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ORIGINAL PAPER

Function characterization of a soybean sucrose transporter GmSUT4.2 involved in plant growth, development, and crop yield

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Abstract

Sucrose transporters (SUTs) play an important role in regulating carbohydrate homogeneity, and well understand of the regulatory control of sugars into demanding sink is important for plant growth and seed yield. Nevertheless, key sucrose transporters that are involved in this process are not identified or characterized in soybean. Here, a sucrose transporter gene, *GmSUT4.2*, belonging to the SUT4 subfamily was cloned and functionally characterized. It encodes a protein of 513 amino acids which is localized in the plasma membrane. Real-time quantitative PCR showed that the expression of this gene was induced by sucrose and the sucrose transport capability could be functionally recovered by the expression of *GmSUT4.2* in a sucrose transport dysfunction mutant yeast SUSY7/ura3. In soybean (*Glycine max* L.), overexpression of *GmSUT4.2* significantly promoted sucrose-stimulated hairy root growth and improved the capacity of sucrose uptake. However, the knockdown of *GmSUT4.2* in soybean by CRISPR/Cas9 caused a small leaf phenotype, which could also be observed in the ethyl methane sulfonate (EMS)-induced *GmSUT4.2* mutant. In addition, the significantly low level of sucrose and soluble sugars were recorded in these mutants compared with wild-type plants (WT). As a result, the soybean yield and biomass in mutants were decreased by more than ~ 30% compared with WT under greenhouse conditions. While overexpression of *GmSUT4.2* (OE) in *Arabidopsis* showed a pleiotropic phenotype with more rosette leaves and branches, resulting in a higher yield (40.07%) than those of wild types. These results suggest that the *GmSUT4.2* played key roles in the regulation of plant growth and development as well as yield formation.

Keywords *GmSUT4.2* · Sucrose transporter · Growth and development · Yield

Introduction

Sucrose is the main form of assimilated carbon produced during photosynthesis and critical for the overall growth and performance of crop plants (Aluko et al. 2021; Bavnhoj et al. 2023), serving as the energy for cellular metabolism, with additional associated roles as a signaling molecule (Koch 2004; Yoon et al. 2021). Sucrose fluxes from source leaf towards demanding sink organs via the phloem was coordinated by sucrose transporters (SUTs) (Carpaneto et al. 2005; Ayre 2011; Nino-Gonzalez et al. 2019; Peng et al. 2020), which are key determinants of plant productivity and crop yield (Ishibashi et al. 2014; Saalbach et al. 2014; Wang et al. 2015; Mathan et al. 2021; Wen et al. 2022; Gong et al. 2023).

SUTs belong to the Major Facilitator Super Family (MFS), which is one of the largest known transporter families (Reddy 2013). Based on phylogenetic relationships analysis, SUTs are divided into five subgroups, namely SUT1-SUT5 (Kuhn and Grof 2010). Among them, the SUT1 subfamily members are indigenous to dicotyledons, which is so far the best characterized SUT members. SUT3 and SUT5 are native to monocotyledons, and SUT5 members have not been characterized. SUT2 and SUT4 clade members are found in both monocotyledons and dicotyledons

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(Reddy et al. 2012), and the SUT4 clade in most species was further divided into two subclades, SUT4.1 and SUT4.2 (Doidy et al. 2019).

Different members of each SUT clade show different functions. The SUT1 clade members are exclusively located in the plasma membrane and plays role in the apoplasmic phloem loading of sucrose (Slewinski et al. 2009; Oner-Sieben and Lohaus 2014; Nieberl et al. 2017; Dobbelstein et al. 2019). In addition, SUT1 genes also play essential roles in a variety of developmental and physiological processes in plants. For example, the SUT1 subfamily participates in regulation of vegetative growth (Gottwald et al. 2000), flowering time(Gu et al. 2020), pollen development (Lemoine et al. 1999; Stadler et al. 1999), anthocyanin accumulation(Sivitz et al. 2008), crop yield(Hackel et al. 2006; Sonnewald and Fernie 2018; Lu et al. 2020; Wang et al. 2021). The SUT2 and SUT3 subfamily were primally described as sugar sensor (Barker et al. 2000; Sauer 2007). However, the subsequent studies confirmed that members of SUT2 clade are involved in phloem loading(Hackel et al. 2006) or as a sucrose transporter (Ma et al. 2016; Ahmed et al. 2018), affecting plant yield, sugar accumulation (Ma et al. 2017) and resistance to abiotic stress (Ma et al. 2019).

In contrast, the SUT4 clade promotes sucrose uptake with low affinity and has high transport capacity within the SUT family (Weise et al. 2000). Members of the SUT4 clade are located at the plasma membrane or vacuolar membrane (Payyavula et al. 2011; Wang et al. 2020; Peng et al. 2020), and SUT4 transgenic plants do not show a consistent phenotype in different species. In *Arabidopsis*, *AtSUC4* releases sucrose from the vacuole into the cytoplasm (Schulz et al. 2011), but the mutants have no observable phenotype. *OsSUT2* mutant of rice exhibits a significantly reduced transport ability to export sucrose from the adult plant leaves compared with the wild type, resulting in severely delayed plant growth and reduced grain yield (Atkins et al. 2011). Similarly, the loss function of the *ZmSUT2* gene in maize resulted in hyperaccumulation of foliar sucrose and greatly reduced growth (Leach et al. 2017). In addition, down-regulation of the *StSUT4* gene resulted in early flowering, fewer leaves at flowering time, shorter internodes, and higher tuber production (Chincinska et al. 2008). In *StSUT4*-RNAi plants, the delayed export of sucrose and the accumulation of soluble sugars and starch in source leaves, suggest that *StSUT4* is involved in circadian clock control (Garg et al. 2022). Thus, these results suggest that SUT4 clade members seem to have overwhelming importance for sucrose transport and plant development. In soybean, there are very few sucrose transporters functionally characterized to date. Doidy et al. (2019) indicated that two SUT4 accessions, *GmSUT4.1* and *GmSUT4.2*, was identified in soybean. *GmSUT4.1* is homologous to *Ccjanus cajan CcSUT4.1* while *GmSUT4.2* has the closest homology relationship to *Ccjanus cajan CcSUT4.2*, *Vigna unguiculata VuSUT4.2* and *Phaseolus vulgaris PvSUT4.2*.

At present, the function of *GmSUT4* in sucrose transport has not been demonstrated yet. In this context, a plasma membrane-localized sucrose transporter *GmSUT4.2* gene was cloned from soybean, and its function was analyzed using yeast, *Arabidopsis*, and soybean. The results showed that GmSUT4.2 could transport sucrose and regulates leaf expanding and seed production, as well as sucrose homeostasis in *Arabidopsis* and soybean. Our findings improve the understanding of the SUTs mechanisms by which they mediate sucrose transport in plants to support the growth and development process.

Materials and methods

Plant materials and growth conditions

The soybean cultivar 'Guixia1', used for the analysis of the spatio-temporal expression levels of *GmSUT4.2*, was grown in potting soil, in a climate chamber. Tissues including those of root, steam, leaf, flower, source leaf, and seed tissues at the different developmental stages of trefoil, flowering, and seed development were sampled and kept in -80 °C.

For the analysis of the sucrose-dependent response, the soybean seedlings with the same growth rate were selected and treated with 1/4 Hoagland nutrient solution containing 1% sucrose. Subsequently, samples of root were collected at 0, 1, 2, 4, 8, 12, 24 and 48 h, for qRT-PCR analysis.

The EMS-induced *GmSUT4.2* gene mutation lines NJAU0264 (GmSUT4-M1) and NJAU1191 (GmSUT4-M2) were provided by Nanjing Agricultural University, these two mutants were developed by using the cultivar Williams 82 (Zhang et al. 2022). All soybean plants used in this study were grown in pot cultures in a greenhouse (27 °C with a 14/10 h, light/dark).

Molecular cloning of *GmSUT4.2* gene

The coding sequence of the *GmSUT4.2* gene (Glyma04g09460) was downloaded from SoyBase (https://www.soybase.org) and primers (Supplementary in Table S1) used to clone the open reading frame (ORF) of *GmSUT4.2* were designed using Premier 5.0. Total RNA was extracted from soybean leaves using TRNzol universal reagent (Vazyme Biotech Co., Ltd) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using a reverse transcription kit (Vazyme Biotech Co., Ltd). Polymerase chain reaction (PCR) products were recovered by FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech Co., Ltd) and cloned using pEASY®-Blunt Cloning

Kit (TransGen Biotech). After screening the positive clones, the strains were sequenced by BGI Genomics Co., Ltd.

Subcellular localization

The coding sequence of *GmSUT4.2* without stop codon was cloned into the pBI121 vector with a green fluorescent protein (pBI121-GFP) between the *Xba* I and *Kpn* I to generate the *GmSUT4.2*-GFP plasmid. The vector of the fusion construct was transformed into the *Agrobacterium* strain EHA105, which was injected into five-week-old tobacco plants as described by Yue et al. (2022).The epidermis of leaves was infiltrated with the suspended bacterial solutions, and the resulting images were captured 48 h later using a fluorescence microscope (BX63, Olympus, Tokyo, Japan).

Functional analysis of *GmSUT4.2* in yeast

To verify the sucrose transport activity of the SUT4.2 protein, the full length of *GmSUT4.2* was sub-cloned into the pDR196 vector to produce the plasmid of pDR196-GmSUT4.2, and subsequently transformed it into yeast strain SUSY7/ura3 which is deficient in cell sucrose metabolism. Empty pDR196 vector was used as a negative control. Positive transformants were selected and grown in SD liquid medium (containing 6.7 g L^{-1} yeast nitrogen base without amino acids, 1.29 g L^{-1} SD/-ura base, 2% glucose, and a pH of 5.8) at 30 °C with continuous shaking at 220 rpm until an OD_{600} value of 1.2 was reached. The supernatant was removed by centrifugation at 4 000 rpm for 5 min, and the pellet was resuspended in sterile water to an optical density of 1.0 at OD_{600}. The yeast cell suspension was then diluted 10^1, 10^2, 10^3, and 10^4 fold, and 2 µL of each dilution reaction was added to modified SD agar medium (SD/-ura liquid medium, 20 g L^{-1} agar) supplemented with 2% sucrose or 2% glucose. Yeast growth conditions were observed and recorded after 3 days of incubation at 30 °C in the dark.

Transformation of soybean hairy roots

Hairy roots were generated according to the previously described method with slight modifications (Fan et al. 2020). Briefly, soybean seeds were surface sterilized by treatment with chlorine gas, which was generated by adding 12 N HCl (4 mL) and 5.25% NaClO (50 mL) in an airtight dryer for 14 h. The seeds were cleaned three times with distilled water, and germinated in MS medium for 3 days. The imbibed seeds were used to produce explants in which the cotyledon nodes running parallel to the hypocotyl axis were gently scratched 3 ~ 4 times with a scalpel. The wounded explants were then transferred to *Agrobacterium* co-cultivation medium (CCM) for 30 min at 28 °C with constant shaking at 120 rpm. For the *Agrobacterium* CCM medium, expression of *GmSUT4.2* was mediated by *Agrobacterium rhizogenes* K599 with the exogenous plasmid pCAMBIA1301-*GmSUT4.2*. After infection, 9 explants were placed evenly on each agar CCM coated with filter paper and co-cultured at 24 °C in the dark for 3 days. After co-culture, explants were washed six times with distilled water containing 300 mg L^{-1} cephalosporin. The explants were then transferred to a rooting medium and kept in an incubator at 24 °C on a 16/8 h light/dark cycle for 12 days.

CRISPR/Cas9 expression vector construction and soybean transformation

The online tool CRISPR-GE (http://skl.scau.edu.cn/) was used to predict and design the targets of sequences of the *GmSUT4.2* gene. The single guide RNA (sgRNA) expression cassettes containing target sequences was conducted by overlapping PCR and subsequently built into the pYL-CRISPR/Cas9P_{pubi}-H vector according to the protocol reported by (Ma et al. 2015). The positive plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 for soybean transformation. The soybean cultivar 'Guixia1' was transformed using the *Agrobacterium*-mediated cotyledon node system using established protocols (Zeng et al. 2004). The T0 generation transgenic seedlings were verified by PCR amplification using the specific primer Cas9-F/R and Hpt-F/R. In addition, gene editing status in the confirmed transgenic plants was examined by amplifying and sequencing fragments of the regions spanning the targets using primer Sp-F/R.

GmSUT4.2 overexpression vector construction and transgenic *Arabidopsis* plants acquisition

The One-step Cloning Kit (Vazyme Biotech Co., Ltd) and homologous recombination method was used for the amplification of the full-length *GmSUT4.2* coding region and construction of the vector. The recombinant pCAMBIA1301-*GmSUT4.2* plasmid was transformed into *Agrobacterium tumefaciens* GV3101, which was then used to transfect *Arabidopsis* plants by the floral dip method (Clough 2005). The T1 generation seedlings of the transgenic plants were tested on 1/2 MS media supplemented with 30 mg L^{-1} hygromycin and further verified by PCR and RT-qPCR.

Morphological and biomass parameters measurements

For leaf area measurements, fully expanded leaves at stage V1 (the single leaf is fully extended), R1 (the first flower appeared), and R3 (2 cm pods at the nodes) were photographed in vitro and the area was quantified using ImageJ software (Schroeder et al. 2021). The leaves and stems of

the plants at R3 stage were collected and killed at 105 °C for 30 min before being dried to a constant weight at 85 °C and weighed to determine the dry biomass.

Sugar and starch content determination

Soluble sugar was extracted from 200 mg of fresh leaves using 1 mL of 80% ethanol. The determination of sucrose content was performed using a method modified from Lee et al. (2020). Starch content was determined using the method described by Huang et al. (2020) with some modifications. After sugar extraction, the pellet was dried, suspended with 1 mL of distilled water, and heated at 100 °C for 15 min. The sample was further incubated with 9.2 mol L^{-1} perchloric acid at 100 °C for 15 min. After cooling, the pellet was centrifuged further at 12 000 rpm for 10 min, and the supernatant was used to measure the starch content at 620 nm by the anthranone colorimetric method.

RT-qPCR analysis

The RNA extraction and cDNA transcription were same as mentioned above. Gene expression was quantified with specific primers q-F and q-R. Actin was used as the control. All primers used for assays are shown in Table S2. TransStart Top Green qPCR SuperMix (TransGen Biotech Co., Ltd.,) was used for the RT-qPCR reactions, and the system and operating procedures were performed according to the instructions. The $2^{-\Delta\Delta Ct}$ method (Zhang et al. 2020b) was used to calculate relative transcript levels normalized by actin. The experiments were performed with three biological replicates, each with three plants.

Statistical analysis

Three biological replicates were used for each experiment. All experimental data were analyzed using SPSS version 22.0 and GraphPad 8.2. One-way ANOVA test was used for significance analysis ($*P < 0.05$, $**P < 0.01$).

Results

Molecular cloning and sequence analysis of *GmSUT4.2*

To investigate the function of sucrose transporters in the growth and development of soybean, we cloned the *GmSUT4.2* gene by PCR amplification. The total nucleotide sequence length of the *GmSUT4.2* gene was 1 765 bp, with an open reading frame (ORF) of 1 542 bp (Fig. S1). The encoded protein had a length of 513 amino acid residues and contained 12 transmembrane domains. The predicted molecular weight of the GmSUT4.2 protein was 57 869.35 Da, and the theoretical isoelectric point was 9.43 (Fig. S2a). Conserved domain analysis of the protein revealed the presence of GPH and MFS functional domains in the GmSUT4.2 protein (Fig. S2b).

GmSUT4.2 encodes a plasma membrane protein

The *GmSUT4*-GFP vector of the *GmSUT4.2* gene was constructed and transformed into tobacco leaf subepidermal tissue using *Agrobacterium*-mediated transformation. After 48 h of co-cultivation, the tissues were observed at 480 nm using a fluorescence microscope. GFP signals were visible in the cell membranes of tobacco cells (Fig. S3), but not in other parts of the cells, indicating that GmSUT4.2 is localized in the cell membranes of tobacco cells.

Tissue-specific and exogenous sucrose sensitivity analysis of *GmSUT4.2*

Root, stem, leaf, flower, and seed samples (15, 25, 35, and 45 days after flowering, DAF) of soybean were harvested and used to analyze the tissue-specific expression of *GmSUT4.2* by RT-qPCR. The highest transcript abundance of *GmSUT4.2* was found in roots, followed by seed 35 DAF, and mature leaf, and the lowest expression abundance was found in the stem, seed 15 DAF, and flower (Fig. 1a). Additional experiments to analyze the sucrose-dependent response analysis of sucrose transporter GmSUT4.2 in roots revealed that the expression of *GmSUT4.2* was upregulated by 1% exogenous sucrose and reached a maximum after 24 h (Fig. 1b).

Analysis of sucrose transport activity of GmSUT4.2

To investigate the function of GmSUT4.2 as a sucrose-uptake carrier, we constructed the pDR196-*GmSUT4.2* vector and transformed it into SUSY7/ura3. At a dilution ratio of 10^1 or 10^2, yeast transformed with *GmSUT4.2* gene grew better than yeasts transformed with an empty pDR196 vector on the SD medium containing 2% sucrose, even at a dilution ratio of 10^3 or 10^4, SUSY7/ura3 expressed *GmSUT4.2* could grow normally. However, there was no difference in the growth of the yeast transformed with pDR196-*GmSUT4.2* and pDR196 empty vector at all dilutions on the SD medium containing 2% glucose (Fig. 2). Expression of *GmSUT4.2* in SUSY7/ura3 was able to recover a normal sucrose transportation capacity for yeast, suggesting that *GmSUT4.2* encodes a functional transporter in sucrose transport.

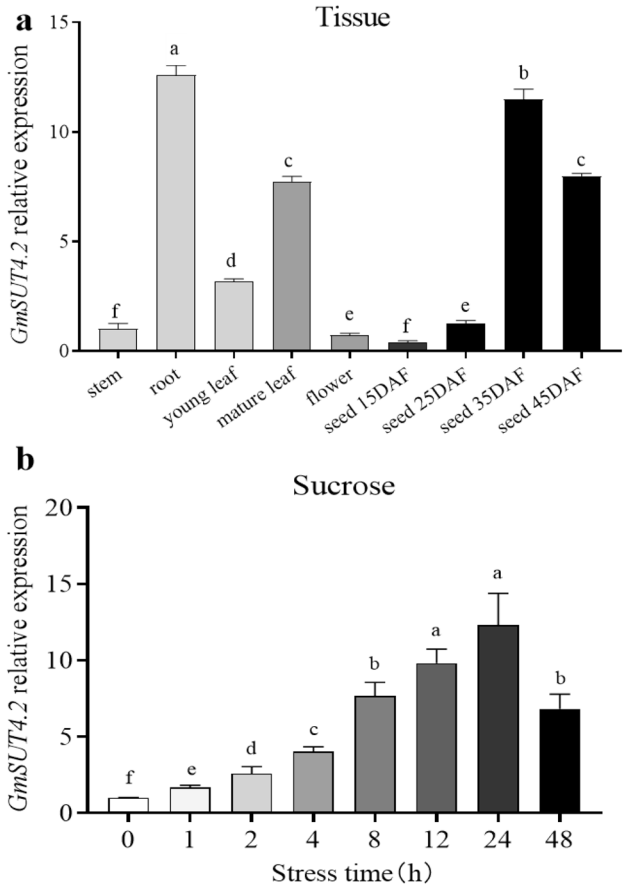

Fig. 1 Analysis of expression patterns of *GmSUT4.2*. **a** The transcript level of *GmSUT4.2* in different soybean tissues (stem, root, young leaf, mature leaf, flower, seed 15DAF, seed 25DAF, seed 35DAF and seed 45DAF) was measured by RT-qPCR (n = 3). **b** Root samples under 1% exogenous sucrose treatment were collected at different time points (0, 1, 2, 4, 8, 12, 24 and 48h) to analyze *GmSUT4.2* expression by RT-qPCR (n = 3). Different letters represent significant differences according to One-way ANOVA ($P < 0.05$)

Overexpression *GmSUT4.2* promotes sucrose uptake in soybean hairy roots

We exposed soybean hairy roots overexpressing *GmSUT4.2* (OE) and expressing empty vector (VC) to sucrose concentrations of 0.1% (MS_0), 1% (MS_1), and 3% (MS_3) in MS medium following confirmation of *GmSUT4.2* expression (Fig. S4). The transgenic roots overexpressing *GmSUT4.2* grew well on MS_1 and MS_3 medium compared with control roots under normal conditions. However, no significant difference was observed in the growth rate between OE and VC grown on MS_0 medium (Fig. 3). Furthermore, the metabolic conversion of exogenous sucrose to soluble sugar and endogenous sucrose was investigated. The results showed that the content of soluble sugar and sucrose in both roots overexpressing the *GmSUT4.2* gene and control roots gradually increased with increasing sucrose concentration.

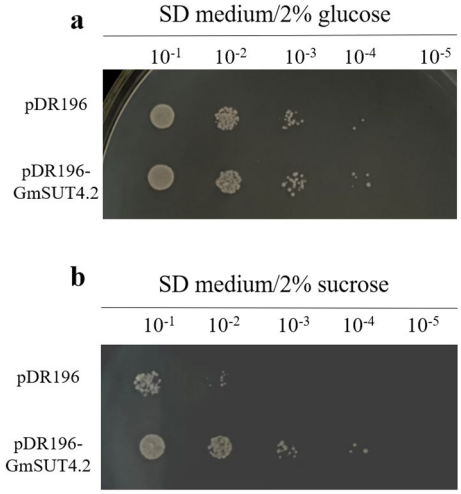

Fig. 2 Function analysis of GmSUT4.2 in SUSY7/ura3. **a** The transformants with pDR196 (up) and pDR196-GmSUT4.2 (down) grew on SD medium supplemented with 2% glucose. **b** Transformants with pDR196 (up) and pDR196-GmSUT4.2 (down) grew on SD medium supplemented with 2% sucrose

Compared with the control roots, *GmSUT4.2* overexpressing roots grown on MS_1 and MS_3 medium showed an increase in soluble sugar content of 78.43% and 155.35%, and sucrose content of 9.85% and 28.08% respectively. Similar content of sucrose (1.22 mg. g^{-1} and 1.25 mg. g^{-1}) and soluble sugar (4.40 mg. g^{-1} and 4.49 mg. g^{-1}) was observed in OE and VC grown on MS_0 medium (Fig. 3). These results suggest that the expression of the sucrose transporter *GmSUT4.2* leads to the rapid uptake of sucrose in overexpressing hairy roots.

Loss of function of *GmSUT4.2* affects leaf size and seed production in soybean

GmSUT4.2 mutations in the EMS-induced mutants *GmSUT4*-M1 and *GmSUT4*-M2 were confirmed by sequencing (Fig.S5). *GmSUT4*-M1 carries an EMS-induced G to A mutation in *GmSUT4.2* at 204 amino acid position in the coding region. *GmSUT4*-M2 carries an EMS-induced C to T mutation in *GmSUT4*.2 at 446 amino acid that introduces a premature stop codon in place of Asparagine. Phenotypic observation of mutant and wild-type plants in the greenhouse showed that non-synonymous mutations of *GmSUT4.2* in soybean significantly inhibited plant growth (Fig. 4). Leaf area measurements showed that the small leaf phenotype of mutant plants appeared at the development stage V1 (Fig. 4c). The defective phenotype gradually becomes evident as the leaf develops and severely affects the growth of the plants in the R stages. Moreover, we characterized agronomic traits of *GmSUT4*-M1, *GmSUT4*-M2, and WT in the greenhouse (Fig. 4d-f). Growth of the mutants was poor compared with wild-type soybean, with fewer

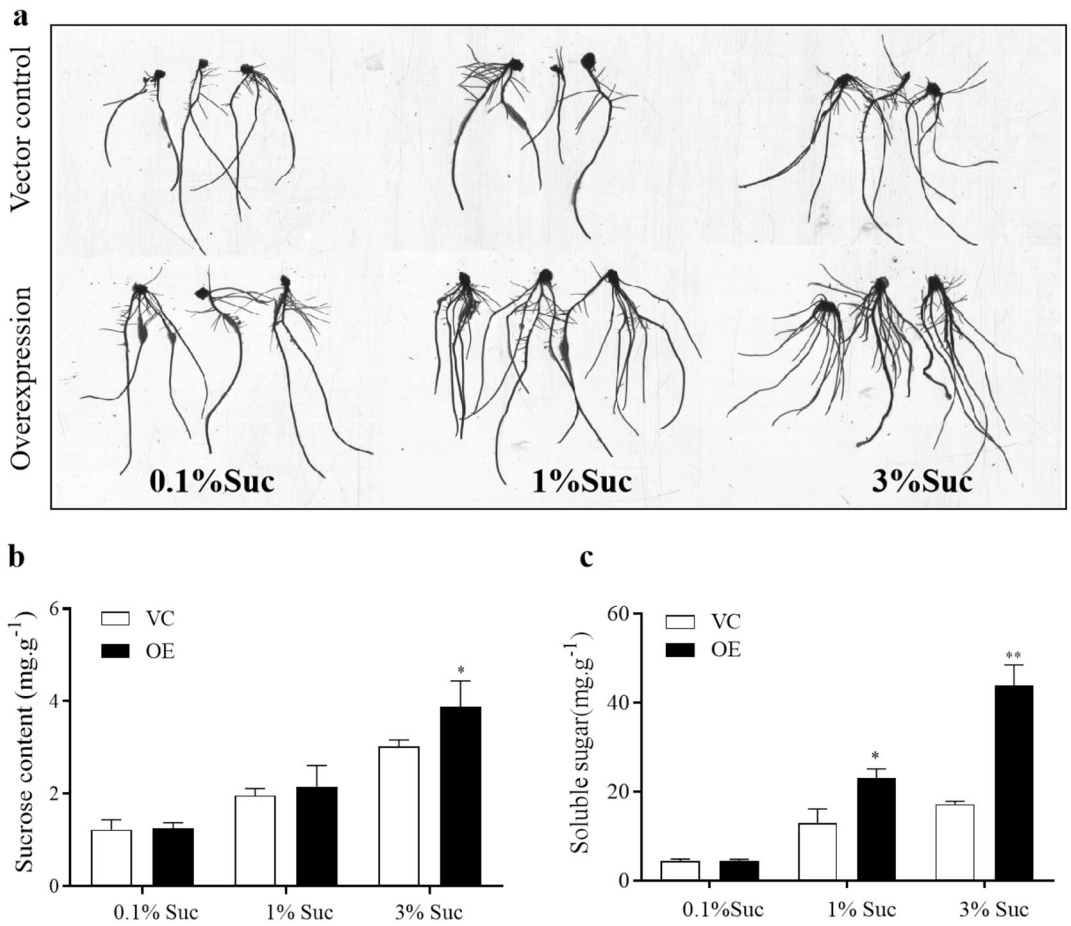

Fig. 3 The sucrose uptake assays in soybean hairy root. **a** Morphology of transgenic hairy roots cultivated in MS medium containing 0.1%, 1% or 3% sucrose content. **b** Sucrose content; **c** Soluble sugar content. Data are expressed as mean ± SE (n = 3). *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

pods, seeds, and lower aerial biomass. Accordingly, seed weight per plant was significantly lower in the mutants than in the wild-type plants.

To confirm that the mutant phenotypes (*GmSUT4*-M1 and *GmSUT4*-M2) were directly caused due to the mutation of *GmSUT4.2* and independent of other gene(s) mutations in NJAU0264 and NJAU1191, we constructed a CRISPR/Cas9-*GmSUT4.2* vector containing two target adaptors, which were chosen for mutagenesis of this gene in soybean using the CRISPR/Cas9 technology (Fig. 5). The *Arabidopsis* U3b, and U6-1 promoters were used to drive the individual expression of the 2 sgRNA expression cassettes containing the designed target sites. We transformed this vector into *Agrobacterium tumefaciens* strain EHA105 to produce soybean transgenic plants. After screening the progeny by PCR and sanger sequencing, we obtained a Cas9-null homozygous mutants with frameshifts in the coding region of Cas-*GmSUT4* (1-bp deletion) (Fig. 5a). Consistent with the *GmSUT4*-M1 and *GmSUT4*-M2, the plant growth and seed weight were decreased in mutant Cas-*GmSUT4* (Fig. 5b-g). Compared to the wild type, seed weight per plant of T1 generation of Cas-*GmSUT4* plants was decreased by 31.25%. Therefore, the combined evidence points to the importance of *GmSUT4.2* in regulating plant growth, development, and agronomic yield of soybean plants.

GmSUT4.2 mutants have decreased sugar content in leaves

The function of sucrose transport of GmSUT4.2 makes us to hypothesize that differences in sugar distribution in mutants and wild types could account for the plant growth differences. To investigate this possibility, the sugar content of mature adult source leaves in both mutants and wild-type was examined (Fig. 6a). The contents of primary metabolites at the R3 stage showed a decrease of 57.63%, 43.91%, and 36.56% in sucrose content and 60.19%, 51.18%, and 29.97% in total soluble sugar content in the lines of *GmSUT4*-M1, *GmSUT4*-M2, and Cas-*GmSUT4*, respectively, which were significantly lower than those of wild-type plants (Williams

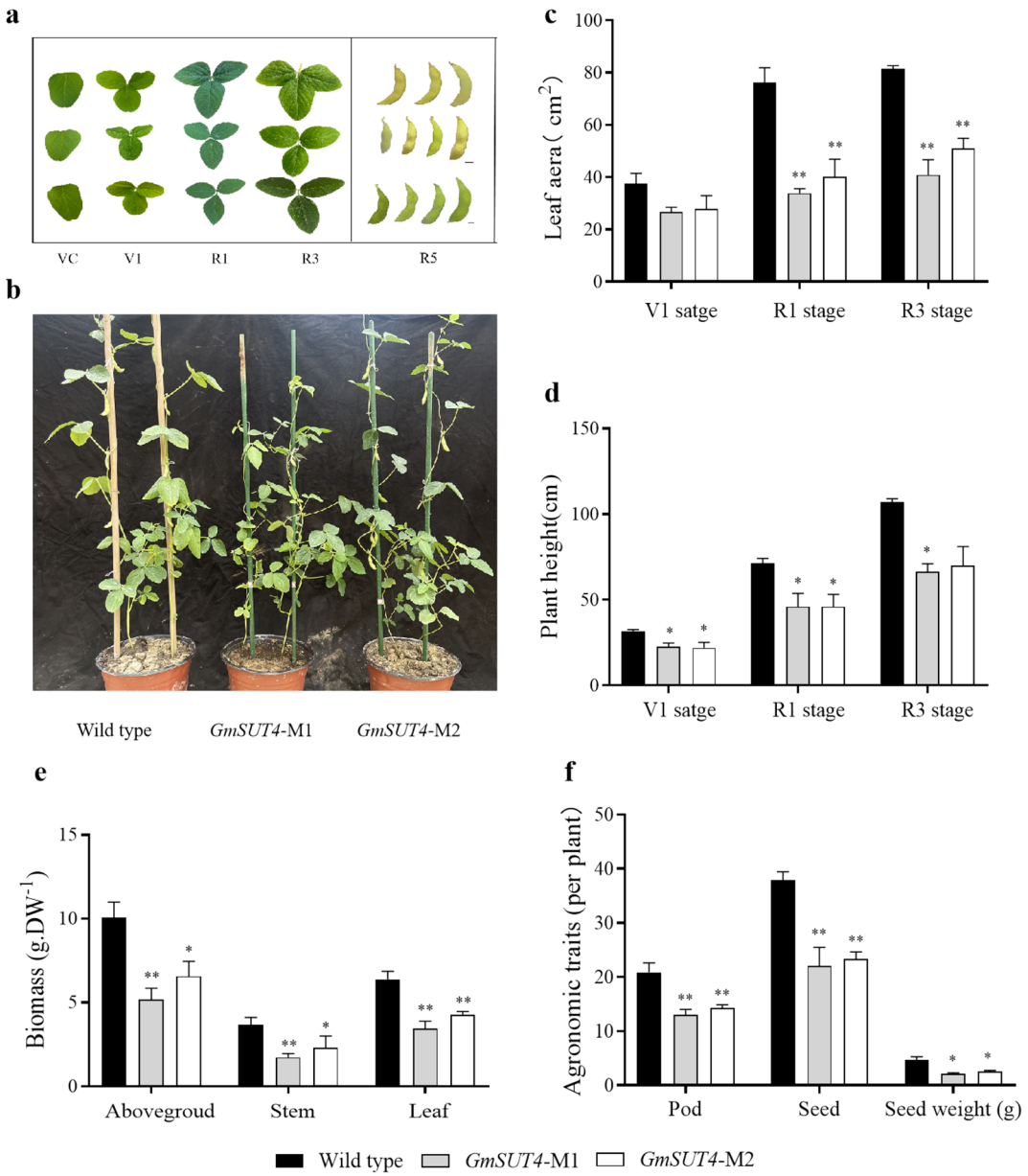

Fig. 4 Organs phenotype and measurement of agronomic traits of EMS-induced soybean mutants. **a** The organs phenotype of *GmSUT4*-M1 and *GmSUT4*-M2 at different stages. **b** Phenotype of soybean plants at R3 stage. **c, d** Leaf area and plant height of WT and mutants at stage V1, R1 and R3 (n = 3). **e** Biomass of mutants and WT (n = 4). **f** Agronomic traits of mutant *GmSUT4*-M1 and *GmSUT4*-M2 (n = 6). *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

82, Guixia1). Similarly, the starch content of the mutant plants was also decreased compared with the wild type. Furthermore, RT-qPCR analysis revealed the differences in the expression levels of genes involved in sugar transport (*GmSUT2*, *GmSWEET6*, and *GmSWEET11*) and sucrose metabolism (*GmSPP2*, and *GmCInv1*), and sucrose signaling (*GmSnRK1*) in the mutants and wild type. The results showed that the expression of *GmSPP2*, *GmCInv1,* and *GmSnRK1* was significantly down-regulated in the mutants compared with the wild type, but the expression of three sucrose transportation genes was up-regulated in *GmSUT4*-M1 and *GmSUT4*-M2 (Fig. 6b).

GmSUT4.2 regulates the development of rosette leaves, branches, and yield in *Arabidopsis*

To further elucidate the functional role of GmSUT4.2 in sucrose transport, the homozygous T3 generation of *Arabidopsis* lines overexpressing the *GmSUT4.2* gene was generated. The transgenic plants with hygromycin resistance

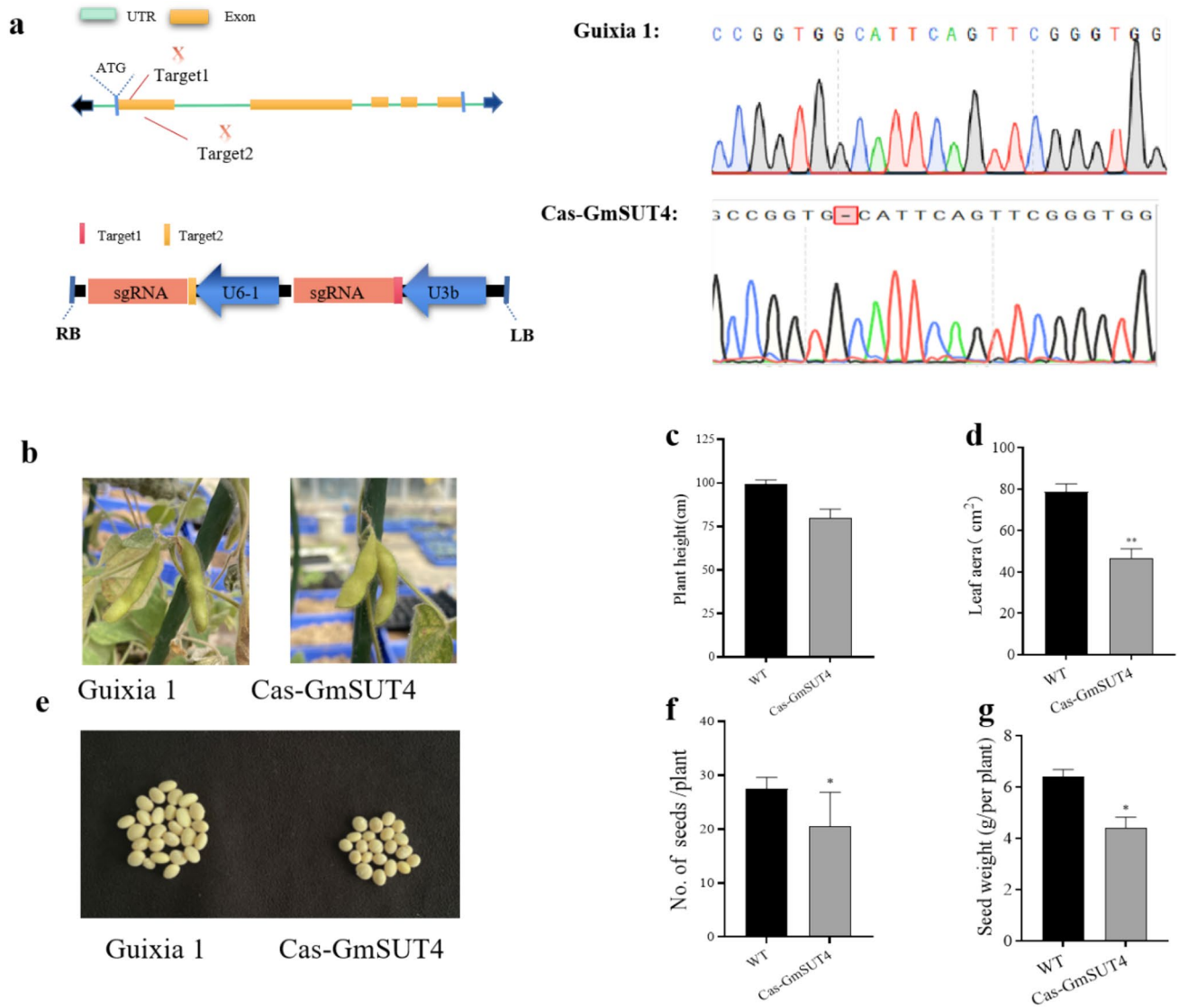

Fig. 5 Phenotypic identification and agronomic trait analysis of *GmSUT4.2* mutant mediated by CRISPR/Cas9. **a** Targets location, diagram of the dual vector gRNA CRISPR/Cas9 and PCR-based identification of T_0 transgenic plants; **b** Pods phenotype; **c** Plant height (n = 6); **d** Leaf size (n = 6); **e** Seeds phenotype; **f, g** Seeds number and seeds weight (n = 2) *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

were further verified by PCR and RT-qPCR. Fragments of the required length could be amplified in the DNA genome of the transgenic plants, but not in the wild types (Fig. S7). Real-time fluorescence quantification results showed that transcription of *GmSUT4.2* was significantly up-regulated in transgenic *Arabidopsis* line OE1, OE2 and OE4 (Fig. S8), and then we used these three lines for further experiments.

Phenotypic observation showed delayed leaf expansion in the transgenic plants compared with the wild type (Fig. 7a). Compared with the fully expanded rosette leaves of the wild type, the leaves of the transgenic plants were contracted in the middle and formed a central bundle, resulting in numerous rosette leaves (Fig. 7b). Transgenic line OE1, OE2 and OE4 had 25, 19, and 21 rosette leaves respectively, with an average of 22 leaves, which was significantly more than the number in WT (average of 13.5 rosette leaves). The number of rosette branches (average of 2.5 RI branches) was also higher in the transgenic plants than in the wild type (average of 1.5 RI branches) (Fig. 7c, d). Furthermore, we also evaluated the seed development of the transgenic lines. Generally, the number of pods per plant was significantly higher (38.44%) in the transgenic *Arabidopsis* than in the wild type. Consequently, the seed yield of the transgenic plants per plant was 40.07% higher than that of wild-type plants. However, we found no

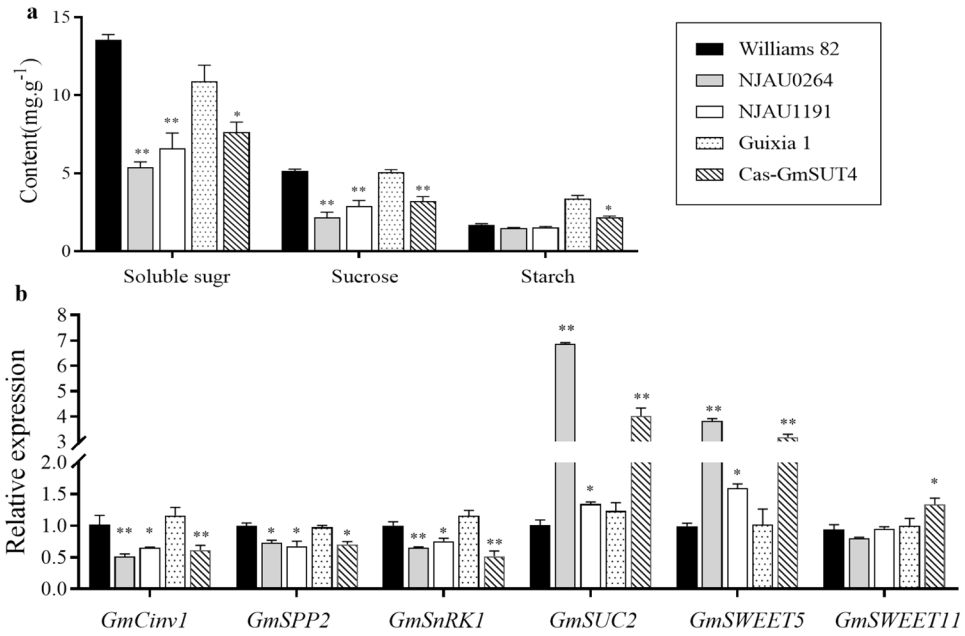

Fig. 6 Determination of sugar and starch content in *GmSUT4.2* mutants (n = 3) and the relative expression of related-genes of sucrose metabolism. *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

significant difference in 100-seed weight in the transgenic plants compared with the wild type (Fig. 7e, f).

Sugar metabolism level in transgenic *Arabidopsis* plants

The measurement of the sucrose content of rosette leaves on day 30 (at the time of axillary bud formation) was conducted. In transgenic lines, the leaves had a higher accumulation of sucrose than the wild-type plants (Fig. 8a). Additionally, we measured photosynthetic activity and the transcript levels of genes related to sucrose metabolism and transport in line OE1 and OE2. The Photosynthetic rate and the expression of *AtSPP2, AtCinv1,* and *AtSnRK1* were enhanced in transgenic lines compared with wild types (Fig. 8b, c). Further research found that transcript levels of sucrose efflux genes, such as *AtSWEET11, AtSWEET12,* and *AtSWEET13*, were also markedly up-regulated in transgenic plants compared with WT (Fig. 8d).

Discussion

GmSUT4.2 is a functional protein localized to the plasma membrane

Subcellular localization is pivotal for the function of transport proteins (Schulz et al. 2011). Here, we observed that the fluorescence signal of GmSUT4-GFP was clearly present in the cell membranes of tobacco cells (Fig.S3). Previously reported that the results for subcellular localization of sucrose transporter genes belonging to the SUT4 subfamily have been mixed in different plants and even in the same plant species, with most of the members being reported to possess at least two different compartments (Schulz et al. 2011). For GeSUT4 from *Gastrodia elata*, it was identified as a plasma membrane protein in functional characterization of yeast, but GFP fusion showed that fluorescence was present in the plasma membrane and the tonoplast(Ho et al. 2021). Furthermore, a variety of SUT4 subfamily members, such as *Arabidopsis* AtSUT4 (Endler et al. 2006; Weise et al. 2000), *Nicotiana tabacum* NiSUT4 (Okubo-Kurihara et al. 2011), barley HvSUT2 (Endler et al. 2006), potato StSUT4 (Chincinska et al. 2013) and lotus LjSUT4 (Reinders et al. 2008), have been shown to reside in the vacuolar membrane and plasma membrane. The expression of *GmSUT4.2* in SUSY7/ura3 yeast restored the sucrose-uptake ability which requires at least the localization of the protein to the plasma membrane (Fig. 2b). This result provided additional evidence that GmSUT4.2 functioned as a transporter in the plasma membrane.

Generally, SUTs transport sucrose; however, AtSUT5 also mediates the transport of biotin (Ludwig et al. 2000), AtSUT9 and LjSUT4 transport a variety of glucosides (Reinders et al. 2008), but not sucrose (AtSUT9) (Sivitz et al. 2007). In the current study, *GmSUT4.2* was transferred into sucrose uptake-deficient yeast mutant SUSY7/ura3 and partially restored sucrose uptake in yeast implying that GmSUT4.2 to be a functional protein with sucrose transport activity (Fig. 2b).

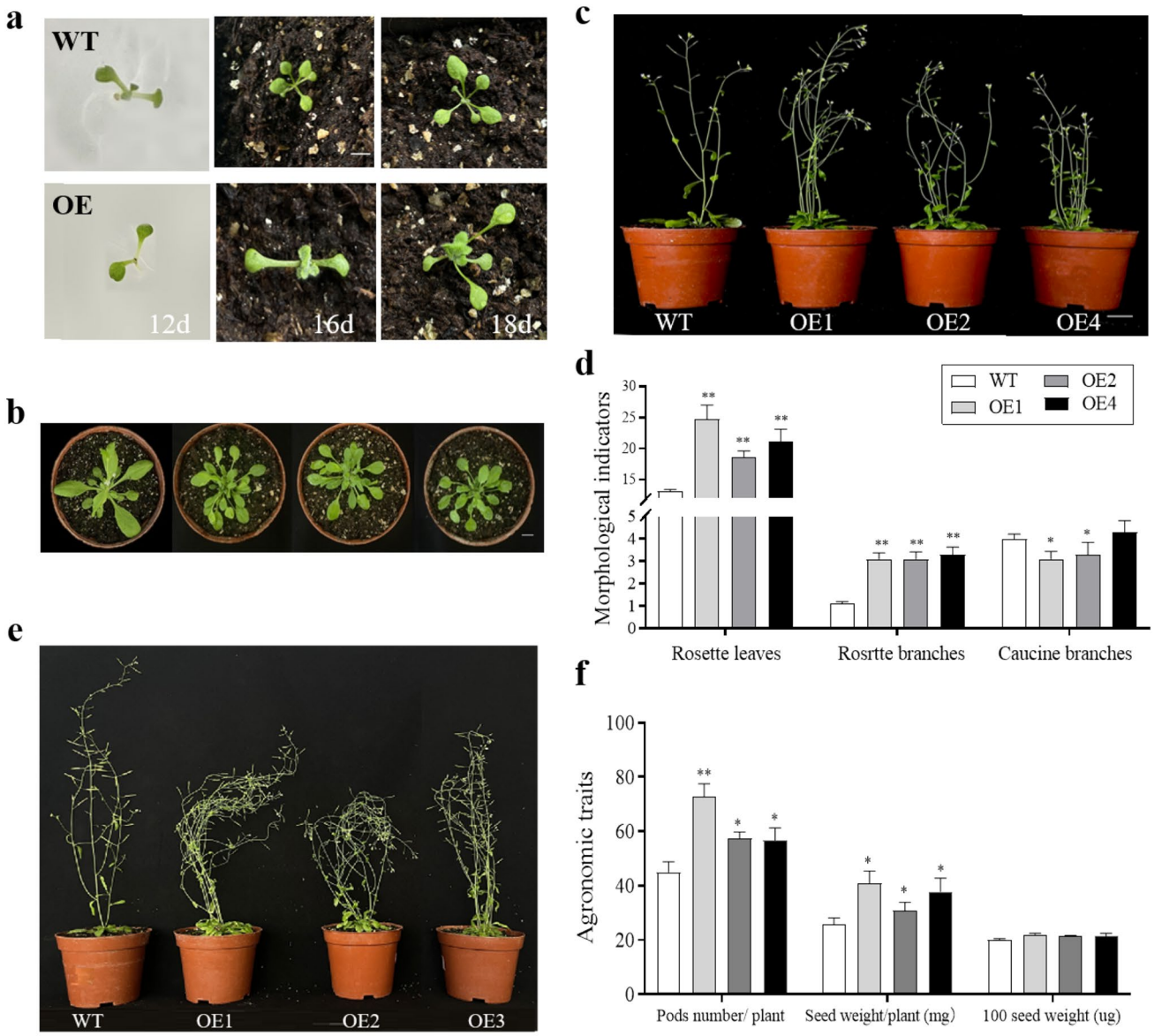

Fig. 7 Morphological analysis of the transgenic and wild-type plants. **a** Phenotype of the 12, 16 and 18 days seedings of WT and transgenic *Arabidopsis* plants. **b**, **c** Phenotype of the rosette leaves and branches of the transgenic and wild-type plants. **d** Wild-type plants and transgenic lines were grown under 16-h photoperiods. Measurement of morphological indicators including rosette leaves, rosette branches and cauline branches (n = 12). **e** Comparison of silique development between 6-week-old wild type and transgenic plants. **f** Agronomic traits (pods number per plant, seeds weight per plant and 100-seed weight) were quantified from transgenic ($n = 36$) and WT ($n = 12$) plants. *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

GmSUT4.2 function is essential for the normal growth of soybean

Mutants play an important role in identifying gene function (Greene et al. 2003; Kuhn and Grof 2010). In our study, we observed that loss function of *GmSUT4.2* display severe growth defects such as reduced leaf area, plant height, and seed yield compared with the wild type (Fig. 4), similar to previous observations for the mutants of SUT4 clade members (*ZmSUT2*, *StSUT4* and *OsSUT2*) in other species (Chincinska et al. 2008; Eom et al. 2011; Leach et al. 2017). Thus, these results suggested that *GmSUT4.2* function is necessary for normal plant growth in soybean. In parallel, mutation of *GmSUT4.2* resulted in decreased sugar capacity in leaf and led to a lower biomass compared with wild-type plants (Fig. 6a). In previous studies, sucrose loading function of SUT4 members in leaves has been thoroughly studied in maize and rice (Eom et al. 2011; Leach et al.

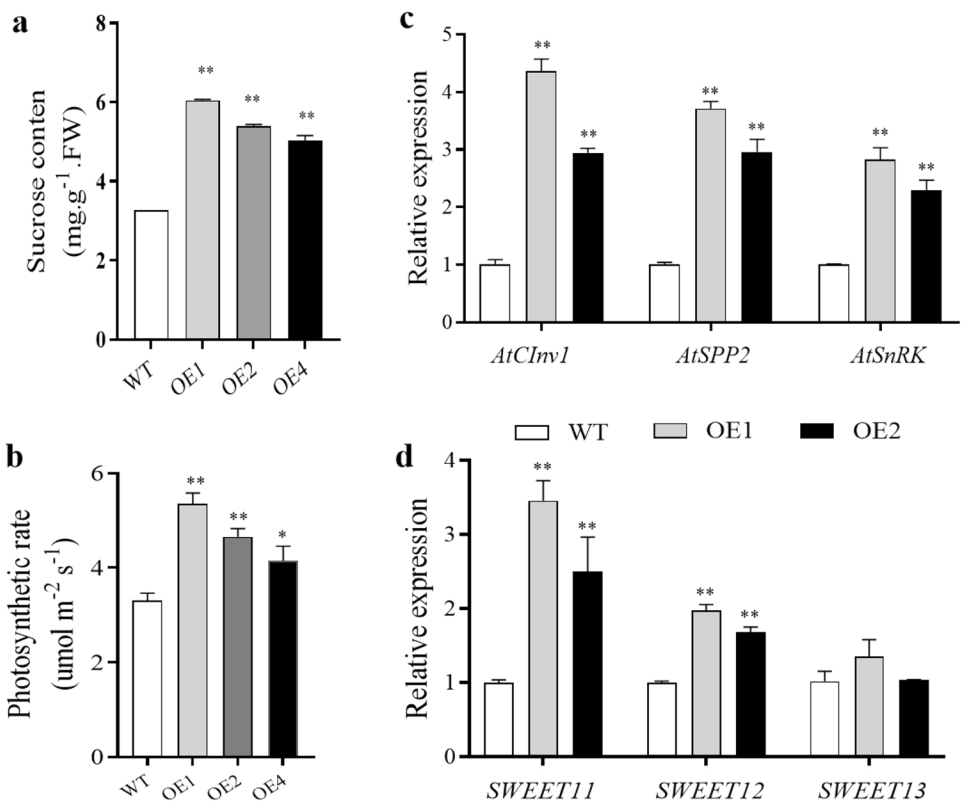

Fig. 8 Physiological and molecular feedbacks in transgenic plants and WT. **a, b** Determination of sucrose content and photosynthetic activity in plants. **c, d** The transcriptional levels of genes related to sugar metabolism in wild-type and transgenic plants (n = 3). *, $P < 0.05$, **, $P < 0.001$, according to One-way ANOVA

2017). Limitations in sucrose export promote accumulation of sugars in leaves, thereby inhibiting leaf photosynthesis. In recent years, increasing evidence shows that SUT4 members function as sugar transporter and contribute to sugar accumulation (Zheng et al. 2014; Zhang et al. 2018; Peng et al. 2020). In addition, several SUT4 clade members, including pear *PbSUT2* (Wang et al. 2016), rice *OsSUT2* (Zhang et al. 2020a) and *Populus PtaSUT4*(Frost et al. 2012), were found to be potentially associated with the photosynthetic rate. Our results support the hypothesis that *GmSUT4.2* functions as a source leaf sucrose phloem loader and is involved in leaf photosynthesis, and additional research is necessary to test this hypothesis.

Manipulation of the source-sink interaction determines the seed formation. In this study, the expression of the sucrose transporter gene *GmSUC2* and *GmSWEET6* is upregulated in the mutants (Fig. 6b). These results suggested that sucrose export is not blocked from the source leaf. There is little evidence that SUT4 orthologs somehow mediate sucrose export from source leaves to sink organs. The growth is severely reduced in null mutants of SUT4 members in rice and maize, while the sucrose export and long-distance translocation are normal (Julius et al. 2017). An explanation is that the loss of function of sucrose transporters and the imbalance of sugar accumulation in plants may induce compensatory expression of other sugar supply or transformation genes.

GmSUT4.2 regulates *Arabidopsis* growth in a sucrose-dependent manner

Overexpression of *GmSUT4.2* strongly affects leaf development in the transgenic *Arabidopsis* plants, resulting in cluster growth in the middle of the leaves and eventually more rosette leaves (Fig. 7a, b). Leaf growth and development are primarily driven by cell proliferation and expansion (Lee et al. 2022). Recent studies have reported sugar availability is closely to apical meristem cell proliferation, affecting overall growth responses and developmental transitions in short-lived plants such as *Arabidopsis* (Zhang et al. 2019). Determination of sugar content showed that the overexpressing lines accumulated more sucrose in leaves compared with the wild type (Fig. 8a). This result is consistent with those observed in the transgenic tobacco overexpression of *RuSUT2* (Yan et al. 2021). This also confirms recent work in peas and other crops demonstrating that the expression of sucrose transporters in plants had higher photosynthesis capacity than control plants, increased sugar accumulation, and significantly improved the storage of sinks (Ding et al. 2019; Lu et al. 2020; Wang et al. 2016). The intrinsic mechanism by which sucrose transporters affect photosynthetic activity in plants is unclear. Recent studies suggest that sucrose transport depends on transporter activity in leaves, which in turn regulates the transport load of the phloem and ultimately controls photosynthesis (Bush 2020).

Moreover, ectopic expression of *GmSUT4.2* promoted the buds to be released from a dormant state, resulting in increased rosette branches, and finally has a strong additive effect on the yield of *Arabidopsis* (Fig. 7c, f; Fig. 8a, b). Given the established function of sucrose as a signal for the availability of plant bud branches at the top advantage (Barbier et al. 2015, 2019; Fichtner et al. 2017; Martin-Fontecha et al. 2018). At our consideration, whether sucrose sensing and signaling functions or transport ability was altered by the expression of *GmSUT4.2* in *Arabidopsis thaliana*. T6P is a sucrose signaling metabolite and acts as a negative feedback regulator of sucrose levels (Yadav et al. 2014; Figueroa and Lunn 2016). SnRK1 is a sugar signaling node and appears to be repressed by high-energy signals such as trehalose-6-P (T6P) (Crepin and Rolland 2019), and CINV1 activity is an important checkpoint for coordinating sucrose catabolism plant growth and development mediated by sugar signaling and metabolism (Meng et al. 2021). As a result, RT-qPCR analysis revealed that the expression of *SnRK1* and CINV1 was higher in transgenic plants than in wild-type plants (Fig. 8c). We assumed that the development of the plant growth and development was influenced by the interaction of SnRK1 and *GmSUT4.2*-mediatied sucrose signaling and metabolism because the final development of organs is primarily under the control of genetic programs. These findings call for further research to verify this possibility.

Conclusion

In this study, a soybean *GmSUT4.2* gene was isolated and functionally characterized. Subcellular localization revealed that GmSUT4.2 localized to the plasma membrane, and the heterologous expression of *GmSUT4.2* enabled SUSY7/ura3 yeast cells to grow on a sucrose medium. Moreover, loss function of *GmSUT4.2* in soybean inhibits plant growth, resulting in a decrease in biomass and seed yield under greenhouse conditions. While *GmSUT4.2* overexpression in *Arabidopsis* showed the opposite trend, with more rosette leaves, branches, and higher seed yield compared to the wild-type plants. Thus, our findings provide evidence that the *GmSUT4.2* gene is involved in the regulation of plant growth and development as well as seed yield. *GmSUT4.2* could be used as a candidate gene for future soybean seed yield and quality genetic improvements.

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Author contributions PC, conceived and designed the research. XW performed the experiments and analyzed the data**.** XW wrote the manuscript and JY, SM revised the manuscript. DJL, SC, CCW, QQW, HZ, CNC, MW, JZN participated in experiments and data collection. All authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study were included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors declare no known competing financial interests.

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