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Impaired phloem loading in zmsweet13a, b, c sucrose transporter triple knock-out mutants in Zea mays

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Summary

• Crop yield depends on efficient allocation of sucrose from leaves to seeds. In Arabidopsis, phloem loading is mediated by a combination of SWEET sucrose effluxers and subsequent uptake by SUT1/SUC2 sucrose/H+ symporters. ZmSUT1 is essential for carbon allocation in maize, but the relative contribution to apoplasmic phloem loading and retrieval of sucrose leaking from the translocation path is not known.
• Here we analysed the contribution of SWEETs to phloem loading in maize.
• We identified three leaf-expressed SWEET sucrose transporters as key components of apoplasmic phloem loading in Zea mays. ZmSWEET13 paralogues (a, b, c) are among the most highly expressed genes in the leaf vasculature. Genome-edited triple knock-out mutants were severely stunted. Photosynthesis of mutants was impaired and leaves accumulated high levels of soluble sugars and starch. RNA-seq revealed profound transcriptional deregulation of genes associated with photosynthesis and carbohydrate metabolism. Genome-wide association study (GWAS) analyses may indicate that variability in ZmSWEET13s correlates with agronomical traits, specifically flowering time and leaf angle.
• This work provides support for cooperation of three ZmSWEET13s with ZmSUT1 in phloem loading in Z. mays.

Introduction

Crop yield is critical for human nutrition, yet the underlying machinery that ultimately determines yield potential is still not understood. Crop productivity under ideal conditions is determined by the efficiency with which plants intercept light, convert it into chemical energy, translocate photosynthates and convert these to storage products in harvestable organs (Zhu et al., 2010). In many crops, sucrose is the primary form for translocation and retrieval of sucrose along the translocation path (Chen et al., 2015a).

Sucrose is thought to follow one of three routes during phloem loading: (1) apoplastic loading via plasma membrane transporters, (2) symplasmic loading via diffusion through plasmodesmata or (3) polymer trapping via enzymatic addition of galactose, which is thought to impede back-diffusion through plasmodesmata (Turgeon & Wolf, 2009; Chen et al., 2015a). Some mechanisms may coexist, as suggested by anatomical studies which have found thin- and thick-walled sieve tubes in monocots, cell types that may differ regarding the primary loading mechanism (Botha, 2013).

In Arabidopsis, a SWEET/SUT-mediated apoplastic mechanism appears to be important for phloem loading (Chen et al., 2012, 2015a). SWEETs are a class of transporters with seven transmembrane helices that function as hexose or sucrose uniporters (Xuan et al., 2013). Multiple SemiSWEETs and SWEETs have been crystallized, and AtSWEET13 has been proposed to function in complexes via a ‘revolving door’ mechanism to accelerate transport efficacy (Feng & Frommer, 2015; Han et al., 2017; Latorraca et al., 2017). In Arabidopsis, SWEET roles include phloem loading, nectar secretion, pollen nutrition and seed filling (Chen et al., 2012; Sun et al., 2013; Lin et al., 2014; Sosso et al., 2015). In rice, cassava and cotton, SWEETs act as susceptibility factors for pathogen infections (Chen et al., 2010; Cohn et al., 2014; Cox et al., 2017). AtSWEET11 and 12 are probably responsible for effluxing sucrose from the phloem parenchyma into the apoplast (Chen et al., 2012). Sucrose is subsequently loaded against a concentration gradient into the sieve element companion cell complex (SECC) via the SUT1 sucrose/H+ symporter (a.k.a. AtSUC2), powered by the proton gradient created by co-localized H+/ATPases (Riesmeier et al., 1994; Gottwald et al., 2000; Slewin-ski et al., 2009; Srivastava et al., 2009). Although the fundamental
involvement of SUT transporters in phloem loading has been demonstrated using RNA interference (RNAi) and knock-out mutants in Arabidopsis (also in potato, tobacco, tomato and maize) (Riesmeier et al., 1994; Bürkle et al., 1998; Srivastava et al., 2009; Chen et al., 2015a), atsweet11.12 and atsuc2 (sut) mutants were able to produce viable seeds and only showed a slight reduction in plant growth (Chen et al., 2012).

In monocots, including all cereal crops, the situation is less clear. In maize, the phloem-expressed ZmSUT1 (Baker et al., 2016) (phylogenetically in the SUT2 clade) appears to be critical whereas rice ossut1 whereas rice (Slewinski et al., 2009), whereas rice ossut1 mutants and RNAi lines had no apparent growth or yield defects (Ishimaru et al., 2001; Scofield et al., 2002; Eom et al., 2012). As a result, there is an ongoing debate regarding the mechanisms behind phloem loading in cereals (Braun et al., 2014; Regmi et al., 2016).

Here we identified a set of three close paralogs of SWEET13 from Zea mays L. as essential transporters for efflux of sucrose into the apoplasm before phloem loading.

Materials and Methods

Plant material and growth conditions

zmsweet13a, zmsweet13b and zmsweet13c alleles were obtained with a CRISPR-Cas9 construct targeting a sequence (5’-GCATCTACAAGAGCAAGTCGA-3’), the underlined CGG for PAM) conserved in all three paralogs in the 3rd exon using a CRISPR system and associated method as described (Char et al., 2017). Briefly, a pair of 24-nt oligonucleotides matching to the target site were synthesized and annealed into a double-stranded DNA fragment. The DNA fragment was subcloned into an intermediate vector pgRNA1 and the resulting guide RNA expression cassette was mobilized into the Cas9 expressing binary vector pGW-Cas9 through the Gateway recombination reaction using the recombinase, resulting in pCas9-gRNA_SWEET13. The CRISPR construct was transformed into the Agrobacterium tumefaciens strain EHA101 for plant transformation. Maize transformation was performed at the Iowa State University Plant Transformation Facility. Hi-II calli were derived from the F1 immature embryos of Hi-IIA and Hi-IIB plants, which are independent lines of a B73xA188 cross. Calli were transformed with A. tumefaciens containing plasmids for expressing guide RNAs and the Cas9 construct. T0 plantlets grown on sterile media from successfully transformed calli were transplanted to soil when 1 inch in height. T0 plants were selfed or outcrossed to B73, and plants which did not contain the CRISPR construct were selected by performing PCR using three different primer pairs targeting Cas9 (Supporting Information Table S1). T1, T2 and T3 plants homozygous for all three mutated genes (zmsweet13abc) were selected along with wild-type siblings. Height was assessed by weekly measurement from the soil surface to the top of the highest fully developed leaf. Wild-type ‘siblings’ were descendents of the Hi-II plants transformed and outcrossed once to B73, which in the T1 generation did not carry the CRISPR-Cas9 construct or any detectable mutations. Triple mutant plants either descended from selfed T0 Hi-II plants or outcrossed once to B73. The mutant phenotype was unaffected by the difference in genetic background. Mutants and wild-type plants were grown side by side, in glasshouses under long-day conditions (16 h : 8 h, day : night, 28–30°C), and in 2016 in a summer field at Carnegie Science (Stanford, CA, USA).

Genotyping of maize plants

Genomic DNA was extracted from leaves using a Qiagen Biotum 96 device. PCR was performed with the Terra PCR Direct Red Dye Premix Protocol (Clontech Laboratories, Palo Alto, CA, USA) with melting temperatures of 60, 64 and 62.5°C for ZmSWEET13a, b and c, respectively (for primers see Table S1). Amplicons of relevant regions of the CRISPR-Cas9 targeted ZmSWEET13 alleles were sequenced by Sequetech (Mountain View, CA, USA). Chromatograms were analysed using 4Peaks (www.nucleobytes.com/4peaks/).

Plastic embedding and sectioning

Flag leaves collected at 07:00 h were placed in 0.1 M cacodylate-buffered fixative with 2% paraformaldehyde and 2% glutaraldehyde, vacuum infiltrated for 15 min and incubated overnight. Sample dehydration was performed by a graded ethanol series (10, 30, 50, 70 and 95%). Sample embedding was performed according to the LR White embedding kit protocol (Electron Microscopy Science, Hatfield, PA, USA). Cross-sections (1.5 μm) were obtained on an Ultracut (Reichert, Depew, NY, USA), stained for 30 s with 0.1% toluidine blue and washed with double distilled H2O (2×), followed by 5 min of starch staining with saturated Lugol’s solution. Sections were mounted with CytoSeal 60 (Electron Microscopy Science).

Phylogenetic analyses

The evolutionary history was inferred by using maximum likelihood with a JTT matrix-based model. The tree with the highest log likelihood (−3000.1) is shown. The percentage of trees in which associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by neighbour-joining to a matrix of pairwise distances with the JTT model used for estimation. The analysis involved 16 polypeptide sequences, derived from Phytozome (https://phytozome.jgi.doe.gov) and Gramene (http://www.gramene.org/) using ZmSWEET13a as a template in a search for similar sequences in the genomes of Z. mays, Sorghum bicolor, Setaria italic, Hordeum vulgare, Triticum urartu (progenitor of A-genome of bread wheat Triticum aestivum), Brachypodium distachyon and Oryza sativa. A minimum of 95% site coverage was required so that no more than 5% alignment gaps, missing data and ambiguous bases were allowed at any position. There were a total of 252 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Soluble sugar analyses

Flag leaves were harvested from mature plants at 07:00 h. In total, 70 mg of liquid nitrogen-ground tissue was incubated for
1 h with 1 ml of 80% ethanol on ice with frequent mixing. Samples were centrifuged for 5 min at 4°C at 13 000 g, and supernatant was removed. This step was repeated once. The liquid supernatant was subsequently dried in a vacuum concentrator and re-suspended in water. Sucrose, glucose and fructose were measured using NADPH-coupled enzymatic methods using an M1000 plate reader (Tecan, Männedorf, Switzerland), with measured values normalized to fresh weight. Starch quantification was performed as previously described (Sosso et al., 2015).

Starch staining

Flag leaves collected at 07:00 h were boiled in 95% ethanol for c. 30 min (until chlorophyll pigments disappeared). Cleared leaves were submerged in saturated Lugol’s iodine solution for 15 min, rinsed twice with H2O and imaged with a Lumix GF1 camera (Panasonic, Kadoma, Osaka, Japan). The IKI solution used for starch staining was made by adding 1 g of iodine and 1 g of potassium iodide to 100 ml H2O. The ZmSWEET13a, b coding sequences were cloned into the Gateway entry vector pDONR221f1, followed by LR (arbL, attrR) recombination into pcDNA3.2V5 for expression in HEK293T cells. HEK293T cells were co-transfected with ZmSWEET13a, b or c in pcDNA3.2V5 and the sucrose sensor FLIPsuc90μΔ1V (Chen et al., 2012) using Lipofectamine 2000 (Invitrogen). For fluorescence resonance energy transfer (FRET) imaging, Hank’s balanced salt solution medium was used to perfuse HEK293T/FLIPsuc90μΔ1V cells with defined pulses containing 20 mM sucrose in buffer. Image acquisition and analysis were performed as previously described (Chen et al., 2012). AtSWEET12 was used as a positive control. Negative controls were empty vector transfectants.

Transient gene expression in Nicotiana benthamiana leaves

The A. tumefaciens strain GV3101 was transformed with the binary expression clone (pAB117) carrying ZmSWEET13a, b or c C-terminally fused with enhanced green fluorescent protein (eGFP) and driven by the CaMV 35S promoter. Agrobacterium culture and tobacco leaf infiltration were performed as described (Sosso et al., 2015). Chloroplast autofluorescence was detected on a Leica TCS SP8 confocal microscope with 488 nm excitation (eGFP) and 561 nm excitation (chlorophyll). Emission was detected at 522–572 nm (eGFP fluorescence) and 667–773 nm (chloroplast fluorescence). Epidermal leaf chloroplast fluorescence (Dupree et al., 1991) allowed us to determine eGFP vacuolar localization (lining chloroplasts on the vacuolar side) and plasma membrane localization was deduced (peripheral to chloroplasts; according to bright-field image). Image analysis was performed using Fiji software (https://fiji.sc/).

Analyses of photosynthetic rates

Licor LI-6800 measurements were taken at mid-day under glasshouse conditions (28°C, photosynthetically active radiation (PAR) 1000 μE m−2 s−1, 60% relative humidity). Two-centimetre-diameter discs of leaves were clamped in the Licor measurement chamber and relative concentrations of CO2 inside and outside of the chamber were measured. CO2 absorbed (μmol m−2 s−1) by leaf segments in the chamber was used as a proxy for photosynthetic rate. Measurements were made at the tips of leaf 7 to leaf 10 at midday.

Candidate gene association study

To test whether sequences at SWEET loci are associated with phenotypic variations in the maize population, we analyzed a maize diversity panel composed of 282 inbred lines (HapMap3 SNP data (Bukowski et al., 2017) for the panel from the Panzea database (www.panzea.org)). We filtered single nucleotide polymorphism (SNP) data (minor allele frequency (MAF) > 0.1; missing rate < 0.5) using PLINK (Purcell et al., 2007) and calculated a kinship matrix with GEMMA (Zhou & Stephens, 2012) using the filtered SNP set. A genome-wide association study (GWAS) was performed by fitting a mixed linear model using GEMMA, where the kinship matrix was fitted as random effects in the model. A false discovery rate (FDR) approach (Benjamini & Hochberg, 1995) was used to control the multiple test problem with a cut off of 0.05. Linkage disequilibrium of SNPs in our candidate genes with significant association SNPs was calculated using PLINK (Purcell et al., 2007).
uniquely mapping reads, these were filtered by mapping quality (q20), and PCR duplicates were removed using SAMtools (v.1.3.1). Gene expression was analysed in R (v.3.4.1) using DESeq2 software (v.1.16.1) (Love et al., 2014). Genes were defined as differentially expressed by a two-fold expression difference with a P-value, adjusted for multiple testing, of < 0.05 (Fig. S7; Table S2). RNA-seq data are available in the NCBI Gene Expression Omnibus database.

**Results**

To test whether SWEETs are involved in phloem loading in maize, we evaluated the role of leaf-expressed maize SWEETs in carbon allocation. We identified three SWEET13 paralogs (GRMZM2G173669: ZmSWEET13a, GRMZM2G021706: ZmSWEET13b, GRMZM2G179349: ZmSWEET13c) as the most highly expressed SWEETs in maize leaves based on published expression values in four publicly available datasets (Denton et al., 2017) (Fig. S1). ZmSWEET13a and b are located in tandem on chromosome 10 in a region syntenic with the OsSWEET13 locus in rice, while ZmSWEET13c is on chromosome 3 (Fig. S2). Interestingly, maize is one of the few cereals having three SWEET13 paralogs, along with Sorghum bicolor and Triticum urartu (Figs 1a, S3). Similar to ZmSUT1, ZmSWEET13a, b and c mRNA preferentially accumulated in bundle sheath/vein preparations rather than mesophyll (Fig. 2a). If the SWEETs were involved in phloem loading, one would expect that their mRNA levels would be highest in leaf domains that serve as sucrose sources, as compared to sink tissues. Consistent with a role in phloem loading, mRNA levels of all three SWEET13s (as well as SUT1) were highest in leaf tips (Fig. 2b).

Analysis of independent RNA-seq experiments that had differentiated source and sink regions of maize leaves on the basis of radiotracer experiments also found ZmSWEET13 transcripts to be ~ five-fold higher in source vs sink domains (Fig. S4) (Wang et al., 2014). We tested the transport activity of the three SWEETs in human HEK293T cells coexpressing a genetically encoded sensor (Chen et al., 2010, 2012). All three SWEETs mediated sucrose transport (Fig. 1b). To test whether these SWEETs were part of (1) intercellular translocation or (2) intracellular sugar sequestration similar to Arabidopsis SWEET2, 16 or 17 (Chardon et al., 2013; Klemens et al., 2013; Guo et al., 2014; Chen et al., 2015b), we tested their subcellular localization in transiently transformed tobacco cells, and found that they localized preferentially to the plasma membrane (Fig. 1c).

Recently, ZmSWEET13 had been implicated as a possible key player in C4-photosynthesis in grasses (Emms et al., 2016). To test their role in maize, we designed guide RNAs that target a conserved region within a transmembrane domain, assuming that defects in the membrane domain would lead to complete loss of function. We generated single knock-out mutants, as well as

![Fig. 1](image-url) Phylogeny, functional analysis and subcellular localization of SWEET13a,b,c. (a) Phylogenetic relationship between putative orthologs in the following grasses: Zea mays, Sorghum bicolor, Setaria italica, Hordeum vulgare, Triticum urartu (progenitor of A-genome of bread wheat Triticum aestivum), Brachypodium distachyon and Oryza sativa. Chronogram branch divergence time-points are in million years (Emms et al., 2016). Red dots represent the number of SWEET13 paralogs for each species. (b) Sucrose transport activity by ZmSWEET13a, b and c in HEK293T cells coexpressing FLIpsuc90µM1V (fluorescent sucrose sensor). Cells were transfected to express sensors only as negative control, or to co-express AtSWEET12 as a positive control. HEK293T cells were perfused with buffer, then subjected to a 3-min pulse of 20 mM sucrose (mean ± SEM, repeated independently four times with comparable results). (c) Confocal images (maximum projection of Z-stack) of Agrobacterium-infiltrated Nicotiana benthamiana epidermal leaf cells transiently expressing ZmSWEET13a-eGFP, ZmSWEET13b-eGFP or ZmSWEET13c-eGFP fusions. The eGFP emission (green, 522–572 nm) was merged with chloroplast fluorescence (blue, 667–773 nm). ZmSWEET13x-eGFP derived fluorescence between chloroplasts and the cell periphery indicates localization to the plasma membrane. Bars, 50 µm.
combinations of mutant alleles, using CRISPR-Cas9 (Fig. 3). We recovered two mutant alleles of ZmSWEET13a, four of ZmSWEET13b and three of ZmSWEET13c. The majority of mutations were caused by single nucleotide insertions in the target sequence. All mutations created premature stop codons leading to truncated polypeptides at amino acid 129 in the fourth of seven transmembrane domains (Fig. 3). T2 lines carrying homozygous mutations in all three genes were characterized by severe growth defects (Fig. 4a). The growth phenotype was analyzed in subsequent generations in the glasshouse and in a single field season. Single and double mutants showed slight growth defects, while triple mutants had substantial defects: plants were severely stunted and three of ZmSWEET13s, consistent with impaired photosynthesis and chlorosis (Figs S8, S9). Furthermore, in line with the accumulation of starch and soluble sugars in leaves, transcripts related to carbohydrate synthesis and degradation, in particular starch biosynthesis were substantially reduced in triple mutants, consistent with impaired photosynthesis and degradation, in particular starch biosynthesis and degradation, in particular starch biosynthesis were substantially reduced in triple mutants, consistent with impaired photosynthesis and chlorosis (Figs S8, S9). Furthermore, in line with the accumulation of starch and soluble sugars in leaves, transcripts related to carbohydrate synthesis and degradation, in particular starch biosynthesis and degradation, were affected in the triple mutants (Fig. S10; Table S3).

A recent study has found that the Arabidopsis homolog AtSWEET13 (although phylogenetically not the closest homolog of ZmSWEET13) can also transport gibberellin (Kanno et al., 2016). The observed phenotypes of the triple zmsweet13 knock out mutants in maize are consistent with a primary role in sucrose transport and distinct from those observed in the Arabidopsis...
sucrose concentrations. The high sucrose contents in the loading zone are thought to create a pressure gradient that drives phloem translocation. Inhibition of the expression of the SUT1 sucrose/H+ symporter by RNAi or T-DNA insertion typically leads to stunted growth and accumulation of carbohydrates in leaves (Riesmeier et al., 1994; Bürkle et al., 1998; Gottwald et al., 2000; Slewinski et al., 2009; Srivastava et al., 2009). Chlorosis and inhibition of photosynthesis, which often accompany defects in phloem translocation, may either be due to feedback inhibition of photosynthesis or be a consequence of nutrient deficiencies caused by the reduced supply of carbohydrates to the root system (Ainsworth & Bush, 2011). SUTs function as sucrose/H+ symporters and, at least in maize, appear to fulfill two roles: (1) loading of the SECC with sucrose in source leaves, and (2) retrieval of sucrose that diffuses out of the SECC, as a consequence of the high sucrose concentration in the SECC, relative to surrounding tissues. SUTs import sucrose from the cell wall space, implying the existence of transporters that efflux sucrose into the cell wall space preceding uptake by SUTs. AtSWEET11 and 12 are candidate mutants for such an efflux role in Arabidopsis: they appear to function as uniporters and can thus serve as cellular efflux systems when sucrose gradients are suitable. Both SWEETs were highly expressed in leaves, localized most likely to the phloem parenchyma, and atswee11;12 mutants were smaller and accumulated starch in leaves (Chen et al., 2012). However, the phenotype of atswee11;12 mutants was relatively weak, implying leaky mutations, compensation by other transporters or the coexistence of other phloem loading mechanisms. Other mechanisms could include symplasmic transport, or yet unknown processes.

Here, we show that maize has three closely related clade III SWEETs (named SWEET13a, b and c) that are encoded by some of the most highly expressed genes in the leaf. The three genes possibly derive from relatively recent gene duplication events: sorghum and wheat have three copies per genome, while

**Discussion**

The phloem sap of many monocots and dicots contains high sucrose concentrations. The high sucrose contents in the loading zone are thought to create a pressure gradient that drives phloem translocation. Inhibition of the expression of the SUT1 sucrose/H+ symporter by RNAi or T-DNA insertion typically leads to stunted growth and accumulation of carbohydrates in leaves (Riesmeier et al., 1994; Bürkle et al., 1998; Gottwald et al., 2000; Slewinski et al., 2009; Srivastava et al., 2009). Chlorosis and inhibition of photosynthesis, which often accompany defects in phloem translocation, may either be due to feedback inhibition of photosynthesis or be a consequence of nutrient deficiencies caused by the reduced supply of carbohydrates to the root system (Ainsworth & Bush, 2011). SUTs function as sucrose/H+ symporters and, at least in maize, appear to fulfill two roles: (1) loading of the SECC with sucrose in source leaves, and (2) retrieval of sucrose that diffuses out of the SECC, as a consequence of the high sucrose concentration in the SECC, relative to surrounding tissues. SUTs import sucrose from the cell wall space, implying the existence of transporters that efflux sucrose into the cell wall space preceding uptake by SUTs. AtSWEET11 and 12 are candidate mutants for such an efflux role in Arabidopsis: they appear to function as uniporters and can thus serve as cellular efflux systems when sucrose gradients are suitable. Both SWEETs were highly expressed in leaves, localized most likely to the phloem parenchyma, and atswee11;12 mutants were smaller and accumulated starch in leaves (Chen et al., 2012). However, the phenotype of atswee11;12 mutants was relatively weak, implying leaky mutations, compensation by other transporters or the coexistence of other phloem loading mechanisms. Other mechanisms could include symplasmic transport, or yet unknown processes.

Here, we show that maize has three closely related clade III SWEETs (named SWEET13a, b and c) that are encoded by some of the most highly expressed genes in the leaf. The three genes possibly derive from relatively recent gene duplication events: sorghum and wheat have three copies per genome, while
Brachypodium and rice each have only one. The comparatively high number of SWEET13s had been attributed to specific roles in C4 photosynthesis (Emms et al., 2016), but the presence of three SWEET13s in *T. urartu* (Fig. 1a), the progenitor of the A-genome of bread wheat *T. aestivum*, both of which are C3 plants, puts this interpretation into question. Evidence that maize SWEET13s cooperate in phloem loading is based on two key observations: the severe growth defect of *zmsweet13abc* mutants is similar to that of *zmsut1* mutants (Slewinski et al., 2009), and a massive accumulation of free sugars and starch in leaves is also consistent with a defect in phloem translocation. These phenotypic effects are also similar to the RNAi-mediated SUT1 knockdown phenotypes in potato and tobacco (Riesmeier et al., 1994; Bürkle et al., 1998). The observed growth defect in maize is much more severe than that of the *atsweet11;12* mutant in Arabidopsis, and comparable to that of the *zmsut1* mutant (Slewinski et al., 2009; Chen et al., 2012). We thus propose that the three ZmSWEET13s and ZmSUT1 play dominant roles in phloem loading, probably in the same pathway.

Notably, the combined *zmsweet13abc* mutations were not lethal, because the plants still produced fertile viable offspring, implying compensatory or alternative mechanisms for phloem loading. While it is possible that other transporters might compensate, it is unlikely that other clade III SWEETs take over such roles, as judged by the lack of induction of other clade III SWEET genes in the mutants. Maize may thus either also have parallel symplasmic or other yet unknown loading mechanisms.

It is still not clear whether SWEET13 triplication mainly serves to increase the amount of SWEET protein in the same cells (e.g. phloem parenchyma), or if each SWEET13 transporter mediates

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**Fig. 4** Characterization of ZmSWEET13abc triple *Zea mays* L. mutants. (a) Mature wild-type and same-age *zmsweet13a1b1c1* triple mutant, showing reduced growth and leaf chlorosis. Bar, 50 cm. (b) Leaf phenotype of plants presented in Fig. 2(a), showing reduced length, width and chlorosis in leaves of the *zmsweet13a1b1c1* triple mutant. (c) Growth of wild-type and triple mutants in glasshouse conditions (mean ± SEM, n = 17 and 15, two-tailed t-test performed between wild-type and *zmsweet13abc*: *, P < 0.005; **, P < 0.001). (d) Relative mRNA levels (by qRT-PCR) of ZmSWEET13 paralogs in maize flag leaves from wild-type and *zmsweet13abc*. Samples were harvested at 16:00 h (mean ± SEM, n = 3 technical replicates with expression normalized to 18S levels, repeated independently five times with comparable results, see Fig. S13(e, f)). Two-tailed t-test performed between wild-type and *zmsweet13abc* for each gene: four pools of four (16 plants) for each genotype: *, P < 0.0001. (e) Mature field-grown *zmsweet13a1b4c1* plants under field conditions (Carnegie field 2016). Mutants were stunted, showed severe chlorosis of all leaves, and anthocyanin accumulation in the oldest leaves.
efflux from a specific cell type and loading is achieved in a multilayer manner. This question is of particular interest because in situ hybridization experiments identified SUT1 in companion cells, xylem and phloem parenchyma, as well as bundle sheath (Baker et al., 2016). With the intent of localizing SWEET13 paralogs, we had generated translational reporter gene fusions that included the first three introns. However, neither GUS activity nor GFP fluorescence were detectable in any of the transformants carrying fusions for either of the three SWEET13s (data not shown). We therefore hypothesize that additional regulatory elements that were lacking from our chimeras must be required for proper expression.

Another interesting question is whether maize can serve as a model for phloem loading in rice, barley and wheat. Surprisingly, RNAi of the rice homolog of ZmSUT1 did not lead to a detectable effect on the phenotype of the sporophyte (Ishimaru et al., 2001). Thus, it remains a matter of debate whether rice uses predominantly apoplasmic and symplasmic or other mechanisms simultaneously (Eom et al., 2012; Braun et al., 2014). It will therefore be important to study the role of SWEET homologs in rice and other crops. It is noteworthy in this context that the clade III sucrose transporters OsSWEET11 and 15 are expressed preferentially in the caryopsis and act as key players in apoplasmic unloading processes in developing rice grains (Ma et al., 2017; Yang et al., 2018).

Data from the GWAS analysis indicate that genetic variation at the ZmSWEET13 loci in the maize diversity panels is significantly associated with several phenotypic traits, including ear- and developmental-related traits. Although the causality needs to be validated, the identified SNP markers might be useful for marker-assisted selection for further crop improvement. A better understanding of the role of SWEET sugar transporters in phloem loading in maize may guide future engineering efforts to improve yield potential.

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Author contributions

Competing financial interests
The authors (D.S. and W.B.F., on behalf of Carnegie Science) have filed a PCT patent application based in part on this work with the US Patent and Trademark Office.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

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Fig. S13 Biological repeats of sugar quantification and qRT-PCR of Zea mays L. leaves.

Table S1 List of primers

Table S2 Read statistics of RNA-seq experiments

Table S3 Significantly affected pathways in Zea mays L. zmsweet13abc mutants

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SUPPORTING INFORMATION

Impaired phloem loading in zmsweet13a,b,c sucrose transporter triple knock-out mutants in Zea mays

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The following Supporting Information is available for this article:

**Figure S1.** Expression pattern of SWEET genes in *Zea mays* L. across four leaf-specific RNA-seq datasets.

**Figure S2.** Chromosomal localization and potential synteny of *ZmSWEET13s* in *Zea mays* L. and *Oryza sativa*.

**Figure S3.** SWEET13 orthologues in different grasses.

**Figure S4.** Expression of *ZmSWEET13a, b*, and *c* in sink and source tissues of *Zea mays* L.

**Figure S5.** Photosynthetic rates in *Zea mays* L. *zmsweet13abc* mutants.

**Figure S6.** *ZmSWEET* mRNA levels in *Zea mays* L. *zmsweet13abc* mutants.

**Figure S7.** Distribution of RNAseq samples and differentially expressed genes in *Zea mays* L.

**Figure S8.** Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in light harvesting processes.

**Figure S9.** Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in chlorophyll and tetrapyrrole biosynthesis.

**Figure S10.** Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in sucrose and starch biosynthesis.

**Figure S11.** GWAS results for *ZmSWEET13c* genes in *Zea mays* L.

**Figure S12.** GWAS results for *ZmSWEET13a* and *ZmSWEET13b* genes in *Zea mays* L.

**Figure S13.** Biological repeats of sugar quantification and qRT-PCR of *Zea mays* L. leaves.

**Table S1.** List of primers

**Table S2.** Read statistics of RNAseq experiments in *Zea mays* L.

**Table S3.** Significantly affected pathways in *Zea mays* L. *sweet13abc* mutants
Figure S1: Expression pattern of SWEET genes in *Zea mays* L. across four leaf-specific RNA-seq datasets.

ZmSWEET1a b c mRNAs preferentially accumulate in the bundle sheath and veins, rather than mesophyll cells in all four independent databases (Li et al., 2010; Chang et al., 2012; Tausta et al., 2014; Denton et al., 2017).

| ZmSWEET1a | GRMZM2G039365 | 0.51 | 2.14 | 0.00 | 0.00 | 0.66 | 3.40 | 0.52 | 0.38 |
| ZmSWEET1b | GRMZM2G0303356 | 12.74 | 2.16 | 0.07 | 0.04 | 7.61 | 32.21 | 1.08 | 0.32 |
| ZmSWEET2  | GRMZM2G0324903 | 1.44 | 2.46 | 0.34 | 0.21 | 7.12 | 5.58 | 2.47 | 0.92 |
| ZmSWEET3a | GRMZM2G179679 | 0.00 | 0.01 | 0.00 | 0.00 | 0.11 | 0.47 | 0.00 | 0.00 |
| ZmSWEET3b | GRMZM2G060974 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET4a | GRMZM2G000812 | 0.10 | 0.60 | 0.03 | 0.03 | 0.19 | 0.17 | 0.15 | 0.00 |
| ZmSWEET4b | GRMZM2G144581 | 0.22 | 3.09 | 0.31 | 0.85 | 2.21 | 32.33 | 1.16 | 3.07 |
| ZmSWEET4c | GRMZM2G137954 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.05 | 0.00 | 0.00 |
| ZmSWEET6a | GRMZM2G127675 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 | 0.25 | 0.00 | 0.12 |
| ZmSWEET6b | GRMZM2G169665 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET11a | GRMZM2G368827 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.00 |
| ZmSWEET11b | GRMZM50872141 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET12a | GRMZM2G133322 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET12b | GRMZM2G099809 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET13a | GRMZM2G173669 | 110.60 | 582.15 | 29.11 | 83.33 | 92.68 | 106.79 | 59.77 | 173.79 |
| ZmSWEET13b | GRMZM2G021706 | 25.32 | 127.24 | 53.56 | 199.05 | 35.41 | 512.93 | 79.02 | 428.84 |
| ZmSWEET13c | GRMZM2G179349 | 19.25 | 479.85 | 1.72 | 10.22 | 109.45 | 1406.79 | 8.55 | 62.31 |
| ZmSWEET14a | GRMZM2G094955 | 0.00 | 0.00 | 0.00 | 0.00 | 0.03 | 0.28 | 0.09 | 0.00 |
| ZmSWEET14b | GRMZM2G015976 | 0.00 | 0.11 | 0.08 | 0.00 | 1.98 | 2.41 | 0.00 | 0.00 |
| ZmSWEET15a | GRMZM2G156636 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.20 | 0.00 |
| ZmSWEET15b | GRMZM50872392 | 0.00 | 0.07 | 0.02 | 0.00 | 0.07 | 0.08 | 0.00 | 0.00 |
| ZmSWEET16  | GRMZM2G107957 | 0.00 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| ZmSWEET17a | GRMZM2G106462 | 19.33 | 10.48 | 0.08 | 0.19 | 0.00 | 0.00 | 0.78 | 1.74 |
| ZmSWEET17b | GRMZM2G111926 | 5.36 | 4.81 | 0.43 | 0.22 | 2.84 | 2.42 | 0.00 | 0.00 |
Figure S2. Chromosomal localization and potential synteny of \textit{ZmSWEET13s} in \textit{Zea mays} L. and \textit{Oryza sativa}. \textit{OsSWEET13} locus on rice chr12 (dark green box) is syntenic with the corresponding locus on maize chr10 (red box), where \textit{ZmSWEET13a} and \textit{ZmSWEET13b} are located. \textit{ZmSWEET13c} is located on maize chr3, and might be the result of a more recent translocation event in maize or its ancestors.
Figure S3. SWEET13 orthologs in different grasses.
Phylogenetic tree of SWEET proteins collected from different grasses: Zea mays L. (Zm), Oryza sativa (Os, rice), Sorghum bicolor (Sb, sorghum), Setaria italica (Si; foxtail millet), Brachypodium distachyon (Bd, Brachypodium), Hordeum vulgare (Hv, barley) and Triticum urartu (Tu, wheat). A. thaliana was used as sole dicot out-group. Tree was inferred using Maximum likely-hood. Bootstrap values are out of 1000 replicates. Schematic of the SWEET13 gene models across all species used in the tree (black boxes represent exons, lines represent introns).
Figure S4. Expression of ZmSWEET13a, b, and c in sink and source tissues of Zea mays L.

Relative levels of ZmSWEET13 transcripts in 3rd leaf of juvenile maize plants. Wang et al 2014 determined source and sink segments using $[^{14}\text{C}]$-labelling and found that segments 1-4 represent sink tissue while 5-15 represent source tissue “Comparative analyses of C4 and C3 photosynthesis in developing leaves of maize and rice”. SWEET13a, SWEET13b and SWEET13c all show dramatic increase in transcript levels at transition to from sink to source tissue. Scales show absolute transcript counts.
Figure S5. Photosynthetic rates in *Zea mays* L. *zmsweet13abc* mutants.
Licor LI-6800 measurements on greenhouse-grown plants were made in the 7th-10th leaf tip at mid-day (mean ± s.e.m., n=3 (*zmsweet13abc*), 3 (wild-type Hi-II) and 1 (wild-type B73). Lower and upper limits of boxes represent first and third quartile, respectively. Whiskers represent highest and lowest values, diamonds the median.
Figure S6. *ZmSWEET* mRNA levels in *Zea mays* L. *zmsweet13abc* mutants.

Relative expression of *SWEET* clade I-IV genes in *zmsweet13abc* mutant plants relative to wild type siblings. baseMean was calculated as the average of normalized count values taken over all samples. Error bars represent standard error of three biological replicates.
Figure S7. Distribution of RNAseq samples and differentially expressed genes in *Zea mays* L.

Principal component analysis of genes differentially expressed genes in *zmsweet13* mutants and wild type

(a) Principal component analysis plot using the rlog-transformed values for three replicates obtained with *zmsweet13abc* (turquoise) and wild type (red). (b) MA-plot of differentially expressed genes in *zmsweet13abc* versus wild type. Log2 fold change for mRNA levels in *sweet13abc* and wild type is plotted on the y-axis and the average of the counts normalized by size factor is shown on the x-axis. Each gene is represented with a dot. Differentially expressed genes with a *p*-value adjusted for multiple testing below 0.05 and above a twofold change in expression are shown in red.
Figure S8. Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in light harvesting processes.

Differentially expressed genes (>2 fold, adj. p<0.05) in *zmsweet13abc* mutant plants relative to wild type siblings, were mapped (ZmB73_5b_2012) to MapMan (v. 3.6.0RC1) pathway of “ChloroPlast_CustomArray” in maize. Scale bar indicates fold change of downregulated (red) and upregulated (blue) genes in the mutant.
Figure S9. Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in chlorophyll and tetrapyrrole biosynthesis. Differentially expressed genes (>2 fold, adj. p<0.05) in *zmsweet13abc* mutant plants relative to wild type siblings, were mapped (ZmB73_5b_2012) to the MapMan (v. 3.6.0RC1) pathway of “tetrapyrrole biosynthesis” in maize. Scale bar indicates fold change of downregulated (red) and upregulated (blue) genes in the mutant.
Figure S10. Differentially expressed genes in *Zea mays* L. *zmsweet13abc* mutants involved in sucrose and starch biosynthesis.

Differentially expressed genes (>2 fold, adj. p<0.05) in *zmsweet13* mutant plants versus wild type siblings, were mapped (ZmB73_5b_2012) to the MapMan (v. 3.6.0RC1) pathway of "sucrose and starch biosynthesis" in maize. Scale bar indicates fold change of downregulated (red) and upregulated (blue) genes in the mutant.
Figure S11. GWAS results for ZmSWEET13c genes in Zea mays L.

Horizontal red dashed lines denote the statistical threshold (FDR = 0.05). Significant SNPs in LD with ZmSWEET13 genes are highlighted in green. Vertical dashed lines denote the physical location of ZmSWEET13c genes. Coordinates are based on AGPv3.
Figure S12. GWAS results for ZmSWEET13a and ZmSWEET13b genes in Zea mays L.
Horizontal red dashed lines denote the statistical threshold (FDR = 0.05). Significant SNPs in LD with ZmSWEET13a and ZmSWEET13b genes are highlighted in green. Vertical dashed lines denote the physical location of the SWEET genes. Coordinates are based on AGPv3.
Figure S13. Biological repeats of sugar quantification and qRT-PCR of *Zea mays* L. leaves.
(A, B) Biological replicates of starch assay from Figure 5. Starch quantification of flag leaf tip from VT plant. The triple mutant shows 3x more starch than wild-type in the leaf tip (mean ± s.e.m., two-tailed t-test performed between wild type and zmsweet13abc; * p<0.05, ** p<0.001, *** p<0.0001)

(C,D) Biological replicates of soluble sugar assay from Figure 5. Soluble sugar measurements of wild-type and zmsweet13a2b1c1 in maize flag leaf sections. The triple mutant shows higher sugar concentration in both the tip and the base of the leaf. Base of leaf measurements not available for second data set. Samples were harvested at 7:00 am (mean ± s.e.m., two-tailed t-test performed between wild type and zmsweet13abc for each sugar in the respective leaf sections; * p<0.05, ** p<0.001, *** p<0.0001)

(E,F) Biological replicates of qRT-PCR from Fig 2d. Relative expression (by qRT-PCR) of ZmSWEET13 paralogs in maize flag leaf from wild-type and zmsweet13abc. Samples were harvested at 4:00 pm, expression normalized to 18S levels (mean ± s.e.m; two-tailed t-test performed between wild type and zmsweet13abc for each gene; *p<0.0001)
### Table S1. List of primers

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<th>Primers</th>
<th>Sequence</th>
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### Table S2. Read statistics of RNAseq experiments in Zea mays L.

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Table S3. Significantly affected pathways in *Zea mays* L. *zmsweet13abc* mutants

For pathway enrichment analysis the Plant MetGenMAP (Joung et al., 2009) database with a significance cut-off of \( \leq 0.05 \) was used and included all differentially expressed genes (fold change \( \geq 2 \), adjusted p-value \( \leq 0.05 \)) between WT and sweet13abc plants.

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References


