Stem vacuole-targeted 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 (*S*) gene, driven by stem-specific promoters *A1* (*A*) or *LSG2* (*L*) with a signal peptide, was designed to target the stem vacuole in grain sorghum inbred line (Tx430).

Results: The study demonstrated that transgenic lines of grain sorghum can accumulate isomaltulose which accounted for 50-60% of total sugar (up to 1012 mM) in stalks. While the average sugar content is 118 mM in the control Tx430. Subsequently, the best-engineered line (L9) was crossed with an elite sweet sorghum variety (Rio). The total sugar contents were significantly higher in both F₁ (up to 763 mM) and F₂ (up to 821 mM) progenies than the sweet sorghum Rio (485 mM), representing 57% and 69% increase respectively. Those total sugar contents in those engineered sorghum lines are higher than in the field-grown sugarcane (600-700 mM). Physiological characterization demonstrated that the superior progenies of F₂ hybrids had notably higher rates of photosynthesis, sucrose transport, and sink strength than controls.

Conclusion: The genetic engineering approach has significantly enhanced total sugar content in grain sorghum and hybrids of (sweet X grain) 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 content is **heritable** 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 transgenic sorghum.

Background

Sugar yield is a key determinant of economic sustainability for sugar crops. In recent decades, the 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 content, 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 mM) compared with sugarcane (600-700 mM), urgently requires breeders to improve sugar accumulation in stalks for biofuel [13].

It is reported that some bacteria can 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 process of isomaltulose digestion. This phenomenon 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 a 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 [$\sim 15 \mu\text{mol/g}$ 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 the 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 mM sugar content in stalks. 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 content could be accumulated in stems of engineered lines. Since the efficient transformation system of grain sorghum has been well established in our lab [31]. We strategically avoid transforming sweet sorghum directly due to its recalcitrance to tissue culture and transformation [32]. However, investigation on hybrids of (sweet X grain) sorghum would provide 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. **A representative chromatogram (Fig. 1a) is shown the detection of sugar profile including glucose, fructose, sucrose, trehalulose, and isomaltulose, by high-performance liquid chromatography (HPLC).** Among these transgenic lines, 16 of 20 showed detectable isomaltulose levels. Isomaltulose was accumulated up to 446 mM in stalk juice, which was four-fold higher than the total sugar content of the untransformed Tx430 (118 mM). There were substantial differences in isomaltulose content between transgenic lines (Fig. 1b). As for isomaltulose accumulation, similar patterns were observed in two transgenic populations driven by promoters of *A* or *L* (Fig. 1b).

Because of the high specificity of the UQ68J SI for producing isomaltulose [23], trehalulose concentrations in stem juice of most positive samples were below 5.0% of the isomaltulose concentrations in corresponding internodes (**Table S1**). Most transgenic lines with the vacuole-targeted, silencing-optimized NTPP-68J SI, expression driven by a stem-specific promoter (*A* or *L*) [33], were morphologically similar and equivalent in measured growth parameters to the untransformed control Tx430 grown at the vegetative growth stage in the PC2 glasshouse (Fig. S1). 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 content increased with age in leaves to a maximum of 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. Despite substantial isomaltulose accumulation in stalks, SI enzyme activity in stalk 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 transgenic lines notably 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 (from the top) 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 total sugar contents were comparable or even higher than that of the field-grown sugarcane (normally 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 control Tx430 (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).

Accumulating high sugar contents across transgenic stalks

Three transgenic lines with high-sugar contents, A2, A5 driven by *A1(A)* promoter and L9 driven by *LSG2* (*L*) promoter), were selected for further characterization on sugar profiles of all internodes (Fig. 3) in developmental stages (20 days post anthesis). The control Tx430 accumulated higher sugar content in the first internode (close to panicle) than the rest internodes (Fig. 3a). On the contrary, the transgenic lines accumulated much less sugar in the first internode than the rest internodes (Fig. 3b, 3c, and 3d). Lines A5 and L9 accumulated high levels of isomaltulose in the stalk up to 691 mM in juice from mature internodes (Fig. 3c, 3d). 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. 3).

Surprisingly, isomaltulose could not be detected in any A2 tissues including all internodes of the stalks, but sucrose content accumulated eightfold higher in A2 than in the control Tx430 (Fig. 3b).

Inheriting high-sugar content 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 **cytoplasmic male-sterile** version of Rio **which is used in the sorghum breeding program in Australia**. 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 F₁ seeds were harvested from successful crossing.

Thirty seeds of hybrids were sown in pots along with the controls of Rio and Tx430 in the PC2 glasshouse. Another sweet sorghum cultivar R9188, a version of Rio with an extra dwarf gene, hence almost 50 cm shorter, was used as an additional control. Germination and early plant growth were similar to controls, except the progenies of one hybrid seed which did not germinate. Sugar profiles showed that among 29 progenies of the F₁ 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 that hybrid seeds inherited the *Sl* gene sexually from the parent L9.

Within the isomaltulose positive group, three progenies (10.3%), named LR27, LR17, and LR9, converted almost all sucrose into IM (last three bars in Fig. 4); 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 most isomaltulose positive groups (Fig. 4). The increase of total sugar content in the positive group was from 17% to 57% when compared to the sweet sorghum Rio. The increase ranged from 484% to 932% if compared with the grain sorghum Tx430, which is in agreement with the results of the first transgenic T₀ generation (Fig. 2).

Based on isomaltulose production, total sugar content, stalk weight, and seed production, progenies P3 from LR3, P19 from LR19, and P20 from LR20 outperformed the rest and were selected for further characterization. With the parental controls of sweet sorghum Rio, **a null segregant progeny P24 from LR24 was also selected as a hybrid control because it was negative of the *Sl* gene and no detectable isomaltulose**, with comparative high sugar content. Seeds were produced by self-pollination of the selected progenies and harvested at maturity.

Sugar profiles of the isomaltulose positive plants displayed that they inherited the phenotype of both isomaltulose production and high-sugar accumulation (Fig. 5). In all three *Sl*/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 (P3, P19, and P20) and controls (Rio, and P24). 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 progeny 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). Analysis of variance (ANOVA) with Bonferroni post-tests showed that all three selected *Sl*-positive progenies had significantly increased sugar content and reduced water content ($P < 0.001$) in all tested internodes, compared with the Rio and hybrid P24 controls. 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 content 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 CO₂ assimilation, were examined to understand the mechanisms of enhanced sugar accumulation. Rates of leaf electron transport and CO₂ 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 CO₂ 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). Analysis of variance (ANOVA) with Bonferroni post-tests showed significant differences ($P < 0.001$) in both electron transport and CO₂ fixation rates in all tested light intensities between any control and P3, P19 or P20. 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 of 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 apoplasmic [36]. Cell wall invertase (CWI) activity is a determinant of the 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 restricted to the storage parenchyma (Fig. 8d), indicating the abilities on assimilation was increased within the sink tissues of the high-sugar progenies.

Discussion

The present study demonstrated that significantly higher total sugar contents are achievable in sorghum (over 800 mM in grain and hybrids of [sweet X grain] sorghum) by genetic engineering of the *S1* gene. The results of F₁ hybrids and F₂ progenies displayed that the phenotype of high-sugar accumulation is stably

inherited. This study demonstrates that sucrose isomerase can efficiently convert sucrose into isomaltulose and dramatically increase total sugar accumulation in sorghum. Besides, 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 in plants and have illustrated the enormous potential for renewable bio-energy and other high-value compounds for plant engineering.

High-value isomaltulose has been successfully produced through genetic engineering, leading to a massive increase in total sugar accumulation in grain sorghum, F_1 , and F_2 hybrids. Lessons from sugarcane were important to the success of this project. Firstly, 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 promoters for the *S/* gene expression [30, 33]. Finally, the *S/* gene sequence was modified to remove the motifs that trigger silencing in plants [30, 38].

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 *S/* driven by the strong stem-specific *L* or *ScR1MYB1A* 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 for producing high isomaltulose content in stalks.

There has been an ongoing discussion as to whether current sugar crops have reached a physiological plateau regards to sugar accumulation [40]. Sweet sorghum has been considered as a biofuel and biomass crop [13]. Our results displayed that total sugar content can be increased by up to 69% in hybrids compared with sweet sorghum, which will boost industrial value at large scale. 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 content by manipulating foreign genes like *S/* 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 content 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 contribute to high-sugar yield. **Further comparative analysis (e.g. a field trial) of the superior 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 crops. In the longer term, sugars ultimately underpin all other biosyntheses in plants. The sugar boosting effect of the *S*/gene may be a foundation for higher sugar yields of many other bioenergy materials such as algae.

Conclusions

The *S*/gene has successfully been transformed into sorghum and significantly improved total sugar content in both grain and hybrid (sweet X grain) sorghum. Remarkably, the total sugar content in grain sorghum increased up to sevenfold compared with the control Tx430. Furthermore, the total sugar content in F₁ and F₂ generations have improved 57% and 69% respectively compared with the parental control 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 not show any negative effect on growth morphologically in the L9 line that was selected as a parent for crossing. **More investigations are needed to value the total sugar yield in the future.** 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 *ScR1MYB1A* promoter (GenBank EU719199) [30] or a sugarcane *loading stem gene* promoter (*L*, 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 *L* (*LSG2*) promoter or *ScR1MYB1A* (*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 young leaves of the transgenic and non-transgenic plants. The 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 carried out 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

After three days hardening-off period, transgenic plantlets and controls were transferred to 20-liter pots with three plantlets per pot. Pots were randomized and grown in a PC2 temperature-controlled glasshouse (18-28 °C). 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). For crossing, starting from the same time when the transgenic plantlets were moved to the glasshouse, seeds of the sweet sorghum Rio (male-sterile line) were sowed in pots with different batches one-week interval to match the flowering of the desired transgenic line. The crossing was performed as described by LR House [51].

Measuring sugar contents by high-performance liquid chromatography electrochemical detection (HPLC-ED)

In the beginning, the main stalks of the wild-type Tx430 as the control and T₀ transgenic plants above the ground were harvested from the PC2 glasshouse 20 days post-anthesis. Leaves including leaf sheaths were removed. Then internodes were cut off separately and marked in order from top to bottom. After that, the middle of each internode, the flag leaf, and root were sampled. After the protocol was established, the fourth internode was selected as a standard stalk sample for the sugar profile measurement.

For stalk, leaf, and root samples, the process of sample preparation for HPLC-ED was described as LG Wu and RG Birch [37].

Gas exchange and chlorophyll fluorescence measurements

The methods of gas exchange and chlorophyll fluorescence measurement were described as LG Wu and RG Birch [37]. Briefly, the photosynthetic electron transport rate was measured by a fiber-optic MINI-PAM/F (Heinz Waltz GmbH, Effeltrich, Germany). The parameters of the MINI-PAM light intensity, saturation pulse intensity, saturation pulse width, leaf absorption factor and illumination time were adjusted at 680 $\mu\text{mol}/\text{m}^2/\text{s}$, 680 $\mu\text{mol}/\text{m}^2/\text{s}$, 0.8 s, 0.84 and 10 s, respectively. An LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) was utilized to measure CO₂ fixation rates on the same leaves. Measurements were taken in the morning from 10 AM to 12 AM. Three plants per progeny were measured as biological replicates.

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 (β aminoethylether)-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 PMV isolation and transport assays were described as LG Wu and RG Birch [37].

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 [52].

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(pH7.5), 10 mM MgCl₂, 2 mM EDTA, 2mM 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.

Declarations

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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 in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Ethical approval and consent to participate are not required.

Consent for publication

All authors agree to publish this article.

Competing interests

The authors declare no competing interests.

Abbreviations

SI: Sucrose isomerase; FW: fresh weight; NTPP: N-terminal pro-peptide; PCR: polymerase chain reaction; HPLC: high-performance liquid chromatography; PAR: photosynthetically active radiation; PMV: plasma membrane vesicles; CWI: cell wall invertase; FT: fructosyltransferase; HPLC-ED: high-performance liquid chromatography electrochemical detection; DW: dry weight; PMV: plasmalemma vesicle; HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; EGTA: ethyleneglycol-bis (β-aminoethylether)-N, N'-tetraacetic acid; DTT: dithiothreitol; BSA: bovine serum albumin; PVP: polyvinylpyrrolidone; PMSF: phenylmethyl sulphonyl fluoride; SBRB: sorbitol-based re-suspension buffer; MES: N-morpholino ethane sulphonic acid; CCCP: carbonyl cyanide m-chlorophenyl hydrazone.

References

1. Mathur S, Umakanth AV, Tonapi VA, Sharma R, Sharma MK: **Sweet sorghum as biofuel feedstock: recent advances and available resources.** *Biotechnology for Biofuels* 2017, **10**.
2. Holou RAY, Stevens G: **Juice, sugar, and bagasse response of sweet sorghum (*Sorghum bicolor* (L.) Moench cv. M81E) to N fertilization and soil type.** *Gcb Bioenergy* 2012, **4**(3):302-310.
3. Soileau JM, Bradford BN: **Biomass and Sugar Yield Response of Sweet Sorghum to Lime and Fertilizer.** *Agron J* 1985, **77**(3):471-475.
4. Jackson PA: **Breeding for improved sugar content in sugarcane.** *Field Crop Res* 2005, **92**(2-3):277-290.
5. Lakshmanan P, Geijskes RJ, Aitken KS, Grof CLP, Bonnett GD, Smith GR: **Sugarcane biotechnology: The challenges and opportunities.** *In Vitro Cell Dev-Pl* 2005, **41**(4):345-363.

6. Manners JM: **Functional Genomics of Sugarcane**. *Adv Bot Res* 2011, **60**:89-168.
7. Jiang SY, Chi YH, Wang JZ, Zhou JX, Cheng YS, Zhang BL, Ma A, Vanitha J, Ramachandran S: **Sucrose metabolism gene families and their biological functions**. *Sci Rep-Uk* 2015, **5**.
8. Capell T, Christou P: **Progress in plant metabolic engineering**. *Curr Opin Biotech* 2004, **15**(2):148-154.
9. Moore PH: **Temporal and Spatial Regulation of Sucrose Accumulation in the Sugarcane Stem**. *Aust J Plant Physiol* 1995, **22**(4):661-679.
10. Grof CPL, Campbell JA: **Sugarcane sucrose metabolism: scope for molecular manipulation**. *Aust J Plant Physiol* 2001, **28**(1):1-12.
11. Bindon KA, Botha FC: **Tissue discs as an experimental system for metabolic flux analysis in the sugarcane culm**. *S Afr J Bot* 2001, **67**(2):244-249.
12. Gutjahr S, Clément-Vidal A, Trouche G, Vaksman M, Thera K, Sonderegger N, Dingkuhn M, Luquet D: **Functional analysis of sugar accumulation in sorghum stems and its competition with grain filling among contrasted genotypes**. In: *Proceedings of Agro 2010 : the XIth ESA Congress: 2010; Montpellier, France*. Agropolis international: 89-90.
13. Mathur S, Umakanth AV, Tonapi VA, Sharma R, Sharma MK: **Sweet sorghum as biofuel feedstock: recent advances and available resources**. *Biotechnol Biofuels* 2017, **10**:19.
14. Wu L, Birch RG: **Characterization of Pantoea dispersa UQ68J: producer of a highly efficient sucrose isomerase for isomaltulose biosynthesis**. *J Appl Microbiol* 2004, **97**(1):93-103.
15. Wu LG, Birch RG: **Physiological basis for enhanced sucrose accumulation in an engineered sugarcane cell line**. *Funct Plant Biol* 2010, **37**(12):1161-1174.
16. Jonker D, Lina BAR, Kozianowski G: **13-week oral toxicity study with isomaltulose (Palatinose (R)) in rats**. *Food Chem Toxicol* 2002, **40**(10):1383-1389.
17. Sawale PD, Shendurse AM, Mohan MS, Patil GR: **Isomaltulose (Palatinose) - An emerging carbohydrate**. *Food Biosci* 2017, **18**:46-52.
18. Takazoe I: **Palatinose – an isomeric alternative to sucrose**. . In: *Progress in Sweeteners* Edited by Grenby TH. Barking: Elsevier; 1989: 143-167.
19. Lina BAR, Jonker D, Kozianowski G: **Isomaltulose (Palatinose (R)): a review of biological and toxicological studies**. *Food Chem Toxicol* 2002, **40**(10):1375-1381.
20. Lichtenthaler FW, Peters S: **Carbohydrates as green raw materials for the chemical industry**. *Cr Chim* 2004, **7**(2):65-90.
21. Schiweck H, Munir, M., Rapp, K.M., Schneider, B. and Vogel, M. : **New developments in the use of sucrose as an industrial bulk chemical**. In: *Carbohydrates as Organic Raw Materials* Edited by Lichtenthaler FW. Weinheim: Wiley-VCH; 1991: 57–94.
22. Buchholz K, Kasche, V., Bornscheuer, U. T.: **Biocatalysts and Enzyme Technology**: Wiley-Blackwell; 2012.
23. Wu LQ, Birch RG: **Characterization of the highly efficient sucrose isomerase from Pantoea dispersa UQ68J and cloning of the sucrose isomerase gene**. *Appl Environ Microbiol* 2005, **71**(3):1581-1590.

24. Loreti E, Alpi A, Perata P: **Glucose and disaccharide-sensing mechanisms modulate the expression of alpha-amylase in barley embryos.** *Plant Physiol* 2000, **123**(3):939-948.
25. Sinha AK, Hofmann MG, Romer U, Kockenberger W, Elling L, Roitsch T: **Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato.** *Plant Physiol* 2002, **128**(4):1480-1489.
26. Bornke F, Hajirezaei M, Heineke D, Melzer M, Herbers K, Sonnewald U: **High-level production of the non-cariogenic sucrose isomer palatinose in transgenic tobacco plants strongly impairs development.** *Planta* 2002, **214**(3):356-364.
27. Bornke F, Hajirezaei M, Sonnewald U: **Potato tubers as bioreactors for palatinose production.** *J Biotechnol* 2002, **96**(1):119-124.
28. Hajirezaei MR, Bornke F, Peisker M, Takahata Y, Lerchl J, Kirakosyan A, Sonnewald U: **Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers (*Solanum tuberosum*).** *J Exp Bot* 2003, **54**(382):477-488.
29. Gnanasambandam A, Birch RG: **Efficient developmental mis-targeting by the sporamin NTPP vacuolar signal to plastids in young leaves of sugarcane and *Arabidopsis*.** *Plant Cell Rep* 2004, **23**(7):435-447.
30. Mudge SR, Basnayake SWV, Moyle RL, Osabe K, Graham MW, Morgan TE, Birch RG: **Mature-stem expression of a silencing-resistant sucrose isomerase gene drives isomaltulose accumulation to high levels in sugarcane.** *Plant Biotechnology Journal* 2013, **11**(4):502-509.
31. Liu GQ, Godwin ID: **Highly efficient sorghum transformation.** *Plant Cell Rep* 2012, **31**(6):999-1007.
32. Raghuwanshi A, Birch RG: **Genetic transformation of sweet sorghum.** *Plant Cell Rep* 2010, **29**(9):997-1005.
33. Moyle RL, Birch RG: **Sugarcane Loading Stem Gene promoters drive transgene expression preferentially in the stem.** *Plant Mol Biol* 2013, **82**(1-2):51-58.
34. Mudge SR, Osabe K, Casu RE, Bonnett GD, Manners JM, Birch RG: **Efficient silencing of reporter transgenes coupled to known functional promoters in sugarcane, a highly polyploid crop species.** *Planta* 2009, **229**(3):549-558.
35. Bush DR, Chiou TJ, Chen LS: **Molecular analysis of plant sugar and amino acid transporters.** *J Exp Bot* 1996, **47**:1205-1210.
36. Bihmidine S, Baker RF, Hoffner C, Braun DM: **Sucrose accumulation in sweet sorghum stems occurs by apoplastic phloem unloading and does not involve differential Sucrose transporter expression.** *Bmc Plant Biol* 2015, **15**.
37. Wu LG, Birch RG: **Doubled sugar content in sugarcane plants modified to produce a sucrose isomer.** *Plant Biotechnol J* 2007, **5**(1):109-117.
38. Basnayake SWV, Morgan TC, Wu LG, Birch RG: **Field performance of transgenic sugarcane expressing isomaltulose synthase.** *Plant Biotechnol J* 2012, **10**(2):217-225.

39. Wu LG, Birch RG: **Isomaltulose Is Actively Metabolized in Plant Cells.** *Plant Physiol* 2011, **157**(4):2094-2101.
40. Inman-Bamber NG, Jackson PA, Hewitt M: **Sucrose accumulation in sugarcane stalks does not limit photosynthesis and biomass production.** *Crop Pasture Sci* 2011, **62**(10):848-858.
41. Nell JS: **Genetic manipulation of sucrose-storing tissue to produce alternative products.** South Africa.: The University of Stellenbosch 2007.
42. Rolland F, Moore B, Sheen J: **Sugar sensing and signaling in plants.** *Plant Cell* 2002, **14**:S185-S205.
43. Fernie AR, Roessner U, Geigenberger P: **The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers.** *Plant Physiol* 2001, **125**(4):1967-1977.
44. Koch K: **Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development.** *Curr Opin Plant Biol* 2004, **7**(3):235-246.
45. Fernie AR, Geigenberger P, Stitt M: **Flux an important, but neglected, component of functional genomics.** *Curr Opin Plant Biol* 2005, **8**(2):174-182.
46. Proels RK, Huckelhoven R: **Cell-wall invertases, key enzymes in the modulation of plant metabolism during defence responses.** *Mol Plant Pathol* 2014, **15**(8):858-864.
47. De Oliveira MED, Vaughan BE, Rykiel EJ: **Ethanol as fuels: Energy, carbon dioxide balances, and ecological footprint.** *Bioscience* 2005, **55**(7):593-602.
48. Long HL, Li XB, Wang H, Jia JD: **Biomass resources and their bioenergy potential estimation: A review.** *Renew Sust Energ Rev* 2013, **26**:344-352.
49. Liu GQ, Campbell BC, Godwin ID: **Sorghum Genetic Transformation by Particle Bombardment.** *Methods Mol Biol* 2014, **1099**:219-234.
50. Liu GQ, Gilding EK, Godwin ID: **A robust tissue culture system for sorghum [*Sorghum bicolor* (L.) Moench].** *South African Journal of Botany* 2015, **98**:157-160.
51. House LR: **A Guide to Sorghum Breeding**, Second edn. Patancheru, India: International Crops Research Institute for the Semi-Arid Tropics; 1985.
52. Albertson PL, Peters KF, Grof CPL: **An improved method for the measurement of cell wall invertase activity in sugarcane tissue.** *Aust J Plant Physiol* 2001, **28**(4):323-328.

Figures

Figure 1

Screening transgenic Tx430 sorghum lines for the presence of isomaltulose (IM) in stem juice. (a) High-performance liquid chromatography (HPLC) profiles. Black curve: Standard solutions contained glucose, fructose, sucrose, trehalulose and IM; Pink curve: Diluted (16,000x) juice from transgenic sorghum stalk internode 4 showing isomaltulose (last peak #5) was accumulated beyond glucose, fructose and sucrose; Blue curve: Diluted (16,000x) juice from a parent control sorghum stalk showing no isomaltulose accumulated. (b) isomaltulose concentrations in juice from the internode 4 of the transgenic lines. The line's label starts with A driven by A1 promoter and with L driven by LSG2 promoter. The plants were the first vegetative generation from tissue culture with around 7 internodes when sampled 20 days post-anthesis. A horizontal line was drawn on the highest total sugar content (sucrose equivalent) among the five Tx430 plants (Controls).

Figure 2

Total sugar profile of the internode 4 in controls Tx430 and transgenic lines. A horizontal line was drawn on the highest sugar content of the control Tx430. G+F: $\frac{1}{2}$ (Glucose plus fructose); Suc: Sucrose; IM: Isomaltulose. T1 to T5: five untransformed Tx430; The line's label starts with A driven by A1 promoter and with L driven by LSG2 promoter.

Figure 3

Sugar profile of internodes in controls Tx430 and transgenic lines. The plants were sampled 20 days post-anthesis with 7-8 internodes. G+F: $\frac{1}{2}$ (Glucose plus fructose); Suc: Sucrose; IM: Isomaltulose. Results from the Tx430 controls are means of five replicates, with standard errors. A horizontal line on each panel was drawn on the highest sugar content of internode 1 of the control Tx430. (a) The controls Tx430; (b) Transgenic line A2; (c) Transgenic line A5; and (d) Transgenic line L9.

Figure 4

Total sugar content in the F1 hybrids (Rio X L9). Sugars were measured 20 days post-anthesis in the middle section of internode 4 (counted from top). L9 is the transgenic line driven by LSG2 promoter. G+F: $\frac{1}{2}$ (Glucose plus fructose); Suc: Sucrose; IM: Isomaltulose. Bars of the controls Rio, R9188, and Tx430, were means of three stalks. Three horizontal lines represent the average of the three controls respectively. The progenies with red ticks (✓) were selected for further testing.

Figure 5

Sugar profile of internodes in controls and selected progenies of the F2 generation (Rio X L9). The first group represents the parent Rio control, the second group 24 (P24 from LR24) represents the transgene negative control, and the rest three groups are progenies 3 (P3 from LR3), 19 (P19 from LR19), and 20

(P20 from LR20) of positive transgenes. The last digit in the label of the X-axis is the internode number counted from the top. G+F: ½ (Glucose plus fructose); Suc: Sucrose; IM: Isomaltulose. Sugars were measured 20 days post-anthesis in the middle section of each internode. Results were means with standard errors from three replicates. The horizontal line was drawn on the highest total sugar content among all internodes of the Rio control.

Figure 6

Total water (a), sugar (b), and fiber (c) contents in the internodes of controls and the F2 progenies. Rio was the parent control, P24 (from LR24) was transgene negative progeny as a hybrid control. P3 (from LR3), P19 (from LR19), and P20 (from LR20) were transgene positive progenies. Internodes were numbered from the top. Results were means with standard errors from three replicate plants.

Figure 7

Photosynthetic electron transport rate (a) and CO2 assimilation (b) in controls and the F2 progenies. Three controls: Rio (parent control), Tx430 (untransformed control), and P24 (from LR24) transgene negative progeny as a hybrid control P24. Three high-sugar progenies: P3 (from LR3), P19 (from LR19), and P20 (from LR20). Photosynthesis was measured after 10-11 days of anthesis in the second leaf from the top. Results were means with standard errors from three replicates.

Figure 8

The relationship of source and sink in controls and the F2 progenies. (a) sucrose transport (source). (b) CWI activity was measured in the central parenchyma-rich zone (sink). (c) in the peripheral vascular-rich zone (sink). (d) in separated vascular bundles and parenchyma tissue from the central zone of internode 5 (sink). Three controls: Rio (parent control), Tx430 (untransformed control), and P24 (from LR24) transgene negative progeny as a hybrid control P24. Three high-sugar progenies: P3 (from LR3), P19 (from LR19), and P20 (from LR20). CCCP: carbonyl cyanide m-chlorophenyl hydrazone. The leaves and internodes were sampled at 20 days after anthesis. Results are means with standard errors from three replicates. Analysis of variance (ANOVA) with Bonferroni post-tests showed significant differences between any control and high-sugar progenies in the sucrose transport rates at all time points. The same statistical analysis showed significant differences between controls and high-sugar progenies in CWI activity of parenchyma cells in the central zone. *P < 0.05; **P < 0.01; ***P < 0.001.

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