Overexpression of GmSnRK1, a soybean sucrose non-fermenting-1 related protein kinase 1 gene, results in directional alteration of carbohydrate metabolism in transgenic Arabidopsis

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ABSTRACT
Sucrose non-fermenting-1-related protein kinase 1 (SnRK1) plays an important role in plant carbohydrate metabolism and starch biosynthesis. The regulatory role of GmSnRK1 from soybean in regulating carbohydrate metabolism and starch accumulation has not been reported. In the present study, a gene encoding the SnRK1 protein, named GmSnRK1, was successfully isolated from soybean, and the functions of this gene were studied. Subcellular localisation analysis in onion epidermal cells indicated that GmSnRK1 protein was localised to the nucleus. The GmSnRK1 gene was introduced into Arabidopsis to obtain transgenic plants. Its overexpression significantly increased the starch content, as well as the sucrose, glucose and fructose content in the transgenic plants compared to the wild-type (WT). Real-time quantitative polymerase chain reaction (PCR) analysis showed that overexpression of GmSnRK1 up-regulated the genes involved in starch biosynthesis, including sucrose synthase (AtSUS), phosphoglucomutase (AtPGM), ADP-glucose pyrophosphorylase (AtAGPase), granule-bound starch synthases (AtGBSS I and AtGBSS II), soluble starch synthases (AtSSS I, AtSSS II, AtSSS III and AtSSS IV) and starch branching enzymes (AtSBE I and AtSBE II) genes. In contrast, the expression of sucrose phosphate synthase (SPS) gene was decreased in the transgenic plants. Meanwhile, the enzyme activity levels of the five starch biosynthetic enzymes (SUS, AGPase, GBSS, SSS and SBE) exhibited higher activities, while the SPS activity was decreased in the transgenic plants compared to WT. These results suggest that the manipulation of GmSnRK1 expression might be used for improving the starch content in engineered plants for biofuel production.

Introduction
Biofuel, which can decrease environmental damage by reducing the extraction and use of fossil fuels, is more and more important with the development of society. Breeding of non-food energy crops for economically viable production as environmentally friendly biofuels is on the way [1,2]. Since the conversion of starch into fermentable sugars is relatively easy, starch has been taken as a major feedstock for first-generation biofuel production [1,3]. Therefore, elucidating how carbohydrates are metabolised in plants, could greatly facilitate the development of crops by means of enhancing starch synthesis, and the improvement of biofuel production efficiency [1].

There are several reports on increasing starch content by genetic engineering in plants. Constitutive expression of the Arabidopsis AtAATP1 increased the starch content in potato tubers [4]. Heterologous expression of MdSnRK1 from apple increased the starch content in transgenic tomato plants [5]. The IbSnRK1/StSnRK1-expressing tobacco plants exhibited increased starch accumulation [6,7]. Overexpression of NtTrxF led to a striking increase of starch accumulation in the leaves of tobacco [1]. The StTrxF/SiTrxF-expressing Arabidopsis plants had higher starch content [8,9]. Wang et al. [10–12] cloned the SIAATP/StAATP/GmAATP gene from tomato/potato/soybean; starch content and soluble sugar content; Arabidopsis GmSnRK1; soybean; starch content

KEYWORDS
Arabidopsis; GmSnRK1; soluble sugar content; soybean; starch content

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carbohydrate metabolism and starch biosynthesis [5,16,17]. It is required for redox modulation of ADP-glucose pyrophosphorylase (AGPase) activity in response to sucrose [18] and is involved in regulating the expression of genes encoding sucrose synthase (SUS) and AGPase, which are two key enzymes involved in the biosynthetic pathway from sucrose to starch [19,20].

The genes encoding SnRK1 were cloned from several plant species, such as rye [21], Arabidopsis [22], maize [23], apple [24], sweetpotato [6] and potato [7]. There are a few reports about the function of SnRK1 in regulating carbohydrate metabolism and starch biosynthesis of plants. Antisense inhibition of SnRK1 in developing pollen grains led to an almost complete loss of starch accumulation [17]. It has shown that SnRK1 played an important role in starch accumulation [26]. The expression of SUS and AGPase was up-regulated and their enzyme activities were also increased in the SnRK1-overexpressing potato plants, in which the starch levels in the tubers were increased by up to 30% [20]. Jain et al. [27] found that the expression of a SnRK1 gene coincided with the onset of starch accumulation in sorghum endosperm and maize endosperm. Wang et al. [5,7] and Ji et al. [6] further demonstrated that overexpression of SnRK1 from apple/sweetpotato/potato increased the soluble sugar and starch content by up-regulating the expression of SUS and AGPase and increasing the activities of SUS and AGPase in transgenic tomato/tobacco plants. It is proposed that SnRK1 channels carbon through the storage pathway to starch [17,20]. Furthermore, it is known that SnRK1 can regulate the carbohydrate metabolism by inactivating sucrose phosphate synthase (SPS) via phosphorylation [17,28]. The activity of SPS was decreased in the transgenic tomato/tobacco plants overexpressing SnRK1 from apple/ sweetpotato/ potato [5–7].

Although the function of SnRK1 from other plant species has been well studied, the regulatory role of GmSnRK1 (Genbank accession No. NM_001251194) from soybean in regulating carbohydrate metabolism and starch accumulation has not been reported. In this study, we isolated GmSnRK1 from soybean and estimated its roles in transgenic Arabidopsis. We also developed GmSnRK1-overexpressing Arabidopsis plants and found that overexpression of GmSnRK1 significantly increased sucrose, glucose, fructose and starch content in the leaves of transgenic plants, indicating a great potential of GmSnRK1 in increasing soluble sugar and starch levels of plants in the future.

**Materials and methods**

**Plant materials**

Soybean cultivar Williams 82 was employed for GmSnRK1 gene cloning in this study. Arabidopsis [ecotype Columbia-0, wild type (WT)] was used as a model plant to investigate the functions of this gene.

**Cloning of the soybean GmSnRK1 gene**

Total RNA was extracted from the leaves of Williams 82 with the RNAprep Pure Kit (Tiangen Biotech, Beijing, China). RNA samples were reverse-transcribed according to the instructions of Quantscript Reverse Transcriptase Kit (Tiangen Biotech, Beijing, China). Based on the sequence of GmSnRK1 (Genbank accession No. NM_001251194), we designed gene-specific primers (GC-F/R) for reverse transcription polymerase chain reaction (RT-PCR) (Supplemental Table S1) to obtain its full-length cDNA sequence. PCR was performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were separated in a 1.0% w/v agarose gel. The target DNA band was recovered by gel extraction, then cloned into PMD19-T (TaKaRa, Beijing, China), and finally transformed into competent cells of Escherichia coli strain DH5α. White colonies were checked by PCR and the positive colonies were used for sequencing (Invitrogen, Beijing, China).

**Sequence analysis of the GmSnRK1 gene**

The open reading frame (ORF) of the cloned GmSnRK1 gene was predicted with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/orf/). The homology of GmSnRK1 protein was identified using protein BLAST in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domain of GmSnRK1 protein was scanned by the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iproscan/). The theoretical molecular weight and isoelectric point (pl) were calculated using ProtParam tool (http://web.expasy.org/protparam/). The nuclear localisation signal (NLS) of the GmSnRK1 protein was predicted by the cNLS Mapper program (http://nls-map.per.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). For multiple sequence alignment analysis, the amino acid sequences of GmSnRK1 and other SnRK1 homologues from different plant species retrieved from NCBI were aligned using the DNAMAN software (Lynnon Biosoft,
Quebec, Canada). Phylogenetic analysis was conducted with the MEGA4 software (http://www.megasoftware.net/).

**Subcellular localisation of GmSnRK1**

The subcellular localisation of GmSnRK1 in onion (*Allium cepa*) epidermal cells was analysed as described by Wang et al. [29]. The ORF of GmSnRK1 was cloned and then inserted into the pMD838 vector containing the green fluorescent protein (GFP) gene at Spe I and Asc I restriction sites under the control of the CaMV35S promoter and NOS (nopaline synthase) terminator (Table S1). Both the fusion construct (3SS-GmSnRK1::GFP) and the control vector (3SS::GFP) were transformed into living onion epidermal cells by particle bombardment with a GeneGun (Biorad HeliosTM), according to the instruction manual (helium pressure 260 psi). After incubation on MS (Murashige and Skoog) [30] medium (pH 5.8) solidified with 3% agar at 28 °C for 24 h, the onion cells were observed with bright field and fluorescence using confocal microscopy (Nikon Inc., Melville, NY).

**Generation of transgenic Arabidopsis plants**

The coding region of GmSnRK1 was amplified using a pair of specific primers with terminal BamH I and Sac I restriction sites, and then inserted into the same enzyme sites in pCAMBIA1301 to create the plant expression vector pCAMBIA1301-GmSnRK1, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. This vector also contained β-glucuronidase (gusA) and hygromycin resistance (hpt II) genes driven by the CaMV 35S promoter. Both pCAMBIA1301-GmSnRK1 and the control vector (VC) pCAMBIA1301 were transformed into the Agrobacterium tumefaciens strain LBA4404 cells by the electroporation method for *Arabidopsis* transformation [31]. Transgenic plants were produced according to methods described previously [32]. Transformants were selected based on their resistance to hygromycin (Hyg). Putative transformant seeds were germinated on agar-solidified MS medium containing 25 mg/L Hyg. Positive transgenic seedlings were grown in pots containing a mixture of soil, vermiculite and humus (1:1:1, v/v/v) for T2 and T3 seed selection. The incubation and growth conditions of *Arabidopsis* were the same as described previously [32].

**Molecular confirmation of transgenic plants**

The presence of GmSnRK1 in hygromycin-resistant plants was assessed by PCR analysis using specific primers (Table S1) to amplify fragments of the hpt II coding sequence according to the method of Wang et al. [29]. DNA was first extracted from *Arabidopsis* leaves, according to the instructions of EasyPure Plant Genomic DNA Kit (Transgen, Beijing, China). PCR amplifications were performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in a 1.0% (w/v) agarose gel.

**Analyses of sucrose, glucose, fructose and starch content**

The content of sucrose, glucose and fructose were measured using the anthrone method, according to the method of Zhang et al. [33]. Starch extraction and quantification were performed as described previously [34]. Seeds were grown on MS medium for 2 weeks and transferred to pots containing a mixture of soil, vermiculite and humus (1:1:1, v/v/v). Plants were grown in growth chamber for 4 weeks at 22 °C under standard long day conditions (14 h light and 10 h dark). Leaves of four-week-old plants were harvested to determine sucrose, glucose, fructose and starch content in light at 10–11 am. All treatments were performed in triplicate.

**Southern blot analysis**

Genomic DNA was extracted from the leaves of transgenic, VC and WT plants by the cetyltrimethylammonium bromide (CTAB) method [35]. Approximately 20 μg genomic DNA of each sample was digested by EcoR I. The restriction fragments were size-fractionated by 1.0% (w/v) agarose gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, UK). A 532 bp GmSnRK1 fragment coding sequence generated with specific primers (Table S1) was used as the probe. The labelling of probe, prehybridisation, hybridisation and detection were performed by the protocol of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Germany).

**Expression analysis of the related genes**

The expression of GmSnRK1 and starch biosynthesis related genes was analysed by real-time quantitative PCR (qRT-PCR). Transgenic, VC and WT plants were grown in pots for 4 weeks under normal condition. Total RNA was extracted from the leaves of these plants, respectively, using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China). RNA samples were
reverse-transcribed using Quantscript Reverse Transcriptase Kit (Tiangen Biotech, Beijing, China). The cDNA solution was used as templates for PCR amplification with gene-specific primers (Table S1). The Arabidopsis AtActin gene (Genbank accession No. NM112764) was used as an internal control [36] (Table S1). PCR amplifications were conducted by ABI PRISM 7500 (Software for 7500 and 7500 Fast Real-Time PCR Systems, V2.0.1, USA) using SYBR Green PCR Master Mix (Tiangen Biotech, Beijing, China). The amplifications were performed with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantification of gene expression was done with the comparative C\(_T\) method [37]. All experiments were repeated three times and each data point represents the average of three experiments.

**SUS, SPS, AGPase, GBSS, SSS and SBE activity analysis**

The activity of SUS, SPS, AGPase, GBSS, SSS and SBE in the leaves of four-week-old transgenic, VC and WT plants was performed according to the methods described by Wang et al. [5] and Nakamura et al. [38].

**Statistical analysis**

All experiments were repeated three times and the data are presented as mean values with standard error (±SEM). Where applicable, data were analysed by Student’s t-test in a two-tailed analysis. Values of \(P < 0.05\) or \(P < 0.01\) were considered to indicate statistically significant differences.

**Figure 1.** Multiple sequence alignment of the predicted GmSnRK1 protein with its homologous proteins from other plant species.
Results and discussion

Cloning and sequence analysis of GmSnRK1

The GmSnRK1 gene, containing a 1545 bp ORF and encoding a polypeptide of 514 amino acids, was cloned by RT-PCR. The molecular weight of the protein was 58.95 kDa and the theoretical isoelectric point (pI) was 6.88. Sequence analysis via the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/interproscan/) showed that the GmSnRK1 protein contained the Serine/Threonine-protein kinase catalytic domain (S-TKc) and the Serine/Threonine-protein kinase active site at amino acid residues 139–151, which are the features of plant SnRK1 proteins. A putative nuclear localisation signal sequence was also identified at amino acid residues 223–235.

A BLAST search showed that the amino-acid sequence of GmSnRK1 showed a high amino-acid identity with predicted protein products of Cajanus cajan (KYP60405, 93.00%), Vigna radiata (XP_014512885, 92.43%), Phaseolus vulgaris (XP_007151266, 92.23%), Arachis duranensis (XP_015946108, 90.10%), Cicer arietinum (XP_004489425, 88.74%), Nicotiana tomentosiformis (XP_009621306, 86.58%), Malus domestica (XP_008365408, 85.47%), Theobroma cacao (XP_007032620, 85.47%), Solanum lycopersicum (NP_001304105, 82.95%), Arabidopsis thaliana (NP_566130, 82.33%) and Solanum tuberosum (NP_001274953, 81.78%) (Figure 1). Phylogenetic analysis revealed that GmSnRK1 had a close relationship with the predicted protein product of Cajanus cajan (KYP60405) (Figure 2).

Nuclear localisation of GmSnRK1

To investigate the subcellular localisation of GmSnRK1, an Agrobacterium-mediated transformation system was used for a transient assay. Two constructs (35S::GFP and 35S-GmSnRK1::GFP) (Figure 3a) were individually introduced into onion epidermal cells. As shown in Figure 3b, the GFP fluorescence of GmSnRK1-GFP was exclusively located in the nuclei of the cells, whereas the GFP control was distributed throughout the whole onion cells. These results indicate that GmSnRK1 is a nuclear-localised protein. This localisation may aid in the determination of both the function and the molecular mechanism underlying the function of this protein. Vincent et al. [39] found that SnRK1 was localised to the nucleus and identified a novel signaling pathway that controlled this nuclear localisation in response to glucose phosphorylation in yeast.

Increased sucrose, glucose, fructose and starch content in Arabidopsis overexpressing GmSnRK1

The SnRK1 protein belongs to a subfamily of Serine/Threonine kinases [40] and its name is derived from sucrose nonfermenting-1 (SNF1), its homologue in yeast [20,41]. SnRK1 is reportedly involved in plant carbohydrate metabolism and starch biosynthesis in plants. The study of Kanegae et al. [26] provided evidence that SnRK1 had a role in starch accumulation in rice. Antisense inhibition of SnRK1 in developing pollen grains resulted in an

![Figure 2. Phylogenetic tree of the predicted GmSnRK1 protein with its homologous proteins from other plant species.](image-url)
almost complete loss of starch accumulation and viability [25]. Overexpression of SnRK1 from apple/sweetpotato/potato increased the soluble sugar and starch content in transgenic tomato/tobacco plants [5–7].

In this work, the ORF of GmSnRK1 was ectopically expressed in Arabidopsis (Col-0, WT) using the binary vector pCAMBIA1301-GmSnRK1 (Figure 4a). Eight independent transgenic lines overexpressing GmSnRK1 (T1 generation) were obtained by Hyg-resistance selection, named #1–#8, respectively, and their progenies (T2 generation) were generated. The PCR analyses of their genomic DNA confirmed the successful integration of the transgene (Figure 4b). qRT-PCR analysis showed that the highest expression levels of GmSnRK1 were observed in transgenic lines #1, #4, #5 and #7, while no transgene expression was observed in VC and WT (Figure 4c). Therefore, transgenic lines #1, #4, #5 and #7 were selected for further analysis.

Two-week-old WT, VC and transgenic plants (lines #1, #4, #5 and #7) were grown in pots under normal condition for 4 weeks. The sucrose, glucose, fructose and starch content in the leaves of these plants were quantified. The results showed that the plants overexpressing GmSnRK1 had significantly higher sucrose, glucose, fructose and soluble sugar and starch content, which were increased by 65%–192%, 68%–297%, 9%–112% and 63%–145%, respectively, compared to WT (Figures 5 and 6), whereas no significant difference was observed between VC and WT plants (Figures 5 and 6).

Southern blot analysis of Arabidopsis overexpressing GmSnRK1

The transgene integration patterns of the four transgenic plants (lines #1, #4, #5 and #7) with higher starch content were analysed by Southern blot. The genomic DNA of the transgenic plants, VC and WT was digested with EcoRⅠ, which has a unique cleavage site in the T-DNA region in the vector, and was hybridised with the GmSnRK1 gene probe. The transgenic plants displayed different patterns and the copy number of integrated GmSnRK1 gene varied from 1 to 2 (Figure 7). No hybridising band was observed in WT and VC as expected (Figure 7). A clear relationship between the starch accumulation and the copy number was also found, similar to the results reported by Wang et al. [10], in which the copy number of the integrated SIAATP gene ranged from 1 to 2 in transgenic plants exhibiting higher starch content.

Gene expression and enzyme activity assay in Arabidopsis overexpressing GmSnRK1

To dissect how the expression of GmSnRK1 increased the starch content in the transgenic plants, the transcript levels of 15 starch biosynthetic genes in WT, VC and transgenic plants (lines #1, #4, #5 and #7) were examined by qRT-PCR (Figure 8). Meanwhile, the activities of SUS, SPS, AGPase, GBSS, SSS and SBE involved in starch biosynthesis were also investigated in the leaves of WT, VC and transgenic plants (lines #1, #4, #5 and #7) (Figure 9).

SUS, a key enzyme involved in the biosynthetic pathway from sucrose to starch, catalyses the reversible conversion of sucrose and UDP to UDP-glucose (UDPG) and fructose [6,42,43]. The activity of SUS was closely correlated with sink strength and starch accumulation [6,44]. SPS catalyses the conversion of UDPG and fructose into sucrose [17,28]. In plants, plastidial phosphoglucomutase (PGM), which catalyses glucose-6-phosphate (G-6-P) to glucose-1-phosphate (G-1-P), provides the substrate G-1-P for the committed pathway of starch synthesis [45]. AGPase is a major regulatory enzyme of starch biosynthesis which converts G-1-P to ADP-glucose (ADPG) [18,46]. It is reported that AGPase is subjected to post-
translational redox regulation, which provides a mechanism to adjust the rate of starch synthesis in potato tubers to sucrose supply [18].

Purcell et al. [19] provided clear evidence that SnRK1 is involved in the control of sucrose synthase gene in potato, and for the first time confirmed that SnRK1 was involved in the regulation of several aspects of carbon metabolism in plants through the transcriptional regulation of SUS. SnRK1 can regulate carbohydrate metabolism by phosphorylating and inactivating SPS [17,28]. McKibbin et al. [20] reported that the expression of SUS and AGPase was up-regulated and their enzyme activities were also increased in the SnRK1-overexpressing potato plants, in which starch levels in the tubers were increased by up to 30%. Wang et al. [5,7] and Jiang et al. [6] demonstrated that overexpression of SnRK1 from apple/sweetpotato/potato up-regulated the expression of SUS and AGPase, and increased the activities of SUS and AGPase and decreased the activity of SPS, leading to increased soluble sugar and starch content in transgenic tomato/tobacco plants. In our work, the expression of AtSUS, AtPGM, AGPase small subunit (AtAGPase-S1 and AtAGPase-S2) and AGPase large subunit (AtAGPase-L1 and AtAGPase-L2) was up-regulated, while the expression of AtSPS was slightly inhibited in transgenic plants (Figure 8). Consistently, the enzymatic activity of SUS and AGPase were also significantly increased, and the SPS activity was also decreased in the GmSnRK1-overexpressing Arabidopsis plants (Figure 9).

The higher level of starch content is related to the increased expression of starch biosynthesis genes [6,7,47]. Starch synthase can be grouped into five types,
granule-bound starch synthase (GBSS) and four types of soluble starch synthases (SSS): SSS I, SSS II, SSS III and SSS IV [47–49]. Up-regulation of these genes could increase starch accumulation in plants [6,7,49–52]. In our study, systematic up-regulation of these genes, granule-bound starch synthase (AtGBSS I and AtGBSS II), soluble starch synthases (AtSSS I, AtSSS II, AtSSS III and AtSSS IV) and starch branching enzyme (AtSBE I and AtSBE II) involved in the starch biosynthesis pathway, was observed in transgenic plants (Figure 8). Consistent with this phenomenon, the activities of the major enzymes (GBSS, SSS and SBE) were also increased in transgenic plants (Figure 9). Thus, it is thought that overexpression of GmSnRK1 up-regulates the expression of AtSUS and AtAGPase and increases the activities of SUS and AGPase, which further increase the expression of the genes and the activity of the major enzymes involved in starch biosynthesis, leading to increased starch accumulation in transgenic plants (Figure 10).

In addition, the availability of sufficient substrate (sucrose) is a very important factor in the starch biosynthesis pathway. Genes encoding SUS and AGPase in potato tubers are inducible by sucrose [6,53,54]. The present results indicated that sucrose levels and SUS and
AGPase activities were significantly increased in the transgenic tobacco plants (Figures 5 and 8). Our work found that the high level of sucrose can induce the expression of SUS and AGPase, which further modulated the starch metabolism in transgenic plants (Figure 10).

All of the results suggest that SnRK1 channels carbon through the storage pathway to starch by regulating the activities of SUS, SPS and AGPase. Our works support the hypothesis proposed by Halford and Hey [17] and Jiang et al. [6] that SnRK1 is activated by sucrose, and then SnRK1 increases the flux through the starch biosynthesis pathway by up-regulating SUS and AGPase and down-regulating SPS (Figure 10). These results suggest that the potential application of GmSnRK1 expression might serve to improve the starch content in engineered plants for biofuel production.

Conclusions

Taken together, the GmSnRK1 gene was successfully isolated from soybean. Overexpression of GmSnRK1 was found to significantly increase the soluble sugar and
starch content in transgenic Arabidopsis plants. Our results suggest that GmSnRK1 plays a crucial role in starch metabolism, and has great potential in the engineering of alternative energy crop plants with improved starch accumulation.

Disclosure statement
No potential conflict of interest was reported by the authors.

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