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* L. 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, especifically 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 inside the conduit (i.e. the phloem). A combination of SWEET-mediated efflux from phloem parenchyma and subsequent secondary active sucrose import by SUT sucrose/H^+ symporters is thought to create the driving force for pressure gradient-driven phloem transport and retrieval of sucrose leaking along the translocation path (Chen *et al.*, 2015a).

Sucrose is thought to follow one of three routes during phloem loading: (1) apoplasmic 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 impair 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 apoplasmic 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 apoplasm (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; Slewinski *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 (sut1)* 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 critically important for phloem translocation (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′-GCATCTACAAGAGCAAGTCGACGG-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 F_1 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 descendants 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 Biosprint 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 H_2O (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 italica*, *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 H_2O and imaged with a Lumix GF1 camera (Panasonci, 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 H_2O.

qRT-PCR RNA isolation and transcript analyses

RNA was extracted using the Trizol method (Invitrogen). First-strand cDNA was synthesized using a Quantitect reverse transcription kit (Qiagen). Quantitative reverse transcriptase PCR (qRT-PCR) to determine expression level was performed using a LightCycler 480 (Roche), and the $2^{-\Delta Ct}$ method for relative quantification. Wild-type maize and *zmsweet13abc* flag leaves were sampled at 17:00 h. Primers in the last exon and the 3′ untranslated region of *ZmSWEET13a, b* and *c* (Table S1) were used for qRT-PCR to determine gene expression levels. Internal references were *Zm18s* and *ZmLUG*.

FRET sucrose sensor analysis in HEK293T cells

ZmSWEET13a, b and *c* coding sequences were cloned into the Gateway entry vector pDONR221f1, followed by LR (*att*L, *att*R) 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 CO_2 inside and outside of the chamber were measured. CO_2 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).

RNA-seq and data analysis

zmsweet13abc triple mutants and wild-type siblings were grown in soil under glasshouse conditions. Total RNA was isolated from flag leaf tissues using acidic phenol extraction as described previously (Eggermont *et al.*, 1996). Purification of poly-adenylated mRNA using oligo(dT) beads, construction of barcoded libraries and sequencing using Illumina HiSeq technology (150 bp paired-end reads) were performed by Novogene (https://en.novogene.com/) using the manufacturer's recommendations. Trimmed and quality control-filtered sequence reads were mapped to the B73 AGPv3 genome using Star (v.2.54) (Dobin *et al.*, 2013) in two pass mode (parameters: –outFilterScoreMinOverLread 0.3, –outFilterMatchNminOverLread 0.3, –outSAMstrandField intronMotif, –outFilterType BySJout, –outFilterIntronMotifs RemoveNoncanonical, –quantMode TranscriptomeSAM GeneCounts). To obtain

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 *SWEET13*s (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 C_4-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

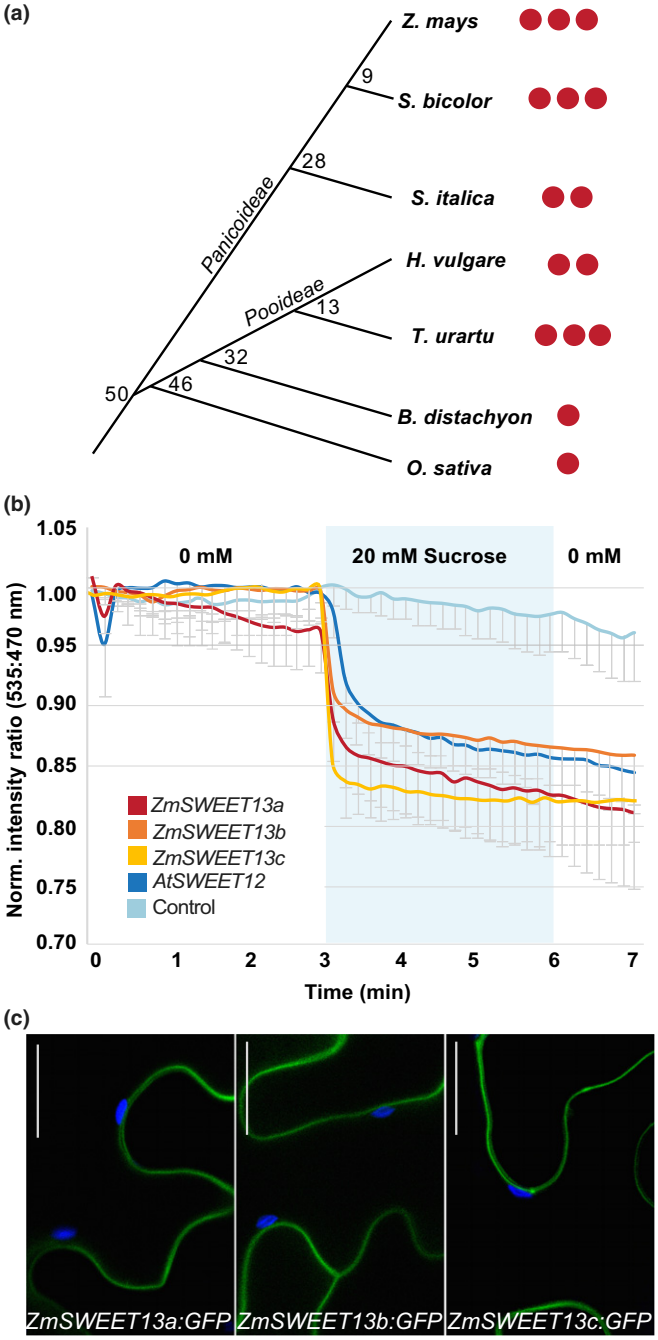

Fig. 1 Phylogeny, functional analysis and subcellular localization of SWEET13a,b,c. (a) Phylogenetic relationship between putative orthologs in the following grasses: *Zea mays* L., *Sorghum bicolor*, *Setaria italica*, *Hordeum vulgare, Triticum urartu* (progenitor of A-genome of bread wheat *Triticum 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 FLIPsuc90μΔ1V (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.

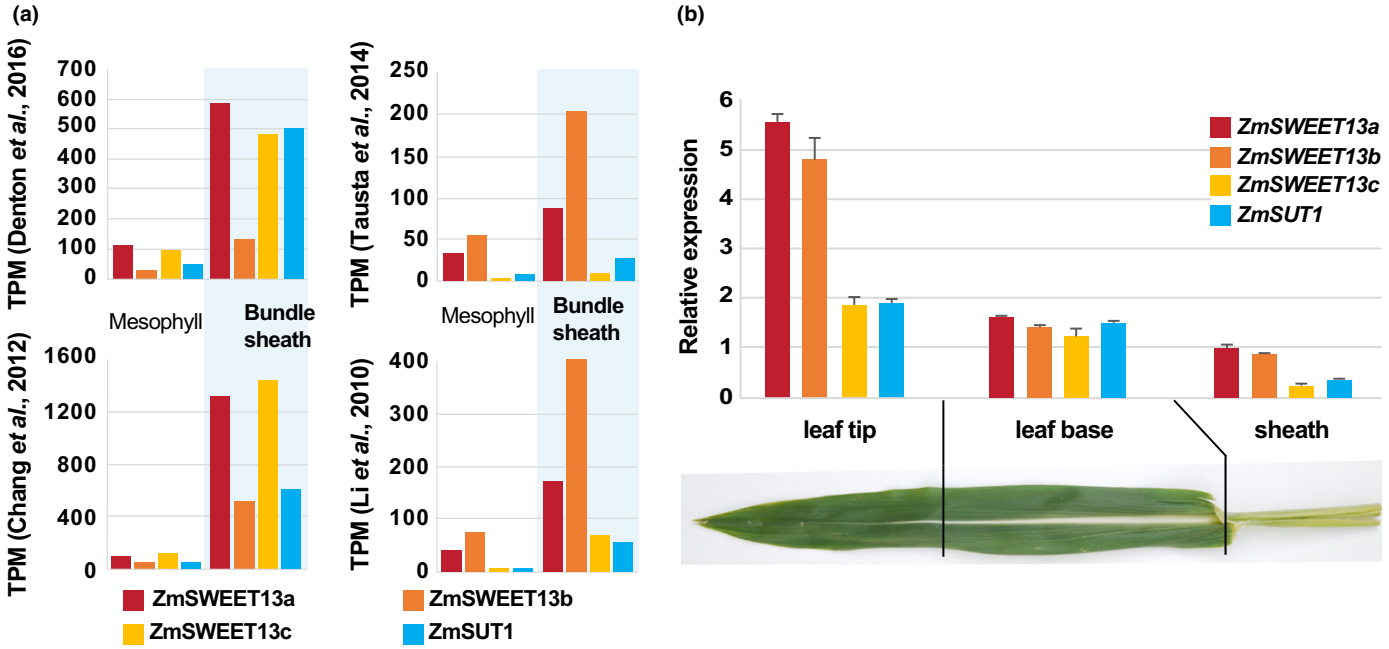

Fig. 2 *ZmSWEET13s* and *ZmSUT1* mRNA levels in flag leaves of *Zea mays* L. (a) *ZmSWEET13*a,b,c and *ZmSUT1* mRNAs accumulate preferentially in the bundle sheath and vein preparations, relative to mesophyll in four independent datasets (Li *et al.*, 2010; Chang *et al.*, 2012; Tausta *et al.*, 2014; Denton *et al.*, 2017) (TPM, transcripts per million; see also Supporting Information Fig. S1). (b) Relative mRNA levels (by qRT-PCR) of *ZmSWEET13a, b, c* and *ZmSUT1* in maize flag leaves. All four genes had highest mRNA levels in leaf tips (mean ± SEM, $n = 3$ technical replicates with expression normalized to *Zm18S* transcript levels, repeated independently four times with comparable results, see Fig. S13e, f).

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 with shorter, narrower leaves (Fig. 4a–c). Leaves were chlorotic, and accumulated ~5× more starch and ~4× more soluble sugars compared to the wild-type (Fig. 5a–c), consistent with symptoms expected for impaired phloem loading. Accumulation of starch occurred primarily in mesophyll and bundle sheath cells (Fig. 5d, e). As observed in plants with impaired phloem loading, photosynthesis was also strongly impacted in glasshouse-grown *zmsweet13a,b,c* mutants (Fig. S5). In the field, triple mutants from five independent allelic combinations presented even more severe phenotypes, with extreme chlorosis, massive anthocyanin accumulation and extremely stunted growth; in several cases this resulted in lethality (Fig. 4e). *SWEET13* mRNA levels were drastically reduced in all three *ZmSWEET13s*, as quantified by RNA-seq and qRT-PCR (Figs 4d, S6). In summary, the strong phenotype of the triple mutants is consistent with maize using predominantly an apoplasmic phloem loading mechanism.

Despite the severe defects, triple mutant plants grown in the glasshouse (as well as a subset in the field) exported sufficient sugars from leaves to produce viable seeds. A possible explanation for the viability of the triple mutants could be compensation by other sucrose-transporting clade III SWEETs. To test this hypothesis and to obtain insights about possible physiological changes in the mutants, we performed an RNA-seq analysis of flag leaves of wild-type (Hi-II transformants outcrossed once to B73 and selfed that neither contain SWEET13 mutations nor contain Cas9 as verified by PCR) and triple mutant plants (Hi-II background) (Fig. S7). Notably, we did not observe significant enrichment of mRNA of any of the clade III *SWEETs*, arguing against transcriptional compensation by other clade III *SWEETs* (Fig. S6). Our data do not exclude the possibility that compensation occurs at the post-transcriptional level. We performed a pathway enrichment analysis using the Plant MetGenMap database (Joung *et al.*, 2009) and found that mRNA levels of multiple genes encoding functions in the light-harvesting complex and in chlorophyll/tetrapyrrole 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 sucrose 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

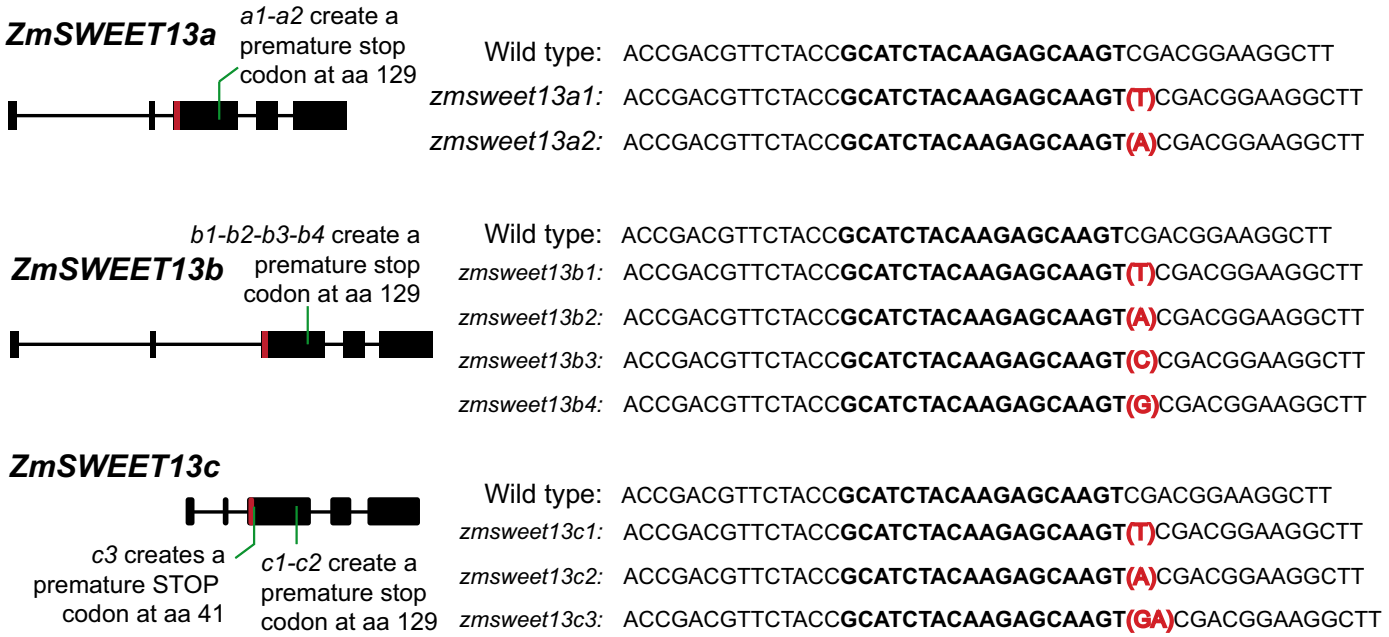

Fig. 3 CRISPR-Cas9-induced *ZmSWEET13a, b* and *c* mutations in *Zea mays* L. Schematic representation of *ZmSWEET13a*, *b* and *c* gene models, with exons displayed as black boxes. Schematics of the target site within the third exon (red) and sequences of the insertions obtained by genomic editing by CRISPR-Cas9, as determined in T3 homozygous lines, with guide RNAs marked in bold. The nine alleles carry frameshift mutations with insertions of either 1 or 2 nt, resulting in premature stop codons, as indicated within the gene model by a green line.

sweet13;14 double mutant, namely male sterility, and increased seedling and seed size (Kanno *et al.*, 2016).

To determine if variation in the *ZmSWEET13* genes may account for differences in agronomically important traits in existing maize lines, we conducted a GWAS using phenotypic traits obtained from a maize diversity panel (Flint-Garcia *et al.*, 2005). We obtained genotypic data from maize HapMap3 SNPs (Bukowski *et al.*, 2017) and filtered out SNPs with a minor allele frequency < 0.1 and missing rate > 0.5, leaving ~13 million SNPs for analyses. We performed GWAS using a mixed linear model approach (Zhou & Stephens, 2012), where kinship calculated from the genome-wide SNPs was fitted as the random effects. The SNPs that passed the FDR threshold of 0.05 and showed linkage disequilibrium ($R^2 > 0.8$) with *ZmSWEET13a,b,c* genes were considered significant associations. SNPs in *ZmSWEET13s* were significantly associated with ear-related traits (i.e. ear rank number and ear height) and developmental traits (i.e. days to silk, days to tassel, middle leaf angle and germination count) (Figs S11, S12). While these results are compatible with a key role of *ZmSWEET13s* in carbon allocation, it will be necessary to determine whether polymorphisms in these genes or flanking regions are causative for these traits.

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 fulfil 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 candidates 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 *atsweet11;12* mutants were smaller and accumulated starch in leaves (Chen *et al.*, 2012). However, the phenotype of *atsweet11;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

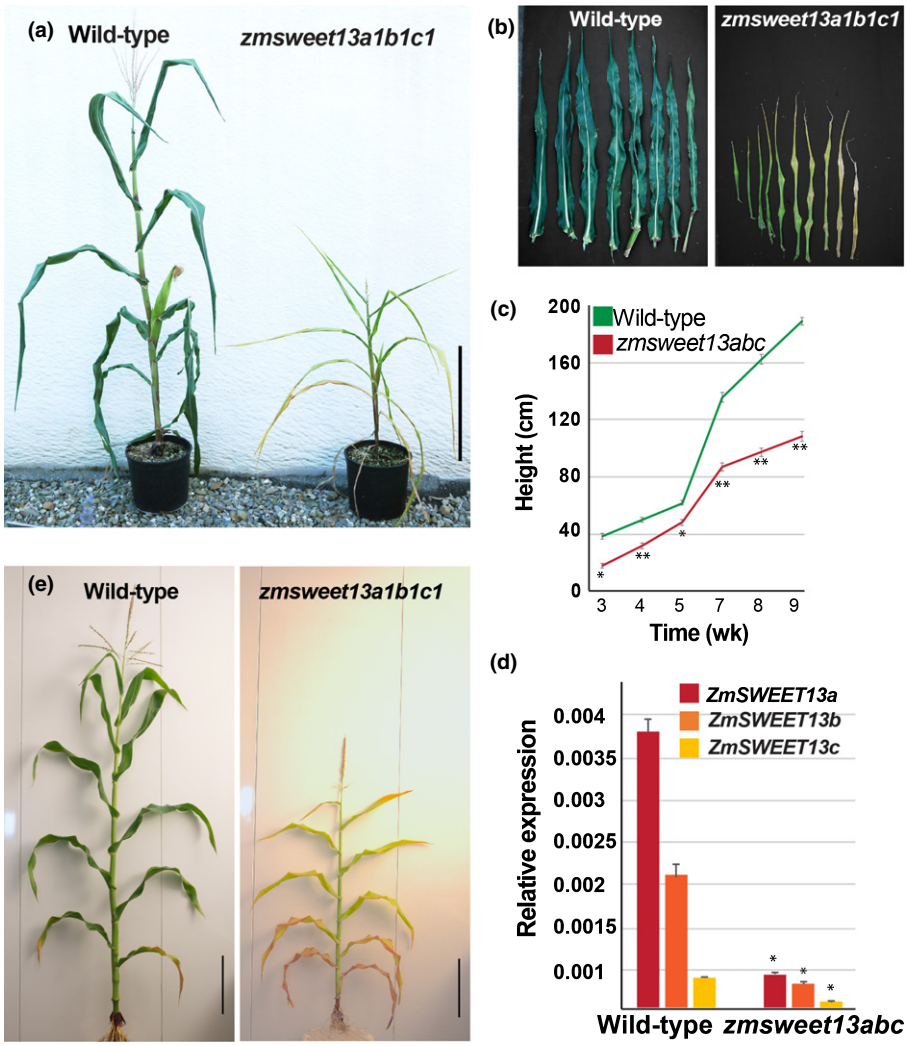

Fig. 4 Characterization *of ZmSWEET13abc* triple *Zea mays* L. mutants. (a) Mature wild-type and same-age *zmsweet13a1b1c1* triple mutant, showing reduced growth and leaf chlorosis. Bar, 50 cm. (b) Leaf phenotype of plants presented in Fig. 2(a), showing reduced length, width and chlorosis in leaves 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: *, $P < .0001$. (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.

Brachypodium and rice each have only one. The comparatively high number of *SWEET13s* had been attributed to specific roles in C_4 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 C_3 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

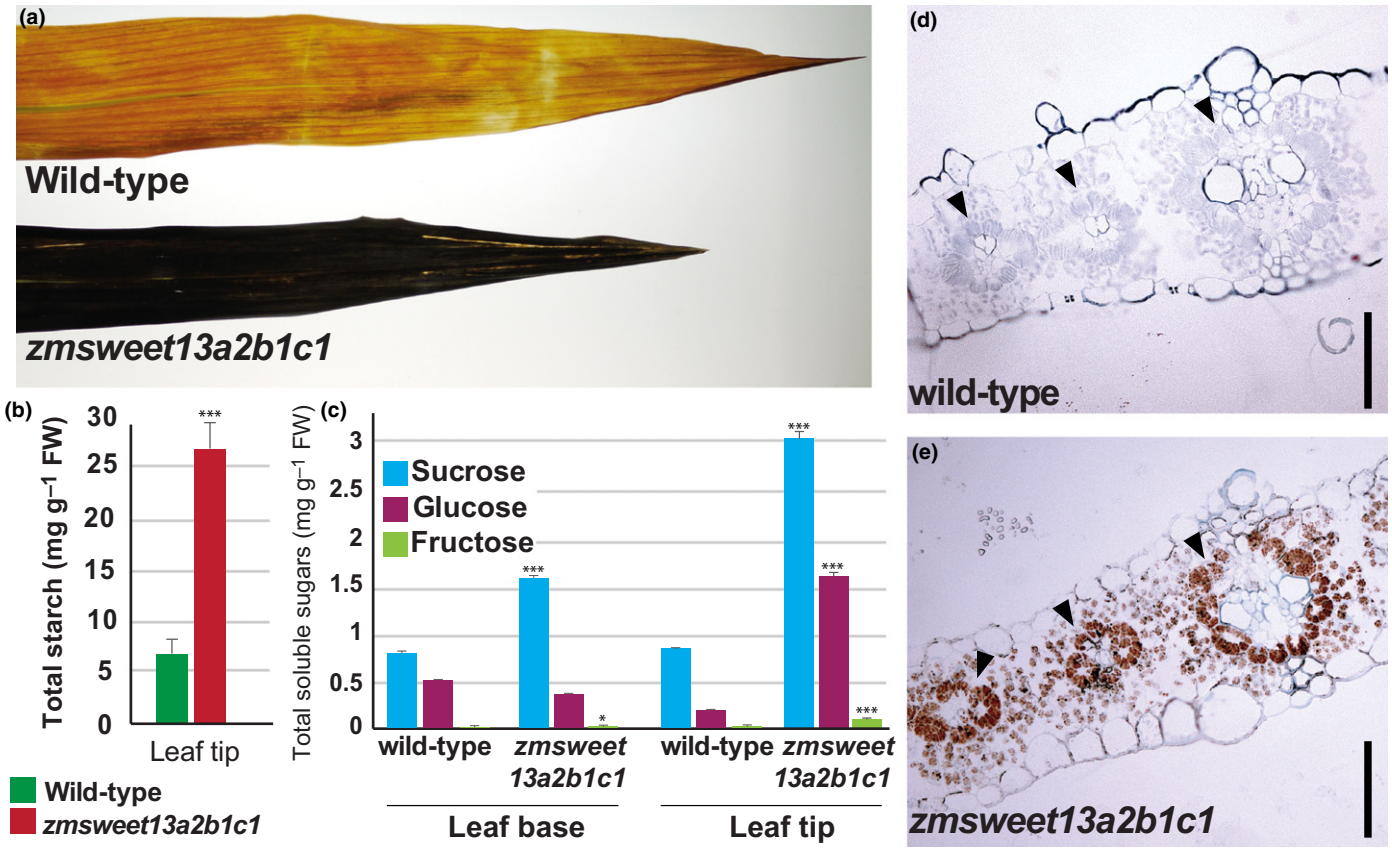

Fig. 5 Starch and soluble sugar accumulation in *zmsweet13abc Zea mays* L. triple mutants. (a) Flag leaves were collected at dawn (07:00 h), cleared with boiling ethanol and stained for 15 min with Lugol's solution. (b) Starch quantification from the same leaves as displayed in (a). The triple mutant contained ~5× more starch compared to wild-type in leaf tips. No significant differences were measured in the sheath or base (mean ± SEM, two-tailed *t*-test performed between wild-type and *zmsweet13abc*: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; $n = 3$ technical replicates, repeated independently three times with comparable results, see Fig. S13a, b). (c) Quantification of soluble sugars in the flag leaf of *zmsweet13a2b1c1* mutants. The triple mutant contained higher sugar levels in both tip and base of the leaf. Samples were harvested at 07:00 h (mean ± SEM, two-tailed *t*-test performed between wild-type and *zmsweet13abc* for each sugar: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$; $n = 3$ technical replicates, repeated independently three times with comparable results, see Fig. S13b,c). (d, e) Starch accumulation in leaves from wild-type (d) and the triple mutant (e) harvested at the end of the night. Leaves were fixed, embedded and stained with Lugol's solution for starch. Starch grains accumulated in both mesophyll and bundle sheath cells in the *zmsweet13a2b1c1* mutant, while wild-type controls showed no detectable starch accumulation at this time point (arrowheads indicate vascular bundles). Bar, 75 mm.

efflux from a specific cell type and loading is achieved in a multi-tier 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 *ZmSWEET13s* 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

M.B., S.N.C., B.Y., W.B.F. and D.S. conceived and designed experiments. M.B., M.H., T.H., S.N.C., B.Y. and D.S. performed experiments. M.B., T.H., B.Y., J.Y., W.B.F. and D.S. analysed the data. D.S. and W.B.F. wrote the manuscript, M.B. and B.Y. helped with revisions.

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:

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

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

Fig. S3 SWEET13 orthologs in different grasses.

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

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

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

Fig. S7 Principal component analysis of differentially expressed genes in *zmsweet13* mutants and wild type

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

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

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

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

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

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. zm*sweet-13abc* mutants

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