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Carbon partitioning and export in transgenic *Arabidopsis thaliana* with altered capacity for sucrose synthesis grown at low temperature: a role for metabolite transporters

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ABSTRACT

We investigated the role of metabolite transporters in cold acclimation by comparing the responses of wild-type (WT) *Arabidopsis thaliana* (Heynh.) with that of transgenic plants over-expressing sucrose-phosphate synthase (SPSox) or with that of antisense repression of cytosolic fructose-1,6-bisphosphatase (FBPas). Plants were grown at 23 °C and then shifted to 5 °C. We compared the leaves shifted to 5 °C for 3 and 10 d with new leaves that developed at 5 °C with control leaves on plants at 23 °C. At 23 °C, ectopic expression of SPS resulted in 30% more carbon being fixed per day and an increase in sucrose export from source leaves. This increase in fixation and export was supported by increased expression of the plastidic triose-phosphate transporter AtTPT and, to a lesser extent, the high-affinity Suc transporter AtSUC1. The improved photosynthetic performance of the SPSox plants was maintained after they were shifted to 5 °C and this was associated with further increases in AtSUC1 expression but with a strong repression of AtTPT mRNA abundance. Similar responses were shown by WT plants during acclimation to low temperature and this response was attenuated in the low sucrose producing FBPas plants. These data suggest that a key element in recovering flux through carbohydrate metabolism in the cold is to control the partitioning of metabolites between the chloroplast and the cytosol, and *Arabidopsis* modulates the expression of AtTPT to maintain balanced carbon flow. *Arabidopsis* also up-regulates the expression of AtSUC1, and to lesser extent AtSUC2, as down-stream components facilitate sucrose transport in leaves that develop at low temperatures.

Key-words: carbon flux; cold acclimation; frost tolerance; photosynthesis.

INTRODUCTION

Low temperature is one of the most important environmental factors affecting plant growth (Levitt 1980) and crop productivity (Boyer 1982) and therefore limits the geographical range of many economically important species. A rapid drop in growth temperature results in fast, severe inhibition of photosynthesis in a range of plant species (Somersalo & Krause 1989; Holaday *et al*. 1992; Hurry & Huner 1992; Streb, Feierabend & Bligny 1997), affecting carbon metabolism and allocation of carbon to developing sink tissues. However, cold-tolerant species such as *Arabidopsis thaliana* and winter cereals are able to recover their photosynthetic capacity and resume growth after several days to weeks at low temperature through the process of cold acclimation (Levitt 1980; Hurry & Huner 1991; Strand *et al*. 1997). This ability to restore photosynthesis is essential for the long-term survival of over-wintering herbaceous plants (Stitt & Hurry 2002; Ensminger, Busch & Huner 2006).

Low temperature repression of photosynthesis is the result of the loss of activity by cold-sensitive Calvin cycle enzymes and by an over-proportional inhibition of end-product synthesis, leading to an accumulation of phosphorylated intermediates that in turn exert a Pi limitation on photophosphorylation (Stitt 1986; Stitt & Grosse 1988; Labate & Leegood 1989). During cold acclimation, carbon metabolism is reprogrammed to release the inhibition of photosynthesis (Hurry *et al*. 1995, 1996, 2000; Strand *et al*. 1999). These data have recently been expanded to demonstrate a role for β-amylase activity and starch degradation during short-term abiotic stress events (Kaplan & Guy 2004; Yano *et al*. 2005; Kaplan, Sung & Guy 2006), along with new data supporting a role for the accumulation of raffinose-series sugars in cold acclimation (Cook *et al*. 2004; Kaplan *et al*. 2004). This cold-induced reprogramming of carbon metabolism by frost-tolerant plants such as winter rye and *Arabidopsis* requires the development of new leaves at low temperature, and full acclimation is only achieved by these cold-developed leaves (Hurry *et al*. 2002; Strand *et al*. 2003; Ensminger *et al*. 2006). Therefore, one determinant of the rate or ability of a plant to cold acclimate is its ability to continue to supply carbohydrates and other substrates to the developing sink leaves (Strand *et al*. 2003; Takagi *et al*. 2003). Analysis of carbon fluxes in over-expressing sucrose-phosphate synthase (SPSox) transgenic

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Arabidopsis (Signora *et al*. 1998; Strand *et al*. 2003), tobacco (Baxter *et al*. 2003) and rice (Ono *et al*. 2003) indicates that SPS activity and Suc export rates may be closely linked. This suggests that increases in the rate of Suc synthesis during cold acclimation may not only increase the production of compatible solutes at low temperature but this may also be a mechanism to enhance Suc export to developing sinks at low temperature.

Arabidopsis belongs to the type 1–2a phloem-loading group (Gamalei 1991) that shows few plasmodesmata connecting the sieve element-companion cell complex (SE-CCC) of the minor veins to the surrounding cells (Haritatos, Medville & Turgeon 2000). The SE-CCCs are therefore largely symplastically isolated from the surrounding cells and *Arabidopsis* utilizes an active step involving high affinity Suc/proton cotransporters to load sucrose into the SE-CCCs from the apoplast against a steep concentration gradient. These transporters are localized in the plasma membrane of the SE-CCC, together with H^+-ATPases that generate the required protonmotive force (Riesmeier, Frommer & Willmitzer 1994; Sauer & Stolz 1994; Truernit & Sauer 1995; Kuhn *et al*. 1999; Gottwald *et al*. 2000). In addition to these disaccharide transporters, plants also posses a number of transporters responsible for metabolite traffic across the chloroplast envelope. These include the triose-phosphate transporter responsible for exporting the triose phosphates synthesized in the Calvin cycle to the cytosol to support the immediate metabolic requirements of the cell or for Suc synthesis (Riesmeier *et al*. 1993; Schneider *et al*. 2002; Flügge *et al*. 2003) and the recently discovered maltose transporter that is essential for nocturnal starch mobilization (Niittyla *et al*. 2004).

At this point, little is known about what role the metabolite transporters in the chloroplast envelope may play in the reprogramming of carbon metabolism in the source cell at low temperatures or what role the disaccharide transporters play to facilitate phloem loading at low temperatures. We have used transgenic *Arabidopsis* plants with modified capacities for sucrose synthesis resulting in enhanced or reduced ability to cold acclimate, respectively. These transgenic lines were used as tools to elucidate how changes in Suc synthesis and in the ability to translocate the assimilated carbon to developing sinks at both warm and low temperatures are related to changes in abundance of a number of plastid metabolite transporters and vascular sucrose transporters.

MATERIALS AND METHODS

Plant material

A. thaliana L. (Heynh.) ecotype Colombia, wild-type (WT) and two transgenic lines were selected from multiple lines characterized previously (Strand *et al*. 2000, 2003). For the construction of the antisense line, a cDNA insert of the EST clone 169K9T7 (At1g43670) encoding the entire 1060 bp structural protein of cFBPase was ligated in an antisense orientation under the control of the 35S CaMV promoter into the binary vector pBI120 (Jefferson, Kavanagh & Bevan 1987). From the three antisense cFBPase lines studied previously (Strand *et al*. 2000, 2003), line 12 (FBPas) was selected for these experiments. Arabidopsis plants with elevated amounts of SPS were made using the pCGN3812 construct, containing the maize SPS gene (NP003949) cDNA, belonging to SPS gene family B (Lunn & MacRae 2003), under the control of the RBCS promoter of tobacco (Signora *et al*. 1998). From the lines studied previously (Strand *et al*. 2003), SPSox line 5 was selected for these experiments.

Seeds were germinated under non-hardening (NH) controlled environmental conditions: 150 μmol photons m^{-2} s^{-1}; day/night temperature regime 23/18 °C; photoperiod 8 h. After 49 d, when fully mature source leaves had developed, the plants were shifted to a day/night temperature regime of 5/5 °C. After 3 and 10 d at 5 °C, source leaves that had completed expansion before transfer to low temperature were used to analyse acclimation in pre-existing leaves (3 and 10 d plants, respectively). After 40 d at 5 °C, source leaves that had developed at 5 °C were sampled to investigate acclimation processes requiring modification during leaf development (Dev plants).

Protein isolation and Western blot analysis

Leaf samples were snap frozen and ground at the temperature of liquid N_2, and 25 mg of frozen tissue was solubilized by adding 250 μL of hot (70 °C) LDS-PAGE sample buffer [65 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 2% (w/v) LDS, 0.005% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol], heated at 70 °C for 10 min, centrifuged at 20 000 *g* for 10 min and the supernatant used for SDS-PAGE. For comparisons of relative protein content for SPS and cFBPase, separation of extracted proteins was carried out using denaturing 4–12% Bis-Tris polyacrylamide gels and MOPS-SDS running buffer (Novex, San Diego, CA, USA). After electrophoresis, the proteins were transferred to a PVDF membrane (Immun-Blot; Bio-Rad, Hercules, CA, USA), SPS and cFBPase were immunostained with polyclonal rabbit antibodies and visualized using enhanced chemiluminescence (ECL; Amersham Biosciences Ltd, Buckinghamshire, UK).

Whole-plant CO_2 exchange

Whole-plant gas exchange was measured on intact rosettes in an open flow gas-exchange system. Purpose built, whole-plant gas exchange cuvettes were connected to an infrared gas analyser (IRGA; Li-Cor 6262, Lincoln, NE, USA) and each cuvette was sampled for 1 min every 15 min for 72 h. During the measurements NH control, 10 d 5 °C cold-stressed and Dev 5 °C cold-acclimated plants were maintained at their respective growth conditions. Prior to measurement, the plants were given 12 h to adjust to the cuvettes.

RNA isolation and reverse transcriptase (RT)-PCR

Total RNA was isolated using the Trizol reagent according to the manufacturers protocol (Life Technologies, Rockville, MD, USA). The total RNA samples were DNase-treated with DNA-free (Ambion Inc., Austin, TX, USA) prior to cDNA synthesis. The full-length first-strand cDNA was generated using $Pd(N)_6$ primers (First-Strand cDNA Synthesis Kit; Amersham Biosciences Ltd). Five micrograms of total RNA was used in each reaction. In order to compensate for variability in RNA quality and random tube to tube variation in RT reactions, we ran multiplex RT-PCR using Universal 18S as an endogenous standard, and by using a competimer, the amplification efficiency of the 18S standard was reduced without the primer becoming limiting and without loss of relative quantification (QuantumRNA 18 Internal Standards, Ambion Inc.). RT-PCR reactions were run using the following primer pairs: AtSUC1 (At1g71880), Fwd-5′GTCTTGGAGTCCAATCTGG3′ Rev-5′CATGG TCGTTGCCTTGGG3′; AtSUC2 (At1g22710), Fwd-5′GA GCCAACAGCCGATGG3′ Rev-5′GGTGGACCTGT TTTAGCGCC3′; AtSUC3 (At2g02860), Fwd-5′-TCAT TCTGATTCGGCTGATG-3′ Rev-5′AAGGCCTTTG GACTGGAGAT-3′; AtSUT4 (At1g09960), Fwd-5′TACTG AGAATGATAATCGC3′ Rev-5′AGACGCTTAGGA TCGTAGTT3′; AtTPT (At5g46110), Fwd-5′GCACTGC ATCGTCTTCCTCGTT3′ Rev-5′CGTGACAGACTGC GACTGGTAT3′; AtMEX1 (At5g17520), Fwd-5′TCATCC TCAATACCCAGAA3′ Rev-5′AGACCCTACAAACC TAACACC3′; AtG6P (At5g17630) Fwd-5′GTCGCTGTTT CGTTCACTCA3′ Rev-5′-ATCCATCGCCTTGGTGTT AC3′; AtPHS1 (At3g29320) Fwd- 5′GAGGCACTGGAGA AGTGGAG3′ Rev- 5′ATCCATGCGCTTGGTGTTAC3′; AtBMY6 (at5g55700) Fwd-5′TTCCATCTGGAGTTCCG TTC3′ Rev- 5′-TGCAACCAGCATTACAGAGC3′; AtBMY7 (At3g23920) Fwd-5′GACACCCAGTTCTTC AAAA3′ Rev- 5′CTCAACTTCTTCCCGACA3′; AtBMY8 (At4g17090) Fwd-5′GGAACAAGTGGACC TCAT3′ Rev- 5′TCTCAGCGATCTTGCCTT3′; AtBMY9 (At4g00490) Fwd 5′GCTGGCAGGCGTAACACT3′ Rev 5′CGGTTTGAGGAGTTGTAGAAG3′. Ten microliters of the PCR reaction was run on a 1.5% agarose gel and the bands were digitized and quantified by absolute integrated optical density (IOD) using the Gel-Pro Analyser software (MediaCybernetics, Silver Spring, MD, USA). The amount of product from the gene of interest was then normalized against the product from the 18S control.

$^{14}CO_2$ labelling of intact plants

Intact *Arabidopsis* plants were place in an airtight plexiglas box in the growth rooms and whole rosettes were labelled with $^{14}CO_2$ released by acidification of sodium ^{14}C-bicarbonate at three time points during the day; 30 min into the photoperiod, in the middle (4 h) of the photoperiod and 30 min before the end of the photoperiod. After a 10 min pulse (20 min for the 10 d cold-stressed plants because of the low photosynthetic rate), the aerial parts of the plants were snap frozen in liquid N_2. The plant material was extracted at 80 °C in 80% ethanol. The amount of ^{14}C present in the ethanol soluble and insoluble fractions was determined as described previously (Nielsen & Veierskov 1990).

Metabolites

At the same time that samples were collected for ^{14}C incorporation, plus an additional time point 30 min before the lights came on in the morning, unlabelled leaf material was harvested by freeze-clamping into liquid N_2. For metabolite determination, freeze-clamped leaf material was ground to a fine powder at the temperature of liquid N_2 and extracted in TCA. Phosphorylated intermediates were determined spectrophotometrically (Stitt *et al*. 1989). Fru2,6bisP was assayed as described previously (Stitt 1990) with the assay modified for microplate wells (Trevanion 2000) using a Spectramax 190 plate reader (Molecular Devices, Wokingham, UK).

Sucrose efflux

Efflux of sucrose from source leaves of control and transgenic plants was determined by cutting the leaves of mature plants and incubating them in 5 mM EDTA pH 6.0 at the respective growth conditions, throughout the period, taking sample once every hour (Riesmeier *et al*. 1994). The amount in the incubation solution was determined enzymatically as described in Strand *et al*. (2003).

RESULTS

Plant material

A. thaliana L. (Heynh.) ecotype Colombia, WT and two transgenic lines were selected from multiple lines characterized previously (Strand *et al*. 2000, 2003). Under warm (23 °C) growth conditions, the plants expressing the transcript for cFPBase in the antisense direction (FBPas) contained less than 5% of the WT cFBPase protein and the data show that in the FBPas plants, the cold-induced increase in the cFBPase protein was largely blocked. The plants expressing maize SPS transcript in the sense direction (SPSox) showed a several-fold increase in SPS protein compared with WT at 23 °C (Fig. 1).

Whole-plant gas exchange

We measured the daytime CO_2 uptake, averaged over three consecutive days, of entire intact *Arabidopsis* rosettes, grown under ambient irradiance (150 μmol photons m^{-2} s^{-1}, 8 h photoperiod) and CO_2 concentrations (380 μL L^{-1}), and at either 23/18 °C day/night temperatures (NH) or 5/5 °C day/night temperatures (10 d and Dev). Under these growth conditions, the SPSox plants consistently take up more carbon over a full photoperiod than WT plants

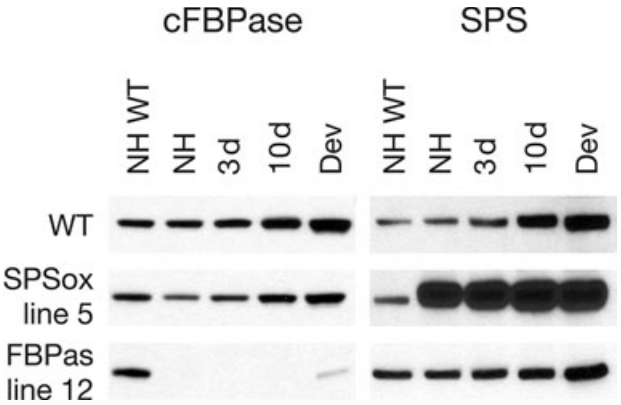

Figure 1. Western blots showing the amount of protein detected using cytosolic fructose-1,6-bisphosphatase (cFBPase) and sucrose-phosphate synthase (SPS)-specific antibodies in wild type (WT) and the two different transgenic lines grown at 23 °C (non-hardened, NH), following exposure to 5 °C for 3 d , 10 d, and after development of new leaves at 5 °C (Dev). NH WT protein extracts were used as an exposure control for all immunoblots. The protein samples shown are from a mixture of four separate plants within each line. Samples were collected 4 h into the light period.

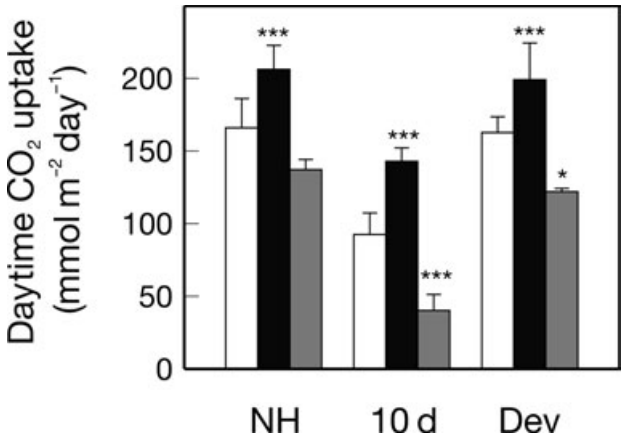

Figure 2. Photosynthetic CO_2 uptake by whole, intact rosettes of wild-type (open bar), over-expressing sucrose-phosphate synthase (filled bar) and antisense cytosolic fructose-1,6-bisphosphatase (grey bar) plants. Measurements were made under ambient growth conditions: non-hardened (NH), plants grown at 23 °C and measured at 23 °C; 10 d, plants grown at 23 °C and shifted to 5 °C for 10 d and measured at 5 °C (data are averaged from measurements on day 9, 10 and 11); Dev, plants shifted to 5 °C until a new rosette of leaves had developed and measured at 5 °C. Plants were measured every 15 min for 3 d and the data shown are the average daytime uptake rates expressed on a leaf-area basis. Each bar represents the mean (± SD) of four different plants within each line. Statistical significance was tested first by two-way ANOVA with Bonferroni post-test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). $*P < 0.05$, $***P < 0.001$.

regardless of growth temperature or acclimation phase. Conversely, the FBPas plants are consistently impaired, relative to WT, and show stronger low-temperature depression of photosynthesis in the 10 d shifted plants (Fig. 2).

Carbon partitioning, metabolites and sucrose export

To determine how these changes in whole-plant photosynthesis are related to changes in carbon partitioning and export, we also investigated diurnal partitioning of newly fixed carbon by these *Arabidopsis* rosettes (Fig. 3). The partitioning of newly fixed carbon into starch plus other insoluble compounds and into soluble (neutral) sugar pools

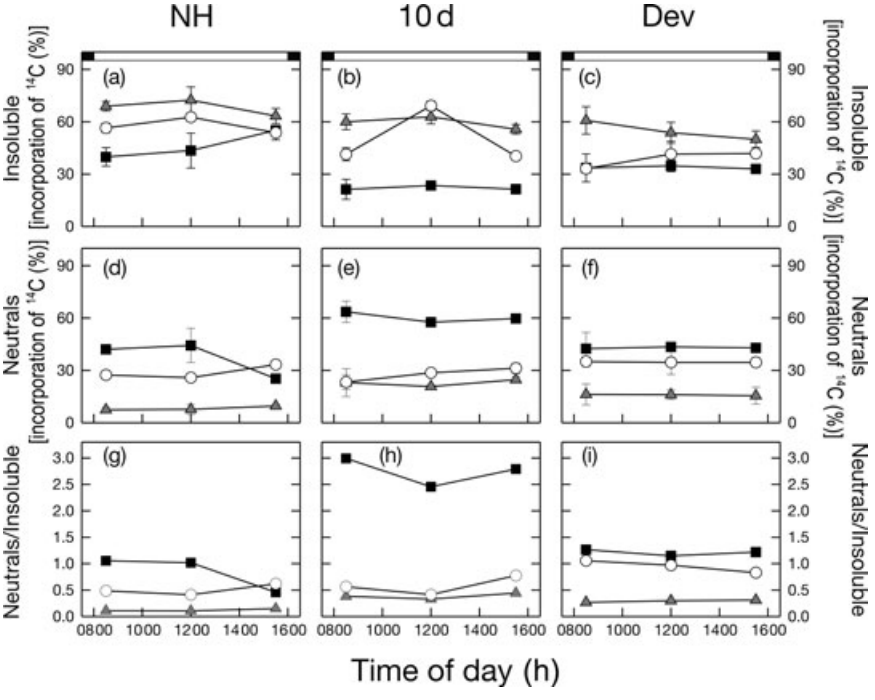

Figure 3. Diurnal variation in ^{14}C-assimilate partitioning in wild-type (open circles), over-expressing sucrose-phosphate synthase (filled squares) and antisense cytosolic fructose-1,6-bisphosphatase (grey triangles) plants. Incubations were performed under ambient growth conditions on intact rosettes [10 min labelling pulse at 23 °C for non-hardened (NH) plants, 10 min at 5 °C for Dev plants and 20 min at 5 °C for 10 d plants]. Each point represents the mean (± SD) of four different plants within each line. Top bar indicates when light was off (black bars) and on (white bars).

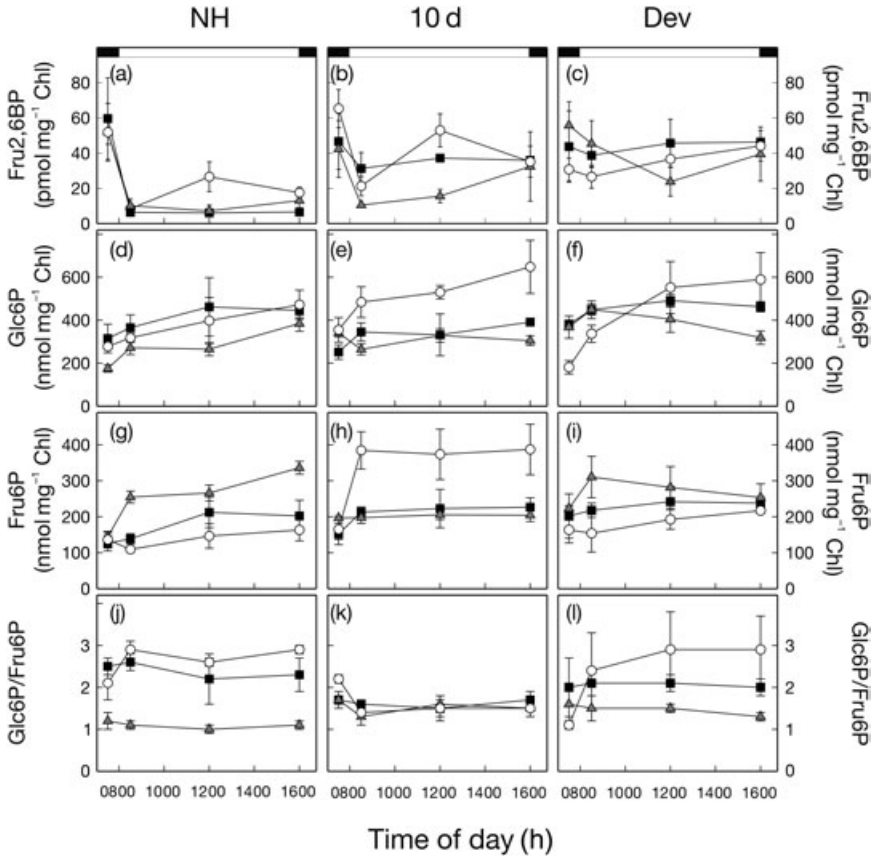

Figure 4. Diurnal variation in Fru2,6bisP and major hexose-phosphate pools for wild-type (open circles), over-expressing sucrose-phosphate synthase (filled squares) and antisense cytosolic fructose-1,6-bisphosphatase (grey triangles) plants. Samples were frozen at various times during the photoperiod at the temperature of liquid N_2. Each point represents the mean (± SD) of four different plants within each line. Top bar indicates when light was off (black bars) and on (white bars).

was generally stable throughout the photoperiod in WT and both transgenic lines regardless of growth temperature (Fig. 3a–f), and this was associated with stable hexose monophosphate and Fru2,6bisP pools during the day (Fig. 4). However, as shown previously under saturating CO_2 and light (Strand *et al*. 2003), in these experiments under conditions of ambient CO_2 and light, there is a consistent shift in partitioning towards soluble sugars in the SPSox plants and a shift towards starch in the FBPas plants (Fig. 3g).

When the plants were shifted to 5 °C for 10 d, there were minor increases in the partitioning of newly fixed carbon into soluble compounds at the expense of starch and other insoluble compounds in WT and FBPas plants, and this was strongly enhanced in the SPSox plants (Fig. 3e & h). However, following the development of new leaves at 5 °C, WT plants showed a clear shift in partitioning towards soluble sugars, similar to that shown by the SPSox plants, as the capacity for the Suc synthesis pathway was up-regulated in the WT plants during the development of new leaves (Fig. 1). However, the FBPas plants were never able to fully modify their partitioning in favour of soluble sugars, even after the development of a new rosette of leaves at 5 °C. Thus, despite generally keeping lower levels of Fru2,6bisP in their leaves at 5 °C (Fig. 4b & c) and increasing SPS protein content (Fig. 1), the severe loss of cFBPase protein was sufficient to exert strong control over flux through this pathway and to inhibit normal acclimation to low temperature.

To assess whether these changes in photosynthesis and the shift in partitioning into soluble sugars resulted in detectable changes in carbon export from leaves, we measured Suc efflux from excised leaves (Riesmeier *et al*. 1994) maintained under ambient growth conditions (Fig. 5). These data show an increase in Suc export in the SPSox plants and a decrease in the FBPas plants grown at 23 °C (Fig. 5a), in agreement with the measured photosynthetic (Fig. 2) and carbon partitioning data (Fig. 3). However, when the three genotypes were shifted to 5 °C for 10 d, we could measure no significant Suc efflux from excised WT and FBPas leaves (Fig. 5b). This limited export capacity was associated with the maintenance of large starch and free Glc pools (Strand *et al*. 2003), and with the down-regulation of photosynthesis (Fig. 2). While better than WT, Suc export from the SPSox leaves was also severely limited at 5 °C in the 10 d shifted leaves (Fig. 5b). In contrast, Suc export capacity at 5 °C was strikingly recovered by all genotypes following the development of new leaves at low temperature (Fig. 5c). This recovery of export capacity correlated with the strong recovery of photosynthesis by these new leaves (Fig. 2).

Effect of the transgenes on transcript abundance of genes involved in starch mobilization and metabolite transport

Under warm growth conditions, the increase in partitioning of newly fixed carbon into Suc by the SPSox plants was

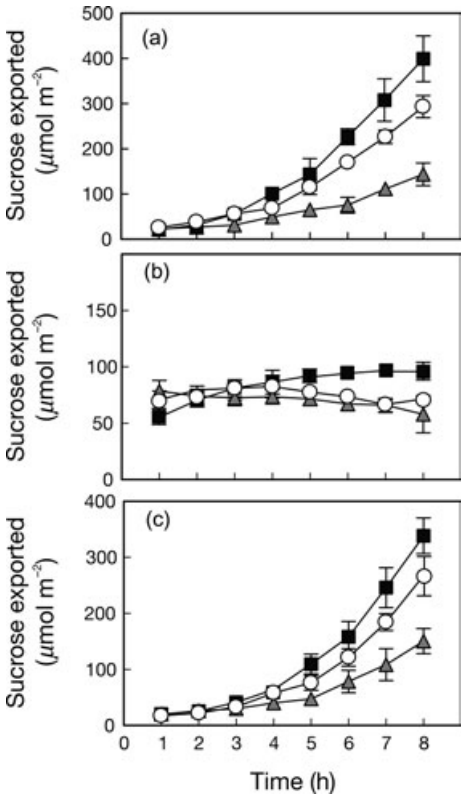

Figure 5. Sucrose efflux from (a) excised non-hardened (NH) 23 °C-grown control leaves, (b) leaves shifted to 5 °C for 10 d and (c) leaves that developed at 5 °C from wild-type (WT) and the two transgenic lines. WT (open circles), over-expressing sucrose-phosphate synthase (filled squares) and antisense cytosolic fructose-1,6-bisphosphatase (grey triangles) plants. Each point represents the mean (± SD) from at least four different leaves from different plants within each line.

associated with significant reductions in transcript abundance of three of the four known plastidic β-amylases (Kaplan & Guy 2004; Kaplan *et al.* 2006), AtBMY7, AtBMY8 and AtBMY9 (Fig. 6a) that are responsible for hydrolytic degradation of starch (Kossmann & Lloyd 2000) but did not result in any change in expression of the plastidic α-glucan phosphorylase (AtPHS1) involved in phosphorylitic starch degradation (Fig. 6a) (Zeeman *et al.* 2004). In contrast, the FBPas plants showed significant increases in transcript abundance for both AtBMY9 and AtPHS1 (Fig. 6a). The increase in the relative importance of Suc synthesis in the SPSox plants was further supported by an increase in expression of AtTPT, the transporter responsible for exporting triose phosphate to the cytosol (Riesmeier *et al.* 1993), and a decrease in abundance of AtMEX1 transcript, the transporter responsible for maltose transport across the chloroplast envelope (Niittyla *et al.* 2004) (Fig. 6b), but there was no effect on transcript abundance of the glucose-6-phoshate transporter AtGTP (Fig. 6b). The increase in Suc synthesis and Suc efflux in the SPSox plants was also supported by a significant increase in expression of the high affinity Suc/proton cotransporter AtSUC1 (Fig. 6b). In contrast, the reduction in Suc synthesis and flux through the Suc pool in the FBPas plants did not significantly alter transcript abundance for any of the transporters tested (Fig. 6b). Although these changes in transcript abundance were relatively minor (twofold or less), they were reproducible in independent experiments and they indicate that the large changes in flux through the Suc and starch pools, especially in the SPSox line, have resulted in transcriptional fine-tuning of metabolite transport and of the enzymes responsible for starch turnover.

Effect of temperature on transcript abundance of genes involved in starch mobilization and metabolite transport

We also evaluated the transcriptional responses of these genes during cold acclimation. Of the 12 genes studied, four (AtBMY6, AtBMY7, AtGTP and AtSUC3) showed no significant response and were not considered for further analysis (data not shown). The remaining eight all showed

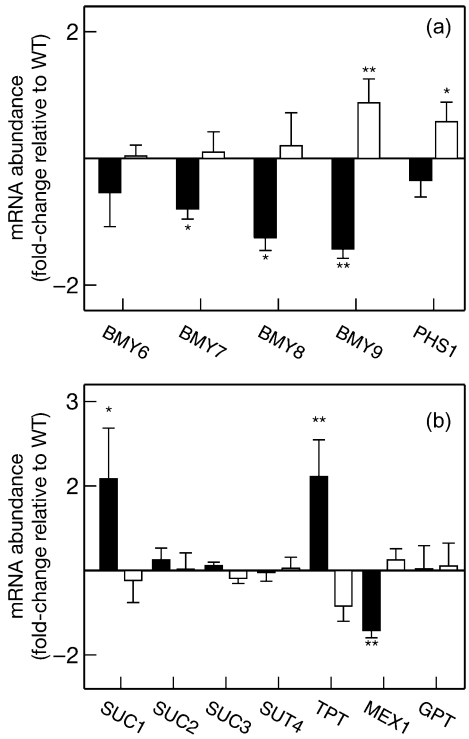

Figure 6. mRNA abundance of genes involved in starch mobilization and metabolite transport in source leaves from over-expressing sucrose-phosphate synthase (filled bars) and antisense cytosolic fructose-1,6-bisphosphatase (open bars) plants grown under control (23 °C) temperatures. Total RNA was extracted from mature source leaves collected in the middle of the photoperiod. Data are expressed relative to wild type (WT) and each point represents the mean (± SD) of mRNA extracted individually from four to six different plants within each line. Statistical significance was tested first by one-way ANOVA with Dunnett's post-test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). $*P < 0.05$, $**P < 0.01$.

statistically significant (assessed by two-way ANOVA) responses to low temperature (Fig. 7). All three genotypes showed a significant increase in transcript abundance for AtBMY8 and AtBMY9 (Fig. 7a & b), similar to earlier reports (Kaplan & Guy 2004; Kaplan *et al*. 2006). In addition, AtPHS1 showed a significant response to temperature as a result of a twofold reduction in transcript abundance by the WT and FBPas plants after 3 d at 5 °C (Fig. 7c). When we investigated the response of the metabolite transporters to low temperature, we show that AtMEX1 mRNA, which was initially less abundant in SPSox plants (Fig. 6a), increased in abundance in the SPSox plants but was unchanged in WT and FBPas plants (Fig. 7d). Furthermore, all genotypes showed a pronounced reduction in transcript abundance for AtTPT, and this was significantly more pronounced in the SPSox line (Fig. 7e). Beyond the source cell, we show that transcript abundance for AtSUC1, which was already higher in the high Suc-producing SPSox plants at warm temperatures (Fig. 6b), increased further during acclimation to low temperature, and also increased in abundance in WT and FBPas leaves (Fig. 7f). AtSUC2 transcript abundance also increased significantly in response to temperature in WT and SPSox, but not FBPas leaves (Fig. 7g). AtSUT4, a low affinity/high-capacity Suc transporter, which together with AtSUC3, appears to be involved in sucrose retrieval into enucleated sieve elements (Weise *et al*. 2000; Reinders *et al*. 2002), showed a significant increase in transcript abundance only in the high Suc-producing SPSox line following transfer to 5 °C (Fig. 7h). The low temperature-induced increase in transcript abundance for AtSUC1 (up to threefold) and AtSUC2 (1.5-fold) is low relative to the changes observed for AtBMY8 and AtTPT, however, these

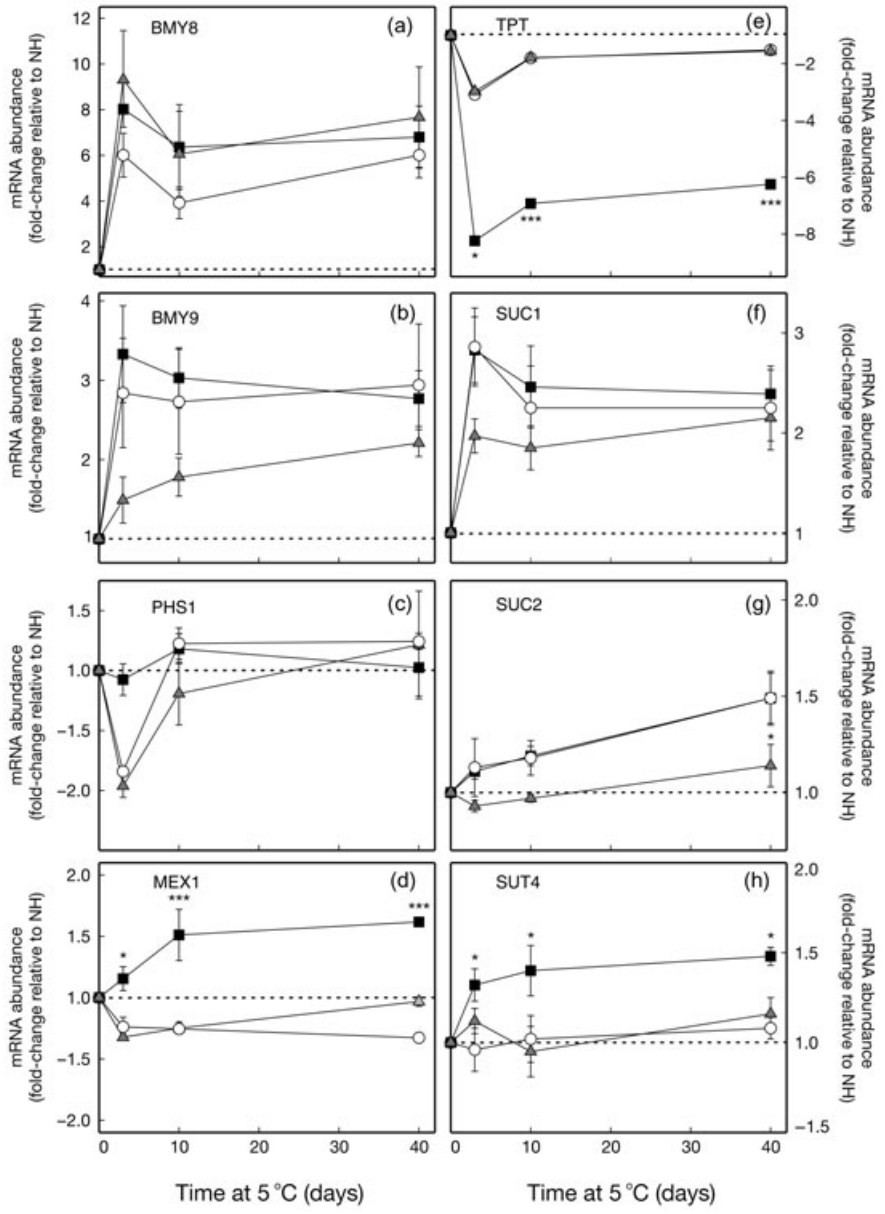

Figure 7. Effect of low temperature on mRNA abundance of genes involved in starch mobilization and metabolite transport in source leaves from wild-type (WT, open circles), over-expressing sucrose-phosphate synthase (filled squares) and antisense cytosolic fructose-1,6-bisphosphatase (grey triangles) plants. (a) AtBMY8; (b) AtBMY9; (c) AtPHS1; (d) AtMEX1; (e) AtTPT; (f) AtSUC1; (g) AtSUC2; (h) AtSUT4. Total RNA was extracted from mature source leaves collected in the middle of the photoperiod. Data are expressed relative to the non-hardened (NH) sample and each point represents the mean (± SD) of mRNA extracted individually from four to six different plants within each line. Statistical significance was tested first by two-way ANOVA using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA) and all genes shown in the figure significantly responded to temperature ($P < 0.05$). Differences between WT and transgenic lines at individual time points were tested with Bonferroni post-test (GraphPad Software). $*P < 0.05$, $***P < 0.001$.

changes were significant and reproducible and indicate temperature- and/or Suc-mediated modulation of transcription for these important Suc carriers.

DISCUSSION

In herbaceous winter annuals, such as *Arabidopsis*, the acquisition of full freezing tolerance requires the development of new leaves at low temperatures, and the development of these new leaves is strongly correlated with a reprogramming of metabolism, involving a change in carbon partitioning in favour of sucrose synthesis (Strand *et al*. 1997, 1999, 2003; Hurry *et al*. 2002). Essential to cold-acclimating plants therefore is the ability to not only fix carbon but also to transport this fixed carbon to sink tissues to support growth and acclimation processes. To address how metabolite flux, and metabolic balance between the cellular compartments, is recovered during cold acclimation, we have used transgenic *Arabidopsis* plants with altered capacities for Suc synthesis to test the effect of low temperature and increased flux through the Suc pool on the regulation of metabolite transporters across the chloroplast envelope and for those involved in loading of Suc into the SE-CCC. The different transgenic lines used in these experiments have either increased sucrose synthesis as a result of ectopic expression of maize SPS (Signora *et al*. 1998; Strand *et al*. 2003) or decreased sucrose synthesis as a result of antisense expression of cytosolic FBPase (Strand *et al*. 2000, 2003).

Under warm growth conditions, we show that transgenic plants ectopically expressing SPS (SPSox) fix 30% more carbon per day than WT (Fig. 2). This result contrasts to earlier reports, based on single-leaf photosynthetic measurements in the middle of the photoperiod, showing no increase in net photosynthesis for the SPSox plants (Strand *et al*. 2003). However, in the current experiments, photosynthesis was measured on the entire rosette every 15 min over a period of 3 d and therefore these measurements take into account normal fluctuations in photosynthesis throughout the light period and the different photosynthetic rates of leaves of different ages. Using this rosette photosynthesis and the ^{14}C partitioning data (Fig. 3), we calculate that the SPSox plants produce around 34 μmol g^{-1} FW day^{-1}, more hexose units (as Suc, Glc and Fru) than WT but only accumulate an additional 4 μmol g^{-1} FW day^{-1} in their source leaves. The remaining additional 30 μmol g^{-1} FW day^{-1} is exported from the source leaves and this increased export can be seen as increased Suc efflux from cut petioles (Fig. 5). To facilitate the increase in export, SPSox *Arabidopsis* plants specifically increase the expression of AtSUC1, a high-affinity Suc transporter with close homology to AtSUC2. AtSUC2 has been localized to the SE-CCC (Riesmeier *et al*. 1994; Sauer & Stolz 1994; Stadler *et al*. 1995; Truernit & Sauer 1995; Stadler & Sauer 1996) and shown to play a major role in loading Suc into the companion cells (Gottwald *et al*. 2000). The increase in expression of AtSUC1 in source leaves of the SPSox plants was unexpected as AtSUC1 has previously been reported to have flower-specific expression (Stadler *et al*. 1999). We confirmed AtSUC1 expression by sequencing the product of the RT-PCR reaction (data not shown) and there are RT-PCR (Furuichi *et al*. 2001) and microarray (Lloyd & Zakhleniuk 2004) data also showing AtSUC1 expression in leaves but as of yet it has not been localized to any specific cell type. Without localization data, it is not possible to assign a clear physiological function for the increase in AtSUC1 expression, however, homozygous T-DNA insertion knockout lines of AtSUC2 show a severe stunted, sterile, phenotype (Gottwald *et al*. 2000) suggesting a limited capacity for AtSUC1 to complement AtSUC2 function in phloem loading. AtSUC1 may therefore have a role in Suc retrieval into the sieve elements, supporting the function of AtSUC3 and AtSUT4 in regulating the partitioning of Suc between the cytoplasm and the apoplast.

In addition to these changes in Suc symporter expression, we showed that to support the increased photosynthesis and associated Suc synthesis, the SPSox plants also upregulated the plastidic triose phosphate-phosphate translocator (AtTPT). This response suggests that this antiporter may not be present in excess *in vivo*, a conclusion that is supported by data from transgenic potato (Riesmeier *et al*. 1993; Heineke *et al*. 1994; Hattenbach *et al*. 1997), and tobacco (Hausler *et al*. 1998; 2000c; Hausler, Schlieben & Flügge 2000b) and *Arabidopsis* mutants (Schneider *et al*. 2002; Flügge *et al*. 2003) demonstrating that the TPT is capable of exerting control over the flux of carbon between starch and Suc and over photosynthesis (Poolman, Fell & Thomas 2000). In contrast, the SPSox plants showed lower expression of AtMEX1, the transporter necessary for nocturnal starch mobilization (Niittyla *et al*. 2004). It is noteworthy that under these conditions of increased photosynthesis, lower rates of partitioning into starch and reduced AtMEX1 expression the SPSox plants end up accumulating similar amounts of starch as WT (Strand *et al*. 2003). The SPSox plants also mobilize their starch pool during the long (16 h) night (Strand *et al*. 2003), suggesting that AtMEX1 abundance may not be limiting for nocturnal starch mobilization under these long night growth conditions. This conclusion is supported by the observation that AtMEX1 expression was not significantly induced in the high starch-accumulating FBPas plants (Fig. 6). Nevertheless, it is possible that the increase in supply of Suc in the SPSox plants has resulted in these plants modulating AtMEX1 expression but a more targeted study, including detailed analysis of nocturnal starch mobilization, will be required before it is possible to draw any firm conclusions on whether AtMEX1 expression is subject to metabolite regulation.

In contrast to the simpler path for increased Suc synthesis and export outlined for the SPSox plants grown at warm temperatures, cold-acclimating *Arabidopsis* plants face a much more complex regulatory problem. We have shown previously that during cold acclimation there is an increase in the availability of Pi and phosphorylated intermediates in both the pathway for Suc synthesis and the Calvin cycle, and that there are also increased capacities of enzymes in both pathways (Hurry, Gardeström &

Öquist 1993, Hurry *et al*. 1994, 2000; Strand *et al*. 1997). However, one consequence of these long-term changes in cytosolic Pi availability and the capacity for Suc synthesis could be to pull too much carbon out of the chloroplast via the triose-phosphate transporter. This would, in turn, reduce the capacity of the Calvin cycle to regenerate RuBP and inhibit photosynthesis. The changes described for the two compartments during cold acclimation could therefore be viewed as competing rather than complementary responses because both require an increased utilization of triose phosphates. The data we present in this paper suggest that an important site for regulation during cold acclimation could be the TPT. To date, there is no evidence that the activity of the TPT is modulated by post-translational modifications and no regulatory metabolites have been identified. However, the TPT has previously been shown to be transcriptionally down-regulated in tobacco grown on Suc (Knight & Gray 1994). Growth of plants on Suc-containing media, or feeding leaves Suc, have been shown to result in large increases in leaf soluble carbohydrate and phosphorylated intermediate pools that are similar to what we show in cold-exposed leaves (e.g. Strand *et al*. 1999). The results from both transgenic lines and WT during cold acclimation indicate that in order for source-leaf metabolism to recover proper function in the cold, the increase in phosphorylated metabolites and Pi that supports the increase in flux through Suc (Strand *et al*. 1999; Hurry *et al*. 2000) and the recovery of RuBP-regeneration (Hurry *et al*. 1996) must be balanced by tighter control of metabolite exchange between the chloroplast and the cytosol, and one component of this control is the low temperature-induced down-regulation of AtTPT expression.

Earlier studies of antisense TPT tobacco (Hausler *et al*. 1998, 2000a,b) and an *Arabidopsis* T-DNA insertional mutant (Schneider *et al*. 2002) have also demonstrated that plants with severely limited triose-phosphate export capacity are able to increase starch degradation during the day and utilize hexoses exported to the cytosol, presumably via either the maltose (Niittyla *et al*. 2004) or glucose (Weber *et al*. 2000) transporter for Suc synthesis. Whether cold-acclimated plants utilize a similar daytime starch mobilization route is unknown but the lack of significant starch accumulation during the day in cold-stressed and cold-developed leaves (Strand *et al*. 2003) despite clear data showing substantial daily incorporation of ^{14}C into the insoluble pool (Fig. 3) suggests that this is a possibility. Recent data from *Arabidopsis* show that β-amylase gene expression and enzymatic activity are enhanced, and that maltose does accumulate, during cold stress (Kaplan & Guy 2004; Kaplan *et al*. 2004, 2006). We show here that the cold enhancement of AtBMY8 and AtBMY9 expression is sustained even after development of new leaves at 5 °C (Fig. 7). In the cytosol, maltose is metabolized via a transglucosylation reaction catalysed by DPE2 (Chia *et al*. 2004) and the glucose released is then converted to hexose phosphates via hexose kinase (Smith, Zeeman & Smith 2005). This mechanism might help to explain why the FBPas plants maintain relatively high photosynthesis and Suc-export rates despite the strong repression of cFBPase activity. Utilization of such a pathway for daytime carbon export from the chloroplast would enable the cold-stressed and cold-acclimated plants to avoid the TPT and Fru2,6 BP regulatory steps, maintain chloroplastic phosphorylated intermediates and Pi, and also maintain high rates of Suc synthesis. The changes in β-amylase and TPT gene expression, combined with the carbon flux data reported here for cold-stressed and cold-developed leaves, suggest that this alternate pathway for carbon mobilization from the chloroplast during the day at low temperatures is a possibility that should be explored further.

When we look beyond source cell metabolism, during low-temperature acclimation WT plants up-regulate Suc biosynthesis and demonstrate a similar induction of AtSUC1 as the SPSox plants grown at warm temperatures. The SPSox plants also show a further cold-induced up-regulation of AtSUC1. However, in the experiments reported here, we were unable to measure significant Suc export from excised 10 d cold-stressed WT leaves, despite the enhancement of Suc biosynthetic pathway and the increased abundance of AtSUC1 symporter transcript. This failure to maintain Suc export in the cold in the 10 d shifted leaves results in a reduction in photosynthesis of the intact rosette (Fig. 2). In contrast, leaves that developed in the cold showed near complete recovery of Suc-export capacity and photosynthetic rates under ambient growth conditions (Figs 2 & 5). This recovery is associated with stable increases in expression of AtSUC1 and, to a lesser extent, AtSUC2 (Fig. 7). Why the 10 d cold-stressed leaves show such reduced export capacity while the cold-developed leaves show such strong recovery, remains unclear. It seems unlikely that it can be explained as a 'sink-limited' feedback mechanisms such as suggested for BvSUT1 (Chiou & Bush 1998; Vaughn, Harrington & Bush 2002) because we see no loss of symporter transcript in the 10 d cold-stressed leaves. It may instead be a result of the plants having problems maintaining membrane energization at the low temperature either because of inappropriate lipid/fatty acid composition (Uemura, Joseph & Steponkus 1995) or because of a reduced ability to generate the proton-motive force required. At this point it is not clear what signal(s) symporter expression responds to or from where the signal(s) originate (source or sink tissues) but it is becoming clear that metabolite-mediated transcriptional regulation of the symporter genes plays a key role in balancing photosynthetic carbon uptake and utilization. Furthermore, recent studies with sugar beet BvSUT1 have demonstrated high turnover rates of both symporter mRNA and protein, indicating that there is tight transcriptional control over phloem-loading capacity (Vaughn *et al*. 2002). Thus, our results suggest that ectopic expression of the maize SPS gene and cold acclimation result not only in an increased Suc synthesis but also in integrated changes in expression of the major metabolite transporters involved in intermediate export from the chloroplasts and in Suc export.

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