

Article

Functional Studies and Expression Characteristics of the Vacuolar Sugar Transporter CoSWEET2a in *Camellia oleifera*

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Abstract

Sugar transporters of the SWEET family are essential for plant growth, development, yield formation, and stress responses by regulating sugar transport and distribution. This study characterizes the function and expression characteristics of CoSWEET2a, a Clade I SWEET gene in *Camellia oleifera*. We conducted subcellular localization, functional complementation in *Arabidopsis*, sugar response assays, drought tolerance tests, and hormone induction analysis. A key finding is CoSWEET2a, which that is localized on the vacuolar membrane in *Camellia oleifera*. Heterologous expression in *Arabidopsis atsweet2* mutants revealed sugar-specific effects on root growth. Moreover, expression of CoSWEET2a increased soluble sugar content in *Arabidopsis* seeds. Additionally, CoSWEET2a overexpression enhanced drought stress tolerance by augmenting sugar content. The expression of CoSWEET2a is regulated by gibberellin (GA) and abscisic acid (ABA), and its promoter contains corresponding hormone response elements. In conclusion, CoSWEET2a functions as a “sugar buffer” on the vacuolar membrane, regulating sugar accumulation, root development, and drought stress responses. This discovery not only reveals that vacuolar SWEET plays an important role in maintaining cytoplasmic sugar homeostasis in plants but also provides a direct genetic target for engineering high-quality, drought-tolerant crops.

Keywords: *Camellia oleifera*; SWEET transporter; sugar transport; hormone response; drought stress



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1. Introduction

Sugar is a fundamental component in plant energy metabolism, acting as the principal form of photosynthetic products and a key mediator in cell osmoregulation and signaling [1]. The transport of photosynthetic products in plants follows a three-stage process: (1) phloem loading in the leaves, (2) long-distance transport within the sieve element–companion cell complex (SE/CC), and (3) phloem unloading in sink organs [2]. This unloading process takes place through symplasmic transport, the apoplasmic pathway, or a combination of the two, with the apoplasmic pathway relying on sugar transporter proteins to facilitate sugar transmembrane transit [3].

The SWEET (Sugars Will Eventually be Exported Transporters) family has received a lot of attention for its bidirectional transport capabilities [4–6], playing vital roles in

plant growth, development, yield formation, and responses to biotic and abiotic stresses by regulating sugar allocation [7,8]. A typical SWEET protein consists of seven transmembrane helices. These helices connect two MtN3/saliva structural domains. SWEET proteins allow substrates like as sugars and hormones to be transported across membranes based on concentration gradients [9]. Phylogenetic analysis divided the SWEET family into four subfamilies: Clades I–IV. Clade I is principally responsible for hexose transfer. Proteins that transport glucose include AtSWEET2 [10], OsSWEET2b [11], and StSWEET1g [12]. Critically, several SWEETs localized to the vacuolar membrane have been implicated in stress tolerance mechanisms. AtSWEET2 limits the accumulation of glucose in vacuoles, which imparts resistance to pathogens [10]. AtSWEET17 reduces drought stress by encouraging the development of lateral roots through vacuolar fructose efflux [13]. Moreover, germination and cold tolerance are improved by overexpressing AtSWEET16, which is located in the vacuolar membrane [14]. In woody plants, CsSWEET16 overexpression in *Arabidopsis* decreases fructose buildup leaves at low temperatures, indicating that it plays a role in the vacuolar fructose efflux pathway [15]. These findings underscore the significance of vacuolar membrane-localized SWEETs in plant adaptation.

Camellia (*C.*) *oleifera* Abel. is a member of the *Camellia* genus and is mostly found in subtropical areas [16]. The seeds of *C. oleifera* are rich in oil, with high-quality content and a significant proportion of unsaturated fatty acids [17]. These seeds can be converted into edible oil, which has enormous potential [18]. Droughts in the summer and fall have become increasingly common in recent years due to global warming and abnormalities in air circulation within the primary oil tea production areas [19]. *C. oleifera* grows in hilly areas where artificial irrigation is difficult. The drought stress reduces the photosynthetic rate, number of flower buds, fruit set rate, fruit weight, and seed oil content of *C. oleifera*, significantly lowering the yield and economic benefits of *C. oleifera* orchards [20–22]. Sugar enhances the plant's tolerance to stress at the levels of physiological homeostasis and molecular pathways by acting as a signaling switch and an osmotic pressure buffer [23]. Therefore, it is imperative to look into the mechanisms behind the development and stress tolerance of *C. oleifera* in order to cultivate high-yielding, drought-resistant types that will assure the industry's sustained growth.

Sugar transport to developing seeds involves complex apoplast–plasmodesmata transitions, where vacuolar sugar homeostasis in transport cells plays a balancing role [24]. It is thought that CoSWEET2a, currently the only SWEET protein found in *C. oleifera* that is located in the vacuolar membrane, is essential to this process [24]. CoSWEET2a is highly expressed in seeds and responds to drought stress [25], suggesting that it may perform a similar function in stress resistance as the characterized vacuolar Clade I SWEET proteins. Additionally, vacuoles play a key role in material storage and homeostasis regulation [26]. Therefore, elucidating the molecular function and physiological mechanisms of CoSWEET2a not only holds promise for revealing its specific contributions in *C. oleifera* but also offers new insights into how woody perennial plants optimize energy storage and respond to environmental challenges such as drought through adaptive sugar transport mechanisms.

In this work, we examined the protein structure and oligomerization characteristics of CoSWEET2a. We directly localized CoSWEET2a to the vacuolar membrane using mesophyll protoplasts from the woody oil crop *C. oleifera*. This represents the first structural validation of tonoplast targeting for a SWEET transporter in a perennial oil plant. When CoSWEET2a was overexpressed in *Arabidopsis*, the level of soluble sugar in the seeds increased. CoSWEET2a also affected the growth of root length under treatments with sucrose, glucose, and fructose. It also increased seedling survival under drought stress, as well as an overall increase in soluble sugar content throughout the plant. We established

a causal chain linking CoSWEET2a-mediated vacuolar sugar buffering to systemic sugar accumulation and drought resilience. GA and ABA increase the expression of *CoSWEET2a*, providing evidence for hormonal regulation of vacuolar SWEET activity. As a “vacuolar sugar buffer” for hormone signal response, *CoSWEET2a* is critical in coordinating seed sugar buildup, root development, and drought tolerance.

2. Results

2.1. *CoSWEET2a* Is Localized to the Vacuolar Membrane in *C. oleifera*

Through heterologous transient transformation in tobacco (*Nicotiana benthamiana*), Du et al. [24] discovered that the CoSWEET2a protein is localized to vacuolar membranes. To precisely determine the subcellular localization within *C. oleifera* tissues, we performed transient transformation in protoplasts derived from callus and examined the subcellular localization of *CoSWEET2a*. Confocal microscopy revealed that CoSWEET2a-GFP fluorescence exhibited an invaginated structure distally located from the nucleus (Figure 1), indicating consistency with the characteristic morphology of the tonoplast. However, in the control group (transformed with an empty vector), fluorescent signals were diffusely distributed throughout the cell of leaves. These findings support CoSWEET2a’s location in the vacuolar membrane.

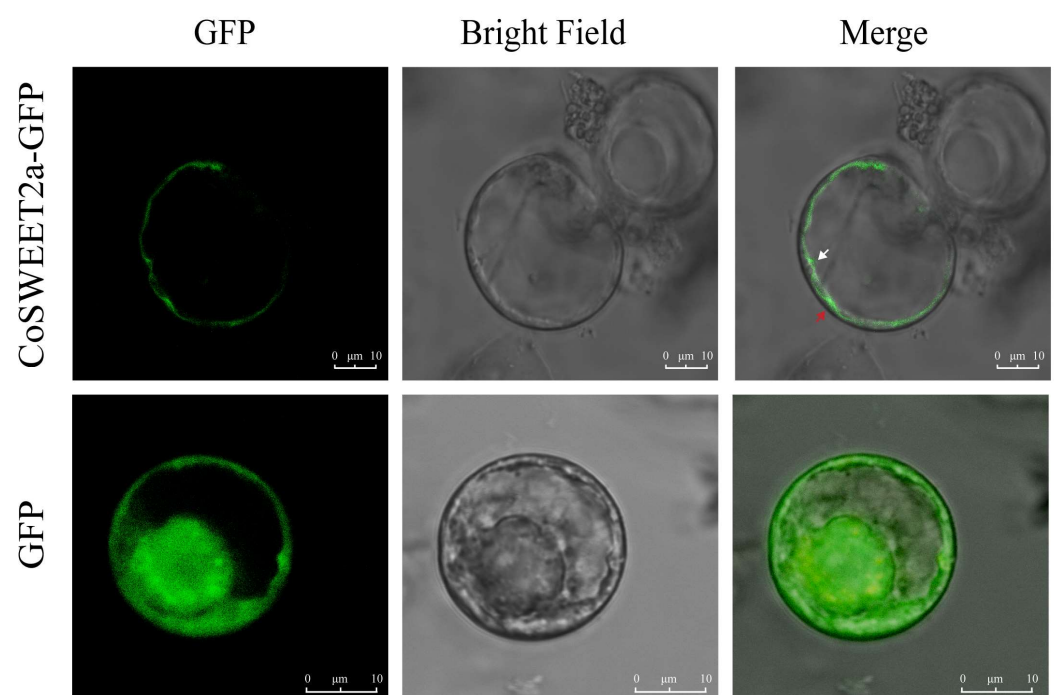


Figure 1. The localization of CoSWEET2a in protoplasts of *C. oleifera*. White arrows point to the vacuolar membrane, and red arrows mark the plasma membrane. The empty vector (GFP) was a positive control. Scale bar = 10 μ m. The experiment was conducted independently three times, and three images were captured each time.

2.2. Basic Characteristics and Phylogenetic Analysis of *CoSWEET2a*

The CoSWEET2a gene’s coding sequence (CDS) spans 708 nucleotides and codes for a 235 amino acid protein (Tables S1 and S2). This protein has a molecular mass of 25.99 kDa, with an average hydrophilicity index of 0.926, indicating that it is extremely hydrophobic and may be involved in membrane-associated processes (Figure S1A). By aligning the CoSWEET2a’s sequence with the NCBI database to find homologous SWEET proteins in order to categorize it and forecast its possible function (Table S2). According to sequencing research, CoSWEET2a and its related proteins have a significant degree of conservation,

especially in the amino acid sequence areas 18–100 and 138–220. These areas match the SWEET family's distinctive MtN3/sativa domain (Figure 2A), which is made up of three transmembrane domains (TMs) at the front and back (Figure 2B). Additionally, the transmembrane domains correspond to the tertiary (Figure S1B). Additionally, a phylogenetic tree comparing CoSWEET2a with members of the SWEET family from *A. thaliana* and *C. sinensis* was created using the maximum likelihood method (Table S2). The findings showed that AtSWEET2 and CsSWEET2a/b/c, both of which are members of Clade I, are the most closely related to CoSWEET2a (Figure 2C).

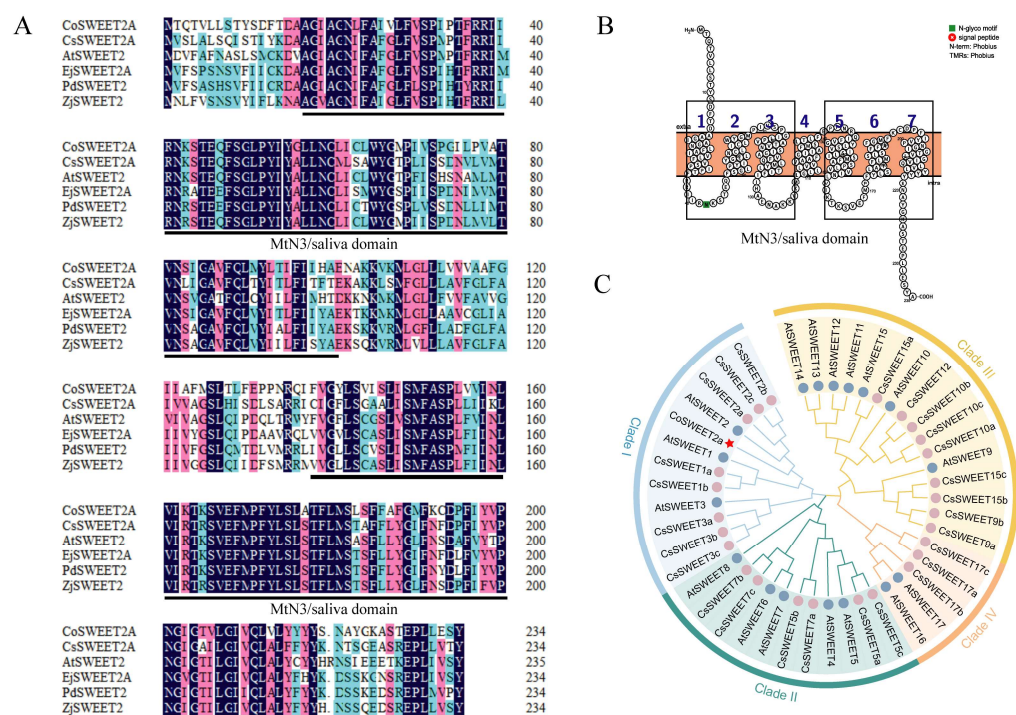


Figure 2. Bioinformatics analysis of CoSWEET2a. (A) Multiple sequence alignment of the CoSWEET2a amino acid sequence with homologous sequences from other plants. The two conserved MtN3_slv domains are indicated by black lines. (B) Transmembrane domains of CoSWEET2a. The seven transmembrane domains (TMD1–TMD7) are numbered sequentially from the N-terminus to the C-terminus. The MtN3_slv domains are highlighted with black boxes. (C) Phylogenetic analysis of CoSWEET2a with proteins from other species. CoSWEET2a is highlighted in red star. *Arabidopsis thaliana* is marked with a blue circle, and *Camellia sinensis* is marked with a red circle.

2.3. CoSWEET2a Forms Homodimers at the Vesicular Membrane

Prior research has shown that some SWEET proteins can increase the pace or efficiency of macromolecular sugar transport by forming homo- or heterodimers with other transporters or with themselves [27]. We used a bimolecular fluorescence complementation (BiFC) assay for insight into the transport form of CoSWEET2a in plants. In this experiment, CoSWEET2a was fused to the N-terminal and C-terminal ends of YFP proteins, respectively, and then transformed into tobacco epidermal cells. YFP fluorescence localized to the vacuolar membrane was clearly evident when CoSWEET2a-cYFP and CoSWEET2a-nYFP were co-transformed, according to fluorescence observation. This fluorescence overlapped with the red fluorescence of the vacuolar membrane marker vac-CD3-971. Notably, neither vac-CD3-971 [28] nor YFP fluorescence went to the nucleus (Figure 3). However, no green fluorescence was seen in either scenario when CoSWEET2a-cYFP and CoSWEET2a-nYFP were injected separately. These findings imply that CoSWEET2a can form a homodimer with itself at the vacuolar membrane.

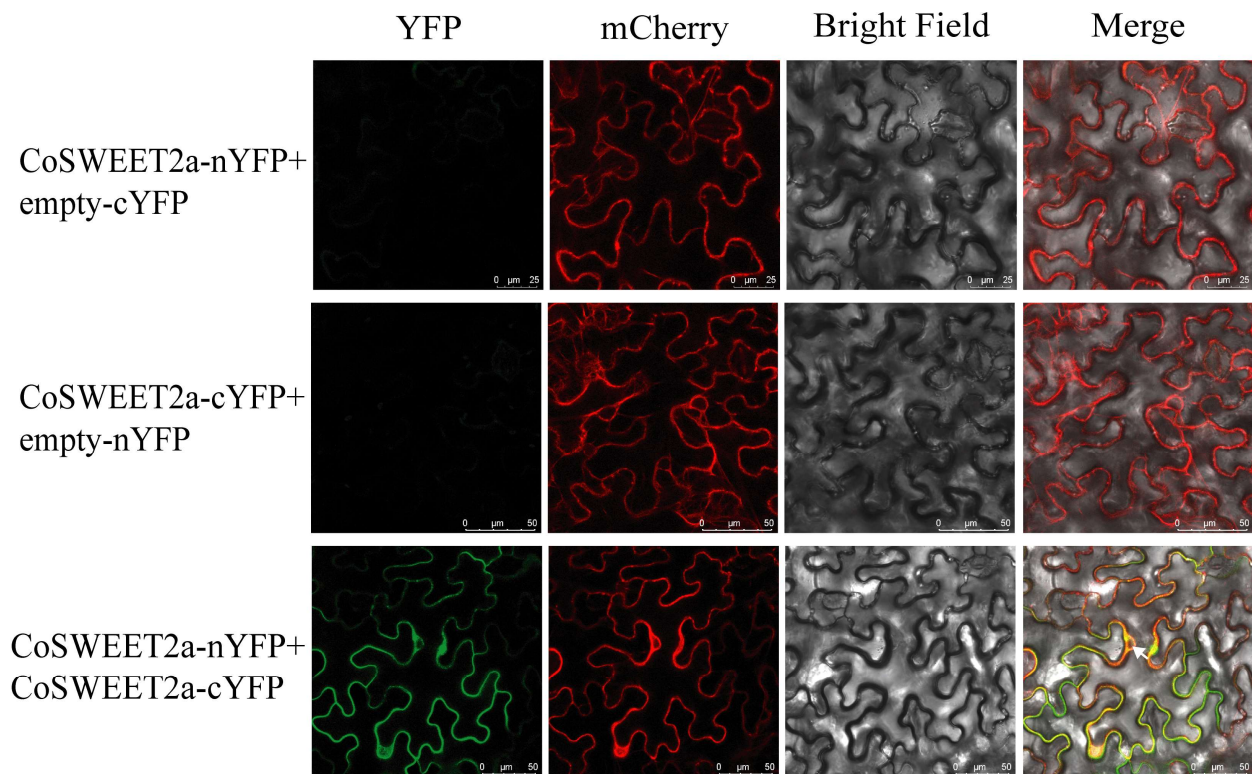


Figure 3. Bimolecular fluorescence complementation analysis of CoSWEET2a self-interaction. The green signals indicate YFP, whereas the red signals indicate the plasma membrane marker vac-CD3-971 vector (mCherry). The yellow signals represent co-localization of YFP and mCherry signals. White arrowheads indicate the cell nucleus. The experiment was conducted independently three times, and three images were captured each sample.

2.4. CoSWEET2a Overexpression Enhances Sugar Accumulation in Seeds

To investigate the function of *CoSWEET2a* and its impact on plant growth, development, seed yield, and other aspects, we generated the homozygous *atsweet2* mutant in *Arabidopsis*. After that, the 35S::*CoSWEET2a* overexpression vector was constructed and introduced into the mutants. After identification by PCR and RT-qPCR, two homozygous restoration lines, *CoSWEET2a*-R6 and *CoSWEET2a*-R8 were selected for further studies (Figure S2).

Arabidopsis wild-type, *atsweet2* mutant, and *CoSWEET2a* restoration lines were germinated and cultivated under the same circumstances. During the vegetative and maturation stages, there were no discernible variations in the lines' growth (Figure 4A,B). R6's average height at maturity (24.22 cm) was much shorter than that of the other strains. Compared to the wild-type, mutant, and R8, the decrease was roughly 8.3%, 6.2%, and 10.1% (Figure 4C). We also took measurements of the sugar content and seed production. Neither the oil content nor the weight of the thousand seeds changed significantly (Figure 4D,F). In contrast, the sugar content of the restoration lines was 14.14% higher on average than that of the wild-type and mutant (Figure 4E). These data show that *CoSWEET2a* expression increased seed sugar content without markedly affecting plant growth or yield.

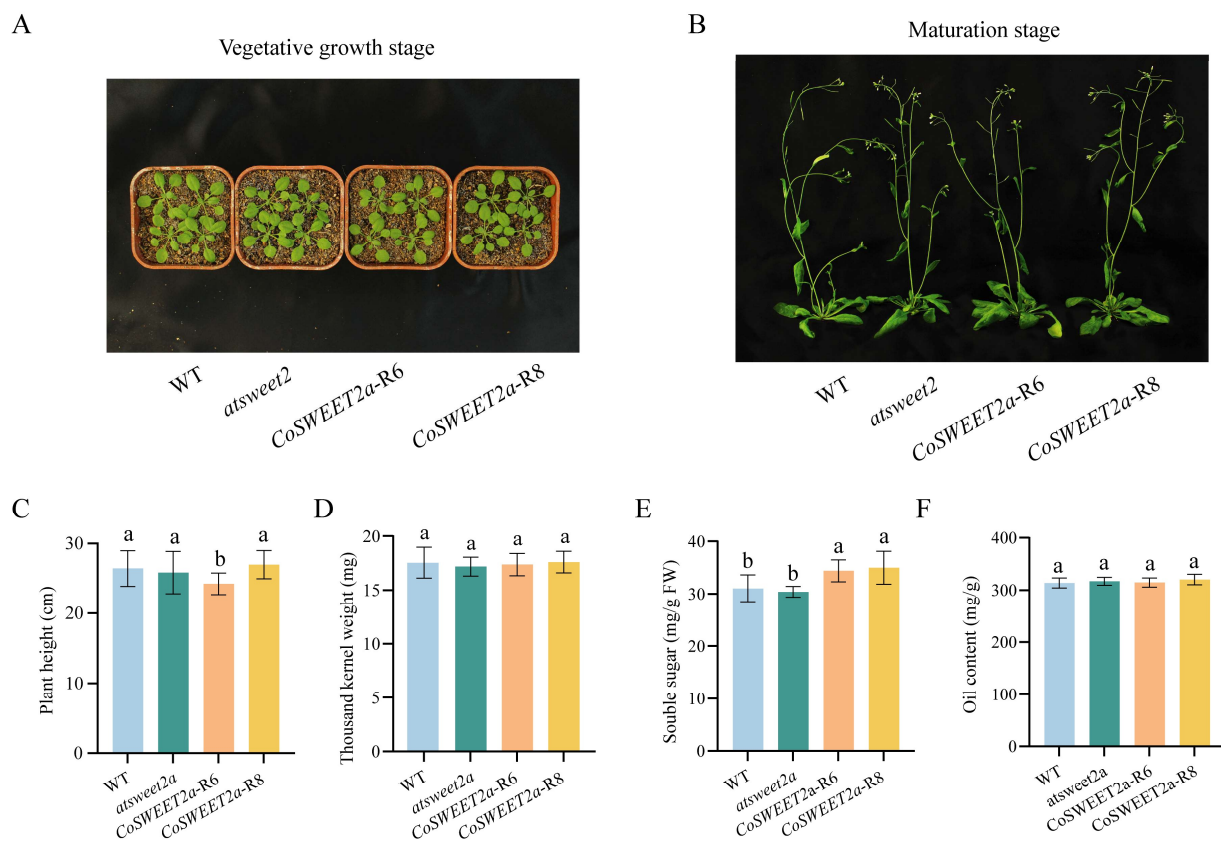


Figure 4. Phenotypic and physiological characterization of *Arabidopsis* transgenic lines. (**A,B**) Phenotypes of *Arabidopsis* wild type (WT), *atsweet2* mutants, and restoration lines (CoSWEET2a-R6, and CoSWEET2a-R8) during vegetative growth stage (**A**) and maturation stage (**B**). (**C**) Measurement of plant height in mature *Arabidopsis* plants. (**D–F**) Statistics of thousand-seed weight (**D**), determination of sugar content (**E**), and determination of oil content (**F**) in seeds from different *Arabidopsis* lines. Different letters indicate significant differences between groups ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$). Three independent biological replicates were used for the experiment.

2.5. Analysis of Seedling Root Growth of *Arabidopsis* Lines Under Different Sugar Treatments

WT, *atsweet2* mutant, CoSWEET2a-R6, and CoSWEET2a-R8 seedlings were used as study materials to examine how CoSWEET2a responds to various sugar types in plants. The seedlings were vertically cultured for one week on sugar free (no sugar), low-sugar (1.5%), and high-sugar (4%) sucrose, glucose, and fructose media, respectively. Under low-sugar conditions (Figure 5A), *atsweet2* showed a significant root length shortening phenotype, which could be rescued by CoSWEET2a. Compared to the WT, the restoration lines (R6, R8) showed an average increase of 16.1% in root length under fructose treatment. Both WT and the restoration lines' root length was reduced to varying degrees under high-sugar stress (Figure 5B). In contrast, the mutant exhibited minimal inhibition, suggesting that *AtSWEET2* deletion may lessen the effects of high-sugar stress. The greatest root length shortening in R6 and R8 was observed under 4% glucose conditions, suggesting that CoSWEET2a heterologous expression enhances high sugar stress and encourages glucose translocation. To sum up, the CoSWEET2a protein facilitates the uptake of hexose (glucose and fructose) and exhibits a response to sucrose.

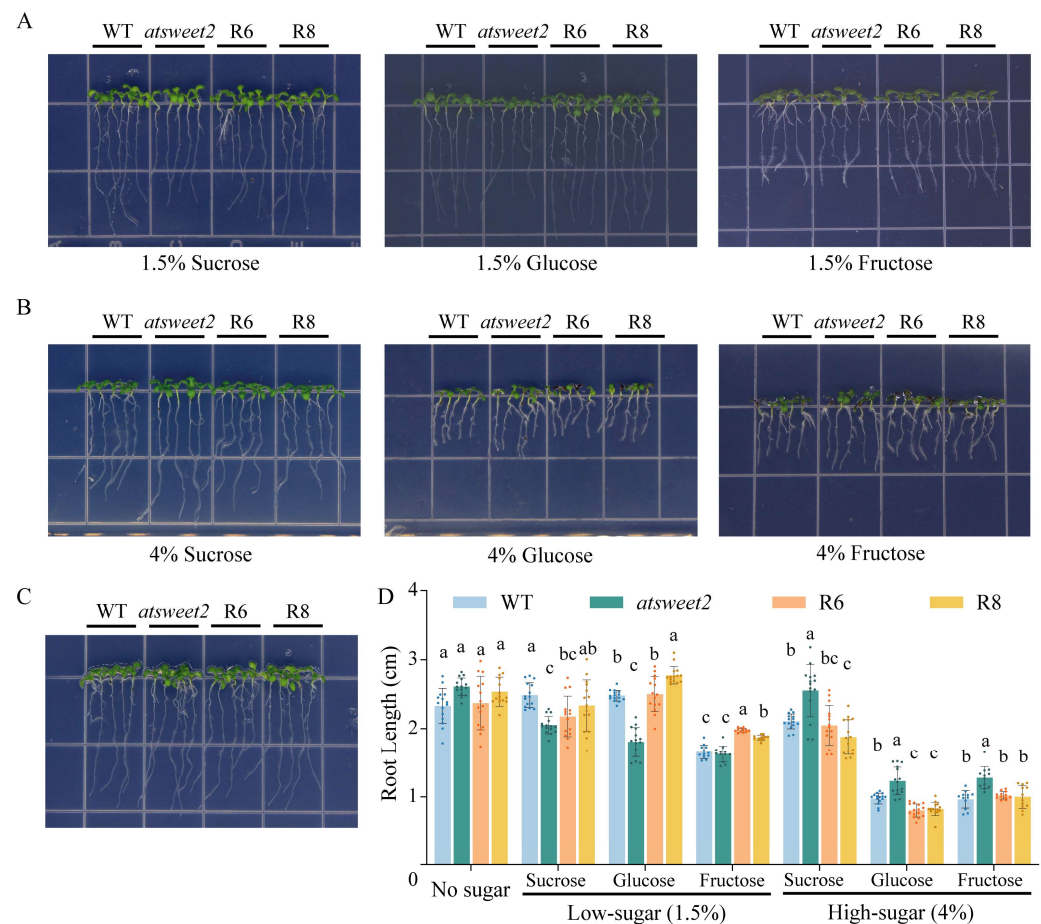


Figure 5. Phenotypic and root length analysis of *Arabidopsis* wild type (WT), *atsweet2* mutant, and two restoration lines (R6 and R8) under different sugar treatments. (A–C) Above-ground and root phenotypes of *Arabidopsis* treated with sugar free (no sugar) (C), sucrose, glucose, and fructose at concentrations of 1.5% (A) and 4% (B). (D) Main root length between *Arabidopsis* lines under different sugar treatments. The color circles represent individual data points. The seven treatments in the figure were analyzed independently using one-way ANOVA ($\alpha = 0.05$); multiple comparisons were performed using Duncan's method. Different letters indicate significant differences between groups ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$). Three independent biological replicates were used for the experiment.

2.6. Phenotypic Observation and Analysis of *Arabidopsis* Strains Under Drought Treatment

We evaluated drought resistance in several *Arabidopsis* lines for insight into how *CoSWEET2a* affects the plant's reaction to drought stress. Drought treatment was applied to four-week-old *Arabidopsis* seedlings for around two weeks. The *atsweet2* mutant's leaves were severely wilting (Figure 6A), and a reduced survival after recovery (Figure 6B). These findings imply that *CoSWEET2a* considerably improves plants' ability to survive drought.

Soluble sugar levels were measured in drought-treated and untreated plants across different lines (Figure 6C). Under drought, all *Arabidopsis* lines showed a considerable increase in soluble sugar content. Interestingly, the *CoSWEET2a* restoration lines showed greater increases (~45–51%) compared to wild-type (31.1%) and mutant (26.1%) lines.

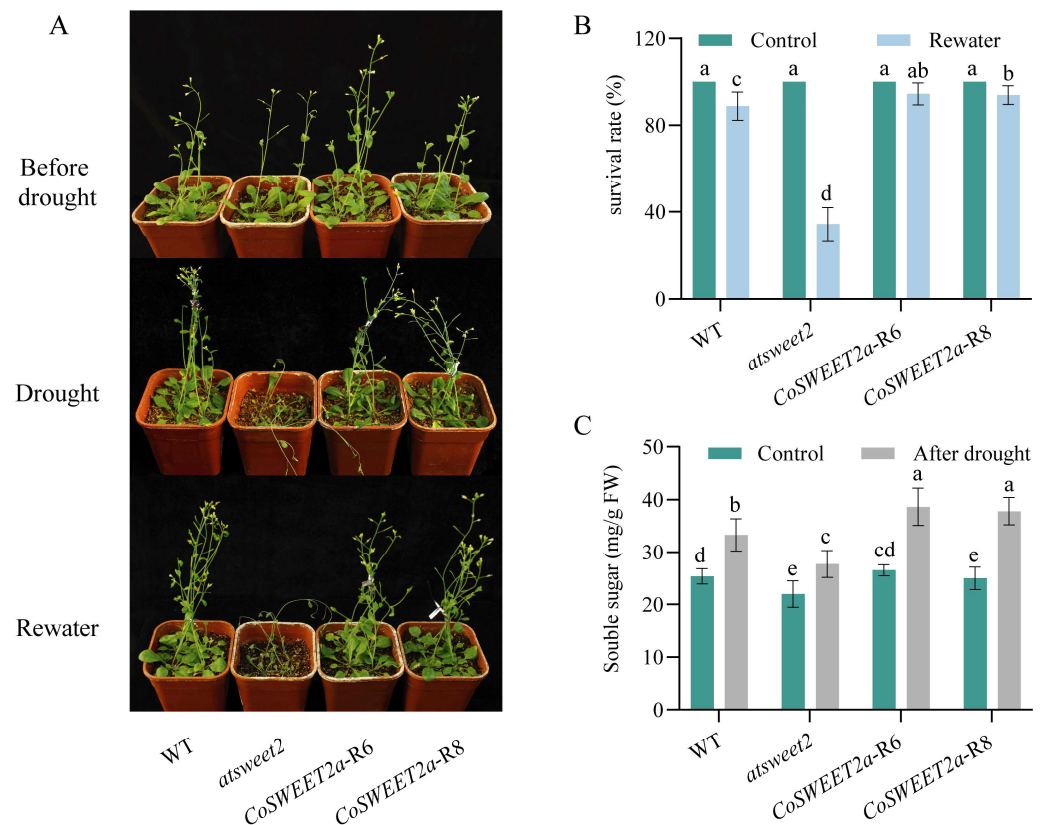


Figure 6. Phenotypic analysis of transgenic *Arabidopsis* under drought stress. **(A)** Phenotypic responses of *Arabidopsis* lines to drought treatment. Drought treatment for 14 days followed by rehydration for 5 days. **(B)** Survival rate of *Arabidopsis* seedlings on the fifth day of rehydration. Control is untreated group. Plants were considered survivors if their leaves turned green again or if new green leaves emerged. **(C)** The soluble sugar contents of WT, *atsweet2*, *CoSWEET2a-R6*, and *CoSWEET2a-R8* in whole *Arabidopsis* plants. Different letters indicate significant differences between groups ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$). Three independent biological replicates were used for the experiment.

2.7. Analysis of *CoSWEET2a* Gene Promoter Elements and Response to Different Hormones

Plants react to abiotic stressors through a variety of intricate processes, such as hormone synthesis, ROS scavenging, stress-responsive gene expression, and other tactics. By controlling downstream target genes, phytohormone signaling is one of them that can coordinate the stress response. This study first examined the upstream 2000 bp sequence of the *CoSWEET2a* gene. In addition to the fundamental elements, this region has four kinds of cis-acting elements (Figure S3). These include hormone-responsive elements like gibberellin response element (GARE motif), salicylic acid-responsive element (TCA-element) and abscisic acid-responsive elements (ABRE and AAGAA-motif), as well as regulatory elements for the abiotic stress response like the wound-responsive element (WUN-motif) (Figure 7A).

By providing exogenous hormones to immature oil tea seeds in vitro, the regulatory mechanism was further confirmed based on the aforementioned predictions (Figure 7B). After eight days of dark incubation, it was found that the *CoSWEET2a* gene was highly upregulated by both GA₃ and ABA, with relative expression increasing by around six and three times, respectively. The change in expression was not significantly impacted by treatments like SA and IAA (Figure 7C).

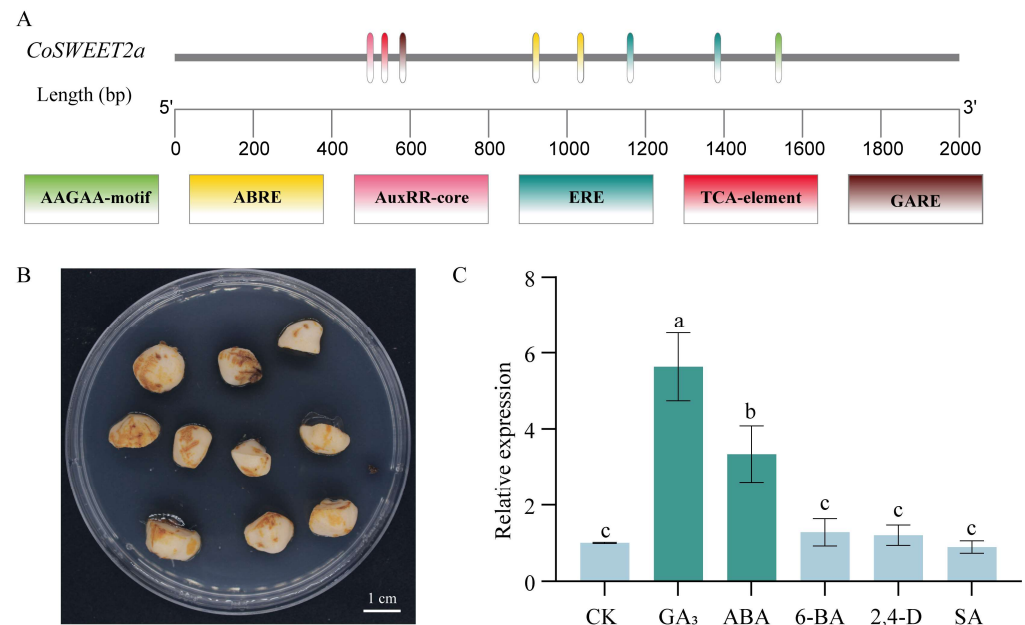


Figure 7. Promoter element analysis of *CoSWEET2a* and expression of *CoSWEET2a* under different hormone treatments. (A) Hormone-responsive elements in the *CoSWEET2a* promoter sequence. (B) Immature *C. oleifera* seeds cultured in vitro. (C) Expression patterns of *CoSWEET2a* under different hormone treatments: GA₃ (Gibberellin), ABA (Absciscic acid), 2,4-D (Auxin), and SA (Salicylic acid). Different letters indicate significant differences between groups ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$). Three independent biological replicates were used for the experiment.

3. Discussion

SWEET family proteins are found in both prokaryotes and eukaryotes, and they are essential for organ development, stress responses, and photosynthetic assimilate partitioning [29,30]. According to prior research, *CoSWEET2a* is localized to the vacuolar membrane in tobacco epidermal cells and protoplasts [24], but is found throughout the yeast cell [24]. This species-specific localization pattern motivated us to do the first subcellular localization investigation of *CoSWEET2a* in the native *C. oleifera* system. Our results confirmed the vacuolar localization of *CoSWEET2a* (Figure 1), which is consistent with the results of previous studies on homologous genes, such as *AtSWEET2* [31] and *OsSWEET2b* [11]. These results imply that the location of the vacuolar membrane may be a characteristic shared by some SWEET2 members in different plant species.

According to phylogenetic analysis, *CoSWEET2a* is a member of Clade I (Figure 2C). The primary function of SWEET proteins in Clade I is glucose transport [32]. However, yeast assays revealed *CoSWEET2a* transports glucose, sucrose, and fructose [24]. Our results confirm that *CoSWEET2a* responds to sucrose, fructose, and glucose in *Arabidopsis* (Figure 5A–C). This broad substrate specificity diverges from canonical Clade I functions and mirrors multisubstrate transporters like *Camellia sinensis* *CsSWEET1a* (Clade I), which transports hexoses and sucrose [15]. BiFC assay further indicates *CoSWEET2a* forms homodimers (Figure 3)—a feature previously linked to enhanced proteins' stability and transport effectiveness [27]. Taken together, *CoSWEET2a* represents an evolutionarily distinct Clade I transporter with expanded substrate range, likely enabled by its homodimeric state for sucrose transport.

Traditional sink-strengthening SWEETs directly enhance yield traits. For instance, rice *OsSWEET4c* promotes endosperm starch storage [33], and soybean *GmSWEET10a/b* supports embryo sugar supply [5]. In *C. oleifera*, *CoSWEET10* plays a comparable role in

seed development and oil accumulation [34]. By contrast, *CoSWEET2a* serves a distinct physiological role. Heterologous expression in *Arabidopsis* elevated seed sugar content without altering oil content or thousand-seed weight (Figure 4C–E), indicating its role in sugar translocation, but it had no discernible impact on plant growth or production.

CoSWEET2a is expressed in the transfer cell layer of *C. oleifera* seeds, according to in situ hybridization results [24]. And its subcellular localization to vacuolar membranes (Figure 1). We propose that *CoSWEET2a* might serve as a “vacuolar sugar buffer”. We speculate that it may regulate sugar transport within vacuoles in response to changes in inside the cell sugar concentrations. Vacuoles temporarily store extra sugars when their influx to plasma membrane surpasses the embryo’s capacity for utilization [25]. However, further experiments are needed to verify the transport properties of *CoSWEET2a*, such as in vitro verification using an African clawed frog oocyte expression system. In summary, *CoSWEET2a* demonstrates a decoupling of vacuolar sugar accumulation and biomass formation, representing a unique mechanism in sugar transport regulation.

Sugar enhances the plant’s tolerance to stress at the levels of physiological homeostasis and molecular pathways by acting as a signaling switch and an osmotic pressure buffer [30]. Under drought stress, the *CoSWEET2a* restoration lines showed high survival rates and elevated sugar content (Figure 6A–C), and the relative expression level of *CoSWEET2a* was upregulated in *C. oleifera* [25]. Drawing from prior studies, we postulate that *CoSWEET2a* could enhance drought resistance via two possible pathways: (1) *CoSWEET2a* contributes to the maintenance of cytoplasmic osmotic pressure during drought by exporting vacuolar sugar [35]. This vacuolar buffering mechanism allows plants to tolerate greater fluctuations in sugar concentrations, which could be an adaptive strategy for woody plants in variable environments. (2) Vacuolar sugar transporters may prioritize the redistribution of stored sugars from vacuoles to critical regions, such as meristematic tissues, under drought stress, ensuring essential metabolic needs are met. This aligns with the hypothesis proposed for *AtSWEET17* [13,36]. These results confirm that *CoSWEET2a* contributes to drought tolerance by regulating sugar homeostasis, consistent with its “vacuolar sugar buffer” strategy for sugar dynamic balance. *CoSWEET2a* represents a promising genomic target for developing drought-resistant *C. oleifera* cultivars through precision breeding. However, its long juvenile phase and tetraploid genome complicate transgenic approaches. We recommend marker-assisted selection of natural variants using CAPS/dCAPS markers as a faster alternative to develop stress-tolerant varieties.

Phytohormonal signaling pathways, which alter target genes involved in drought adaptation, are commonly used by plants to control stress responses [37]. In this study, gibberellin and abscisic acid significantly increased the expression of *CoSWEET2a* in *C. oleifera* seeds treated with exogenous hormones (Figure 7C), consistent with their antagonistic roles in seed development [38]. We speculate that drought stress causes ABA accumulation, which in turn triggers *CoSWEET2a* expression. Several ABA-responsive bZIP transcription factors, such as rice *OsZIP72* [39], apple *MdbZIP23/46* [40], have been shown to regulate SWEET gene expression. Since *CoSWEET2a* enhances drought resistance, identifying its regulators in *C. oleifera* could benefit breeding. Additionally, while certain SWEET family members (including *AtSWEET13/14* and *OsSWEET3a*) exhibit gibberellin transport activity [41,42], the capacity of *CoSWEET2a* to transport GA remains unconfirmed.

4. Materials and Methods

4.1. Plant Materials, Growth Conditions, and Stress Treatment

Fruit samples of *C. oleifera* ‘Huashuo’ were collected from the large-fruited oil tea test base in Changsha, Hunan Province. The fruits were harvested 230 days post-pollination (July), and the seeds were immediately frozen in liquid nitrogen and stored at -80°C .

for subsequent RNA extraction. Concurrently, fruit samples with attached branches were collected and moisturized for seed culture experiments.

Arabidopsis thaliana and *Nicotiana benthamiana* were grown in an artificial climate chamber under a 16 h light/8 h dark cycle at 25 °C with approximately 60% relative humidity. Four-week-old *Arabidopsis* seedlings of similar growth status were subjected to drought treatment by water withholding for 14 days followed by 5 days of rewatering, with normal watered plants serving as controls. The drought treatment duration was referenced from Ye et al. [34]. For sugar treatment experiments, *Arabidopsis* seeds were sown on 1/2 MS medium and incubated in the dark at 4 °C. After germination, the seedlings were transferred to vertical culture plates containing different sugars and continued to grow for 5 days.

Callus tissue was induced from axillary buds of *C. oleifera* and cultured in dark conditions at 24 °C in a medium containing 4.42 g/L MS, 30 g/L sucrose, 5 g/L 2,4-D, and 6 g/L agar.

4.2. Cloning and Subcellular Localization of CoSWEET2a Gene

Total RNA was extracted from *C. oleifera* seeds using the Total RNA Extraction Kit for Polysaccharides and Polyphenols (Tiangen, Beijing, China). Reverse transcription was performed using the AccuRT gDNA Removal Kit (Applied Biological Materials, Richmond, BC, Canada). Gene-specific primers were designed with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) (Table S3), and the target gene sequence was amplified using Primer STARMax DNA Polymerase (Takara Bio Inc., Kusatsu, Shiga, Japan), which offers high fidelity. The PCR products were separated by electrophoresis, and the target bands were subsequently recovered and ligated into the cloning T vector. The recombinant vector was then transformed into *E. coli* cells, and positive clones were selected using antibiotic resistance and sent for sequencing at Beijing Kinko Biotech.

The subcellular localization of CoSWEET2a was determined using an *C. oleifera* protoplast transient expression system. The CDS of CoSWEET2a, with the stop codon removed, was inserted into the overexpression vector pCAMBIA1300-GFP. Negative control was pCAMBIA1300-GFP empty vector. Protoplast isolation and PEG-mediated transformation were performed as described by Li et al. (2021, 2022) [43,44], except that the incubation time after transfection was shortened to 16 h in darkness. Fluorescence signals were observed with a Zeiss LSM 780 laser-confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using the GFP channel (440–500 nm).

4.3. Bioinformatics Analysis

The sequence of the CoSWEET2a gene is provided in Supplementary Table S2. The physicochemical properties of the CoSWEET2a protein were analyzed using the Expasy server (<https://web.expasy.org/> (accessed on 20 January 2025)). The tertiary structure of the CoSWEET2a protein was modeled using SWISS-MODEL (<https://swissmodel.expasy.org/> (accessed on 20 January 2025)). Transmembrane structural domains were predicted using the Protter tool (<http://wlab.ethz.ch/protter/start/> (accessed on 20 January 2025)). The homologous protein sequence of CoSWEET2a was retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/> (accessed on 20 January 2025)), and conserved structural domains were identified using the CDD website (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (accessed on 20 January 2025)). Amino acid sequence comparisons were performed with DNAMAN version 9 (Lynnon Biosoft, San Ramon, CA, USA), and the phylogenetic tree was constructed using MEGA11 version 11.0.13 (Pennsylvania State University, PA, USA). The amino acid sequences employed for this phylogenetic analysis are listed in Supplementary Table S3. Sequences 2000 bp upstream of the CoSWEET2a transcriptional

start site were extracted using TBtools v1.098 (<https://github.com/CJ-Chen/TBtools>), and the promoter region was analyzed for cis-acting elements using the online tools PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/> (accessed on 20 January 2025)) and New PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace> (accessed on 20 January 2025)).

4.4. Bimolecular Fluorescence Complementation Experiments

The determination of the homodimerization ability of CoSWEET2a in plants was performed using tobacco leaf transient transformation technology. The CDS of *CoSWEET2a* was amplified without the stop codon and subsequently ligated into the vectors pSPYCE (cYFP) and pSPYNE (nYFP). The correctly sequenced CoSWEET2a-cYFP and CoSWEET2a-nYFP plasmids were transferred into *A. tumefaciens* GV3101. Tobacco epidermal cells were co-transfected with a vacuolar membrane localization marker (vac-CD3-971) [28]. According to the experimental setup described in Zhang et al. [45]. Negative controls consisted of CoSWEET2a-nYFP paired with empty-cYFP and empty-nYFP paired with CoSWEET2a-cYFP. After 36–48 h of transient expression, fluorescence signals were observed with a Zeiss LSM 780 laser-scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) at 510 nm and 550 nm.

4.5. Generation of *Arabidopsis* Homozygous Mutants and Restoration Lines

Seeds of the *Arabidopsis atsweet2* mutant (SALK_034060) were obtained from the Arabidopsis Biological Resource Center (ABRC). The homozygous mutants were identified using a three-primer method: LP and RP primers were used to test the two insertion sites, while LBal was used to amplify the insertion fragment. Table S3 lists the primers that were used in this study. The recombinant *CoSWEET2a*-pCAMBIA1300 plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101. The *atsweet2* mutant was then stably transformed using the inflorescence infiltration method [34]. After identifying heterozygous overexpression-positive plants by PCR and RT-qPCR, T3-generation purity restoration lines were selected and further screened using Timentin. Two homozygous restoration lines, *CoSWEET2a*-R6 and *CoSWEET2a*-R8, with high expression levels, were selected for further studies (Figure S2). Wild-type *Arabidopsis* plants served as controls.

4.6. Phenotypic Observation and Physiological Analysis of *A. thaliana*

In order to investigate the impact of *CoSWEET2a* on plant-related traits, a comprehensive phenotypic assessment was conducted on the *Arabidopsis* wild type (WT), *atsweet2* mutant, and two restoration lines (R6 and R8). The plant height was measured from the soil surface to the highest point of the plant at the time of seed maturity and recorded accordingly. One thousand seeds were randomly selected using the quadrat method, and their total weight was measured. The total soluble sugar content in the seeds was determined using anthrone colorimetry. The same method was applied to measure the sugar content in the whole plants of drought-treated *Arabidopsis*. The root length of *Arabidopsis* seedlings from each line was measured on culture plates.

4.7. In Vitro Culture of *C. oleifera* Seeds Under Different Hormone Treatments

To examine the effects of phytohormones on sugar metabolism-related gene expression during seed development, fruits of *C. oleifera* were harvested at 230 days after pollination (DAP), rinsed under running water, and disinfected in a sterile environment. Seeds with smooth, glossy epidermises and uniform sizes were selected and placed in culture medium for in vitro cultivation, with 10 seeds per medium. Seed culture conditions followed the protocol established by Sosso et al. [33], with minor modifications.

Seeds were cultured on modified MS medium (4.42 g/L MS, 1 g/L acid-hydrolyzed complex protein, 1 mg/L streptomycin, 30 g/L sucrose, and 5.8 g/L agar.) Experimental groups received basal medium supplemented with 1 μ M dichlorophenoxyacetic acid (2,4-D), 1 μ M gibberellic acid (GA₃), 0.1 mM abscisic acid (ABA), and 10 mM salicylic acid (SA) in addition to the control medium. All cultures were incubated in the dark for 8 days.

4.8. RT-qPCR Analysis of CoSWEET2a Gene

To analyze the effect of hormone treatment on *CoSWEET2a* expression levels, RNA was extracted from *C. oleifera* seeds after 8 days of dark culture and reverse-transcribed into complementary DNA (cDNA) for subsequent RT-qPCR analysis, following the method outlined in Section 4.2. *CoActin* was used as the internal reference gene [46]. Primers for real-time quantitative PCR were designed as indicated in Table S1. The SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used as the reaction reagent, and quantitative reverse transcription PCR was conducted on the Step One Plus system, with an assay volume of 20 μ L. The relative expression levels of *CoSWEET2a* were calculated using the $2^{-\Delta\Delta CT}$ method [47].

4.9. Data Processing

SPSS 27.0 software was used for statistical analysis. Significance was analyzed by one-way analysis of variance (ANOVA) and multiple comparisons test. Different letters indicate significant differences between groups ($p < 0.05$), while the same letters indicate no significant differences ($p > 0.05$). Data are expressed as the mean of at least 3 replications $\pm$ SD. At least five plants were used for each replicate. GraphPad Prism 9.0 and Adobe Illustrator 2020 were used for plotting.

5. Conclusions

CoSWEET2a acts as a “vacuolar sugar buffer”, integrating sugar transport and hormone response to improve drought tolerance. By simultaneously boosting seed soluble-sugar content and drought tolerance, *CoSWEET2a* exemplifies the critical role that vacuolar sugar transporters play when they modulate sugar homeostasis, a process which is vital for environmental adaptation. Our findings identify *CoSWEET2a* as a precise target for drought-tolerant transgenic breeding, as well as a direct candidate gene for marker-assisted natural variation selection.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/plants14172618/s1>, Table S1: Coding sequence (CDS) of *CoSWEET2a*, Table S2: Amino acid sequences used for homologous protein alignment and phylogenetic tree construction, Table S3: Sequences of primers used in this study; Figure S1: Hydrophilicity analysis (A) and tertiary structure prediction (B) of the *CoSWEET2a* protein, Figure S2: Identification of mutant and restoration lines of *Arabidopsis*, Figure S3: Analysis of cis-acting elements in the promoter region of the *CoSWEET2a* gene.

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