Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit

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Abstract
Sucrose unloading and sink activity were examined in tomato plants (Lycopersicon esculentum) overexpressing sucrose phosphate synthase (SPS; EC 2.3.1.14). Like the leaves, the fruit of the transformed tomato plants had elevated (2.4-fold) SPS activity. SPS overexpression in tomato fruit did not significantly change acid invertase activity, and only slightly reduced ADP-glucose pyrophosphorylase activity, but enhanced sucrose synthase activity by 27%. More importantly, the amount of sucrose unloaded into the fruit was considerably increased. Using [³H]-fructosyl-sucrose in in vitro unloading experiments with harvested 20-d-old fruit, 70% more sucrose was unloaded into the transformed fruits compared to the untransformed controls. Furthermore, the turnover of the sucrose unloaded into the fruit of transformed plants was 60% higher than that observed in the untransformed controls. Taken together, these results demonstrate that SPS overexpression increases the sink strength of transformed tomato fruit.

Key words: Sucrose phosphate synthase (SPS), starch, sucrose unloading, tomato fruit, carbon metabolism.

Introduction
Sucrose phosphate synthase (SPS) catalyses net sucrose synthesis in plants. SPS activity is generally high in source tissues and low in sink organs (Huber and Huber, 1992). SPS activity can be a controlling factor for sucrose synthesis and also for photosynthesis (Stitt et al., 1988). However, in transformed potato plants, a decrease in SPS activity of 60–70% caused no significant inhibition of photosynthesis compared to the untransformed controls (Geigenberger et al., 1995). To increase photosynthesis and sucrose synthesis, Worrell et al. (1991) genetically manipulated tomatoes to overexpress a maize SPS cDNA, under the control of the promoter of the small subunit of Rubisco from tobacco (rbcS). Several rbcS lines showed a higher SPS activity in their leaves than untransformed controls (Worrell et al., 1991). These were characterized and plant growth evaluated under different conditions (Galtier et al., 1993, 1995; Micallef et al., 1995; Laporte et al., 1997). The SPS activity in the transformed leaves was six times greater than that of the controls. At low irradiances no increase in net photosynthesis was observed, but the light- and CO₂-saturated rates of photosynthesis in transformed leaves were increased by about 20% (Galtier et al., 1993, 1995; Micallef et al., 1995). Carbon allocation within the plant was not modified except in very young plants where an increase in root dry weight was observed (Galtier et al., 1993; Laporte et al., 1997). Furthermore, SPS overexpression caused an increase in the sucrose/starch ratio of leaves (Galtier et al., 1993, 1995; Signora et al., 1998), but this was due more to increased turnover of starch than to increased sucrose biosynthesis (Galtier et al., 1993). Most interestingly, the total fruit number in the SPS transformants was increased to 1.5 times that of the control (Micallef et al., 1995). The fruit on the trans-
formed plants matured earlier and total fruit dry weight was increased by up to 32% when these plants were grown in air and high light (1000 μmol m⁻² s⁻¹). However, no increase in total fruit yield was observed in conditions of CO₂ enrichment (Micaleff et al., 1995).

In sink tissues, unloaded sucrose has to be hydrolysed for metabolism. Depending on the plant species unloaded sucrose can be hydrolysed either by invertase or by sucrose synthase. Several studies have shown that unloaded sucrose can undergo a futile cycle of degradation and resynthesis in sink tissues (N’chobo, 1998a, b; ap Rees and Hill, 1994; Dancer et al., 1990; Geigenberger and Stitt, 1993; Hargreaves and ap Rees, 1988; Viola, 1996; Wendler et al., 1990). In such a cycle, the nature of the enzyme that catalyses the resynthesis of sucrose remains to be elucidated. Geigenberger and Stitt (1993) suggested that sucrose resynthesis in sink tissues is catalyzed by both SuSy and SPS. In sink tissues, SPS is present at low levels: 2.5–10% of SuSy activity in rapidly growing-sinks (Dali et al., 1992; Klann et al., 1993; Weber et al., 1996). Sucrose phosphate synthase has been considered to be the limiting factor in the regulation of sucrose accumulation in tomato fruit (Dali et al., 1992; Miron and Schaffer, 1991). However, this hypothesis was not supported by later observations which suggest that the sucrose concentration of tomato fruit was determined by acid invertase activity (Klann et al., 1993; Ohyama et al., 1995; Scholes et al., 1996). Increased sucrose accumulation in transgenic potato tubers with low ADPglc pase activity, however, was correlated with increased SPS activity (Geigenberger et al., 1995).

While the role of SPS in relation to putative futile cycles has been studied in sink tissues (Geigenberger and Stitt, 1993), the role of SPS in determining sink strength and sucrose unloading remains to be elucidated. This study has examined sucrose metabolism in tomato fruit where SPS is overexpressed. It has shown that overexpression of SPS in young fruit favours increased sucrose synthesis, resulting in increased sink cleavage and sink activity.

Materials and methods

Plant material and growing conditions

Tomato (Lycopersicon esculentum var. UC82B) seed of untransformed and transformed (Line 3812–9) lines was obtained from Calgene Inc (USA). The transformed tomatoes used in the present study are an homozygous line (T4) with only one transgene insertion (verified by Southern blot; data not shown). This transgenic clone was used in previous studies for analysis of photosynthesis capacity (Galtier et al., 1993) and fruit yield (Micalef et al., 1995) under the name: SSU-9. Expression of the maize SPS cDNA was under the control of the Rubisco small subunit promoter. The plants were grown in a greenhouse with a 14 h photoperiod, 25/22 °C day/night. Irradiance was 700 μmol m⁻² s⁻¹.

Enzyme extraction

Freshly harvested tomato fruits were ground in liquid nitrogen. Proteins were extracted into buffer A consisting of 200 mM HEPES (pH 7.0) 3 mM magnesium acetate, 0.5 mM EDTA, 1 mM PMSF, 5 mM DTT, 20 mM 2-mercaptoethanol, 10% ethylene glycol, 1% PVPP, and 1% Dowex-1 (chloride form) as previously described (Sun et al., 1992). The ratio of fruit fresh weight (g) to extraction volume (ml) was 1:5. The homogenate was incubated on ice for 5 min, passed through one layer of Miracloth (InterScience, Markham, Ontario, Canada) and centrifuged at 20 000 × g for 10 min. The supernatant was desalted through Sephadex G-25 superfine (Pharmacia Biotech, Baie D’Urfé, Québec, Canada) with buffer B which consisted of 50 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 2 mM magnesium acetate.

Enzyme assays

Sucrose phosphate synthase activity was determined at limiting substrate concentrations (2 mM UDP glucose, 2 mM F6P and 10 mM G6P) with 5 mM Pi, as described by Huber and Huber (1991). Sucrose formation was determined by the modified anthrone method (Van Handel, 1968).

Sucrose synthase and neutral invertase activities were assayed in the direction of sucrose breakdown by a coupled enzyme assay as described previously (Huber and Akazawa, 1986). The reaction mixture (1 ml total volume) consisted of buffer B containing 100 mM sucrose, 1 mM ATP, 0.4 mM NAD, 4 units hexokinase, 4 units glucose isomerase, 2 units 6-phosphoglucose dehydrogenase (Leucomostoc mesenteroides; NAD-dependent form) without invertase contamination obtained from Boehringer Mannheim, Laval, Québec, Canada), and the change in absorbance at 340 nm at 25 °C was measured. This provided an estimation of the background invertase activity arising from contamination of the coupling enzymes. After 2 min, 20 μl of sample was added allowing estimation of the neutral invertase activity. Sucrose synthase activity was measured by the subsequent addition of 1 mM of UDP (final concentration).

Acid invertase was assayed in acetate buffer (pH 4.5). 100 μl of sample was mixed with 500 μl of 100 mM acetate buffer containing 3% sucrose. After 15 min incubation at 30 °C, the reaction was neutralized with 50 μl of 1 M TRIS-HCl buffer (pH 8.0). Further enzyme activity was prevented by boiling the mixtures for 5 min. The hexose formed in the reaction was measured in an oxygen electrode (commercial YSI model select 2700, Yellow Spring Instrument Co. Inc., Ohio, USA) with a glucose membrane where glucose oxidase is immobilized. By the action of glucose oxidase in the presence of O₂, glucose was converted to d-glucosone-lactone and H₂O₂. The amount of H₂O₂ formed was measured using a platinum anode (see the YSI manual for 2700 SELECT analyser).

ADPglc pase activity was measured in the direction of G1P formation in a coupling reaction as previously described by Rocher et al. (1989). Desalted extract (40 μl) was added to 450 μl HEPES-NaOH buffer (50 mM, pH 7.5) containing 3 mM Mg-acetate, 0.1 mg BSA, 1 mM ADPglucose, 0.3 mM NAD, 1 unit phosphoglucose mutase, and 1 unit 6-phosphoglucose dehydrogenase. The reaction was started by the addition of 1 mM PPI, and the formation of NADH was measured by the change in absorbance at 340 at 25 °C.

Western blotting and tissue printing

For Western blot assay, the total protein in non-desalted extract was immediately precipitated with 3% TCA. After centrifugation, the pellet was solubilized in the electrophoresis loading buffer (pH 6.8). Five μg total protein from each sample were
separated on 10% SDS–PAGE as described by Laemmli (1970). Proteins were electrotransferred to nitrocellulose membrane for immunodetection with polyclonal antibodies raised against maize SPS (Worrell et al., 1991; 1/5000 dilution) or polyclonal antibody against maize SuSy (Nguyen-Quoc et al., 1990; 1/5000 dilution) as primary antiserum with alkaline phosphatase-coupled secondary antibodies (Boehringer Mannheim, Laval, Quebec, Canada) using the NBT/BCIP substrate combination for colour development (Promega, Madison, MN, USA). The density of the Western blot band was determined by NIH image (version 1.61) application on the scan image. Tissue printing was as described by Cassab and Varner (1987) with a slight modification as follows. The tomato fruit was cut in half along the centre and proteins were printed onto a nitrocellulose membrane. The membrane was washed in the TBS buffer containing 0.1% SDS and boiled for 10 min in TBS buffer to denature the endogenous phosphatase. The enzyme immunodetection protocol was identical to that used for Western blotting.

In vivo sucrose unloading and carbohydrate determination

Unloading was assayed by a modification of the technique described by Dali et al. (1992). The tomato fruit with their peduncles attached were harvested. The peduncles were plunged immediately into 1 ml of 2% unlabelled sucrose and 3 μCi (6.106 DPM) [3H]- (fructosyl)-sucrose (Dupont Canada Inc., Mississauga, Ontario, Canada). After a 1 h pulse, the fruits were transferred to 2% unlabelled sucrose (chase). Whole fruit samples were removed from the solution at different times (0, 30, 60, 90, 120 min). The peduncle was removed before analysis. Fruits were ground in liquid N2 and then homogenized in 5 vols of 80% ethanol. The homogenate was boiled for 10 min at 80 °C. After 10 min centrifugation at 12 000 g, the supernatant was collected and used for determination of sucrose and hexoses and the pellet was retained for starch analysis. For the soluble sugar determination, the supernatant was lyophilized, resuspended in water and purified by ion exchange chromatography (cation and anion AG resin; Biorad, Mississauga, Ontario, Canada). The sucrose and hexose fractions were separated on carbohydrate HPLC column (Millipore, Ville Ste Laurent, Quebec, Canada). Sucrose, glucose and fructose fractions corresponding to the standards were collected and the radioactivity was measured in a liquid scintillation counter (Wallac, Turku, Finland). To determine the symmetrical labelling of the sucrose molecule, the purified sucrose fraction was hydrolysed by yeast invertase and re-fractioned by HPLC.

Data analysis

Statistical analyses were performed using analysis of variance (ANOVA).

Results

Analysis of sucrose metabolising enzymes and carbohydrate composition of fruit

Enzyme activity. Previous studies have shown that sink activity in tomato fruits is maximum at 20 DAA (Ho et al., 1987). In transformed tomato plants with altered sucrose metabolism enzyme activities (SuSy, SPS, invertase or ADPGlc ppase), the timing of fruit development was not changed (data not shown). Therefore SPS, SuSy, acid invertase and ADPGlc ppase activities in 20-d-old fruits from transformed and untransformed (control) plants were analysed. Table 1 shows that the SPS activity of fruit from transformed plants, measured with limiting concentrations of substrate, was 2.4 times higher than that of untransformed controls. This increase was accompanied by a significant increase in SuSy activity (27%; P ≤ 0.05). The increase in activity of these two enzymes was comparable in terms of the capacity to cleave or synthesise sucrose (Table 1). Acid invertase and ADPGlc ppase activities were not significantly different from those in untransformed controls.

The expression of transgene in the fruit was demonstrated by Western blot (Fig. 1A, lane 2). One protein band was detected with a molecular mass of 120 kDa, equivalent to the size of the maize SPS peptide. This result suggests that the increase in SPS activity in the transformed fruits was due to over-expression of SPS. Similarly, the increase in SuSy activity in transformed tomato fruits correlated with increased enzyme protein as determined by semi-quantitative scanning of the SuSy peptide in Western blot (Fig. 1B). The band intensity of SuSy peptide in the transformed fruit (Fig. 1B, lane 2) was 57% higher than that in untransformed (Fig. 1B, lane 1) controls.

The increases in SPS and SuSy activities in transformed tomato fruits correlated with increased enzyme protein as determined by Western blotting with maize SPS (Fig. 1A) and SuSy (Fig. 1B) antibodies. Maize SPS antibodies did not crossreact with endogenous tomato SPS (Fig. 1A), while the antibody for SuSy from maize cross-reacted with the native tomato SuSy isoforms. No bands of native SPS protein were detected with the antibodies to the maize enzyme (Fig. 1A, track 1), but in the transformed line (Fig. 1A, lane 2) one band was detected which corresponded to a molecular mass of 120 kDa which is equivalent to the size of the maize SPS peptide. The density of staining of this SPS band was equivalent to that detected in extracts from transformed leaves when gels were loaded on an equal protein basis (data not shown). Figure 1B shows the immunodetection of SuSy in tomato fruits from untransformed (track 1) and transformed plants (track 2). The SuSy band in the transformed line was clearly much more intense, for an equivalent protein concentration, than in untransformed plants indicating that the quantity of this enzyme protein was increased in the former compared to the latter. The molecular mass of tomato SuSy was estimated to be about 90 kDa by comparison with standard protein molecular markers (BioRad, Massachusetts, USA).

The expression of SPS in the different tissues of the tomato fruit from transformed and untransformed plants was compared by tissue printing. The tissue prints from transformed fruit obtained with maize SPS antibodies (Fig. 2B) shows that the amounts of these enzyme proteins are relatively homogeneous in all types of tissue within the fruit and are similar in both the external and
Table 1. Activities of SPS (measured at limiting substrate concentrations), sucrose synthase, acid invertase, and ADPglc ppase in 20 DAA fruits from untransformed and transformed tomato plants; values represent the mean ± SE, n = 3 plants

<table>
<thead>
<tr>
<th></th>
<th>SPS (nmol min⁻¹ g⁻¹ FW)</th>
<th>SuSy (nmol min⁻¹ g⁻¹ FW)</th>
<th>Acid invertase (nmol min⁻¹ g⁻¹ FW)</th>
<th>ADPglc ppase (nmol min⁻¹ g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed</td>
<td>96 ± 3</td>
<td>1254 ± 35</td>
<td>1397 ± 335</td>
<td>719 ± 15</td>
</tr>
<tr>
<td>Transformed</td>
<td>233 ± 26</td>
<td>1593 ± 147</td>
<td>1422 ± 21</td>
<td>639 ± 21</td>
</tr>
</tbody>
</table>

Carbohydrate analysis. When the carbohydrate composition (sucrose, hexose and starch) of 20 DAA fruit was analysed (Table 2), the soluble sugar concentrations and the sucrose/hexose ratios in transformed fruit were found to be similar to those of the controls. However, the amount of starch in the transformed fruit was significantly higher than that in the controls (Table 2; P ≤ 0.05).

In vitro unloading of sucrose

In order to distinguish between effects due to SPS overexpression in the source leaves from those occurring in the sink tissues alone, fruits from transformed and control plants were detached and used for unloading experiments. The detached 20 DAA fruits were supplied with labelled sucrose. Figure 3 shows the unloading patterns of transformed and control fruit. After 1 h of labelling and subsequent 30, 60, 90, or 120 min chase periods with unlabelled sucrose, the total radioactivity (Fig. 3A) unloaded in the transformed fruit was about 70% higher than that found in the controls. In the shortest chase times (up to 30 min) the majority of the radioactivity was found in sucrose (data not shown). The differences between the observed amount of radioactivity in transformed and control fruit was due to variations in radioactivity in soluble sugars (Fig. 3A, C). No differences in radioactivity were found in the starch fractions. With longer chase times (from 60 min onwards), however, the increase in radioactivity in the transformed fruit was caused by differences in both the soluble sugar and starch fractions. Moreover, as the period of the chase increased the ratio of soluble sugar to starch became similar in both lines.

Sucrose turnover and sucrose to starch conversion

In SPS overexpression in the fruit may accelerate the rate of sucrose synthesis or sucrose turnover. Since the unloaded

Table 2. Sucrose, hexose and starch contents of 20 DAA fruits from transformed and untransformed tomato plants; values represent the mean ± SE, n = 3 plants

<table>
<thead>
<tr>
<th></th>
<th>Sucrose (mg g⁻¹ FW)</th>
<th>Hexoses (mg g⁻¹ FW)</th>
<th>Starch (mg g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed</td>
<td>1.76 ± 0.13</td>
<td>13.03 ± 0.67</td>
<td>13.04 ± 0.54</td>
</tr>
<tr>
<td>Transformed</td>
<td>1.88 ± 0.05</td>
<td>12.15 ± 0.30</td>
<td>14.82 ± 0.45</td>
</tr>
</tbody>
</table>
Overexpression of SPS

Fig. 4. Symmetry of labelling in the sucrose molecule in control and transgenic tomato fruits. $[^{3}H]$-(fructosyl)sucrose was supplied to 20 DAA tomato fruits. After a 1 h pulse the fruits were transferred to unlabelled sucrose and after 30, 60, 90, and 120 min of chase, the symmetry of labelling of sucrose was analysed. Values are means ± SD for three independent fruits.

Discussion and conclusions

In tomato fruit overexpression of SPS leads to an increase in SuSy and sink activity

The rate of import of assimilate in the rapid growth phase of tomato fruit development (about 20 DAA) has been considered to be crucial for determining final fruit weight (Grange and Andrews, 1993; Ho, 1996; Ho et al., 1987). This period in fruit development is characterized by maximum SuSy and ADPglc ppase activities. Moreover, sucrose unloading also attains a maximum rate at this point (Dali et al., 1992). The correlation observed between the rate of sucrose breakdown and the quantity of sucrose unloaded into the fruit has led to the hypothesis that either invertase or sucrose synthase can limit sucrose unloading and, in turn, determine yield (Ho, 1996). In contrast to the original hypothesis that suggested that invertase is the limiting step in the regulation of assimilate
import in tomato (Walker et al., 1978), Sun et al. (1992) have suggested that SuSy is one of the principal factors regulating fruit growth. This hypothesis is supported by the observations of Wang et al. (1993), who reported a good correlation between SuSy activity and relative fruit growth. Such observations suggest that the cleavage of sucrose in the cytosol may be the limiting step for sucrose import into tomato fruit. However, sucrose imported into this sink organ may undergo rapid turnover involving both degradation and resynthesis (Geigenberger and Stitt, 1993; N’tchobo, 1998a, b).

In young tomato fruits, where sucrose is unloaded principally via the symplast (N’tchobo, 1998a, b; Ruan and Patrick, 1995; Yelle et al., 1988; Yin et al., 1995), SuSy and SPS could together catalyse a cycle of cleavage and resynthesis in the cytosol (Geigenberger and Stitt, 1993). SPS overexpression resulted in a 2.4-fold increase in SPS activity in the fruit. The cleavage of sucrose by SuSy was also enhanced. In the transformed fruit, the observed increase in SPS led to a 27% increase in SuSy activity. The increase in maximal extractable SuSy activity (339 nmol min^{-1} g^{-1}FW) was in the same range as that of SPS activity measured in conditions of limiting substrates (137 nmol min^{-1} g^{-1}FW). Hence, the increase in the capacity for sucrose synthesis in the transformed fruit led to an even greater increase in the capacity for sucrose cleavage.

**Increase in SPS and SuSy activities led to an increase in sucrose turnover and sucrose import**

Since the capacity of both sucrose synthesis and sucrose degradation was increased in the fruits of the transformed plants, it is pertinent to consider whether this leads to increased sucrose turnover. Sucrose turnover in fruit from the SPS transformants was found to be 60% higher than that measured in the untransformed controls. The sucrose used in these experiments was labelled in the fructose moiety. The resynthesis of sucrose by SuSy or SPS must lead to similar changes in the symmetry of labelling in sucrose molecules in both transformed and untransformed lines. Increased SPS activity would only result in enhanced sucrose import if the rate of sucrose turnover was related to the rate of import. This is indeed what was observed since the increase in SuSy and SPS activities in transformed fruit led to an increase in the sucrose turnover rate of about 60%. This, in turn, led to a calculated increase in the rate of sucrose import of 70%.

The increase in sucrose import by the sink organs may have been caused by a change in the rate of sucrose export from source leaves (Ho, 1996; Micallef et al., 1995). However, in the rbcS transformants SPS activity was increased in both leaves and fruit. A change in sink activity involving increased SPS or SuSy activity may be sufficient to increase sink strength. The recent patent application by Calgene, showing that the expression of the maize SPS cDNA under the control of a fruit-specific promoter in tomato plants leads to an increase in yield, confirms this hypothesis.

**Increased sucrose import in tomato fruit leads to an increase in starch synthesis**

ADPGlc ppase activity was not increased in the transformed fruit, but the sucrose/starch ratio was changed by SPS overexpression. The amount of starch and the rate of starch synthesis were higher in transformed fruit than in those from untransformed plants. Starch accumulation is therefore not only dependent on ADPGlc ppase activity, but also on the amount of sucrose unloaded into the fruit. The rate of sucrose resynthesis in tomato fruit may therefore determine the extent of starch synthesis as it does in leaves (Galtier et al., 1993, 1995; Signora et al., 1998). However, overexpression of SPS in fruit increased starch accumulation, while in the leaves the inverse was true.

**Acknowledgement**

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**References**


