of the column and autosampler were 60°C and 4°C, respectively. Each saccharide was separated by increasing the solvent B concentration from 10–20% over 6 min after the first 2 min of the run at 10% using a flow rate of 0.2 mL/min, followed by washing with 10% solvent B for 2 min. Data analysis was performed using MassLynx V4.1 (Waters). Sugar concentrations were calculated based on the standard curve for each sugar and calibrated with the recovery of the whole analysis procedure.

Statistical Analysis

Three biological replicates were analyzed for each treatment. Data values presented as the means $\pm$ SD. Significant differences were determined using Student's t-test with $p < 0.05$ and $p < 0.01$ considered to be statistically significant and highly significant, respectively.

Results

Molecular characterization and sequence analysis of ScGolS1

A previous study reported that the LT_{50} of *S. commersonii* before cold acclimation was -4.5°C , and after cold acclimation reached -11.5°C . In order to verify the freezing tolerance of W3, W3 plants were exposed to -5°C for 24 h, only a few leaves and stems showed wilting after recovery, proving that W3 had strong freezing tolerance (Fig. S1a). The field natural frost test (minimum temperature -5°C) also showed that the freezing tolerance of W3 was stronger than other wild potato materials such as *S. acaule*, *S. demissum*, *S. albicans* and so on (Fig. S1b-c). RNA-seq analysis of W3 in the early stage showed that the *ScGolS1* gene was up-regulated in response to freezing stress. In this study, the full-length cDNA of *ScGolS1* was cloned from W3 based on the EST sequence of the gene (Fig. S3a). *ScGolS1* is located on potato chromosome 1, the open reading frame is 969 bp in length. The *ScGolS1* gene encodes a protein of 322 amino acids with a molecular weight of 36.8 kD and an isoelectric point of 6.61; the instability coefficient is 41.79, which indicates an unstable protein; the average hydrophilicity coefficient is -0.272, which shows that ScGolS1 is a hydrophilic intramembrane protein. Online software predicts that ScGolS1 is located in the cytoplasm, and the main secondary structural features of the protein are alpha helix and random coil.

Using Protein BLAST global alignment, we found that the amino acid sequence identity between ScGolS1 (ADW78839.1) and ScGolS2 (ADW78841.1) and ScGolS3 (ADW78843.1) was 57.74% and 61.13%, respectively, while the similarity between ScGolS2 and ScGolS3 was 77.26%. The amino acid similarity between ScGolS1 and StGolS1 (ADW78846.1) from *S. tuberosum* was 96.89%, and the amino acid consistency between SpGolS1 (XP_015071077.1) from *S. pennellii* and SlGolS1 (NP_001234486.2) from *S. lycopersicum* was >90%. Multi-sequence alignment showed that the GolS protein contained conserved domains, such as a substrate binding domain, a manganese ion (Mn^{2+}) binding site (DGD), and a carboxy (C) terminal typical hydrophobic pentapeptide (A(S)PSAA), indicating that these GolS proteins had the ability to catalyze the synthesis of inositol and UDP-galactose into galactinol (Fig. 1a). However, there were large sequence differences in the conserved domains of the GolS proteins. In addition, there were many serine (S) phosphorylation sites in the GolS1 sequence., our hypothesis was that the function of GolS protein was achieved through phosphorylation.

Phylogenetic analysis showed that the GolS proteins from different Solanaceae species were grouped into three major clades (Fig. 1b), among which ScGolS1, StGolS1, and SlGolS1 were in clade III, ScGolS2, SpGolS1, StGolS2, and NtGolS1 were in clade II, and ScGolS3, StGolS3, SpGolS2, SlGolS2 and NtGolS2 were in clade I. A previous study showed that the activity of SlGolS2 in cultivated tomato was higher than that of SlGolS1 (Liu et al. 2019). The results of phylogenetic analysis and multiple sequence alignment may be helpful to explain how the differences in the sequence of ScGolS1 causes its function to be different from those of ScGolS2 and ScGolS3.

ScGolS1 up-regulated expression in response to freezing stress

We used qRT-PCR to assay the expression of *ScGolS1* in tissue cultured and potted seedlings of W3, the expression level of *ScGolS1* gene in leaves was significantly higher than in roots and stems for both tissue cultured and potted seedlings, and the expression in the young leaves of potted seedlings was

significantly higher than older leaves (Fig. 2a-b), indicating that plants in the seedling stage are more appropriate for subsequent freezing tolerance identification. *Go/S1* gene of wild potato species W3 was up-regulated expression with the decrease of temperature under different low temperature treatments, while they were not expressed or expressed in low level in B44, and there was no obvious expression pattern (Fig. 2c). Under the same continuous low temperature treatment (-1 °C), *Go/S1* gene was still not expressed or expressed in low levels in B44, but in W3, was up-regulated with the extension of freezing stress time (Fig. 2d), indicating that the *Go/S1* gene in W3 responded to freezing stress through up-regulation of expression and resisted adversity.

Overexpression of ScGolS1 improves the freezing tolerance in transgenic plants

The expression vector CaMV35S-pBI121-ScGolS1 was introduced into potato cultivars 'Atlantic' (B44) and 'Desiree' (B7) using *Agrobacterium*-mediated gene transformation, and total 106 transgenic regenerated plants were obtained. The vector primer 73F/211R and a combination of vector primer and gene-specific primers was used to detect transgenic regenerated plants, and we estimated 20 transgenic plants for B44 (Fig. 3a-b). Low-temperature incubator temperature was $-2.0 \pm 0.5^{\circ}\text{C}$; wild-type B44 leaves were frost damaged and chlorotic, and the plants were not able to recover from wilting. However, the leaves of transgenic lines B44-OE2 were still green and vigorous, and the plants grew well (Fig. 3c), indicating that overexpression of the *ScGo/S1* gene could improve freezing tolerance in the transgenic potato plants derived from the cultivars B44.

qRT-PCR analysis showed that the *ScGo/S1* expression level in the transgenic lines B44-OE2 during low temperature treatment was always higher than WT (B44), and was most significant at 12 h. When the low temperature treatment time increased, the expression of *ScGo/S1* showed a pattern in which expression first increased and then decreased (Fig. 3d). Exposure to low temperatures can lead to increased cell membrane permeability and electrolyte leakage. We found that the relative conductivity of the overexpression lines B44-OE2 under low temperature stress was lower than that of the WT (B44), indicating that the overexpression of *ScGo/S1* reduced the degree of damage to the potato cell membrane (Fig. 3e). Malondialdehyde (MDA) is an important product of fatty acid peroxidation, and the MDA content can reflect the degree of cell membrane lipid peroxidation. Before freezing stress, the MDA content in the overexpression line B44-OE2 was lower than that in the WT (B44). Upon exposure to freezing stress, the MDA content in WT (B44) and the overexpression line B44-OE2 increased and then decreased, but the MDA content in the overexpression line B44-OE2 was always lower than in WT (B44) (Fig. 3f). After 12 hours of exposure to freezing temperatures, the reddish brown spots or patches on the leaves of WT (B44) plants following DAB staining were larger than those on the *ScGo/S1*-overexpression lines B44-OE2. The freezing damage to the leaves was obvious, indicating that the H_2O_2 content had increased significantly (Fig. 3g). In addition, we found the similar results in another transformed strain B7. Taking B7-OE4 as an example, the transcript level of *Go/S1* gene was the highest at 12h, and much higher than WT (B7) (Fig. S4a). Physiologically, the relative electrical conductivity of the line OE-4 plants was clearly lower than that of the WT under freezing stress (Fig. S4b). The MDA content was gradually increased with freezing stress treatment in the transgenic and WT plants, but the MDA content of the

transgenic lines was lower than WT (Fig. S4c). DAB dye assays also showed that the color of WT (B7) was darker than that of the transgenic line B7-OE4, indicating that the leaves of WT (B7) were seriously damaged, leading to a large amount of peroxide leakage (Fig. S4d). These results suggested that *ScGolS1* enhanced the antioxidant activity of potato cultivars B44 and B7 under freezing stress.

Obtain the T-DNA insertion position of the transgenic lines

In order to obtain the copy number effect of the introduced *ScGolS1* gene of the introduced *ScGolS1* gene and the effect of copy number on freezing tolerance in transgenic plants, we determined the number of T-DNA insertions in the whole genome of the transgenic lines B44-OE1, B44-OE2, B44-OE6, B44-OE14 and B44-OE19, and found that there were two copies of the *ScGolS1* gene in B44-OE2 and a single copy in the other four transgenic lines. Specifically, the T-DNA insert in B44-OE1 and B44-OE6 was found to be located in two different positions on chr03 (Fig. 4c-d), the T-DNA insert in B44-OE14 and B44-OE19 were located on chr10 and chr01, respectively (Fig. 4e-f), while in B44-OE2 the two inserts were located on chr06 and chr07, respectively (Fig. 4a-b). The preliminary phenotype identification results showed that the freezing-tolerance of B44-OE2 with 2 copies is stronger than that of B44-OE1 with only 1 copy (Fig. S5). This indicated that the increase in potato freezing tolerance might be related to the inserted copy number of the *ScGolS1* gene, that is, the gene dosage plays an important role on freezing tolerance level in *ScGolS1* transgenic lines, but the results have yet to be further verified.

Overexpression of ScGolS1 altered CBF pathway and ERFs transcript level

Experiments on *ScGolS1* overexpression affecting the CBF pathway was carried out. The *CBF* gene family in potato includes four members. The expression levels of *CBF1*, *CBF2*, and *CBF3* in the B44-OE2 line increased rapidly after 12 h of freezing stress treatment and peaked at 24 h, and were significantly higher than WT, but interestingly *CBF4* was not induced by freezing signal (Fig. 5a-d). Moreover, *CBF* downstream cold responsive genes *COR47* and *COR413* were all induced by freezing treatment, while higher levels were observed in *ScGolS1*-overexpressing lines at 12 h compared with WT (B44) (Fig. 5e-f). The results indicated that *CBF* transcription factor genes and their downstream *COR* genes were associated with *ScGolS1* expression, *CBF1*, *CBF2*, and *CBF3* transcripts were induced in response to freezing stress. So *CBFs* might be rapidly activated at low temperature, which in turn induced the expression of a group of downstream cold-responsive genes such as *COR47* and *COR413* and enhances the freezing tolerance in potato plants.

In addition, low temperature stress can also promote the biosynthesis of ethylene (ET). Our qRT-PCR results showed that transcription level of the *ERF3*, *ERF4* and *ERF6* genes in the overexpression line B44-OE2 was higher than WT (B44) at 12 h and 24 h of freezing treatment (Fig. 5g-i). The increase in *ERF* gene expression could promote the biosynthesis of ET, thereby regulating downstream defense responses to enhance freezing tolerance in potato.

Overexpression of ScGolS1 induced sugars accumulation in response to freezing treatment

The sugar accumulation induced by low temperature plays a key role in protection of plants against freezing (Taji et al. 2002; Cunningham et al. 2003; Zhang et al. 2011; Zhuo et al. 2013). Quantitative real-time PCR was used to analyze the expression of genes in the RFOs metabolic pathway, and the results showed that overexpression of *ScGo/S1* under freezing condition increased the expression of *MIPS*, *RS*, and *STS*, especially after 12 h (*RS*) and 48 h (*STS*) (Fig. 6a-c). This showed that overexpression of the *ScGo/S1* could regulate the metabolic balance of inositol, galactinol, inositol, raffinose, and stachyose in the metabolism of RFOs, and thus improved freezing tolerance in potato. Compared to WT (B44), the content levels of fructose, glucose, raffinose, sucrose, and inositol were all higher in *ScGo/S1* overexpression plants (Fig. 6d-h). Moreover, raffinose, sucrose and inositol down-regulated expression after freezing treatment, and galactinol was not detected. These results indicated that *ScGo/S1* expression regulated *MIPS*, *STS*, and *RF* expression level and in turn altered RFOs accumulation and maintained dynamic balance of soluble carbohydrate components in sugar metabolism.

T-DNA insertion analysis indicates gene dosage plays an important role on potato freezing tolerance

In order to obtain the copy number effect of the introduced *ScGo/S1* gene on freezing tolerance in transgenic plants, we determined the number of T-DNA insertions in the whole genome of the transgenic lines B44-OE1, B44-OE2, B44-OE6, B44-OE14 and B44-OE19, and found that there were two copies of the *ScGo/S1* gene in B44-OE2 and a single copy in the other four transgenic lines. Specifically, the T-DNA insert in B44-OE1 and B44-OE6 was found to be located in two different positions on chr03 (Fig. 3c-d), the T-DNA insert in B44-OE14 and B44-OE19 were located on chr10 and chr01, respectively (Fig. 3e-f), while in B44-OE2 the two inserts were located on chr06 and chr07, respectively (Fig. 3a-b). The preliminary phenotype identification results showed that the freezing-tolerance of B44-OE2 with 2 copies is stronger than that of B44-OE1 with only 1 copy (Fig. S5). This indicated that the increase in potato freezing tolerance might be related to the inserted copy number of the *ScGo/s1* gene, that is, the gene dosage plays an important role on freezing tolerance level in *ScGo/s1* transgenic lines, but the results have yet to be further verified.

Discussion

Among the wild potato species, *S. commersonii* and *S. acaule* are considered to be the coldest tolerant, and they are also regarded as important genetic resources for improving freezing tolerance in commercial potato cultivars. *S. commersonii* is a stemless wild potato species. The appraisal of cold-tolerance experiments showed that W3 could still recover its morphology at -5°C, and field experiment also proved that its freezing tolerance was stronger than other wild potato materials in our study. Therefore, *S. commersonii* is an ideal freezing tolerance diploid wild species resource for studying potato freezing tolerance.

RFOs plays an important role in osmotic adjustment in plants, which enables them to resist biological and abiotic stresses. The rate-limiting enzyme *GoS* in the RFOs biosynthetic pathway has also attracted considerable attention. In recent years, *GoS* genes have been cloned from various plant species, and it

has been shown that GolS proteins play an important role in stress resistance of plants (Gu et al. 2013). There are differences in the number of genes encoding GolS family members in different plants. In this study, the three homologous genes *ScGolS1*, *ScGolS2*, and *ScGolS3* in the wild diploid potato species *S. commersonii* were cloned and analyzed, and the function of *ScGolS1* was determined in transgenic plants of *S. tuberosum*. We found that *ScGolS1* and *ScGolS3* has two introns, while *ScGolS2* has three, implying that there might be differences in the functions of these three genes. Further homology analysis revealed the presence of the relatively conserved manganese ion (Mn^{2+}) binding domain DGD that is necessary for the GolS protein to perform its catalytic function. In addition, there are significant differences between the N- and the C-terminal sequences of GolS proteins from four wild *Solanum* species and tobacco (Fig. 1a), indicating that the different GolS proteins may differ in their substrate binding capabilities and catalytic activities. In the phylogenetic analysis, GolS proteins from five different Solanaceae species were found to be closely related and they clustered in three clades (Fig. 1b). Under low temperature stress, tomato *SlGolS2* was found to be up-regulated, but *SlGolS1* was either not expressed or was expressed at a very low level (Liu et al. 2019). We speculate that *ScGolS3*, which is in clade I with *SlGolS2*, has a stronger ability to induce raffinose accumulation than *ScGolS1* and *ScGolS2*. The transcriptional responses of the different *GolS* genes in plants in response to various stresses are quite complex. The *GolS* genes in the same clade can respond to different stresses, and different *GolS* genes can also be induced by the same stress, which is worthy of further study.

The expression characteristics of genes in different tissues are indicative of gene functions. qRT-PCR analysis showed that *ScGolS1* was expressed at the highest level in *S. commersonii* leaves, and that expression in young leaves was higher than in old leaves. This indicates that *ScGolS1* overexpression improves freezing tolerance in potato, and that plants should be assayed for freezing tolerance when they are young. In addition, the relative expression levels of *ScGolS2* and *ScGolS3* in *S. commersonii* was young leaves>old leaves>stems>roots (Fig. S2a-b, 2e-f). In tomato, the expression levels of *SlGolS1* and *SlGolS4* in both old and young leaves were low. The expression of *SlGolS2* in old leaves was higher than in young leaves, while the expression of *SlGolS3* in young leaves was higher than in old leaves. It was speculated that *SlGolS2* is related to sugar transport and storage in senescent leaves, but only *SlGolS2* and *SlGolS4* show a transcriptional response to low temperature stress (Liu et al. 2019). The expression of *VhGolS1* and *VhGolS2* during seed development in hairy vetch (*Vicia hirsuta*) resulted in the accumulation of raffinose in the seed, which gave the seed the ability to resist dehydration (Gojło et al. 2015). Among the nine *GolS* genes in *Populus tomentosa*, *PtrGolS4* and *PtrGolS6* were found in root tips, stems, young leaves and both were expressed in mature leaves, while *PtrGolS3* and *PtrGolS7* were expressed in roots and stems, and the nine genes were also functionally different (Zhou et al. 2014). Three *GolS* genes in *Salvia miltiorrhiza* also showed different tissue expression characteristics (Wang et al. 2012). In our research, the expression characteristics of *ScGolS1* in different tissues implied that its function might be different.

Agrobacterium tumefaciens-mediated transformation is the most widely used system to transfer foreign genes into the genomes of plants (Nadolska et al. 2007). There are three ways to integrate exogenous

genes in the host genome: (1) single copy unit site integration: this integration method is the simplest and easiest to analyze, but not all single copy insertions at individual sites are complete single copy insertions. In most cases, the boundary sequence has been modified (Pawlowski et al. 1998). (2) Tandem concatemer integration of more than one copy at the same site, the transforming plasmid is integrated into the host genome in the form of only one or a few complete multiple copies in tandem, and incomplete plasmids are often attached to one or both ends (Kumar et al. 2000). (3) A single copy or multiple copies of the transgene are integrated at multiple sites, often on more than one chromosome. This integration pattern is more complicated and difficult to analyze, and has been found in transgenic plants obtained by both *Agrobacterium*-mediated and particle gun transformation (Yang et al. 1998; Vaira et al. 2000). In this study, high-efficient thermal asymmetric interlaced PCR (hiTAIL-PCR) was used to clone and analyze the double-flanking sequences of the T-DNA insertion sites in the *ScGo/S1* transgenic lines (Fig. 4). Most of the transgenic lines were found to have one copy of the *ScGo/S1* T-DNA construct, but the integration positions were different. The B44-OE2 line located on potato chromosomes chr06 and chr07, while the remaining transgenic lines have only a single copy. Our analysis showed that the freezing tolerance of B44-OE2 was stronger than that of B44-OE1, which might be related to the number of copies and the relative expression level of *ScGo/S1* in the transgenic lines.

Under low temperature stress, plant growth is reduced, leaves are wilted, flowers and fruits fall off, and death can occur, which seriously affects crop yield and quality. At the physiological and metabolic levels, the damage caused by low temperatures is mainly reflected in changes to the activities of certain enzymes and the reduced photosynthesis rate (Thordal et al. 1997; Ruelland et al. 2010), as well as in the damage to the structure and stability of cell membranes (Seo et al. 2010; Matteucci et al. 2011). In this study, the full-length CDS of *ScGo/S1* was cloned from the diploid freezing-tolerant wild species *S. commersonii* (Fig. S3a), and the function of the *ScGo/S1* gene was studied through *Agrobacterium*-mediated genetic transformation of *S. tuberosum* cultivars. PCR amplification was used to identify the transgenic lines of B44 and B7 that carry the *ScGo/S1* gene. Under freezing stress, the leaves of the transgenic lines were less damaged and the plants had a higher survival rate (Fig. 3c). qRT-PCR analysis showed that the *Go/S1* expression level in the overexpression strains was higher than WT (Fig. 3d; Fig. S4a). Measurements of physiological and biochemical indicators showed that the transgenic lines had significantly reduced leaf electrolyte permeability and MDA content in response to freezing stress (Fig. 3e-f; Fig. S4b-c). In conclusion, the determination of various physiological indicators showed that overexpression of *ScGo/S1* gene could significantly improve freezing tolerance of potato cultivars B44 and B7.

CBF cold response pathway plays an important role in freezing tolerance of plants, and it regulates 10 to 20% of *COR* genes during cold acclimation (Park et al. 2015; Jia et al. 2016; Zhao et al. 2016). *CBF* pathway is downstream of cold triggered Ca^{2+} signal (Ding et al. 2019). *CBF1*, *CBF2*, and *CBF3* play key roles in the freezing stress-response signaling pathway in *Arabidopsis thaliana* as transcriptional activators, and could improve freezing tolerance in plants by combining with CRT/DRE elements to regulate the expression of downstream genes such as *RD29A*, *COR15a*, and *COR15b* (Chinnusamy et al.

2007). In our study, qRT-PCR analysis showed that overexpression of *ScGo/S1* promoted the expression of the *CBF* gene family, showing that *ScGo/S1* might improve freezing tolerance in potatoes by relying on the *CBF* pathway.

Ethylene participates in the regulation of plant growth and development and in the response to biotic and abiotic stresses. Ethylene responsive transcription factors (*ERFs*) play an important role in the plant response to unfavorable environmental conditions and pathogens. A previous study showed that galactosyl inositol acts as a sugar molecule signal in tomato to induce the up-regulation of *ERF* gene expression in the ethylene signaling pathway to increase freezing tolerance in tomato (Liu et al. 2019). Overexpression of both grape *VaERF057* and *VaERF080* genes in *Arabidopsis* significantly improved the freezing tolerance of transgenic *Arabidopsis* lines (Sun et al. 2016). Overexpression of the wheat *TaPIE1* gene activated the expression of defense and stress-related genes in the ethylene signaling pathway, and improved the tolerance of transgenic wheat to low temperature stress (Zhu et al. 2014). In this study, the transcription levels of *ERF3*, *ERF4*, and *ERF6* in the *ScGo/S1*-overexpression line B44-OE2 were higher than that in WT (B44) at 12 and 24 h of low temperature stress, and the expression of *ERF* genes was up-regulated. Besides, the expression levels of other genes in the RFOs metabolic pathways such as *MIPS*, *RS*, and *STS* also increased accordingly; this may be one of the reasons for maintaining the balance of sugar metabolism, and the ethylene signaling pathway was involved in this regulation process. Therefore, overexpression of *ScGo/S1* induces the balance of RFOs and other soluble sugars, which further act as sugar signaling molecules to promote the up-regulated expression of *ERF* transcription factors, feedback the regulation of the ET signaling pathway, and enhance the activity of *CBF* transcriptional activators (*CBF1*, *CBF2*, and *CBF3*), and then promotes expression of the downstream freezing-responsive genes *COR47* and *COR413*, improving freezing tolerance in the potato cultivar B44. These findings provide useful information for potato freezing-resistant breeding..

Conclusion

Overexpression of the *ScGo/S1* gene from *S. commersonii* in *S. tuberosum* cultivars can significantly improve freezing tolerance in transgenic potato plants. Physiological and biochemical analyses show that the relative conductivity and MDA content of the transgenic potato plants decreased compared with WT. Our results demonstrate that *ScGo/S1* induces RFOs metabolic pathway related genes expression under freezing stress, and affects the balance of soluble carbohydrate components in sugar metabolism, which feedback regulates the expression of *ERF* transcription factors and the *CBF* pathway of the transgenic potato plants and thereby improves freezing tolerance of potato. These results provide a theoretical basis and genetic resources for potato freezing-resistant breeding.

Declarations

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Author contribution statements

Feiyan He designed the study, performed the experiments, wrote the original draft. Jianfei Xu and Yinqiao Jian performed the data analysis. Shaoguang Duan and Jun Hu performed phenotype test. Liping Jin designed the study. Guangcun Li designed the study, reviewed and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Sequence analysis of GolS. **a** Multiple sequence alignment of GolS proteins. **b** Phylogenetic relationship of GolS from five species in the Solanaceae. Black box, Black double-ended arrow and black horizontal line represent the manganese binding site DGD, the GolS substrate binding area, and C-terminal hydrophobic pentapeptide A(S)PSAA, respectively. ScGolS1 is underlined in red

Figure 2

Expression analysis of GolS1 gene. a Expression analysis of ScGolS1 gene in roots, stems and leaves of W3 tissue cultured seedlings. b Expression analysis of ScGolS1 gene in roots, stems and leaves of W3 potted seedlings c Relative expression levels of GolS1 gene in B44 (*S. tuberosum*) and W3 (*S. commersonii*) under low temperature stress at 20°C, 12°C, 4°C, 2°C and 0°C for 24 h. d Relative expression levels of GolS1 gene in B44 (*S. tuberosum*) and W3 (*S. commersonii*) under freezing stress at -1°C for 0 h, 3 h, 12 h, and 24 h

Figure 3

Identification of freezing tolerance of B44 transgenic lines. a PCR amplification identification with pBI121 vector primer 73F/211R. b PCR amplification identification with pBI121 vector forward primer 73F and the ScGolS1 gene reverse primer. c Seedlings before freezing treatment, after 24 h at -2°C, and after recovery for 24 h at 23°C. d Relative expression levels of ScGolS1 in WT (B44) and transgenic lines (B44-OE2) at 0, 12, 24, and 48 h after exposure to -1°C freezing stress. e Relative electrolyte leakage in WT (B44) and transgenic lines (B44-OE2) after freezing stress at -1°C for 0, 12 and 24 h. f MDA contents after freezing stress at -1°C for 0, 6, 12, and 24 h in WT (B44) and transgenic lines (B44-OE2). g DAB staining of leaves exposed to -1°C freezing stress for 12h in WT (B44) and transgenic lines (B44-OE2). Values represent means of three biological replicates with error bars indicating standard error of the mean. ** indicates a significant difference compared with the control at $p < 0.01$ determined by Student's t-test

Figure 4

T-DNA insertion in transgenic plants. a-b Insertion position of B44 transgenic line OE2, chr06 and chr07. c Insertion position of B44 transgenic line OE1, chr03. d Insertion position of B44 transgenic line OE6, chr03. e Insertion position of B44 transgenic line OE14, chr10. f Insertion position of B44 transgenic line OE19, chr01

Figure 5

Analysis of transcripts of cold responsive genes in transgenic line B44-OE2 and WT (B44) exposed to freezing stress (-1°C). EF-1 α was used to normalize the amount of template. Relative expression levels were calculated by $2^{-\Delta\Delta\text{Ct}}$. Values are means $\pm$ SD ($n = 3$). * indicate a significant difference compared with WT (B44) at $P < 0.05$, by Student's t-test. ** indicates a significant difference compared with WT (B44) at $p < 0.01$ determined by Student's t-test

Figure 6

Analysis of transcripts of RFOs metabolism related genes and sugars concentrations in transgenic potato plant B44-OE2 and WT. a-c Transcript levels of MIPS, STS and RS were determined after 0h, 12h, 24h and 48 h of freezing treatment. d-h Sugars concentrations were determined after 0h, 12h and 24h of freezing treatment. Means of three replicates and standard errors are presented; Values are means $\pm$ SD ($n = 3$). * indicate a significant difference compared with WT (B44) at $P < 0.05$, by Student's t-test. ** indicates a significant difference compared with WT (B44) at $p < 0.01$ determined by Student's t-test

Figure 7

Diagram of a proposed model for regulation of *ScGolS1* in freezing tolerance in transgenic potato

Supplementary Files

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