Stem vacuole-targetted sucrose isomerase enhances sugar accumulation in sorghum

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Abstract

Background: Sugar accumulation is critically important in determining sugar crop productivity. Sorghum, especially high biomass sweet sorghum has shown great potential for biofuel. However, improvement in sugar content has been stagnant among sugar crops for decades. In this study, sorghum was investigated as a C4 diploid model for more complicated genomes such as maize and sugarcane. To promote sugar accumulation in sorghum, the sucrose isomerase (SI) gene, driven by stem-specific promoters (A2 or LSG) with a signal peptide, was designed to target the stem vacuole in grain sorghum inbred line (Tx430) and sweet x grain hybrids (Rio x Tx430). Results: The study demonstrated that transgenic lines of grain sorghum, containing 50-60% isomaltulose, accumulated sevenfold (804 mmol/L) more total sugar than the control Tx430 did (118 mmol/L) in stalks. Subsequently, the best-engineered line (LSG9) was crossed with an elite sweet sorghum variety (Rio). The total sugar concentrations (over 800 mmol/L) measured by HPLC (high-performance liquid chromatography), were significantly higher in both F1 and F2 progenies than the sweet sorghum Rio, representing a 57% and 69% increase respectively. Those total sugar contents in engineered sorghum lines are higher than in the field-grown sugarcane (normal range 600-700 mmol/L). Physiological characterization demonstrated that the superior progenies had notably increased rates of photosynthesis, sucrose transport, and sink strength. Conclusion: The genetic engineering approach has significantly enhanced total sugar concentration in grain sorghum and hybrids of (grain x sweet) sorghum. This research has put sorghum in the spotlight and frontier as a biofuel crop. More importantly, our results prove that the phenotype of high sugar accumulation is inheritable in the grain
sorghum as well as hybrids. The massive increase in sugar accumulation would lead to enormous financial benefits for industrial and biofuel use. This study would have a substantial impact on renewable energy due to the supreme capacity of total sugar accumulation in sorghum.

Background

Sugar yield is a key determinant of economic sustainability for sugar crops. In recent decades, improvement of sugar yield has been achieved almost entirely through increased biomass [1–3], despite the higher commercial value and higher heritability of increased sugar content [4]. Recent studies on the manipulation of plant genes, which are involved in sugar metabolism, have been unsuccessful for increasing sugar accumulation in sugar crops [5–7]. There is significant pathway redundancy in elite cultivars to buffer against increases in stored sucrose levels through the manipulation of a single gene [8]. Multiple mechanisms appear to contribute to the upper limit of sugar concentration, including regulation in signal transduction from specific (e.g. sucrose) or broad (e.g. osmotic) sensors, thermodynamic limitations (e.g. leakage of sucrose through storage compartment membranes), or energetic limitations (e.g. continuous ‘futile’ cycle of sucrose cleavage and synthesis within the storage pool) [9–12].

Among sugar crops, sweet sorghum is at the forefront of global interest as it demonstrates the huge potential to be multiple sources of energy, food and animal feed. It grows quickly in adverse stress conditions of marginal lands in tropical, subtropical and temperate zones. It is a C₄, drought tolerance, high biomass, and high water use efficiency plant that produces a stalk up to five meters tall with sucrose (α-D-glucopyranosyl-1,2-D-fructofuranose) accumulated in the stalks.
However, current sweet sorghum varieties, containing comparatively low sugar content (around 500 mmol/L), urgently requires breeders to improve sugar accumulation in stalks for biofuel [13].

Some bacteria have the ability to convert sucrose to isomaltulose (α-D-glucopyranosyl-1,6-D-fructofuranose) [14]. Unlike sucrose, isomaltulose is resistant to digestion by invertases [15] and is not metabolized by many microbes, including the predominant oral microflora, presenting advantages in many foods as an acariogenic sweetener [16]. However, isomaltulose can be digested by humans with the same glucose/fructose as primary products and have the same final energy value as sucrose. Interestingly, the first step of digestion involves an intestinal disaccharidase rather than salivary invertase, which slows down the isomaltulose digestion. The slow process results in less fluctuation of glucose and insulin concentration in blood [17]. Therefore, isomaltulose has a growing demand as a stable, slowly digestible, acariogenic, non-hygroscopic sugar in the modern world [17-19]. Furthermore, isomaltulose has an accessible carbonyl group, which makes it an attractive renewable starting material for manufacture of biomaterials as eventual petrochemical replacements [20]. The application is currently limited due to the high cost of isomaltulose production through fermentation [21, 22].

Isomaltulose can be produced through a sucrose isomerase (SI) without any cofactor or substrate activation [23], indicating the feasibility of this bio-production by appropriately expressing an SI gene in plants. Compared to sucrose, isomaltulose is very slowly metabolized and can not be transported in plants [24], hence the site of isomaltulose production becomes the storage site. Exogenous application of isomaltulose triggers some plant sugar sensing mechanisms and changes gene expression profiles differently from sucrose [24, 25]. It has been demonstrated that
the efficient conversion of sucrose into the non-metabolized isomer is lethal or creates severe disruption in growing plant tissues [26]. For example, tuber-specific expression of an apoplasm-targeted SI allowed the partial conversion of sucrose to isomaltulose (∼15 µmol/g fresh weight (FW)) in potato tubers without affecting plant appearance, but with a substantial decrease in total non-structural carbohydrate content [27, 28]. Significant progress has been made in last two decades, recent studies have indicated that the N-terminal pro-peptide (NTPP) fragment from sweet potato sporamin can target various proteins to the sugarcane vacuole, but low pH and high protease activity make this a hostile environment to introduced proteins [29]. With the availability of strong stem-specific promoters, a highly efficient SI gene cloned, and silencing motifs circumvented, high concentration of isomaltulose (up to 483 mM or 81% of total sugars in whole-cane juice from plants aged 13 months) has been successfully achieved in sugarcane [14, 23, 30]. To the best of our knowledge, a similar investigation has not been done in other biomass species. In the storage parenchyma cells of mature sweet sorghum stems, the sugar storage vacuole occupies about 90% of the symplast and 80% of the total tissue space. The vacuole stores a correspondingly large proportion of sucrose, which can accumulate up to 500 µmol/g fresh weight (FW). Our objective was to determine the effect of directing SI activity into the sucrose-storage cell compartment to improve sugar accumulation in sorghum. We hypothesized that high isomaltulose concentration could be accumulated in stems of engineered lines. The efficient transformation system of grain sorghum has been well established in our lab [31]. We strategically avoid transforming sweet sorghum directly since it is highly recalcitrant to tissue culture and transformation [32]. However, investigation on hybrids of (grain x sweet) sorghum provides insightful information on isomaltulose accumulation in
sweet sorghum and commercial hybrids sorghum.

Results

Accumulating substantial isomaltulose in transgenic lines

Twenty independent transgenic lines were demonstrated to contain the sucrose isomerase (SI) gene using the polymerase chain reaction (PCR) analysis. Among these lines, 16 showed detectable isomaltulose levels by high-performance liquid chromatography (HPLC) in stalk tissues (Fig. 1a). Isomaltulose was accumulated up to 472 mM in stalk juice, which was four-fold higher than the total sugar content of the untransformed Tx430. There were substantial differences in isomaltulose concentration between lines (Fig. 1b). For isomaltulose accumulation, similar patterns were observed in two transgenic populations driven by different promoters of A1 or LSG2 (Fig. 1b).

Because of the high specificity of the UQ68J SI for producing isomaltulose [23], trehalulose concentrations were below 4% of the isomaltulose concentrations in corresponding internodes (data not shown). Transgenic lines with the vacuole-targeted, silencing-optimized NTPP-68J SI, expression driven by a stem-specific promoter (A1 or LSG2) [33], were morphologically similar and equivalent in measured growth parameters to the untransformed control Tx430 grown in a containment glasshouse (Fig. S1). Two months after moving into the glasshouse, transgenic plants flowered at a similar time as the control Tx430 (Fig. S1).

When the roots and leaves were tested from all the transgenic lines, isomaltulose concentrations were below 5 mM in roots. Isomaltulose concentration increased with age in leaves to a maximum of about 20 mM, which is consistent with the expression patterns for the ‘stem-dominant’ promoters [33, 34]. However, SI
enzyme activity could not be detected from cell extracts of transgenic roots or leaves (data not shown). Despite substantial isomaltulose accumulation in stalks, SI enzyme activity was below the detection threshold in cell extracts, indicating a short half-life of this protein after delivery into the acidic/proteolytic sucrose storage vacuoles.

Enhancing total sugar content in grain sorghum

The majority of the transgenic lines increased their total sugar contents compared to the untransformed control, regardless of which promoter was used (Fig. 2). The total sugar content in internode number 4 of most lines was in a range of 600-1,000 mM, which was equivalent to five to eight folds of the untransformed control. These concentrations were comparable or even higher than that of the field-grown sugarcane (normally around 600–700 mM). The predominant components of sugar were sucrose and isomaltulose in transgenic lines, however, their glucose and fructose contents were similar to the parent (Fig. 2).

Unexpectedly, some transgenic lines such as L4 and A2 had no detectable isomaltulose but sucrose contents were enhanced five-fold to eightfold when compared to the control Tx430 (Fig. 2), regardless of the promoter used.

Accumulating high sugar contents across transgenic stalks

Three transgenic lines, designated A2, A5 (both driven by A1 promoter) and L9 (driven by LSG2 promoter), with high-sugar content were selected for further characterization on sugar profiles in developmental stages. Lines A5 and L9 accumulated high levels of isomaltulose down the stalk up to 691 mM in juice from mature internodes (Fig. 3c, d). Compared to the control Tx430, the transgenic lines with high yields of isomaltulose did not show commensurable reduction but
enhanced levels in stored sucrose concentrations in most internodes (Fig. 2).
Surprisingly, isomaltulose could not be detected in any A2 tissues including all internodes of the stalks, but sucrose content accumulated eightfold higher when compared to the level in the control Tx430 (Fig. 3b).

Inheriting high-sugar contents in hybrids

The elite sweet sorghum cultivar Rio was selected as a female partner for crossing due to its advantages of large biomass and high-sucrose content in stalks. Crosses were performed with the male-sterile lines of Rio. Transgenic line L9 was selected as the male partner based on its isomaltulose accumulation, high total sugar content and normal development in reproductive organs compared to other transgenic lines. Hybrid seeds were harvested from successful crossing.

Thirty seeds of hybrids were sown in pots along with the controls of Rio and Tx430 in the glasshouse. Another sweet sorghum cultivar R9188, a version of Rio with an extra dwarfing gene, hence almost 50 cm shorter, was used as an additional control. Germination and early plant growth were similar to the controls, except the progenies of one hybrid seed which did not germinate. Sugar profiles showed that among 29 progenies of the F$_1$ generation, 15 progenies were isomaltulose positive (51.7%) and 14 had no detectable isomaltulose (48.3%), close to the predicted 1:1 ratio (Fig. 4), indicating hybrid seeds inherited the SI gene sexually from the parent L9 to its progenies.

Within the isomaltulose positive group, three progenies (10.3%) converted almost all sucrose into IM; six (20.6%) converted more than 65% of sucrose; two (6.9%) converted about 33% of sucrose; four (13.8%) had less than 1% sucrose converted (Fig. 4). Notably, the enhancement of total sugar content was observed in all
isomaltulose positive groups (Fig. 4). The increase of total sugar content in the positive group was from 17–57% when compared to the sweet sorghum Rio. The increase ranged from 484–932% if compared with the grain sorghum Tx430, which is in agreement with the results of the first transgenic generation (Fig. 2).

Based on isomaltulose production, total sugar content, stalk weight, and seed production, progenies LR3, 19 and 20 were selected for further characterization. With the parental controls of sweet sorghum Rio, progeny 24, a null segregant with comparative high sugar content was also selected as a hybrid control. Seeds were produced by self-pollination of the selected progenies.

Sugar profiles of the isomaltulose positive plants showed that they inherited the phenotype of both isomaltulose production and high-sugar accumulation (Fig. 5). In all three SI positive progenies, isomaltulose accumulated at high levels in all internodes along the stalk, plus sucrose stored at comparable levels, resulting in enhancement by up to 69% in total sugar content compared to either the parental or the hybrid control (Fig. 5).

Increasing sugar content and decreasing water content in stalk juice
Carbon partitioning into sugars and fiber was estimated in the selected F2 progenies and controls. There was more sugar per unit fresh weight (FW) in all internodes of the tested high-sugar progenies along the stalk than the controls (Fig. 6a). In the sweet sorghum Rio and hybrid control P24, the water content was typically constant around 75% along the stalk with a slight increase in the bottom internodes, however, in the stalks of the three high-sugar progenies, water content was significantly lower at around 70% (Fig. 6b). Moreover, there were no significant changes in the fiber content among all samples, which was around 11% in internode
tissues (Fig. 6). These results indicated that instead of alteration of fiber and sugar, assimilation was improved and more sugar was stored in the progenies P3, P19, and P20 than the controls. Therefore, the commercially important traits of higher sugar concentration in juice from the selected progenies are underpinned by increasing the storage of photosynthate as sugars and decreasing water content in the mature stalk.

**Increasing photosynthesis in high-sugar hybrid lines**

Two key physiological characteristics, including photosynthetic electron transport and CO$_2$ assimilation, were examined to understand the mechanisms of enhanced sugar accumulation. Rates of leaf electron transport and CO$_2$ assimilation of the progenies P3, P19, and P20 were higher than the controls Rio, Tx430 and hybrid P24. The increases in electron transport rates measured by chlorophyll fluorescence (reflecting photosynthetic efficiency in photosystem II) and in CO$_2$ assimilation rates were in the range 20% – 35% improved relative to controls at a photosynthetically active radiation (PAR) level. Light response curves from fully expanded leaf 2 are shown as an example (Fig. 7). Also, the senescence of the bottom leaves on each stalk of the high-sugar progenies was typically delayed by 2-3 weeks, resulting in leaf functional extension in photosynthesis for most of the growth period.

**Improving sugar transport in source leaves and sink tissues**

Rate of proton gradient-dependent sucrose transport into plasma membrane vesicles (PMV) is an indicator for sucrose uploading in the source leaves [35]. The isolated PMVs from leaf 2 and 3 of the selected high-sugar progenies were 20% – 40% higher than that of controls (null segregant P24, parents Rio and Tx430), indicating the driving power of loading assimilation for transport was improved.
(Fig. 8a) in the source leaves of the high-sugar progenies. Sorghum phloem in a stem vascular bundle is symplasmically isolated from the surrounding parenchyma cells, and the sucrose unloading is apoplastic [36]. Cell wall invertase (CWI) activity is a determinant of sucrose gradient in the unloading area. In all tested internodes, CWI activities of the central storage parenchyma-rich zone were significantly higher in the high-sugar progenies than in the controls P24, Rio and Tx430 (Fig. 8b), but not in the peripheral vascular-rich zone (Fig. 8c). When the vascular bundles were dissected from the storage parenchyma cells in the central zone of internode 5 and assayed separately, the increased CWI activity in the high-sugar progenies was clearly restricted to the storage parenchyma (Fig. 8d), indicating the abilities on assimilate was increased within the sink tissues of the high-sugar progenies.

Discussion
The present study demonstrated that significantly higher sugar concentrations are achievable in sorghum (up to 1000 mM in grain sorghum, over 800 mM in hybrids of [grain x sweet] sorghum) by genetic engineering of the SI gene. The results of F₁ hybrids and F₂ segregants displayed that the phenotype of higher sugar accumulation is stably inheritable. This study demonstrates that sucrose isomerase can efficiently convert sucrose into isomaltulose and dramatically increase total sugar accumulation in sorghum. In addition, the superior progenies have significantly higher photosynthesis, higher sucrose transport, and higher sink strength, all of which could be the key drivers for higher sugar accumulation in plants. This approach provides a new perspective on the plant source-sink relationship. It would have a substantial impact on producing high-value sugar
isomaltulose and have enormous potential for renewable feedstocks for bio-energy and other high value compounds.

High-value isomaltulose has been successfully produced through genetic engineering, leading to a massive increase in total sugar accumulation in grain sorghum and F1, and F2 generations of sorghum. Lessons from sugarcane were important to the success of this study. Sucrose depletion was avoided by targeting the SI enzymes into sucrose-storage vacuoles [37]. Secondly, the disturbance on normal growth/functions of other organs was circumvented by using stem-specific expression of the SI gene [30, 33]. Finally, the SI gene sequence was modified to remove the motifs that trigger silencing in plants [30, 38]. To the best of our knowledge, this is the first report on engineering SI in sorghum and illustrated that high sugar concentration is achievable and heritable in sorghum. Sweet sorghum has been considered as a biofuel and biomass crop [13]. Our results displayed that sugar concentration can be increased by up to 69% in hybrids compared with sweet sorghum, which will boost industrial value at large scale.

The activity of the vacuole-targeted SI enzyme was undetectable in cell extracts because the sucrose-storage vacuoles are highly acidic and proteolytic. Rapid degradation of vacuole-targeted SI presumably protects against quick sucrose running down in growing tissues. It is believed that isomaltulose accumulates gradually in the stalk during development, probably because of the followings: (i) constant transcription of SI driven by the strong stem-specific LSG2 or ScR1MYB1 A1 promoter [30, 33]; (ii) high catalytic efficiency allowing occasional isomaltulose production before SI inactivation [23]; and (iii) very slow isomaltulose metabolism by plant enzymes [39]. For efficient commercialization of this valued sugar, it is essential to achieve proper patterns of developmental expression, cell
compartmentation, and enzyme stability in order to yield high isomaltulose content in stalks.

There has been an ongoing discussion as to whether current sugar crops have reached a physiological plateau with respect to sugar accumulation [40]. High-level sugar accumulation (> 1,000 mM disaccharides content), containing isomaltulose production (up to 691 mM) in stalk juice of the transgenic line (compared to the sugar content of 600–700 mM from the field-grown sugarcane juice) in this study, sheds lights on that the assumed ‘ceiling’ above sugar accumulation could be exceeded. Another example of the sugar enhancing effects was demonstrated by expressing a fructosyltransferase (FT) gene in sugarcane from Cynara scolymus [41]. The FT gene transformation in sugarcane led to 78% of stem sucrose being converted to fructants-like 1-ketose and inulin, resulting in a 63% increase in total soluble sugar content compared to the parent controls. The remarkable increases in sugar concentration by manipulating foreign genes like SI and FT can surpass the former ceiling in stored sugar content, indicating that the addition of a new vacuole-compartmentalized metabolic sink for sucrose deregulated prior constraining processes on sugar accumulation. Multiple biochemical processes were altered through analyse of transgenic plants [15, 37, 41].

Transgenic sorghum lines provide new insightful information on mechanisms as to how plants regulate sugar accumulation, a pivotal question in plant biology [42-45]. The phenotype of high total sugar concentration is attributed to delaying leaf senescence, increasing photosynthetic activity, and enhancing sucrose loading rates in source tissues, as well as higher activity in stalk storage parenchyma of CWI, which has multiple roles in sink tissues [44, 46]. Each of these activities will make a contribution to high sugar yield. Further comparative analysis of the superior lines
and their parent lines will help to reveal key molecular and physiological control points in plant source-sink flux. As all the reported experiments were undertaken under well-watered, controlled temperature glasshouse conditions, it is essential that field trial will be undertaken, given the considerable diurnal and seasonal temperature variations, as well as water and nutrient availability.

Sweetness is an important commercial trait in many food crops. Enhanced sweetness through a slowly digested, acariogenic sugar, such as IM, can bring direct health benefits for consumers [17]. Isomaltulose is naturally present at a very low level (0.1–0.7%) in honey and sugarcane extracts which are too small to be extracted [17]. In this study, isomaltulose can be accumulated at a notably high level (691 mM) in transgenic sorghum lines. It could be harvested and extracted at the commercial scale in the future.

The fermentable carbohydrate content is also a key determinant of the economic and environmental feasibility of renewable biofuel production [47, 48]. Sweet sorghum is widely considered as a biofuel crop [1]. Accumulation of higher sugar content would increase the economic value of renewable energy. In the longer term, sugars ultimately underpin all other biosyntheses in plants. The sugar boosting effect of the SI gene may be a foundation for higher sugar yields of many other bioenergy materials.

Conclusions

Our genetic engineering approach has successfully transformed the SI gene into sorghum and significantly improved total sugar content in both grain and sweet sorghum. Remarkably, the total sugar concentration in grain sorghum increased up to sevenfold compared with the control Tx430. Furthermore, the total sugar
concentration in $F_1$ and $F_2$ generations have improved 57% and 69% respectively compared with sweet sorghum Rio. The massive increase of sugar accumulation in sorghum would boost biofuel production at the commercial scale. More importantly, the higher sugar accumulation did show not any negative effect on growth morphologically in the LSG9 line that was selected as a parent for crossing. These results demonstrate that sorghum has considerable potential as a highly competitive biofuel and bio-industrial crop. It could play an important role in future bio-economy.

Materials and Methods

Constructs of sucrose isomerase gene

Constructs were prepared by recombining four parts. The first part is a 1.2 Kb sugarcane $ScR1MYB1$ A1 promoter (GenBank EU719199) [30] or a sugarcane loading stem gene promoter ($LSG2$, GeneBank JQ920356) [33]. The second part is a fragment encoding signal peptide of sweet potato sporamin NTPP as described [29, 37]. The third part is a modified gene version (GenBank KC147726) encoding the UQ68j SI enzyme [23, 30]. The fourth part is a terminator complex including three contiguous plant transcriptional terminator regions [30] intended to block read-through transcription in either direction (Fig. S2).

Sorghum transformation

Sweet sorghum has been considered as one of the most recalcitrant crops in terms of genetic transformation [32]. To successfully introduce the engineered SI construct into the large biomass sweet sorghum lines, an inbred line of grain sorghum Tx430 was first transformed. Then the Tx430 transgenic lines were used as a male partner for crossing with an elite sweet sorghum cultivar Rio as a female
partner. Rio is advantageous for its large biomass and has been used as a male-sterile parent line.

Each of the constructs, with the sucrose isomerase gene driven either by *LSG2* promoter or *Scr1MYB1 A1* promoter, was co-precipitated on gold particles with *pUKN* selectable marker construct [38, 49]. Transformation protocol by particle bombardment, conditions for selection of transgenic lines, plant regeneration, and growth conditions in the glasshouse were described as GQ Liu, BC Campbell and ID Godwin [49]. Briefly, embryogenic calli derived from immature embryos (11-15 days post-anthesis) were used as explants for transformation. Transformed calli were cultured for 8-12 weeks on selective regeneration media containing 30 mg L$^{-1}$ geneticin with subculturing onto fresh media fortnightly. Putative transgenic shoots were subsequently subcultured onto selective rooting media for 4 weeks following by a 3-day hardening off period. Details of the sorghum tissue culture system were used as described by GQ Liu, EK Gilding and ID Godwin [50].

PCR screening

Genomic DNA was extracted from the young leaves of the transgenic and non-transgenic plantlets prior to moving into the glasshouse. Extracted DNA quality and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). To confirm the *sucrose isomerase (SI)* gene, specific primer pairs were designed (Forward: 5’-AGCAACCCGATCTCAACTGG-3’ and Reverse: 5’-ACGGAGTCGTTCCATTGCAT-3’). PCR screening was undertaken in 20 μl reactions each containing 20 ng of template DNA, 0.5 μM of each specific primer and 10 μl of Taq 2× Master Mix (New England BioLabs). PCR reactions were performed using a BIO-RAD T100 Thermal Cycler®. The PCR program comprised of an initial
denaturation at 95 °C for 7 min, followed by 35 amplification cycles consisting of; 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final elongation step of 72 °C for 7 min. PCR products were separated by gel electrophoresis at 120 V for 1.5 h in 1.0% agarose gels (Fig. S3).

Growth conditions and crossing
Following the hardening off period, SI-positive transgenic plantlets and negative controls (Healthy transgenic plantlets with NPTII-positive but SI-negative in genomic PCR) were transferred to 20-liter pots with three plantlets per pot. Pots were randomized and grown in a temperature-controlled glasshouse (18–28 °C) for around 95 days until physiological maturity. Generally, transgenic plants and the controls started flowering 60 days after moving into the glasshouse. The transgenic plants grew as healthily as the control plants and appeared to be normal in morphology (Fig. S1). Starting from the same time when the transgenic plantlets were moved to the glasshouse, seeds of the sweet sorghum Rio were sowed in the same glasshouse in different batches with a one-week interval to match the flowering of the desired transgenic line for crossing. The crossing was performed as described [51].

Measuring sugar concentrations by high-performance liquid chromatography electrochemical detection (HPLC-ED)
For stalk samples, a transverse tissue slice was taken at the mid-point of each designated internode and cut into radial sectors that were proportionately representative of the different stalk tissues by area. Sectors were placed on a support screen (Promega Spin Basket, Madison, WI) within a 1.5-mL microfuge tube, liquid nitrogen frozen for 20 min, and then thawed on ice and centrifuged at 10 000 $g$ for 15 min at 4 °C to collect the juice. After the collected juice was boiled for 5
min to inactivate enzymes, the insoluble material was removed by centrifugation at 16 000 g for 20 min at 4 °C. In comparative tests conducted on internodes, this procedure gave sugar concentrations equivalent to the manual crushing of stalk samples to extract the juice. Moreover, it was adaptable to large scale samples. FWs were recorded before and after juice extraction and residual dry weights (DWs) were measured after 72 h at 75 °C for tissues, or 90 °C for juice samples. Water contents were measured in alternate subsamples to those used for juice extraction and analysis.

The resolution and quantification of IM, trehalulose, sucrose, glucose and fructose were achieved by isocratic HPLC at high pH (120 mM NaOH), using a Dionex BioLC system (Sunnyvale, CA) with PA20 analytical anion exchange column and quad waveform pulsed ED, with calibration against a dilution series of sugar standards for every sample batch [14, 37]. Sugar concentrations were corrected for dilutions in the procedure and presented as sucrose equivalents in juice. Total sugar contents were calculated on an FW and DW basis, taking account of the residual juice in internode tissues after centrifugation (up to 60% of total juice) and assuming 10% reduction in solute concentration in residual juice relative to first expressed juice, as typically observed in the industry [52]. For leaf samples, about 1 g FW of leaf blade without midrib was taken at one-third of the distance from the dewlap to the leaf tip. For root samples, about 0.5 g FW of young roots was taken from the interface between the soil and pot. Fluids were extracted and assayed by the freeze-thaw-centrifuge-HPLC method described above for stalk samples.

Gas exchange and chlorophyll fluorescence measurements

The photosynthetic electron transport rate was estimated from the fluorescence light curve generated using a fiber-optic MINI-PAM/F (Heinz Waltz GmbH, Effeltrich,
Germany) and leaf-clip holder 2030B positioned at one-tenth of the distance from
the dewlap to the leaf tip. The MINI-PAM light intensity, saturation pulse intensity,
saturation pulse width, leaf absorption factor and illumination time were set at 680
µmol/m²/s, 680 µmol/m²/s, 0.8 s, 0.84 and 10 s, respectively. The internal
temperature of the MINI-PAM was controlled between 25 and 30 °C during
measurement. An LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA)
was used to measure CO₂ fixation rates on the same leaves. Measurements were
made on at least three replicate plants per progeny.

Plasmalemma vesicle (PMV) isolation and transport assays

The blades of the second and third leaves from the top without midribs (12.5 g FW)
were homogenized in 50 mL solution which contains 240 mM sorbitol, 50 mM N-2-
hydroxyethylpiperazine-N’ 2-ethanesulphonic acid (HEPES), 3 mM ethyleneglycol-bis
(βaminoethylether)-N, N’-tetraacetic acid (EGTA), 3 mM dithiothreitol (DTT), 10 mM
KCl, 0.5% bovine serum albumin (BSA), 0.6% polyvinylpyrrolidone (PVP) and 2 mM
phenylmethyl sulphonyl fluoride (PMSF) (adjusted to pH 8.0 using solid Bistris
propane) at 4 °C. The homogenate was filtered through four layers of cheesecloth to
remove tissue debris and then centrifuged at 10 000 g for 10 min to remove
mitochondria and chloroplasts. Microsomal membranes were pelleted by
centrifugation at 50 000 g for 60 min. PMVs were purified from the microsomal
fraction by phase partitioning [35], washed in 25 mL of sorbitol-based re-suspension
buffer (SBRB) (330 mM sorbitol, 2 mM HEPES, 0.1 mM DTT, 10 mM KCl, pH 8.0 with
solid Bistris propane), repelleted by centrifugation at 50 000 g for 60 min and
resuspended at 3–5 mg FW mL⁻¹ of re-suspension buffer. The phase-purified PMVs
were layered over a 20%–50% sucrose gradient in 2 mM HEPES, 1 mM HCl and 1 mM
DTT (pH 8.0 with solid Bistris propane), centrifuged for 15 h at 100 000 g and collected in 1-mL fractions. The fractions were washed in 11 mL SBRB and pelleted by centrifugation at 100 000 g for 60 min. The pellet was suspended in 0.4 mL of SBRB, checked for purity using routine tests for enzymatic activities characteristic of other cellular membrane types, and used for transport experiments.

Transport assays were conducted at 12 °C using three replicate reactions per treatment (Bush et al., 1996). Briefly, for each reaction mixture, 20 µL of resuspended PMVs were diluted into 400 µL of assay buffer (as for SBRB, except adjusted to pH 6.0 with solid 2-[N-morpholino ethane sulphonate acid (MES)] containing 0.2 µCi (14C)sucrose and unlabelled sucrose to the desired concentration. At each time point, vesicles from one reaction mixture were collected on 0.45-µm filters and rinsed three times with 0.6 mL of assay buffer containing only unlabelled sucrose (1 mM). The accumulated radioactivity was measured by scintillation spectrometry. The difference between samples with and without 5 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was defined as ΔpH-dependent sucrose transport.

Internode tissue fractionation and enzyme assays

Transverse sections of each internode were divided into the outer rind of 2 mm thickness and two internal concentric cylinders at equal distances along the stalk radius. Of these, the central parenchyma-rich zone and the peripheral vascular-rich zone were examined for invertase activity. Furthermore, vascular bundles were separated by dissection from parenchyma tissue in the central zone for separate assays. The separated tissues were frozen immediately in liquid nitrogen for enzyme extraction, followed by the determination of CWI activity, using three replicate plants or dissected tissue subsamples per assay [53].
SI enzyme was extracted by grinding the frozen cells in a chilled mortar using three volumes of extraction buffer that contained 0.1 M Hepes-KOH buffer (pH 7.5), 10 mM MgCl₂, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 5 mM DTT, 2% polyvinylpolypyrrolidone and 1x complete protease inhibitor (Roche, Mannheim, Germany). The homogenate was immediately centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was immediately desalted on a PD-10 column (GE Healthcare, Buckinghamshire, UK) that was pre-equilibrated and eluted using the extraction buffer. Protein concentration was assayed by the Bradford reaction using a Bio-Rad kit (Hercules, CA, USA) with bovine serum albumin standards. SI activity was measured by incubating enzyme extract with 292 mM sucrose solution in 0.1 M citrate-phosphate buffer (pH 6.0) at 30 °C, and testing for isomaltulose accumulation over 80 min by HPLC-ED as described above.

Abbreviations

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Author contributions

LW, IDG, GL, and HJ designed the experiments. LW, YP, YZ, GL, and CD conducted the experiments and analyzed the data. LW, GL, and IDG wrote the manuscript. LW, IDG, and HJ supervised the project. All authors reviewed, edited, and approved the manuscript.

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Availability of data and materials

All data and materials generated or analyzed during this study are included in this published article and its Additional file.

Ethics approval and consent to participate

Ethical approval and consent to participate are not required.

Consent for publication

All authors agree for the submission and publication of manuscript in the journal Biotechnology for Biofuel.

Competing interests

The authors declare no competing interests.

Declarations

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**Figures**

![Figure 1](image_url)

Screening transgenic Tx430 sorghum lines for the presence of isomaltulose (IM)
Figure 2

Total sugar content in the control Tx430 (T1 to 5) and transgenic lines (A and L li
Figure 3
Sugar profiles of sorghum transgenic lines and the control Tx430. The plants were...

Figure 4
Total sugar content in the F1 hybrids (L9 X Rio). Sugars were measured 20 days p.
Sugar profiles along with the stalks of controls and the selected progenies of F2 č
Total sugar (a), water (b), and fiber (c) contents in the internodes of the control (Rio) and F2 progenies.
**Figure 7**

Photosynthetic electron transport rate and CO2 assimilation. (a) CO2 assimilation

**Figure 8**

Increased sucrose transport rate into PMV of source leaves. (a) increased CWI act
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