Arabidopsis Sucrose Transporter 4 (AtSUC4) is Involved in Root Response of High Content Sucrose-Growth Via IAA- and ABA- Dependent Manner

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Abstract

Source-to-sink transport of sucrose mediated by sucrose transporters (SUCs) is one of the major determinants of plant growth. However, the role of AtSUC4, the only member of Group IV sucrose transporter in *Arabidopsis*, was undervalued in sink organ during seedling period. In our study, the primary root length of the *atsuc4* mutants was significantly longer than that of the wild-type (WT) under exogenous 4% and 6% sucrose treatment. But this phenotype could not be imitated by external application of glucose or mannitol. It means that the *atsuc4* mutants were insensitive to high sucrose stress compared with WT. Meanwhile, HPLC-MS/MS results showed that the root of *atsuc4* mutants accumulated less sucrose and ABA and more IAA content compared with WT on 4% and 6% sucrose supplementation. Transcriptome analysis showed that many key genes involved in IAA and ABA signals were respective stimulated and repressed in the *atsuc4* mutants, respectively. Taken together, we concluded that the deficiency of *AtSUC4*, not only reducing the transported and uptaked of sucrose, but also as a signal, may be collaborated with IAA and ABA to regulate root growth under high sucrose stress. This study confirmed the new function of AtSUC4, and provided an promising candidate gene for improving tolerance to high sucrose stress.

Introduction

In most plants, sucrose plays an important role in stress tolerance, serving as an osmolyte, a signaling molecule and/or a nutritional substance [1]. Most sucrose is derived from photosynthetic source organ leaves, and transported into sink organs, including flowers, stems, roots and seeds. This way of sucrose exchange requires transport proteins to mediate sucrose uptake or release from cells or subcellular compartments. Thus, not surprisingly, sucrose transporters (SUCs or SUTs) are critical for plant growth and development [2]. Sucrose synthesized in the source cells is exported to the apoplasm, probably by the SWEET efflux proteins, then imported into the sieve elements and companion cells complexes (SE-CCCs) via sucrose transporters (SUT or SUC) in most plants [3]. SUCs are H⁺/sucrose symporters that utilize the proton motive force (PMF) present across the plasma membrane (PM) of the SE-CCCs to load sucrose against its concentration gradient into the phloem [4]. Multiple SUT genes have been identified and initially falled into three subgroups (SUT1, SUT2 and SUT4 subfamily) but later into four distinct groups (Group I, II, III and IV) according to phylogenetic analysis [5, 6]. Among them, most members of the Group II are high-affinity/low-capacity sucrose transporters with Km values ranging between 139 μ M and 1.5 mM and described as plasma membrane-localized transporters, whereas members of the Group IV (with the exception of OsSUT2 from rice) are low-affinity/high-capacity sucrose transporters with Km values ranging from 5 mM to 6 mM and suggested as the tonoplast-localized transporters [7]. There are 9 SUCs in *Arabidopsis* (*Arabidopsis thaliana*), among which 7 members (AtSUC1, 2, 5–9) belong to Group II, but only AtSUC1 and AtSUC2 were well studied due to their high expression levels [4]. AtSUC1 could control dehiscence of anthers and pollen tube growth and AtSUC2 was responsible for phloem loading [8, 9]. SUTs in group IV showed distinct functions in different plant species indicating a versatile role of the members in this group involving plant growth and development [7, 10]. AtSUC4, the only member of

Group IV sucrose transporter in *Arabidopsis*, can also catalyze the transport of sucrose from the vacuolar lumen into the cytoplasm in the cells [10]. Due to the low expression level of AtSUC4, the aerial part of *atsuc4.1* (cs856419) mutant behaved essentially as the WT in sucrose content, germination, development and response to cold treatment when cotyledons were fully unfolded. The *AtSUC4*-overexpressing lines showed a 30% reduction in sucrose content in aerial parts compared with WT under 3% sucrose treatment [11]. However, little is known about the role of AtSUC4 in the root, a typical sink organ that relies exclusively on an source leaves and external import of sucrose, especially in the high sucrose surrounding.

In *Arabidopsis*, primary root elongation rate was basically dependent on sugar concentration in the medium, and a good correlation between the elongation rate and sugar content in primary roots was found [12]. In addition, sugars transported into the root also transmit as signals. In young developing seedling root of *Arabidopsis*, Kircher and Schopfer demonstrated that sucrose transported into root acts as a necessary and sufficient signal regulating root elongation together with light [13]. The expression patterns of *AtSUC1* and *AtSUC2* displayed contrasting roles during the night, showing increased transcript accumulation of *AtSUC2* (sucrose loading in phloem) in leaves and *AtSUC1* (sucrose unloading) in roots [14, 15]. However, the function of *AtSUC4* in root development was uncertain, and a clear linking on the interaction between sucrose transport and root growth remains to be elucidated.

In addition to being directly regulated by sucrose, root development also results from the concerted action of multiple plant hormones. Among these, auxin stands out as a key instructive signal on which many other plant hormones converge to regulate root development [16]. Moreover, exogenous sucrose could promote the root growth through sugar hormone crosstalk in *Arabidopsis* and peach [17, 18]. Further, it was found that exogenous sucrose increased the expression of auxin synthesis- and transport-related genes in roots, resulting in auxin accumulation in the root system [18]. Besides, ABA signaling also plays a critical role in regulating root growth and root system architecture [19]. Yu et al revealed that the *OsERF2* was required for the root architecture and ABA-response by regulating the expression of some genes involved in sugar metabolism and hormone signaling pathways [20]. Additionally, Yuan et al reported that excessive exogenous glucose inhibits primary root growth via *ABI5*, which represses PIN1 accumulation and auxin activity in *Arabidopsis* [21]. To date, however, the molecular mechanism of crosstalk among sucrose transporters, IAA and ABA regulating root growth under stress conditions remains unclear.

To gain more information about sucrose transport regulation in root with emphasis on AtSUC4, expression patterns and subcellular localization of AtSUC4 were performed firstly. *atsuc4* mutant lines were generated and showed longer root length under high exogenous sucrose stress. Comparison of sugar and hormone (IAA and ABA) content in roots between WT and mutant lines were analyzed. Transcriptome were carried out, and indicated that expression of several sucrose transporters, IAA- and ABA-related genes were changed in mutant lines. This study confirmed the new function of AtSUC4, and provided insights into the regulation of AtSUC4 in root growth under high sucrose stress.

Materials And Methods

Strains materials and growth conditions

Arabidopsis thaliana (ecotype Col-0) and *atsuc4* mutants were grown in growth chamber in soil under long-day (LD) conditions (16 h light / 8 h dark) at 22 °C and 70% relative humidity, light was present at 100-120 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$. Before germination, plates were incubated at 4 °C for 2 d in the dark and subsequently transferred to the growth chamber. *Escherichia coli* strain DH5 α was used for all cloning steps. Transformation of *Arabidopsis* was performed using *Agrobacterium tumefaciens* strain GV3101 [22].

Generation of *Arabidopsisatsuc4* mutant lines

Three types of *atsuc4* mutant lines were used in this study. The *atsuc4* mutant (cs93419) was obtained from the Nottingham Stock Centre (<http://arabidopsis.info/>), and screened the *AtSUC4* point mutation homozygous with no *ERECTA* gene mutation, which was named as *suc4-1*. To construct *AtSUC4* single gene knockout vectors contained two target sites (TCACAGAGTCACTCGCAACC and CTCGTTGGGCATAGTAGCGA), we adopted the CRISPR/Cas9 system created by Ma et al [23]. The homozygous transformed plants with vector-free were generated, which was called *suc4-2*. The *AtSUC4* T-DNA insertion line named *suc4-3* (WiscDsLox450E10) was obtained from the Nottingham Stock Centre (<http://arabidopsis.info/>). The position of the T-DNA insertion was determined by sequencing a PCR product obtained from *suc4-3* genomic DNA [11]. For the *AtSUC4* complement-expressors (marked as *AtSUC4/ suc4-1*), the *AtSUC4* promoter and gDNA sequence fragments were amplified from the total DNA of leaves, and inserted into the BamH I- and EcoR I-digested plant transformation vector pCambia1300 [24] by ClonExpress MultiS One Step Cloning Kit (Vazyme). The resulting plasmid pCambia1300-pSUC4::gSUC4-GFP was used for *suc4-1* mutant transformation. Primer pairs for vector construction and identification of *atsuc4* mutants were shown in Data S1.

Subcellular localization assay of AtSUC4

Subcellular localization of AtSUC4 was detected by four methods. For the method of *Arabidopsis* stable transformation system, the promoter (998 bp upstream of ATG) and CDS sequence were amplified, and cloned into pCambia1301 vector with Citrine fluorescent protein coding sequence, which was kindly provided by Dr. Erik Nilesen (University of Michigan). As a result, the construct pCambia1301-pSUC4::cSUC4-Citrine was generated, and subsequently transformed into WT plants. Images were obtained using the roots of transgenic homozygous lines. The second method of transient expression system of *Nicotiana benthamiana* leaves was used. In this method, the plasma membrane marker tag AtCERK1-DsRed [25] and tonoplast marker tag AtCBL6-OFP plasmids [26] were used as positive controls, which were provided by Dr. Xian'an Xie (South China Agricultural University). GV3101 were transformed with plasmid pCambia1300-pSUC4::gSUC4-GFP described above and each positive marker respectively, followed by co-infiltration into *Nicotiana benthamiana* leaves with a final OD₆₀₀=0.5-0.7. Plants were incubated for 36-48 h before imaging. We used the transient expression system of *Arabidopsis* protoplast

as the third method to check the subcellular localization of AtSUC4. Protoplast isolation and transformation of rosette leaf were performed according to Robert et al [27]. The vector pEarley101-35S::cAtSUC4-GFP was generated and used for protoplast transient transformation. All the fluorescence were detected using a Leica TCS SP5 confocal microscope. The excitation wavelengths used to detect fluorescences were 488 nm for GFP, 512 nm for Citrine, and 543 nm for DsRed and OFP, while the emitted fluorescences were collected from 500 to 550 nm for GFP, 519 nm to 650 nm for Citrine and 565 to 615 nm for DsRed and OFP. The fourth method was immunegold labeling of AtSUC4 which essentially performed as described previously [28] using the mouse anti-AtSUC4 monoclonal antibody (Xi Qin, unpublished).

Verification of expression pattern of AtSUC4 at tissue level

To verify expression pattern of AtSUC4 during *Arabidopsis* development at tissue level, we created the pAtSUC4::GUS transgenic plants under the control of *AtSUC4* promoter. A 998-bp fragment before coding region of *AtSUC4* was amplified from total DNA of leaf, subcloned into the entry vector pDONR207 and inserted upstream of the GUS into destination vector pHGWFS.7 [29], yielding construct pAtSUC4::GUS, which was used for *Agrobacterium tumefaciens* transformation. Primer pairs used were shown in Data S1. Samples including the whole seedlings (grown for 4 d, 14 d and 25 d period), flowers (grown for 35 d period), siliques and seeds (grown for 50 d period) were stained according to standard protocols [11]. For each sample, we analyzed 10 to 15 independent lines, and representative results were presented. Photograph were captured by stereomicroscope (Leica MZFL III; Leica Microsystems, Bensheim, Germany).

Genes expression level detection

For gene expression analysis, total RNA was isolated from roots of different plants using the Plant RNA Extraction Kit (Omega, Norcross, GA, USA), and cDNA was synthesized using HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme). Reverse transcription-quantitative real time PCR (RT-qPCR) was conducted using FastStart DNA Master SYBR Green I (Roche) on the Roche LightCycler 480 System (Roche Applied Science), with *ACTIN2* as internal control [30]. Three independent biological replicates and four technical replicates were performed. The primer sequences were listed in Data S1.

Measurement of root length and seed germination rate between WT and mutant lines

For sugars tolerance, the seeds of WT and *atsuc4* mutant lines were sown on MS solid media containing different concentrations of sucrose, glucose and mannitol after sterilizing the seeds (0%, 2%, 4% and 6%). Bright-field images of different plants were captured at 6 d after sowing using Canon 60 D camera. Root length was measured by Image J software. For the phenotype comparison between WT and mutants, plants were grown in soil under LD condition. The number of rosette leaves was recorded at 25 d, and the plant height was measured at 50 d. Three biological replicate and more than 30 plants for each replicates were used for analysis. For seed germination rate, the WT and mutant seeds were sown on MS solid media containing different concentrations of sucrose (0%, 2%, 4% and 6%) and seed germination were

calculated after 24 h, 48 h, 60 h and 72 h. Each treatment was performed with three biological replicates, at least comprising 100 seeds in each experiment.

Analysis of carbohydrate content

Preparation of plant materials and determination of carbohydrate content via ion chromatography was performed as previous described [30]. WT, *atsuc4* mutants and *AtSUC4/suc4-1* seeds were sown on MS solid media containing different concentrations of sucrose (0%, 2%, 4% and 6%). Roots were collected and quickly grinded in liquid nitrogen. An aliquot of each individual sample was precisely weighed (20 mg) and transferred to an eppendorf tube. After adding the addition of 500 µL of extract solvent (ethanol-water, 8:1) and including the isotope as internal stand with the fix concentration. The samples were vortexed sonicated. The homogenate and sonicate circle were repeated for 3 times, followed by incubation at 90 °C for 30 min and centrifugation at 12000 rpm for 15 min. The supernatant was transferred to an auto-sampler vial for UHPLC-MS/MS analysis to measure glucose and fructose. Another aliquot was further diluted for 10 times for UPLC-MS/MS analysis to quantitate the sucrose. When the concentrations of metabolites were beyond the standard curve range, the samples were diluted accordingly to place their concentrations within the range. The final concentration in µg/mg.FW equals the calculated concentration multiplied by the dilution factor.

The determination of IAA and ABA content in roots

Roots were collected from WT and *suc4-1* mutant grown on MS solid media with 2% and 6% sucrose supplementation under LD condition at 6 d after sowing, with three biological replicates (approximately 0.5 g fresh weight per sample). And the contents of endogenous IAA and ABA were determined according to a previously reported method with modification [31]. IAA and ABA analysis were performed on a Thermo Scientific Ultimate 3000 UHPLC System equipped with a Thermo Scientific TSQ Quantiva-Stage Quadrupole Mass Spectrometer (<http://www.greenswordcreation.com>).

RNA sequencing and sequence analysis

Total RNA was isolated from the 6 d-old seedling roots of WT, *suc4-1* and *suc4-2* plants which grown on MS solid media containing different concentrations of sucrose (2%, 4% and 6%) in three biological replicates. RNA samples were treated with DNase before being quality checked using an Agilent 2100 Bioanalyzer. Total RNA was prepared for a strand-specific TruSeq™ RNA-seq library, and all 27 samples were sequenced on an Illumina HiSeq 4000, with 150 bp paired end reads. Differential expression analysis was performed using the DEGSeq R package (1.12.0). *P*-values were adjusted using the Benjamini & Hochberg method. A corrected *P*-value of 0.05 and log₂ (Fold Change) of 1 were set as the threshold for significantly differential expression. Gene ontology enrichment analysis of differentially expressed genes was implemented by the Goseq R package, in which gene length bias was corrected. Gene ontology terms with a corrected *P*-value less than 0.05 were considered significantly enriched by differentially expressed genes. All sequences generated in this study have been deposited in the National

Center for Biotechnology Information Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) with project number PRJNA666009.

Statistical analyses

The overall data were statistically analyzed using SPSS 20 software (IBM China Company Ltd., Beijing, China). One-way or two-way ANOVA with Duncan test at the 5% level was used to test differences between multiple samples. Data were presented as means and SEs using Microsoft Excel.

Results

Expression pattern and subcellular localization of AtSUC4

To reveal the spatio-temporal expression of AtSUC4 in *Arabidopsis*, we generated a construct (pAtSUC4::*GUS*) harboring the GUS reporter gene driven by the endogenous *AtSUC4* promoter and transformed into Col-0. As shown in Figure 1A-B, AtSUC4 was mainly expressed in vasculature cells of leaves and mature portion of 4 d and 14 d-old seedling roots. In 25 d-old plants, GUS staining covered the entire root and juvenile leaves but not mature leaves (rosette) (Fig. 1C). In the inflorescence, petals, styles and the developing flower buds showed the presence of GUS staining (Fig. 1D). Specifically, we detected the GUS activity in vasculature of petals and styles in mature flower (Fig. 1E). In addition, Figure 1F showed that strong GUS activity was detected in vascular tissues of siliques at 1 DAF (days after flowering) or 6 DAF and embryo at 15 DAF. To investigate whether the AtSUC4 expression was induced by sucrose, pAtSUC4::*GUS* seedlings were grown on the medium with different concentrations of sucrose (0%, 2%, 4% and 6%) for 7 d and then stained for GUS activity detection. As shown in Figure 2A, with the increase of sucrose concentration in the medium, the activity of GUS became gradually higher, suggesting that *AtSUC4* expression was significantly induced by sucrose. This result were further supported by *GUS* expression analysis using RT-qPCR (Fig. 2B).

To verify the subcellular localization of AtSUC4, the construct encoding AtSUC4-GFP under endogenous promoter was generated and co-expressed with plasma membrane marker or tonoplast marker in *N. benthamiana* leaves by infiltration with *Agrobacterium tumefaciens*. The result demonstrated that AtSUC4 was co-located with both plasma membrane and tonoplast marker, indicating that AtSUC4 was localized to plasma membrane and tonoplast, but compared to the plasma membrane labelling, the signal in tonoplast was quite faint (Supplementary Fig. S1). We further checked the result with the AtSUC4-Citrine fusions protein expressed in the root of *Arabidopsis*. However, AtSUC4-Citrine gave clear and strong labelling of the plasma membrane but not tonoplast (Fig. 3A). Immune colloidal gold assay and transient transformation of *Arabidopsis* protoplasts with AtSUC4-GFP vector showed that AtSUC4 was localized to plasma membrane (Fig. 3B-C).

Atsuc4 mutants exhibited longer roots under high sucrose stress

In order to observe the effect of *AtSUC4* mutation on plant growth, except the *suc4-3* (T-DNA insertion from Nottingham Stock Centre), we generated *suc4-1* by EMS mutagenesis and *suc4-2* by CRISPR/Cas9 mutagenesis (Fig. 4A). The *suc4-1* point mutation (C502A) and *suc4-2* inserted mutation (A was inserted between nucleotides 29 and 30) introduced stop codon (TAA), resulting an early termination of *AtSUC4* protein translation (Fig. 4B). The *AtSUC4* transcript in all the three mutants were significantly lower than WT, whereas it was similar to that of the WT in the *AtSUC4/suc4-1* (Fig. 4C-D). Under the LD conditions, both plant height and leaf number were similar in all genotypes (Fig. 4E-F, Supplementary Fig. S2A-B). The root length of WT was similar to all the three *atsuc4* mutants on medium supplemented with light sucrose concentration, including 0%, 0.5%, 1%, 1.5% or 2% sucrose (Supplementary Fig. S2C). Although the maturation of the embryo in *suc4-1* was 1 day later than WT (Supplementary Fig. S2D), it did not affected the normal development of the embryo. These data demonstrated that there was no difference in phenotype between WT and *atsuc4* mutants plants on the sucrose-free or light concentration of sucrose condition.

To test whether phenotypes of *atsuc4* mutants were altered in response to high content sucrose, we calculated the primary root length and germination rate of *atsuc4* mutants and WT under different concentrations of sugars (0%, 2%, 4% and 6%). The results showed that as the sucrose concentration became higher, the root length in all lines were gradually decreased (Fig. 5A-B), indicating that high concentration of sucrose would inhibit the root growth. Interestingly, the primary root of the *atsuc4* mutants were significantly longer than that of the WT and *AtSUC4/suc4-1* when the sucrose concentrations in the medium were raised to 4% and 6% (Fig. 5A-B). Besides, we explored whether the high concentration glucose and/or osmotic stress would cause the difference of primary root length among WT, *atsuc4* mutants and *AtSUC4/suc4-1* by external application of a gradient concentration glucose and mannitol (simulated osmotic stress). As shown in Supplementary Figure S3 and S4, no differences were found in the root length among these three genotypes of plants under the same treatment. Besides, we found that the germination rate of *suc4-1* was similar to WT on agar medium supplemented with 0%, 2% and 4% sucrose, respectively (Supplementary Fig. S5). However, the germination of *suc4-1* seeds was 1 to 2 days later than WT when the concentration of sucrose was 6%. Taken together, these results suggested that disruption of *AtSUC4* in *atsuc4* mutants repressed the inhibition of high concentration sucrose on root growth, but not on germination rate. The deficiency of *AtSUC4* might be important for tolerance of high sucrose stress.

***Atsuc4* mutants accumulated less sucrose and ABA and more IAA than WT in roots under high sucrose stress**

To investigated whether the change in root length was caused by the sucrose content in *atsuc4* mutants, we then compared the main sugars (including sucrose, glucose and fructose) content in roots of above plants. The results revealed that an obviously reduced sucrose content in root was shown in *atsuc4* mutants compared with WT and *AtSUC4/suc4-1* on medium with 2%, 4% and 6% sucrose supplementation (Fig. 6A), suggesting negatively correlation with the length of roots. To a lesser extent, the glucose content of *suc4-1* and *suc4-3* roots were decreased compared with WT on medium

supplemented with 4% and 6% sucrose, whereas no significant difference between *suc4-2* and WT (Fig. 6B). The content of fructose in three *atsuc4* mutants were significantly lower than that of WT and *AtSUC4/suc4-1* on medium without sucrose. As the concentration of sucrose increases to 6%, markedly reduced fructose content was detected in *suc4-2* compared with WT, but no significant differences in WT, *suc4-1* and *suc4-3* (Fig. 6C). These results showed that *atsuc4* mutants exhibited longer root length compared with WT due to a less sucrose accumulation during high sucrose stress.

In order to verify whether the reduction of the root length of the *atsuc4* mutants crossed with the IAA and ABA signal pathway under high sucrose stress, we detected the IAA and ABA contents in WT and *suc4-1* roots (Tab. 1). The result showed that the content of IAA in *suc4-1* and WT was decreased in the 6% sucrose supplementation compared with 2% sucrose, however, it was significantly higher in *suc4-1* than WT, suggesting that the less reduction of IAA might alleviate the inhibition of root growth under high sucrose stress. On the contrary, 6% sucrose stimulated the accumulation of ABA in both *suc4-1* and WT, and less ABA was accumulated in the *suc4-1* compared with WT.

Analysis of global gene expression change in *atsuc4* mutants under different sucrose condition

To better understand the role of *AtSUC4* at the molecular level under high sucrose stress, comparative transcriptome analysis was performed between two *atsuc4* mutants (*suc4-1* and *suc4-2*) and WT using root which grown 6 d under 2%, 4% and 6% sucrose treatments. A total of 1,259 (581 up, 678 down) and 3,191 (1,474 up, 1,717 down) DEGs (differentially expressed genes) were identified in *suc4-1* and *suc4-2* compared with WT on the medium supplemented with 2% sucrose, respectively. At 4% sucrose condition, 452 (182 up, 270 down) and 1,990 (821 up, 1,169 down) DEGs were found in *suc4-1* and *suc4-2*, respectively. Under high concentration of sucrose condition (6% sucrose), the number of DEGs increased, which 6,485 (2,837 up, 3,648 down) and 6,354 (2,611 up, 3,743 down) DEGs were identified in *suc4-1* and *suc4-2*, respectively (Supplementary Fig. S6A). Besides, only 26 DEGs were overlapped in *suc4-1* under these three condition, whereas 221 common DEGs were detected in *suc4-2* (Supplementary Fig. S6B). KEGG enrichment analysis among the DEGs revealed that ribosome and glucosinolate biosynthesis were commonly enriched in the *suc4-1* and *suc4-2* under 2% sucrose treatment, while three common enriched pathways were identified under 4% sucrose treatment (Supplementary Fig. S7A), including carbon metabolism, glyoxylate and disarboxylate metabolism and pyruvate metabolism. Additionally, there were 12 enriched pathways overlapped in the *suc4-1* and *suc4-2* at 6% sucrose condition (Supplementary Fig. S7B), which was mainly contributed by more DEGs were detected in both two mutants at this treatment than other two treatments.

***Atsuc4* mutants responded to high sucrose stress through sucrose, IAA and ABA signaling pathway**

Our study show that the sucrose accumulation was much lower in *atsuc4* than WT under 6% sucrose, we therefore checked the expression change of *AtSUCs* and *AtSWEETs* in *atsuc4* mutants by RNA-seq analysis. As shown in Figure 7A, *AtSWEET2*, 14, 16 and 17 were significantly up-regulated in *atsuc4* mutants compared with WT at 6% sucrose condition, whereas a reverse expression pattern were found for *AtSWEET1*, 4, 6, 11, 12 and 15, as well as *AtSUC2*.

We further investigated the expression of *AtSUC2*, *AtSWEET11,12* and *15* by qRT-PCR, the result showed consistence with RNA-seq data (Fig. 7B). These results revealed that more sucrose transporters tend to be down-regulated in *atsuc4* mutants, which might contribute to lower level of sucrose in the mutants under high sucrose stress. Except for sucrose transport, sucrose accumulation are also affected by sucrose synthesis and degradation. Hence, we explored the expression of genes related sucrose synthesis and degradation in *atsuc4* mutants under high sucrose stress. As shown in Figure 7A, three genes that encode Sucrose Synthase (*SUS1*, 4, 5) that are important for sucrose degradation, were significantly up-regulated in two mutant lines compared with WT under high sucrose stress. On the contrary, the transcript levels of the genes encoding Sucrose-Phosphate Synthase (*SPS*), which is involved in sucrose synthesis, were reduced significantly. Based on these results, we speculated that the pathway of sucrose degradation was active, but the sucrose synthesis was blocked by the deficiency of *AtSUC4*.

A significant difference in the content of ABA and IAA between *atsuc4* mutants and WT under 6% sucrose stress were detected (Tab. 1). Therefore, expression of IAA and ABA-related genes were investigated by RNA-seq. As shown in Figure 7C, reduced expression was observed in two IAA biosynthesis genes (*YUC2*, 6), two IAA transporter genes (*PIN1*, 4) and five IAA signal transduction genes (*PLT1*, 2, *SHR*, *IAA6*, 19) in two *atsuc4* mutants and WT under 6% sucrose compared with 2% sucrose treatment, while the transcript of those genes were higher in two *atsuc4* mutants than WT. On the contrary, except for a negative regulator in ABA signal transduction (*ABI1*), one ABA biosynthesis gene (*NCED3*), seven ABA signal transduction genes (*PYL2*, 8, *SnRK2.3*, 2.6, *ABI3*, 4, 5) were up regulated in two *atsuc4* mutants and WT under 6% sucrose compared with 2% sucrose treatment, while the transcript of those genes were lower in two *atsuc4* mutants than WT (Fig. 7E). This result corresponded to the broad reduction and increased in accumulation of IAA and ABA in two *atsuc4* mutants and WT under 6% sucrose treatment, respectively. But the content of IAA and ABA significantly higher and lower in *suc4-1* mutants than WT, respectively. To test the reliability of transcriptome data, five sucrose metabolism genes, five IAA signaling pathway genes and five ABA signaling pathway genes were chosen for RT-qPCR assay. Gene expression as elucidated by RT-qPCR exhibited similar trends with that of the transcriptome data, with some variation in the magnitude (Fig. 7B, D, F). This result clearly suggested that deficiency of *AtSUC4* had directly or indirectly stimulated the IAA signaling pathway and repressed the ABA signaling pathway under high sucrose stress.

Table 1. The WT and *suc4-1* were grown on MS solid media with 2% or 6% sucrose supplementation at the 6th day. Each sample was subjected to three biological replicates. Statistically significant differences compared with WT were determined by Duncan tests (* $P < 0.05$).

Table 1 The ABA and IAA contents of different plants

Analyte	IAA	ABA
	/(ng.g ⁻¹ FW ⁻¹)	/(ng.g ⁻¹ FW ⁻¹)
WT (2% Suc)	10.9±1.2	6.3±0.6
<i>suc4-1</i> (2% Suc)	9.6±0.7	4.1±0.2*
WT (6% Suc)	3.2±0.3*	9.6±0.7
<i>suc4-1</i> (6% Suc)	4.3±0.1	7.2±0.4*

Discussion

As an important sucrose transporter, although various studies have focused on AtSUTs in *Arabidopsis*, relatively few have been performed on the effect of AtSUT4 on root development in recent years. Based on our results in this study, the possible function and the regulation mechanism of AtSUC4 in the root under high sucrose stress would be discussed.

In plants, the root as a typical sink organ that relies exclusively on an external import of sucrose for growth. Therefore, the sucrose transporter has been considered as a major determinant of plant growth for a long time [6]. Schneider et al found that no difference in growth between WT and a *atsuc4* mutant in normal condition, and we confirmed this result using two more *atsuc4* mutants (Fig. 4E-F, Supplementary Fig. S2) [11]. In our study, we characterized a new function of *AtSUC4*, which it played an important role under high sucrose stress. As shown in Figure 2, with the increasing of sucrose concentration in the medium, the activity of GUS was gradually higher in pAtSUC4::*GUS* plant, suggesting that *AtSUC4* was strongly induced by sucrose. Thus, it is possible that the sucrose stress might be the key factor in inducing the biological function of *AtSUC4*. Interestingly, we found that the root length of three *atsuc4* mutants were significantly longer than that of WT and *AtSUC4/suc4-1* when the sucrose concentration were 4% and 6% in the medium (Fig. 5A-B), suggesting that the deficiency of *AtSUC4* enhanced the tolerance of high sucrose stress in roots, and *atsuc4* mutants were less sensitive to high sucrose stress compared with WT. Besides, the sucrose content in *atsuc4* mutants roots was lower compared with WT and *AtSUC4/suc4-1* under high sucrose treatment (Fig. 6A), showing the negative relationship with root length. This was consistent with the previous results [32]. However, the above difference between *atsuc4* mutants and WT gradually weakened with the extension of culture time, and disappeared completely in 11 day-old seedlings, which was mainly due to photosynthesis affecting the sugar content in roots. It has been demonstrated that external application of high concentration sucrose could cause the increase of glucose content and osmotic potential on the root surrounding [33]. However, Supplementary Figure S3 and S4 showed that there was no difference in root length among all genotypes under high concentration glucose and osmotic stress (stimulated by mannitol). These data indicate that disruption of *AtSUC4* plays an important role in coping with high sucrose stress in *Arabidopsis*, mainly by reducing the accumulation of sucrose in roots.

Schneider et al characterized AtSUC4 in mesophyll vacuoles and found that sucrose content in *AtSUC4*-overexpressing seedlings was reduced, indicating *AtSUC4*-mediated release of sucrose from the vacuolar lumen into the cytoplasm [11]. Nevertheless, we found that the deficiency of *AtSUC4* lead to less sucrose accumulation in the root of *atsuc4* mutants (Fig. 6A), suggesting mild conflict with the function of *AtSUC4* mentioned above. In this study, AtSUC4 was localized to both plasma membrane and tonoplast by transient expression in tobacco leaves but compared to the plasma membrane labelling, the signal in tonoplast was quite faint (Supplementary Fig. S1). However, AtSUC4-Citrine gave clear and strong labelling of the plasma membrane in roots but not tonoplast by stable expression in *Arabidopsis* (Fig. 3A). Combined with the results of sucrose content in roots, we speculated that AtSUC4 may transport sucrose from extracellular into cytoplasm, resulting less sucrose accumulation in the disruption of *AtSUC4*.

Sucrose metabolism plays pivotal roles in development and stress response, mainly by generating metabolites to fuel growth and as signals to regulate gene expression and for crosstalk with hormonal signaling [34]. This is achieved by the generation of sugar signaling molecules or metabolic process itself [35]. Therefore, sucrose transport, synthesis and degradation are very important in regulating plant growth. To date, SUCs and SWEETs have been characterized as the two main types of protein responsible for sucrose transport in plants [36]. AtSWEET1, 2, 4, 9-15 and 16 have been identified harbour sucrose transport activity [3]. Chen et al found that *sweet11;12* double mutant and *sweet11;12;15* triple mutant exhibited reduced root length when germinated on sugar-free media that could be ameliorated by external sucrose addition [37]. As SWEETs facilitate sugar transport along the sugar gradient, not surprisingly, 6% sucrose treatment induced the expression of five plasma membrane localized *SWEETs* (*AtSWEET1, 4, 11, 12, 15*) in all three plants, but the transcripts of those genes were down regulated in *atsuc4* mutants compared with WT. Meanwhile, the expression of two vacuolar membrane localized *SWEETs* (*AtSWEET2, 16*), which released sucrose from vacuolar lumen to cytoplasm, were shown the opposite tendency on the 6% sucrose supplementation (Fig. 7A). Unlike *AtSWEETs*, the function of *SUCs* to transport sucrose requires energy, so its expression caters to the needs of the owners. The *AtSUC1* in all three plants were down-regulated with the increase of sucrose supplementation. The *AtSUC2* was up-regulated in WT, but down-regulated in two *atsuc4s*. In addition, compared with WT, the transcript level of *AtSUC2* was much lower in two *atsuc4s* under the 6% sucrose supplementation (Fig. 7A). We proposed that the notable decreased sucrose accumulation in the two mutants could be a result of the more down-regulated expression of plasma membrane localized *AtSWEETs* and *AtSUC2* responsible for sucrose transport than that with up-regulated expression pattern.

Sucrose metabolism plays an important role in plant growth and development [38]. On the one hand, sucrose can be hydrolysis by Invertase (INV), producing glucose and fructose. Molecular genetic studies showed that CWIN activity is required for, or even rate-limits, seed development in some instances by controlling cell division [1]. *AtCWINV5* was up-regulated in three genotype plants by the stimulation of high-sucrose. Compared with WT under 6% sucrose supplementation, the expression level of *AtCWINV5* in two *atsuc4* mutants were down regulated (Fig. 7A). Barratt et al found that reduction of CIN activity by about 40% through double mutation *cinvl/cinv2*, drastically reduced root growth [39]. Also, in rice, *OsCyt*-

inv1 was mutated, the plant exhibited short roots [40]. When the two *atsuc4* mutants and WT were planted on the medium containing 6% sucrose, the expression of *AtCINV1* was down regulated. However, compared with WT, the expression of *AtCINV1* was higher in the two *atsuc4* mutants. On the other hand, sucrose can be degraded by Sucrose Synthase (Sus; EC 2.4.1.13) into Hexoses or their derivatives. Compared with WT under 6% sucrose supplementation, the expression level of *AtSUS1*, 4 and 5 in two *atsuc4* mutants were up-regulated (Fig. 7A). In contrast, due to the increase of substrates, two Sucrose-Phosphate Synthase Enzyme (SPS; EC 2.4.1.14) genes (*AtSPS1*, 3) and the Sucrose Phosphatase (SPP; EC 3.1.3.24) gene (*AtSPP2*), which related to sucrose synthesis, were up regulated in three plant as the sucrose level increasing. While their expression level was lower in the two *atsuc4s* compared with WT under 6% sucrose treatment (Fig. 7). Taken together, these data suggest that *AtSUC4* regulates root growth involved in many sucrose metabolic enzymes, which exert their signaling roles partly by reduce sucrose inflow and biosynthetic and increase sucrose degradation in intracellular.

Evidence from many studies highlights the central role of auxin in generation and maintenance of primary root meristems as well as orchestrating the final root architecture [41,42]. Thus, sucrose and auxin may operate coordinately during plant root growth. Our results showed that the roots were shorter with 6% sucrose application in both *atsuc4* mutants and WT (Fig. 5), indicating that root growth was inhibited under high sucrose stress. Interestingly, the roots of *atsuc4* mutants were longer than that of WT (Fig. 5). Furthermore, the IAA content in the *suc4-1* root was significantly higher than that of the WT under the treatment with 6% sucrose (Tab. 1). Consistent with this, our comparative transcriptome data showed that the expression of the genes related to IAA biosynthesis (*YUC2*, 6) and transporters (*PIN1*, 4) were significantly up regulated in *atsuc4* mutants under high sucrose stress (Fig. 7C). The cellular levels of auxin, in turn contribute to the regulation of gene expression that defines cell fate and impacts root patterning. The *PLETHORA* genes are required to define the root-stem cell niche, and the transcript of these genes are strongly correlated with auxin levels [43]. This expression pattern also coincides with that of the radial patterning genes *SCR* and *SHR*, which provide parallel inputs in specifying root stem cells [44]. According to RNA-seq data, *AtPLT1*, 2 and *AtSHR* were significantly up-regulated in *atsuc4* mutants compared with WT at 6% sucrose application (Fig. 7C). These results suggest that the higher content of IAA provoked the expression of *AtPLT1*, 2 and *AtSHR* in *atsuc4* mutants compared with WT, which help to keep the longer primary root under high sucrose stress. Meanwhile, the *PLT* in turn regulate auxin accumulation in the QC both through transport (*PIN4*) and biosynthesis (*YUCs*) [45,46]. In our study, the *AtPLT1* and 2 shown the same expression trend with *YUC2*, 6 and *PIN4*. Since Aux/IAA proteins are auxin-sensitive repressors that have functions in the regulation of auxin-mediated root growth and development. The *aux/iaa* mutants, including *iaa1*, 2, 13, 6, 17, 19 and 28 reduced multiple auxin responses, with smaller in size to various extents than the WT [47]. In our study, the expression of two *Aux/IAAs* (*IAA6*, 19) in *atsuc4* mutants were significantly higher than WT in the treatment with 6% sucrose (Fig. 7C). Taken together, our genome transcript profiling studies revealed that the deficiency of *AtSUC4* declined sucrose content in *atsuc4* mutants root compared with WT under 6% sucrose treatment, which could accumulated more IAA and up regulated the expression of IAA related genes, including IAA biosynthetic genes (*YUC2*, 6), transporters (*PIN1*, 4), signalling transduction (*PLT1*, 2 and *SHR*) and

signalling molecules (*IAA6*, 19). Consistent with this, *atsuc4* mutants had the longer root meristem zones and primary root.

ABA signaling also plays a critical role in abiotic stress and regulating root growth and root system architecture [48]. According to the data of ABA content, when the concentration of sucrose increased, the content of ABA increased significantly in both *suc4-1* and WT, and it rised to a less extent in *suc4-1* under 6% sucrose supplementation (Fig. 5, Tab. 1). This was in line with the situation that *NCED3*, a key genes that control the synthesis of ABA, was significantly decreased in two *atsuc4s* mutants compared with WT (Fig. 7E). The increase of ABA levels in the plant cell leads to PYR1/ PYL/ RCAR receptor-mediated inhibition of PP2C activity, which results in the activation of the SnRK2s and ultimately of the ABA signaling pathway by directly phosphorylate bZIP transcription factors ABIs [49, 50]. In general, PP2C has a negative regulatory effect on ABA signal transduction [51]. Similiarly, we found that the expression seven ABA signaling transduction genes (*PYL2*, 8, *SnRK2.3*, 2.6, *ABI3*, 4, 5) were significantly decreased in two *atsuc4* mutants with 6% sucrose supplementation, while *ABI1*, a member of group PP2C, was significantly up regulated (Fig. 7). Yuan et al suggest that *ABI5* functions in the glucose-mediated inhibition of the root meristem zone by repressing *PIN1* accumulation, thus leading to reduced auxin levels in roots and shorten primary root, indicating that *ABI5/PIN1* functions at the junction between ABA and auxin signaling [21]. Similarly, PIN1 protein levels were reduced to a lesser extent in *abi4* plants treated with ABA than in ABA-treated WT plants [52]. In our study, the reduced expression of *ABI4*, 5 and increased expression of *PIN1* was found in *atsuc4* mutants caompared with WT under 6% sucrose treatment. In sum, our data may indicate that the deficiency of *AtSUC4* repressed the expression of *ABI4* and 5 in the root, which, in turn, stimulated the expression of *PIN1* and accumulation of IAA content. This increased auxin activity and resulted in longer primary root.

Further physiological functional analysis of *AtSUC* genes are needed to explore the possible relationship between sugar signal and the functions of IAA- or ABA-related genes triggered by different stresses.

Declarations

Additional Information

Consent to publish

Not applicable.

Author contributions

S-WL, C-CP and J-ML conceived the project. S-WL, L-DZ and J-YG performed most of the experiments, TZ and T-TD performed the RNA-seq analysis. S-WL and J-ML analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Data availability

All sequences generated in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) with project number PRJNA666009.

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Conflicts of Interest

The authors declare that they have no competing interests.

Ethics

Not applicable.

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Figures

Figure 1

Expression pattern of the AtSUC4 in life cycle of Arabidopsis by pAtSUC4::GUS expression. (A) Seedlings grown for 4 days on MS solid media with 1% sucrose supplementation. (B) Seedlings grown for 14 days on MS solid media with 1% sucrose supplementation. (C) The rosette leaves and root of the whole plant grown for 25 days in soil. (D) The inflorescence for 35 days in soil. (E) The stigma and vascular tissue of petals for 35 days in soil. (F) The siliques of Arabidopsis at 1, 6 and 15 DAF (days after flowering).

Figure 2

Expression level of AtSUC4 in response to different concentrations of sucrose. (A) 7 d-old pAtSUC4::GUS seedling on MS solid media with different concentrations of sucrose (0%, 2%, 4% and 6%). (B) The relative expression level of GUS in roots of pAtSUC4::GUS plants. Values represent the mean $\pm$ SE of three biological replicates. Bars with the different letters indicate a significantly difference among treatments for a same stage according to Duncan test (at $P < 0.05$).

Figure 3

The subcellular localization of AtSUC4. (A) The root imaged of Citrine fusion to the C-terminus of AtSUT4 by stably transformed. (B) The imaged by immune colloidal gold. SE: sieve element, CC: companion cell, PM: plasma membrane; The black arrow represents the gold particles. The right image served as a negative control. (C) The subcellular localization of a GFP fusion to the C-terminus of AtSUT4 by transient transformation of Arabidopsis protoplasts.

Figure 4

Phenotypic characterizations of *atsuc4* mutants and complement-expressor (*AtSUC4/suc4-1*) plants. (A) Schematic diagram of the mutagenesis types of three *atsuc4* mutants and their mutant sites. *suc4-1* from ethyl-methanesulfonate (EMS) mutagenesis, *suc4-2* from CRISPR/Cas9 mutagenesis and *suc4-3* from T-DNA insertion mutagenesis. (B) Comparison of sequencing results of mutant *suc4-1* and *suc4-2* compared with WT. (C) and (D) Reverse transcription-quantitative and semi-quantitative real time PCR (RT-qPCR) expression analyses of *AtSUC4* transcript in three mutants and the complement-expressor line. The phenotype of 25 d-old rosettes (E) and 50 d-old plants (F) from WT, *atsuc4* mutants and complement-expressor plant shown no difference. Values in (C) represent the mean $\pm$ SE of three biological replicates. Bars with the different letters indicate a significantly difference among different plants for a same stage according to Duncan test (at $P < 0.05$).

Figure 5

Atsuc4 mutants reduced the inhibition of high concentration sucrose stress on root growth at 6 days after sowing. (A) The root phenotype of different lines under different concentrations of sucrose treatment (0%, 2%, 4% and 6%). (B) The statistics of root length among various concentrations of sucrose treatment. Values in (B) represent the mean $\pm$ SE of three biological replicates. Bars with the different letters indicate a significantly difference among different genotypes for the same sucrose concentration treatment according to Duncan test (at $P < 0.05$), $n > 50$.

Figure 6

The concentration of different sugars (including sucrose, glucose and fructose) in roots from all genotypes which were cultivated on MS solid media with different concentration of sucrose (0%, 2%, 4% and 6%) at the 6th day. (A) Sucrose content. (B) Glucose content. (C) Fructose content. Values in (A), (B) and (C) represent the mean $\pm$ SE of three biological replicates. Bars with the different letters indicate a significantly difference among different genotypes for the same sucrose concentration treatment according to Duncan test (at $P < 0.05$), $n > 30$.

Figure 7

The expression level of genes related to sucrose, IAA and ABA signaling pathway in root of different plants which were grown on MS solid media with 2%, 4% or 6% sucrose for 6 days. Each sample was subjected to three biological replicates. (A)(C)(E) Heatmap of representative genes expression related to sucrose, IAA and ABA signaling pathway. (B)(D)(F) Gene expression changes as assessed by RT-qPCR. The relative expression detected by RT-qPCR, was represented by a bar graph, and the left ordinate indicated the expression level. FPKM of genes, according to RNA-Seq data, was represented by a line graph, and the right ordinate indicated the FPKM level. The asterisk represented a significant difference compared with WT. The log2 fold change value of the gene expressions from transcriptome is displayed in different colors. Yellow color means high expression and blue color means low expression.

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