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RESEARCH PAPER

Sucrose transporter1 functions in phloem loading in maize leaves

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Abstract

In most plants, sucrose is exported from source leaves to carbon-importing sink tissues to sustain their growth and metabolism. Apoplastic phloem-loading species require sucrose transporters (SUTs) to transport sucrose into the phloem. In many dicot plants, genetic and biochemical evidence has established that SUT1-type proteins function in phloem loading. However, the role of SUT1 in phloem loading in monocot plants is not clear since the rice (*Oryza sativa*) and sugarcane (*Saccharum* hybrid) *SUT1* orthologues do not appear to function in phloem loading of sucrose. A *SUT1* gene was previously cloned from maize (*Zea mays*) and shown to have expression and biochemical activity consistent with a hypothesized role in phloem loading. To determine the biological function of *SUT1* in maize, a *sut1* mutant was isolated and characterized. *sut1* mutant plants hyperaccumulate carbohydrates in mature leaves and display leaf chlorosis with premature senescence. In addition, *sut1* mutants have greatly reduced stature, altered biomass partitioning, delayed flowering, and stunted tassel development. Cold-girdling wild-type leaves to block phloem transport phenocopied the *sut1* mutants, supporting a role for maize SUT1 in sucrose export. Furthermore, application of ^{14}C-sucrose to abraded *sut1* mutant and wild-type leaves showed that sucrose export was greatly diminished in *sut1* mutants compared with wild type. Collectively, these data demonstrate that SUT1 is crucial for efficient phloem loading of sucrose in maize leaves.

Key words: Carbohydrate accumulation, cold-girdling, maize, phloem, *sucrose transporter1*, *sut1*.

Introduction

In most plant species, sucrose is the principal form of carbohydrate translocated through the veins from carbon-exporting source leaves to carbon-importing sink tissues (Turgeon, 2006). Maize (*Zea mays*) leaves contain two photosynthetic cell types, mesophyll and bundle sheath cells, that are concentrically arranged around the veins and co-operatively perform C_4 carbon assimilation (Esau, 1977; Edwards *et al.*, 2001). Sucrose initially follows a symplastic path from its site of synthesis in the mesophyll cells (Lunn and Furbank, 1999), diffusing through plasmodesmata into the bundle sheath cells and into the vascular parenchyma cells (Russin *et al.*, 1996). Maize is an apoplastic phloem loading species (Evert *et al.*, 1978; Heyser, 1980; Gamalei, 1989). Sucrose is exported into the apoplast from the vascular parenchyma cells by an unknown mechanism, prior to being imported into the phloem companion cells and/or sieve elements of leaf minor veins (Fritz *et al.*, 1983). The minor veins feed into the lateral veins which are responsible for the long-distance transport of assimilates out of the leaf (Russell and Evert, 1985; Fritz *et al.*, 1989).

Sucrose transporters (SUTs) import sucrose from the apoplast into the phloem cells (Lalonde *et al.*, 2004; Sauer, 2007; Braun and Slewinski, 2009). SUTs contain 12 transmembrane domains that form a pore through which sucrose crosses the membrane (Lalonde *et al.*, 2004; Sauer, 2007). SUTs function as H^+/sucrose symporters with a 1:1 stoichiometry (Bush, 1990; Boorer *et al.*, 1996; Zhou *et al.*, 1997; Carpaneto *et al.*, 2005). The H^+ gradient that energizes

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Abbreviations: IKI, iodine potassium iodide; *Mu*, *Mutator*; *SUT*, *sucrose transporter*; TIR, terminal inverted repeat; TUSC, trait utility system for corn.

sucrose import is generated by H^+-ATPases located in the plasma membrane of phloem cells (Bush, 1993; DeWitt and Sussman, 1995; Gaxiola *et al.*, 2007). Experiments in *Xenopus laevis* oocytes and in yeast (*Saccharomyces cerevisiae*) have demonstrated biochemical activity for many SUTs (Riesmeier *et al.*, 1992, 1993; Gahrtz *et al.*, 1994; Sauer and Stolz, 1994; Boorer *et al.*, 1996; Zhou *et al.*, 1997, 2007; Chandran *et al.*, 2003). Based on their sequence and affinity for sucrose, SUTs were previously classified into three different types (Aoki *et al.*, 2003; Lalonde *et al.*, 2004). Type I contained high-affinity transporters proposed to function as the major transporters that load sucrose into the phloem in dicots (Kühn, 2003; Lalonde *et al.*, 2004). In recent years, with the greater number of SUT sequences, phylogenetic analysis divided the type II SUT proteins into two clades, establishing four SUT groups (Sauer, 2007). With the recent completion of the draft genome sequences of maize, sorghum (*Sorghum bicolor*), and *Brachypodium distachyon*, and the identification of their corresponding *SUT* genes, five different SUT groups have been established (Braun and Slewinski, 2009).

Members of group 2 (formerly type I) SUT proteins are unique to dicots and have been shown to function in phloem loading. Plants with reduced *SUT* function caused by a T-DNA insertion in Arabidopsis (*Arabidopsis thaliana*) (Gottwald *et al.*, 2000; Srivastava *et al.*, 2008) or antisense RNA expression in potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*) (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Bürkle *et al.*, 1998; Hackel *et al.*, 2006) accumulate excess carbohydrates in their leaves. In addition, these *sut* mutant plants exhibit leaf chlorosis, reduced rates of photosynthesis, diminished shoot and root growth, delayed flowering, and decreased yields due to the reduced export of carbohydrates to developing sink tissues. These studies demonstrate that these group 2 SUT proteins function as sucrose carriers involved in apoplastic phloem loading.

Group 1 (formerly type II) SUT proteins are present only in monocot plants, and their *in vivo* functions are mostly unknown (Braun and Slewinski, 2009). *SUT1* genes have been cloned from a number of grasses and shown to encode proteins with sucrose transport activity when expressed in heterologous systems (Hirose *et al.*, 1997; Weschke *et al.*, 2000; Carpaneto *et al.*, 2005; Rae *et al.*, 2005; Sivitz *et al.*, 2005; Reinders *et al.*, 2006). The SUT1 protein from sugarcane (*Saccharum* hybrid) was immunolocalized to cells outside the phloem in leaf and stem tissues, indicating that sugarcane SUT1 does not function in phloem loading (Rae *et al.*, 2005). To date, mutational studies have not established whether group 1 SUT1 proteins have a role in phloem loading in grass leaves. Two research groups characterized nearly complete antisense RNA knockdowns of *SUT1* in rice (*Oryza sativa*), but neither found any alterations in photosynthetic rates, plant morphology or carbohydrate content in leaves (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002). The principal phenotypes associated with these lines were reduced grain-filling and decreased seed germination (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002). The authors suggested that another SUT may be responsible for phloem loading, which is consistent with *SUT1* being expressed at low levels in rice source leaves (Hirose *et al.*, 1997). Follow-up work has shown that multiple *SUT*s are present in the rice genome (Aoki *et al.*, 2003). Alternatively, rice may utilize a symplastic phloem loading pathway based on the high frequency of plasmodesmata connecting the vascular parenchyma and companion cells (Kaneko *et al.*, 1980). Based on its expression pattern, *SUT1* in rice has been proposed to function in sucrose retrieval from the apoplasm in seedling tissues as well as in the long-distance assimilate transport pathway from the flag leaf blade to the base of the filling grain (Scofield *et al.*, 2007*a*, *b*). Therefore, no grass *SUT* gene has been demonstrated to have a function in phloem loading in leaves.

Maize and rice SUT1 proteins are highly similar, sharing 82% identity (Aoki *et al.*, 1999), and the maize and sugarcane SUT1 proteins are 95% identical (Rae *et al.*, 2005). Maize SUT1 has been shown to have a biochemical activity and sucrose affinity consistent with a role in phloem loading (Carpaneto *et al.*, 2005). In contrast with rice or sugarcane, the maize *SUT1* gene is expressed in a pattern suggestive of a function in phloem loading. Maize *SUT1* is expressed strongly in source leaves in a diurnal pattern with maximal expression at the end of the day and minimal expression during the night (Aoki *et al.*, 1999). In addition, in developing leaves, it is expressed in a tip-to-base gradient reflective of the sink-to-source transition, which is in agreement with a role in exporting photoassimilates (Aoki *et al.*, 1999). However, given that the orthologous rice and sugarcane *SUT1* genes do not appear to function in phloem loading, the *in vivo* role of maize SUT1 in phloem loading is suspect. To test the hypothesis that the maize SUT1 protein functions in phloem loading, a *sut1* mutant was isolated. Here, the effects of the mutation on plant growth, leaf carbohydrate accumulation, and ^{14}C-sucrose export from leaves are reported, and the role of SUT1 in phloem loading of sucrose in maize leaves is discussed.

Materials and methods

Plant growth conditions

Maize (*Zea mays* L.) plants were grown in a greenhouse supplemented with sodium vapour and metal halide lamps at 1400 μmol m^{-2} s^{-1} under a 16 h day (30 °C) and 8 h night (20 °C). Dawn was at 06.00 h and dusk at 22.00 h. Plants were grown in pots in a peat-based potting soil (Premier ProMix BX, Griffin Greenhouse, Morgantown, PA), watered daily and fertilized weekly with Sprint 330 iron chelate and an N-P-K 15:15:15 fertilizer.

Genetic stocks and genotyping

The *sut1* mutant allele was isolated using the Trait Utility System for Corn (TUSC, Pioneer Hi-Bred) (Bensen *et al.*, 1995). Heterozygous plants carrying the *sut1* allele were outcrossed to plants homozygous for the *Mutator (Mu) Killer* locus, introgressed into the B73 inbred line, to silence

Mu activity (Slotkin *et al.*, 2003, 2005). The F_1 plants were self-pollinated to produce segregating F_2 families used for analyses. Plants carrying the *sut1* allele were identified by PCR genotyping using *SUT1* primer No. 3 in Fig. 1A (5′-CCACGGACAGCTCCAGCTCGCCG-3′), or No. 6 in Supplementary Fig. S1 (5′-GAGTGAGTGCTGGTAGGATGGC-3′), and an outward facing primer designed in the terminal inverted repeat (TIR) of *Mu* elements No. 2 in Fig. 1A and Supplementary Fig. S1 (5′-AACGCCTCCATTTCGTCGAATCC-3′). PCR conditions were 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s for 35 cycles with a final concentration of 3% dimethyl sulphoxide and 5% glycerol. The site of the *Mu* insertion was determined by sequencing the resulting PCR products (see Supplementary Fig. S1 at *JXB* online). To identify the wild-type *SUT1* allele, primer pairs No. 6 and No. 3 were used with the same PCR conditions.

Reverse transcription-PCR experiments

RNA was isolated from three biological replicates of fully mature leaf five from 3-week-old plants collected at the end of the day using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA). cDNA was synthesized following the manufacturer's instructions from 1 μg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA was diluted 5-fold and 2 μl used for each RT-PCR reaction. *Ubiquitin* primers (5′-GACCAGCAGCGTCTGATCTTCGC-3′ and 5′-CTGAAAGACAGAACATAATGACGACA-3′) were used as cDNA normalization controls with PCR conditions of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s for 30 cycles. Expression of the wild-type 5′ transcript was determined using primer No. 3 with primer No. 1 from Fig. 1A (5′-GCTCCAAACACCCACACCCACACCAC-3′). For the *sut1* mutant transcript, primers No. 2 and No. 3 were used with the same PCR conditions listed above for the genotyping reactions with the exception that the PCR was run for 32 cycles. Expression of the 3′ end of wild-type and mutant transcripts was determined using primers No. 4 and No. 5 shown in Fig. 1A (5′-GTTCATCCTCTACGACACCGAC-3′ and 5′-GAGGGATGACGATGGAGATGTTG-3′) with PCR conditions of 95 °C for 30 s, 66 °C for 30 s, 72 °C for 30 s for 32 cycles. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

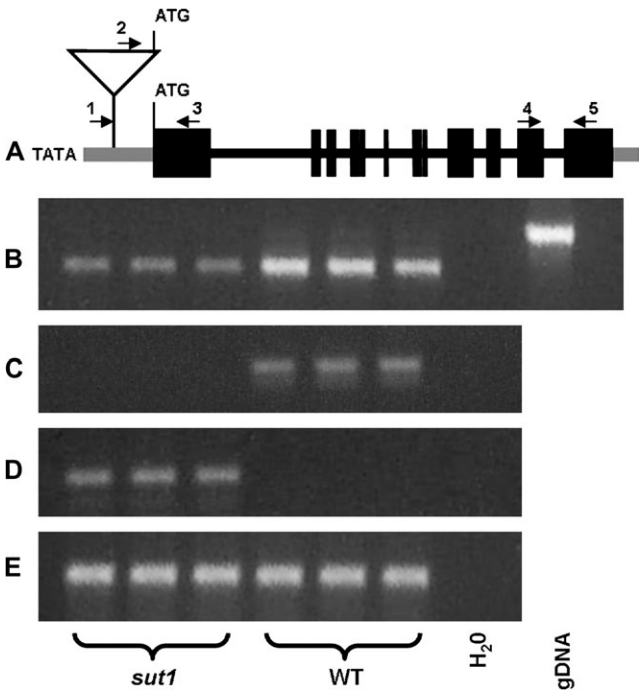

Fig. 1. Molecular characterization of *sut1* mutant RNA expression. (A) Schematic of *sut1* mutant allele showing *Mu1* insertion (inverted triangle) in the 5′ UTR. Exons are shown as black boxes, introns as black lines, UTRs as grey lines and the TATA box is indicated. Arrows indicate location of primers used in RT-PCR experiments. ATG in exon1 shows normal translation start site in wild-type protein. ATG in *Mu1* indicates an upstream, out-of-frame start site in *sut1* mutant transcript. (B–D) RT-PCR of *SUT1* RNA isolated from *sut1* mutant and wild-type (WT) leaves. (B) Expression detected using primers 4 and 5. (C) Expression detected using primers 1 and 3. (D) Expression detected using primers 2 and 3. (E) Expression of *ubiquitin* as a cDNA normalization control. H_2O is no added template control. gDNA is a control showing no genomic DNA contamination in the cDNA pools.

Morphometric analyses

Morphometric studies were conducted on mature plants from two F_2 families segregating *sut1* mutants according to Ma *et al.* (2008). For plant height and leaf number n=10 for each class; for anthesis n=10 for wild type and n=4 for *sut1* mutants as 90% of the mutants did not produce fertile tassels. Shoot and root dry mass were measured from 10-d-old seedlings (n=10) as described by Bürkle *et al.* (1998). The morphometric experiments were repeated twice with similar results, and data from one repeat is shown.

Cold-girdling

For cold-girdling studies, 3-week-old B73 wild-type plants were used. Leaf five had fully expanded and was mature. Half millimetre thick plastic tubing with a 3 mm inner diameter was carefully wrapped around the stalk of the plant to cover 5 cm of leaf five sheath and stem (see Supplementary Fig. S2 at *JXB* online). The stem and tubing were wrapped with aluminium space blankets to provide uniform cooling. The tubing was connected to a small pump that circulated 0 °C water from an ice-water bath. Plants were continuously girdled for 48 h until chlorosis was visible throughout the leaf. For the girdling experiments, four biological replicates were used and the experiment was repeated three times with similar results each time. Data from one repeat are presented. Leaf tissue was collected at 08.00 h at the end of the 48 h period and immediately used for starch staining, or in a separate replicate, at 20.00 h at the end of the 48 h period and quickly frozen at –80 °C for carbohydrate quantification. For all quantifications of leaf samples reported, blade tissue located 2.5–4 cm proximal to the tip, avoiding the midrib, was measured.

Starch staining and carbohydrate quantifications

Starch was visualized in leaf tissue by iodine potassium iodide (IKI) staining (Ruzin, 1999). Carbohydrates were quantified according to Stitt *et al.* (1989). Samples were collected at 08.00 h and 20.00 h from leaves four, five, six and seven of 3-week-old wild-type and *sut1* plants. Leaf tissue was harvested from three biological replicates and each plant was sampled at only one time point. The entire experiment was repeated three times, and data for all replicates are presented. Leaves four and five were fully mature, and leaf six had emerged and expanded to ~55% of its final size. Leaf seven blade tissue had emerged approximately 20 cm out of the whorl (~30% of its final length); the tip had transitioned to source tissue, but the base was still immature, sink tissue (Evert *et al.*, 1996).

^{14}C-sucrose export assays and liquid scintillation counting

Four centimetres below the tip of leaf seven of 3-week-old plants, the adaxial surface was gently abraded with a 5 mm wide piece of fine grain sandpaper to remove the cuticle and epidermis, thereby ensuring a uniform application site for all leaves. A 10 µl solution containing 0.2 µCi of [U-^{14}C]-sucrose (22.2 GBq mmol^{-1}, Amersham, Piscataway, NJ) in 4.5 mM unlabelled sucrose was applied to the abraded site and covered with Parafilm to prevent dehydration. Preliminary experiments determined that little label had exited the leaf within 1 h. After 1 h, a ~22 cm segment (measured from the tip) of leaf seven was removed from the plant, taped to Whatman filter paper, dried at 80 °C on a gel drier, and exposed to X-ray film. Film was typically exposed for 5 d prior to development. For both wild-type and *sut1* mutant plants, nine biological replicates of the experiment were performed with similar results each time.

To quantify ^{14}C transport, 6 mm diameter leaf discs were punched from wild-type and *sut1* dried leaves and placed into separate glass scintillation vials. Due to the tapering of the leaf, one disc was collected at the abraded application site, whereas four discs per sample were harvested across the width of the leaf at both 8 cm and 16 cm proximal to the application site, avoiding the midrib in all cases. Nine biological replicates of the experiment were performed. Photosynthetic pigments were extracted in 1 ml 100% ethanol at 65 °C for 2 h, after which 750 µl of 30% hydrogen peroxide was added to the vials. In a fume hood, the samples were gently heated to evapourate the ethanol, bleach the tissue and de-gas the solution. Once clear, samples were cooled to room temperature, 100 µl of a 1:1 (v/v) glacial acetic acid:water solution added, incubated at room temperature for 10 min and then 8 ml scintillation fluid (Ready Safe, Beckman Coulter, Fullerton, CA) added. Vials were left overnight in the dark to reduce background from chemiluminescence prior to reading. Samples were read on a Beckman LS 6000SE liquid scintillation counter calibrated with a ^{14}C standard.

Statistical analyses

Statistical significance was determined using Student's two-tailed *t* test embedded in Microsoft Excel.

Results

Molecular characterization of a maize sut1 mutant

To characterize the function of *SUT1* in maize, a mutant allele was isolated using TUSC (Bensen *et al.*, 1995). A mutant allele with a *Mutator1 (Mu1)* transposable element insertion in the 5′ untranslated region (UTR) was recovered and designated *sut1-m1::Mu1* (hereafter *sut1*) (Fig. 1A; see Supplementary Fig. S1 at *JXB* online). To determine the consequences of the mutation on gene expression, *SUT1* RNA levels were assayed in mature wild-type and mutant leaf tissues collected at the end of the photoperiod when *SUT1* expression peaks (Aoki *et al.*, 1999). Reverse transcription-PCR (RT-PCR) of *SUT1* RNA was examined using primers located in the 3′ end of the transcript (Fig. 1A, B). Expression of *SUT1* was strongly reduced in the *sut1* mutants compared to wild type. When *SUT1* primers flanking the *Mu1* insertion site were used, no transcripts were detected from *sut1* mutant leaves, while they were present in wild type (Fig. 1A, C). This suggests that the transcript detected in *sut1* mutants using the 3′ primers may be due to initiation from a different site, such as within the transposon. To test this idea, RT-PCR was carried out using the same upstream-facing gene-specific primer with a second primer designed to the *Mu* element TIR. A product was obtained from *SUT1* mutants, but not from wild-type siblings (Fig. 1A, D). This indicates that a portion of the transposon was present in the RNA, most likely due to initiation from an outward-facing promoter located within the TIR (Raizada *et al.*, 2001). Sequencing the *sut1* mutant 5′ RT-PCR product revealed an out-of-frame ATG in the *Mu* TIR located 268 bases upstream of the normal SUT1 translational start site (see Supplementary Fig. S1 at *JXB* online). Therefore, these data suggest that along with altered RNA expression/stability, the *Mu* insertion also results in a novel translation start site which would lead to a frameshift mutation that is predicted to produce a non-functional protein.

sut1 mutant leaves hyperaccumulate carbohydrates

To characterize the contribution of *SUT1* to plant growth and development, the phenotypes of plants homozygous for the *sut1* mutation were analysed. *sut1* mutant plants show numerous growth defects, including greatly reduced plant height (Fig. 2A), chlorotic, senescent, and necrotic leaves (Fig. 2C), and delayed flowering (Table 1; see below). To investigate the basis for these phenotypes, the leaves of *sut1* mutant plants were compared to wild-type siblings. *sut1* mutant leaves display a chlorotic/necrotic phenotype that progressively becomes more severe as the leaf emerges from the whorl. At maturity, leaves of *sut1* plants are strongly chlorotic throughout the leaf blade compared with wild type

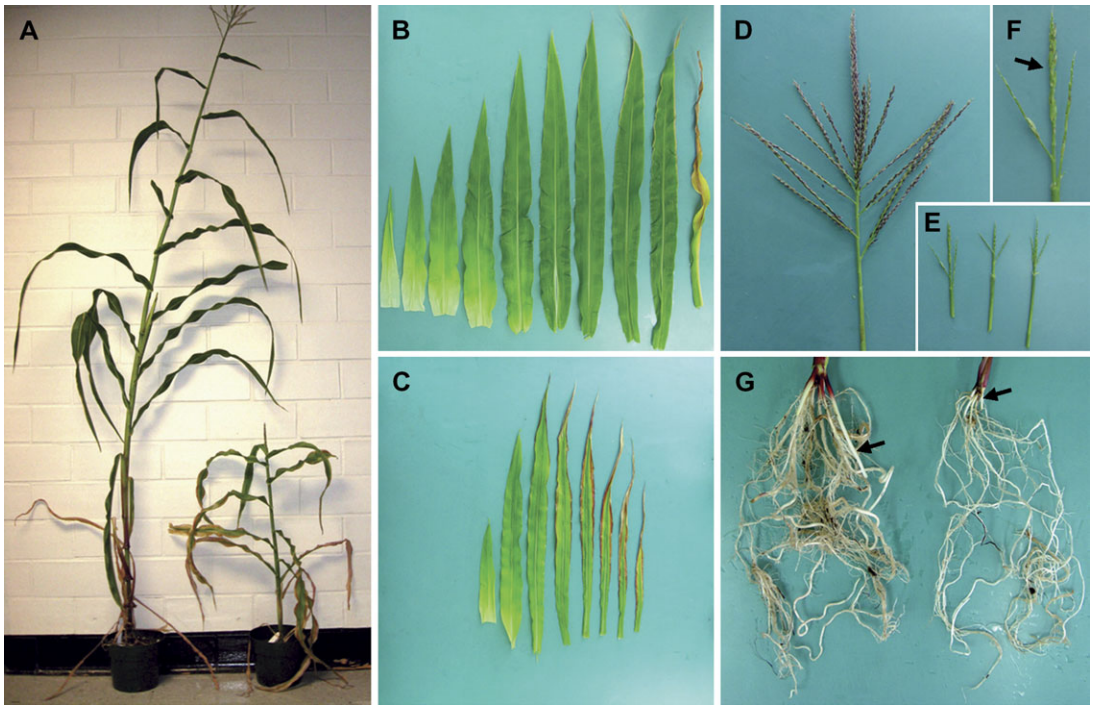

Fig. 2. *sut1* mutants have reduced growth of vegetative and reproductive tissues. (A) Photograph of 10-week-old *sut1* mutant (right) and same age wild-type sibling (left). (B) Leaves of a 6-week-old wild-type plant. (C) Same age *sut1* mutant has fewer leaves that prematurely senesce. In (B) and (C) leaves are arranged from the oldest on the right to the youngest on the left. (D) Tassel of wild type. (E) Tassels from three *sut1* mutants displaying stunted development. (F) *sut1* mutant tassel showing barren patches on branches and limited spikelet development (arrow). (G) Root system of 10-d-old wild type (left) and same-age *sut1* mutant (right) with decreased root number, branching, and growth. Arrows indicate prop roots.

Table 1. Morphometric analyses in wild-type and sut1 *plants*

Plant height, leaf number and days to anthesis are measured at pollen shed. Biomass data are for 10-d-old seedlings. Data represent the mean of ten samples ±SE, except for *sut1* mutants days to anthesis where $n=4$.

Genotype	Plant height[a] (cm)	Leaf number[a]	Days to anthesis[a]	Total mass (g)	Shoot mass[a] (g)	Root mass[a] (g)	Shoot/root ratio
Wild type	237.8±2.3	21.2±0.2	64.1±1.12	2.68	1.92±0.14	0.76±0.09	2.53
sut1 *mutant*	83.1±2.7*	13.6±0.3*	78.4±0.6*	1.53	1.22±0.15*	0.31±0.04*	3.94

[a] Asterisk indicates that the value is significantly different from wild type at P <0.005.

(Fig. 3A, C). To determine if leaf chlorosis was associated with carbohydrate hyperaccumulation, the leaves were cleared of photosynthetic pigments and the tissue was stained for starch with IKI (Ruzin, 1999). Wild-type leaves collected at 08.00 h had little starch whereas *sut1* mutant leaves stained strongly for starch throughout the tissue (Fig. 3B, D). Histological analyses revealed that the bundle sheath cells in wild-type leaves contained trace amounts of starch (Fig. 3G). However, both the bundle sheath and mesophyll cells in *sut1* mutant leaves contained abundant starch (Fig. 3H). Starch does not typically accumulate in mesophyll cells unless the tissue experiences a severe stress that results in inhibition of phloem loading or transport (Rhoades and Carvalho, 1944; Russin *et al.*, 1996; Jeannette *et al.*, 2000). These data suggest that the absence of *SUT1* function results in an increase in starch stored in the chloroplasts.

To test if soluble sugars as well as starch over-accumulate in *sut1* leaves, their levels were quantified at both the beginning and the end of the day. In the 3-week-old plants assayed, leaf seven was the youngest visible leaf and in the process of emerging from the whorl at the top of the plant, leaf six was located one leaf below, had emerged and attained ~55% of its final size, and leaves five and four were fully mature and located one and two leaves further down the plant, respectively. Sucrose, glucose, fructose, and starch were increased in mature leaves four, five, and six of *sut1* mutant plants relative to wild type at both 08.00 h and 20.00 h (Fig. 4A–D). For the four different carbohydrates, at both time points, for leaves four, five, and six, the differences between *sut1* and wild type were highly significant in all cases (P <0.0001). Within either the wild-type or the *sut1* leaves, the levels of glucose, fructose, and starch

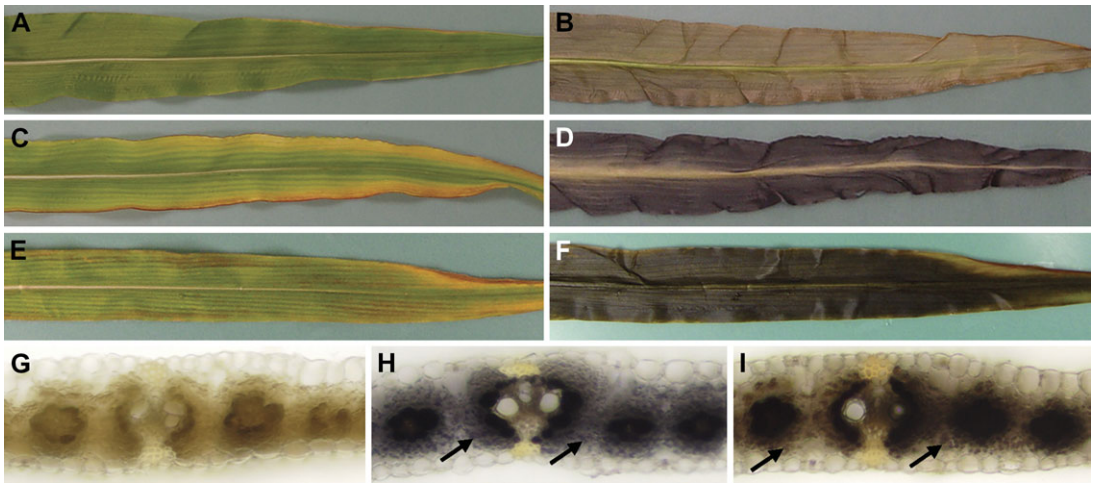

Fig. 3. Starch accumulation in untreated wild-type, *sut1* mutant, and cold-girdled wild-type leaves. Leaf five was collected at 08.00 h from 3-week-old plants, cleared of photosynthetic pigments, and stained with IKI. (A, B, G) Untreated wild type. (C, D, H) *sut1* mutant. (E, F, I) Cold-girdled wild-type leaf. (A, C, E) Photographs of leaves. (B, D, F) Photographs of same leaves after IKI staining. (G–I) Bright-field leaf cross-sections after IKI staining. Arrows indicate starch in mesophyll cells.

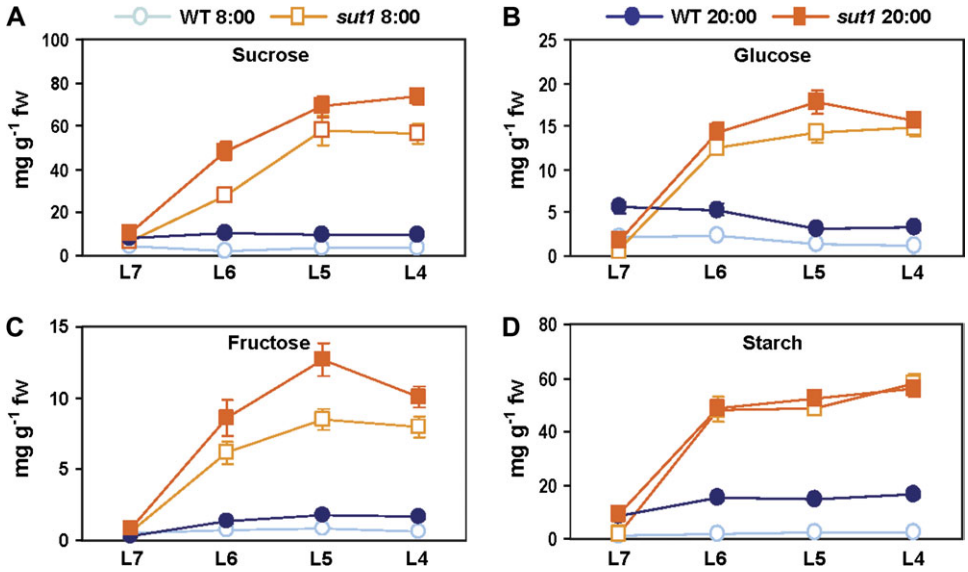

Fig. 4. Carbohydrate accumulation in wild-type and *sut1* mutant leaves. Measurements were made at two time points, 08.00 h and 20.00 h, on leaves four through seven (L4–L7) of 3-week-old plants. Wild type (WT) is represented with circles and *sut1* mutants with squares. Data collected at 08.00 h is represented by open symbols and at 20.00 h with closed symbols. Each value is the mean of nine samples ±SE. Error bars are plotted for all data, but are obscured by the symbols in some cases. (A) Sucrose. (B) Glucose. (C) Fructose. (D) Starch.

were roughly similar in mature leaves four through six (Fig. 4B–D). Sucrose levels in *sut1* gradually increased from leaf six to leaf four (Fig. 4A). The fold differences between the *sut1* and wild-type values were invariably greater at 08.00 h than at 20.00 h, for example, sucrose in leaf six was 12-fold higher at 08.00 h, but only 4.5-fold higher at 20.00 h, in leaf five, 15-fold at 08.00 h, but only 7-fold at 20.00 h, and in leaf four, 14-fold at 08.00 h, but only 7.8-fold at 20.00 h. However, as opposed to the mature leaves, the exposed tip of leaf seven contained similar carbohydrate levels in *sut1* and wild-type plants at both time points (Fig. 4A–D). The only exception was glucose when measured at 20.00 h which was increased in wild-type leaf seven compared with *sut1* (Fig. 4B). Hence, emerging leaf seven appeared virtually equivalent in wild type and *sut1* mutants, but once *sut1* leaves matured, they hyperaccumulated carbohydrates. These data are consistent with *SUT1* functioning in phloem loading in source leaves, and in its absence, the failure to import sucrose into the phloem and the increased retention of carbohydrates in photosynthetic cells.

sut1 *mutants have reduced growth and stunted reproductive development*

Carbohydrate accumulation in leaves has been associated with reductions in plant growth and yield (Riesmeier *et al.*,

1994; Russin *et al.*, 1996; Bürkle *et al.*, 1998; Gottwald *et al.*, 2000; Braun *et al.*, 2006; Baker and Braun, 2008; Ma *et al.*, 2008, 2009; Srivastava *et al.*, 2008). To investigate the effects of the loss of *SUT1* function on plant growth and development further, detailed phenotypic characterization of *sut1* mutant and wild-type plants was performed. Vegetative phenotypes in *sut1* mutants included reduced plant height and leaf number compared with wild-type siblings (Table 1). Further, mutant leaves were smaller and showed premature senescence (Fig. 2B, C). In addition, the retention of carbohydrates in the leaves was associated with decreased total biomass, a 36% reduction in shoot mass, a 59% reduction in root mass and an increased shoot-to-root ratio (Fig. 2A–C, G; Table 1). Concomitant with the reduction in root mass, the elongation of prop roots was severely attenuated in *sut1* mutants (Fig. 2G). Hence, loss of *Sut1* function is correlated with decreased plant vigour.

In addition to the vegetative phenotypes, the *sut1* mutation also conferred reproductive defects. Carbohydrate retention in *sut1* mutant leaves was associated with arrested tassel development (Fig. 2D–F) and a failure to produce ears (Fig. 2A). Approximately 10% of the *sut1* mutants produced tassels with partially barren branches and limited spikelet development (Fig. 2E, F). These stunted tassels yielded very reduced amounts of pollen and were delayed in anthesis by 14 d compared with wild-type siblings (Table 1). All of these phenotypes are presumably caused by the reduced export of sucrose from the leaves, resulting in increased carbohydrate retention within the leaves and decreased delivery to the developing sink tissues. Thus, *sut1* mutant plants display strongly inhibited reproductive development as well as vegetative growth defects.

Cold-girdling wild-type plants results in leaf chlorosis and carbohydrate hyperaccumulation similar to sut1 *mutants*

To investigate if a disruption in phloem loading is consistent with the observed chlorosis and carbohydrate hyperaccumulation seen in *sut1* mutant leaves, cold-girdling of wild-type B73 plants was performed. Cold-girdling dicot plants has been shown to block phloem transport and result in carbohydrate accumulation, down-regulation of photosynthesis, and leaf chlorosis (Krapp *et al.*, 1993; Krapp and Stitt, 1995; Schulz *et al.*, 1998). To test this in maize, plastic tubing containing ice-water was wrapped around the leaf five sheath and allowed to girdle the plant for 48 h (see Supplementary Fig. S2 at *JXB* online). Chlorosis initiated at the tip of the leaf and spread throughout the entire leaf by 48 h (Fig. 3E). To examine if carbohydrates accumulated in the chlorotic tissue, leaves were collected at 08.00 h, cleared, and stained with IKI. Leaves of untreated control plants contained little starch (Fig. 3B). However, the chlorotic leaves of girdled plants contained greatly elevated levels of starch (Fig. 3F). Cross-sectional analyses demonstrated that untreated leaves contained trace amounts of starch only in the bundle sheath cells (Fig. 3G), whereas the girdled leaves accumulated large quantities of starch in both the bundle sheath and mesophyll cells (Fig. 3I). To investigate whether girdled leaves contained different amounts of carbohydrates from wild type near the end of the photoperiod, the levels of soluble sugars and starch were quantified in leaves harvested at 20.00 h. Leaves that had been girdled for 48 h contained significantly higher levels of sucrose, glucose, fructose, and starch relative to untreated controls (Table 2). These data show that cold-girdling leaves results in carbohydrate accumulation and phenocopies the *sut1* mutant phenotype.

In a process known as guttation, maize leaves secrete droplets of water at night when water potential in the roots exceeds the water potential in the leaves (Curtis, 1943). These droplets are secreted from hydathodes, special structures located at the terminal ends of the xylem at the leaf margins (Esau, 1977). Along with water, any solutes present in the apoplasm of the leaf can be carried along the path of water movement and secreted as part of the guttation fluid (Greenhill and Chibnall, 1934; Curtis, 1943; Pilot *et al.*, 2004). Interestingly, it was found that droplets of fluid were secreted from the hydathodes and any wound site on both *sut1* and girdled leaves. The guttation fluid from *sut1* mutant and cold-girdled leaves contained high concentrations of soluble sugars; however, in guttation fluid from untreated, wild-type leaves, only trace amounts of sugars were detected (data not shown). The fact that phloem transport-inhibited cold-girdled wild-type leaves and *sut1* mutant leaves share the novel phenotype of secreting excess sugar in their guttation fluid is supportive of a role for maize *SUT1* in phloem loading of sucrose.

^{14}C-sucrose export is greatly diminished in sut1 *mutant leaves*

The phenotypic data presented thus far is strongly suggestive of a defect in sucrose export in *sut1* mutant leaves. To assess the capability of wild-type and *sut1* mutant leaves to export sucrose directly, radioactively labelled ^{14}C-sucrose was applied to the distal tip of an abraded leaf on an intact plant and allowed to transport for 1 h. The leaf was then excised, dried, and autoradiographed (Fig. 5). For these assays, leaf seven of 3-week-old plants was selected as it was phenotypically similar to wild type with regard to carbohydrate levels, photosynthesis, and gas exchange (Fig. 4 and data not shown). Wild-type leaves showed considerable ^{14}C-sucrose transport down the length of the blade during the 1 h time-frame (Fig. 5B). However, *sut1* mutant leaves

Table 2. Carbohydrate quantification in untreated and girdled wild-type leaves

Leaf five of 3-week-old plants was harvested at 20.00 h, which was the end of the 48 h of cold-girdling. Values represent the mean of four samples ±SE. Units are mg g^{-1} fresh weight.

Phenotype	Sucrose[a]	Glucose[a]	Fructose[a]	Starch[a]
Untreated wild type	5.7±0.48	1.09±0.2	0.18±0.03	8.61±0.84
Cold-girdled wild type	34.3±3.99*	4.48±0.5*	2.48±0.28*	67.75±2.75*

[a] Asterisk indicates that the value is significantly different from untreated wild-type leaves at P <0.002.

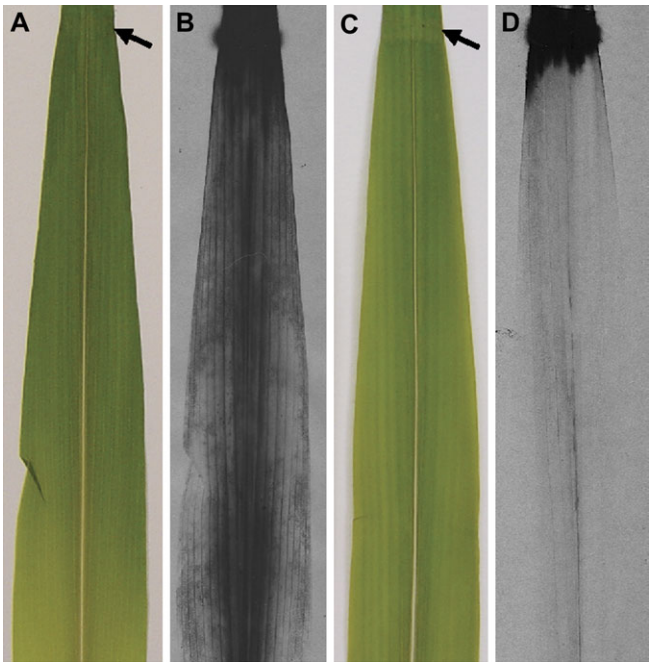

Fig. 5. ^{14}C-sucrose export in wild-type and *sut1* mutant leaves. A ^{14}C-sucrose solution was applied to the abraded tip of leaf seven of 3-week-old plants, the plants were allowed to transport for 1 h, leaf seven was excised, dried, and exposed to X-ray film. (A, B) Wild type. (C, D) *sut1* mutant. (A, C) Photographs of leaves. Arrows indicate application site. (B, D) Autoradiographs of leaves showing ^{14}C-sucrose transport.

displayed a greatly reduced capacity to transport the labelled sucrose, with almost all of the label remaining within 1 cm of the application site (Fig. 5D). Similar results were obtained using mature leaves four through six of *sut1* and wild-type plants (data not shown). Therefore, these data indicate that *sut1* mutant source leaves are severely deficient in sucrose export.

To quantify the differences in sucrose export between wild-type and *sut1* leaves, leaf punches were removed along the length of the blade and the amount of ^{14}C present was counted (Table 3). *sut1* mutant leaves had almost 2-fold higher ^{14}C-sucrose at the application site relative to wild-type leaves, indicating that the *sut1* mutants were less able to export sucrose. At 8 cm proximal to the application site, wild-type leaves had transported 5-fold greater amounts of applied label relative to *sut1*. At 16 cm proximal, the difference was even greater with the wild type showing an approximately 8.4-fold greater amount of ^{14}C-sucrose than the *sut1* mutants. Therefore, these data further support the finding that the *sut1* mutant leaves have a drastically reduced ability to export sucrose compared to the wild type.

Table 3. ^{14}C-sucrose export quantification in wild-type and sut1 *leaves*

The adaxial tip of leaf seven of 3-week-old plants was abraded and a solution containing ^{14}C-sucrose was applied. Plants were left for 1 h, after which leaf seven was excised and dried. Leaf punches were collected at the application site (0 cm), and at 8 cm and 16 cm proximal to the application site, and the amount of radiation was quantified. Values are the mean of nine samples ±SE, and the units are disintegrations per minute.

Genotype	0 cm[a]	8 cm[a]	16 cm[a]
Wild type	32 133±7578	725±48	544±84
sut1 *mutant*	58 277*±3377	134*±22	65*±4

[a] Asterisk indicates that the value is significantly different from wild-type leaves at P <0.01.

Discussion

Maize SUT1 functions in phloem loading

Based on sequence homology and gene expression, it had previously been proposed that the maize SUT1 protein functions in mature leaves to load sucrose into the phloem (Aoki *et al.*, 1999). Biochemical characterization showed that SUT1 possessed sucrose transport activity as well as H^+ and sucrose affinities consistent with its proposed role (Carpaneto *et al.*, 2005). To determine the biological function of the maize *SUT1* gene *in planta*, a *sut1* homozygous mutant was isolated and characterized. It was found that *sut1* mutant plants had carbohydrate hyperaccumulation in leaves and leaves that were chlorotic and prematurely senescent. In addition, *sut1* mutants had reduced growth, displayed altered biomass partitioning and impaired reproductive development. All of these phenotypes are consistent with a defect caused by a failure to transport sucrose from the photosynthetic cells into the phloem. Exogenous ^{14}C-sucrose application confirmed that sucrose export was greatly retarded in *sut1* mutant leaves compared with the wild type, supporting this hypothesis. Furthermore, these phenotypes are similar to those reported in dicot plants mutant for the *SUT1*-type gene that functions in phloem loading (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Bürkle *et al.*, 1998; Gottwald *et al.*, 2000; Hackel *et al.*, 2006; Srivastava *et al.*, 2008). In particular, the defect in ^{14}C-sucrose export observed in maize *sut1* mutant plants is virtually identical to that reported for *Atsuc2* mutant *Arabidopsis* plants (Gottwald *et al.*, 2000; Srivastava *et al.*, 2008). Hence, together with the previous work, the *in vivo* characterization of the *sut1* mutant indicates a critical function for the maize SUT1 protein in loading sucrose into the phloem in leaves for export to sink tissues.

Different roles of closely related grass SUT1 proteins

The SUT1 protein from sugarcane did not localize to the phloem, indicating it does not function in phloem loading (Rae *et al.*, 2005). Further, antisense RNA suppression of the *SUT1* gene in rice did not produce a phloem loading-related phenotype in leaves, suggesting that, at least in rice, SUT1 does not function in leaves to load sucrose into the phloem (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002). However, mutation of the maize *SUT1* gene produced a phloem loading-related phenotype in mature leaves. There are several possible explanations for why a defect in phloem loading may have been observed in maize but not in rice.

First, rice *SUT1* is expressed at low levels in source leaves in comparison to the maize *SUT1* (Hirose *et al.*, 1997; Aoki *et al.*, 1999), so, as previously suggested, it may not have a predominant role in phloem loading in rice. Second, genetic redundancy may mask any phenotype in the rice antisense *SUT1* lines, since two other closely related rice *SUT*s, *SUT3* and *SUT5*, are more highly expressed in source leaves than *SUT1* (Aoki *et al.*, 2003). A third possibility is that the reduction in gene expression achieved using antisense RNA expression in rice was insufficient to eliminate *SUT1* function. However, this explanation is unlikely as some transgenic lines displayed less than 10% of wild-type transcript levels and others had only 1.4% of the wild-type level of SUT1 protein (Ishimaru *et al.*, 2001; Scofield *et al.*, 2002). In contrast to the rice antisense lines, the maize *sut1* mutation is a presumed null allele that eliminates gene function based on the 5′ end of the transcript containing a portion of the *Mu* transposon and encoding an out-of-frame, non-functional protein if translated. Thus, differences in expression and functional requirements in leaves suggest that the orthologous *SUT1* genes in grasses probably play different roles in maize, sugarcane and rice.

Phenotypes observed in sut1 *mutant plants are very likely caused by mutation of* SUT1

As previously noted, the phenotypes of *sut1* mutant plants closely resemble the phenotypes reported in dicot plants that lack a SUT responsible for phloem loading. In addition, the novel phenotype of secreted sugar droplets observed on *sut1* mutant leaves was phenocopied by cold-girdling wild-type leaves (see below). Moreover, this phenotype is unique to the *sut1* mutant as many other independent maize mutants that accumulate excess carbohydrates in their leaves have been characterized, yet they do not exude concentrated sucrose solution in their guttation fluid (Russin *et al.*, 1996; Dinges *et al.*, 2003; Baker and Braun, 2007, 2008; Slewinski *et al.*, 2008). Therefore, although it can not formally be excluded, it is highly improbable that the phenotypes observed in *sut1* mutant plants are caused by an unrelated, closely linked mutation, rather than the *Mu* insertion identified within the *SUT1* gene.

Cold-girdling maize leaves phenocopies sut1 *mutants*

Additional evidence for the maize SUT1 protein functioning in phloem loading of sucrose comes from cold-girdling experiments. Cold-girdling blocks the transport of assimilates in the phloem (Krapp *et al.*, 1993; Krapp and Stitt, 1995; Schulz *et al.*, 1998). Cold-girdling wild-type maize leaves produced a phenotype strongly resembling that seen in *sut1* mutant plants. Cold-girdled leaves became chlorotic and hyperaccumulated starch and soluble sugars, similar to the phenotypes reported in heat-girdled leaves (Goldschmidt and Huber, 1992; Jeannette *et al.*, 2000). Moreover, it was found that inhibition of phloem loading using either genetic or physiological approaches resulted in an identical and novel phenotype of the secretion of a concentrated sugar solution in the guttation fluid. In the case of the *sut1* mutants, sucrose that normally would be loaded into the phloem for transport probably remained in the apoplastic space and was carried along the transpiration path to the hydathodes where the water evapourated, thereby concentrating the sugars. A similar outcome is proposed to have occurred in girdled leaves due to the inhibition of phloem transport in the sheath and stem. The similarity between the *sut1* mutant and phloem transport-inhibited leaf phenotypes supports the hypothesis that *SUT1* functions to load sucrose into the phloem in maize leaves.

Symplastic phloem loading in maize?

Although *sut1* mutants have a severe reduction in plant growth and fitness, they manage to produce a tassel and shed a small amount of pollen. This indicates that some sucrose is transported from the source leaves to the developing sink tissues to sustain their development. This was a somewhat surprising finding and may be explained by several alternatives. One explanation is that other maize *SUT*s may be expressed in source leaves and partially compensate for the loss of the *SUT1* function. A second possibility is that a symplastic pathway for sucrose entry into the phloem still operates in *sut1* mutants, even though the apoplastic path is no longer functional. Plasmodesmata are present at all cellular interfaces from the mesophyll cells to the phloem sieve elements in maize (Evert *et al.*, 1978; Botha, 2005). While the frequency of plasmodesmata is high between the bundle sheath cells and vascular parenchyma cells, it is low between the vascular parenchyma cells and companion cells (Evert *et al.*, 1978; Botha, 2005). However, these data suggest that a limited capability exists for symplastic sucrose movement into the phloem. In support of this possibility, dye labelling studies in maize and rice leaf blades have demonstrated symplastic transport between the vascular parenchyma cells and the phloem sieve elements, indicating that this route is functional (Botha, 2005; Botha *et al.*, 2008). Further support for this hypothesis was recently provided by Srivastava *et al.* (2008). The authors found that *Arabidopsis* plants lacking SUC2, the SUT1-type protein principally responsible for phloem loading, while debilitated, grew and had similar levels of photoassimilated carbon in phloem exudates compared to wild type. In addition, the mutants were unable to load exogenously applied sucrose, suggesting that sucrose entered into the phloem through symplastic transport (Srivastava *et al.*, 2008). Thus, it is conceivable that a symplastic pathway may sustain sucrose entry into the phloem and distribution to developing sink tissues in the *sut1* mutant, albeit at a reduced capacity.

In summary, a role for the maize *SUT1* gene in phloem loading was demonstrated *in vivo*. Plants lacking *SUT1* function display a significant reduction in ^{14}C-sucrose export from leaves, hyperaccumulate carbohydrates in leaves, display the novel phenotype of exuding high concentrations of sugars in their guttation fluid, and are severely impaired in growth and development. These data suggest that maize requires the activity of SUT1 for efficient phloem loading of

sucrose. This report presents the first demonstration of an essential function of a monocot *SUT* in phloem loading in leaves.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Molecular analyses of *Mu1* insertion in *sut1* allele.

Supplementary Fig. S2. Photograph of cold-girdling experimental set-up on a wild-type plant.

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