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ORIGINAL ARTICLE

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Antisense inhibition of sorbitol synthesis leads to up-regulation of starch synthesis without altering CO_2 assimilation in apple leaves

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Abstract Sorbitol is a primary end-product of photosynthesis in apple (*Malus domestica* Borkh.) and many other tree fruit species of the Rosaceae family. Sorbitol synthesis shares a common hexose phosphate pool with sucrose synthesis in the cytosol. In this study, 'Greensleeves' apple was transformed with a cDNA encoding aldose 6-phosphate reductase (A6PR, EC 1.1.1.200) in the antisense orientation. Antisense expression of A6PR decreased A6PR activity in mature leaves to approximately 15–30% of the untransformed control. The antisense plants had lower concentrations of sorbitol but higher concentrations of sucrose and starch in mature leaves at both dusk and predawn. $^{14}CO_2$ pulse-chase labeling at ambient CO_2 demonstrated that partitioning of the newly fixed carbon to starch was significantly increased, whereas that to sucrose remained unchanged in the antisense lines with decreased sorbitol synthesis. Total activities of ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), sucrose-phosphate synthase (EC 2.4.1.14), and ADP-glucose pyrophosphorylase (EC 2.7.7.27) were not significantly altered in the antisense lines, whereas both stromal and cytosolic fructose-1,6-bisphosphatase (EC 3.1.3.11) activities were higher in the antisense lines with 15% of the control A6PR activity. Concentrations of glucose 6-phosphate and fructose 6-phosphate (F6P) were higher in the antisense plants than in the control, but the 3-phosphoglycerate concentration was lower in the antisense plants with 15% of the control A6PR activity. Fructose 2, 6-bisphosphate concentration increased in the antisense plants, but not to the extent expected from the increase in F6P, comparing sucrose-synthesizing species. There was no significant difference in CO_2 assimilation in response to photon flux density or intercellular CO_2 concentration. We concluded that cytosolic FBPase activity in vivo was down-regulated and starch synthesis was up-regulated in response to decreased sorbitol synthesis. As a result, CO_2 assimilation in source leaves was sustained at both ambient CO_2 and saturating CO_2.

Keywords Aldose 6-phosphate reductase · CO_2 assimilation · *Malus domestica* · Photosynthetic carbon partitioning · Sorbitol · Starch synthesis

Abbreviations AGPase: ADP-Glucose pyrophosphorylase · A6PR: Aldose 6-phosphate reductase · FBPase: Fructose-1,6-bisphosphatase · F2,6BP: Fructose 2,6-bisphosphate · F6P: Fructose 6-phosphate · G6P: Glucose 6-phosphate · G6PDH: Glucose 6-phosphate dehydrogenase · PGA: 3-Phosphoglycerate · Pi: Inorganic phosphate · Rubisco: Ribulose 1,5-bisphosphate carboxylase/oxygenase · S6PDH: Sorbitol 6-phosphate dehydrogenase · SPS: Sucrose-phosphate synthase · TPT: Triose phosphate translocator

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Introduction

Photosynthetic end-product synthesis is a divergent metabolic pathway: triose phosphates leaving the Calvin cycle are either retained in the chloroplast for starch synthesis or exported via triose phosphate translocator (TPT) to the cytosol for synthesis of soluble carbohydrates. In most plants, sucrose is the main soluble end-product of photosynthesis in the cytosol. The key regulatory steps in sucrose synthesis are the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate (F6P) catalyzed by a cytosolic fructose-1, 6-bisphosphatase

(cytoFBPase), and the formation of sucrose 6-phosphate from UDP-glucose and F6P catalyzed by sucrose-phosphate synthase (SPS) (Daie 1993; Huber and Huber 1992). CytoFBPase activity is inhibited by the signal metabolite fructose 2,6-bisphosphate (F2,6BP) (Stitt 1990). SPS activity is subject to allosteric regulation by metabolites and post-translational modification via reversible protein phosphorylation (Huber and Huber 1992). Glucose 6-phosphate (G6P) activates whereas inorganic phosphate (Pi) inhibits SPS activity (Doehlert and Huber 1983).

Starch is synthesized in the chloroplast during the day and serves as a transitory storage carbon source. The key enzyme in starch synthesis is ADP-glucose pyrophosphorylase (AGPase), which is activated by 3-phosphoglycerate (PGA) and inhibited by Pi (Sowokinos 1981; Preiss 1988). AGPase is also regulated via redox modification in response to light and sucrose levels in leaves (Hendriks et al. 2003). At night, starch is degraded, and exported as maltose and glucose (Niittylä et al. 2004; Schleucher et al. 1998; Weise et al. 2004) to provide carbon for subsequent sucrose synthesis and export. Studies on transgenic plants or mutants with decreased TPT activity (Häusler et al. 1998, 2000; Heineke et al. 1994; Riesmeier et al. 1993; Schneider et al. 2002), cytoFBPase activity (Sharkey et al. 1992; Strand et al. 2000; Zrenner et al. 1996), phosphoglucose isomerase (Neuhaus et al. 1989), or SPS activity (Krause 1994) have shown that when sucrose synthesis is decreased, starch synthesis is up-regulated via the increased PGA/Pi ratio resulting from accumulation of hexose phosphates in the cytosol. As a result, photosynthetic carbon partitioning to starch is increased without altering photosynthesis at ambient CO_2. However, transgenic plants or mutants with a decreased capacity for sucrose synthesis generally have lower photosynthesis under saturating CO_2 conditions, when the increase in starch synthesis is no longer sufficient to compensate for the reduction in triose phosphate utilization caused by decreased sucrose synthesis (Häusler et al. 1998, 2000; Riesmeier et al. 1993; Schneider et al. 2002; Sharkey et al. 1992).

Sorbitol is a primary end-product of photosynthesis and a major translocated carbohydrate for many economically important tree fruit species in the Rosaceae family, such as apple, pear, peach, cherry, apricot, and plum (Bieleski 1982; Loescher 1987). In these species, sorbitol accounts for 50–90% of the newly fixed carbon and the carbon exported from the source leaves (Bieleski 1982; Bieleski and Redgwell 1985; Escobar-Gutierrez and Gaudillere 1997; Loescher 1987). Sorbitol synthesis shares a common hexose phosphate pool with sucrose synthesis in the cytosol. In source leaves, G6P is first converted to sorbitol 6-phosphate by aldose 6-phosphate reductase (A6PR, EC 1.1.1.200, also called sorbitol-6-phosphate dehydrogenase, S6PDH) (Kanayama and Yamaki 1993; Negm and Loescher 1981; Zhou et al. 2003a), then followed by hydrolysis of sorbitol 6-phosphate to form sorbitol via sorbitol-6-phosphatase (Zhou et al. 2003b). The reaction catalyzed by A6PR is the key regulatory step in sorbitol synthesis. When sorbitol is transported to sink tissues, it is converted to fructose by sorbitol dehydrogenase for further metabolism (Loescher et al. 1982). In addition, sorbitol is implicated in responses of sorbitol-synthesizing plants to abiotic and biotic stresses, such as osmotic adjustment under water stress (Lo Bianco et al. 2000; Ranney et al. 1991; Wang and Stutte 1992), cold hardiness (Raese et al. 1978), mobility of boron in the phloem and tolerance of boron deficiency (Brown et al. 1999; Hu et al. 1997), and disease resistance (Suleman and Steiner 1994).

Although considerable efforts have been made to elucidate the carbon partitioning between sucrose and starch in sucrose-synthesizing species, the biochemical regulation of photosynthetic carbon partitioning between sorbitol, sucrose, and starch in sorbitol-synthesizing species is poorly understood. Because sorbitol synthesis adds another branch to end-product synthesis, the biochemical regulation of photosynthetic carbon partitioning in sorbitol-synthesizing species is likely to be complex. The objectives of this study were to use transgenic apple plants with decreased expression of A6PR to understand the regulation of photosynthesis and carbon partitioning by sorbitol synthesis.

Materials and methods

Vector construction and *Agrobacterium*-mediated transformation

The construction of binary plasmid vector pDU93.0330 for antisense expression of A6PR (S6PDH) cDNA was described in detail by Tao et al. (1995). The A6PR cDNA was ligated in the antisense orientation with respect to the 35S cauliflower mosaic virus (CaMV) promoter and the 3′ regulatory sequence. The vector also contains a β-glucuronidase (GUS) gene and a neomycin phosphotransferase (nptII) gene driven by the CaMV 35S promoter.

The binary plasmid vector, pDU93.0330, was introduced into a disarmed strain of *Agrobacterium tumefaciens*, EHA 101. Leaf discs of 'Greensleeves' apple (*Malus domestica* Borkh.) were transformed with EHA101 following the protocol of James and Dandekar (1991). The transformants were then screened by detection of GUS in tissues via histochemical analysis and quantitative estimation of GUS activity in plant extracts (Jefferson 1987). Shoots were rooted on a medium containing 50 mg l^{-1} kanamycin. A total eight independently transformed lines were obtained. Untransformed 'Greensleeves' plants that were cultured in the same way as the transformed lines were used as controls.

Plant growth conditions

Eight transgenic lines along with the untransformed control were propagated in the spring by grafting onto

M26 rootstocks. The plants were grown in 3.8-l pots containing a mixture of peat moss, pumice and sandy loam soil (1:2:1 by volume) outside under natural conditions at Oregon State University in Corvallis (Ore.). They were supplied with 20 mM N using Plantex NPK (20-10-20) with micronutrients (Plantex, Ontario, Canada) twice weekly during the growing season. Leaf number and leaf area were determined after cessation of stem elongation and leaf expansion. Plant height and stem diameter were measured after natural leaf fall.

One-year-old grafted plants of the transgenic lines and the control were shipped to Cornell University and pruned back to three buds during dormancy. After budbreak, extra shoots were removed and only one shoot was allowed to grow on each plant. The plants were grown in 7.6-l plastic containers in a medium of 1 sand:2 MetroMix 360 (v/v) (Scotts, Marysville, Ohio) outdoors under natural conditions in Ithaca, N.Y. They were fertilized with 15 mM N using Plantex NPK (20-10-20) with micronutrients twice weekly during the growing season. Fungicides and pesticides were sprayed at regular intervals throughout the growing season to protect the plants from diseases and insects. Recently fully expanded, mature leaves (11th or 12th leaf from the shoot tip) of actively growing trees were used for all the molecular, biochemical and physiological measurements.

RNA preparation and gel blotting

RNA was extracted from mature leaves of the control and antisense lines according to the modified hot borate method of Wan and Wilkins (1994). Duplicate, 20 μg samples of total RNA were separated by formaldehyde-agarose gel electrophoresis. The RNA was transferred to a Hybond N nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) by downward capillary with a Turboblotter (Schleicher & Schuell, Keene, N.H.), following the manufacturer's instructions. RNA was fixed to the membrane by UV light cross-linking. Single-stranded, antisense, ^{32}P-labeled A6PR probes were synthesized via primer extension. Hybridization and washing were performed in phosphate-SDS buffer. Autoradiography film was subsequently exposed to the membrane at −70°C for 2 days without an intensifying screen.

Measurements of CO_2 assimilation

Leaf CO_2 assimilation was monitored with a CIRAS-1 portable photosynthesis system (PP systems, Herts, UK). Measurements of CO_2 assimilation in response to PFD were made in descending order, at incident PFDs of 1,800, 1,500, 1,200, 900, 600, 400, 250, 150, or 75 μmol m^{-2} s^{-1} at the leaf surface. Response curves of CO_2 assimilation to intercellular CO_2 concentration (Ci) were constructed at an incident PFD of 1,600 μmol m^{-2} s^{-1} by altering air CO_2 concentrations (Ca) from 75 to 1,350 μmol mol^{-1} in nine steps, until the highest Ci reached approximately 1,050 μmol mol^{-1}. At each PFD or Ca, CO_2 assimilation and stomatal conductance were monitored to ensure that they reached a steady state before a reading was taken. CO_2 assimilation at both ambient CO_2 (360 μmol mol^{-1}) and saturating CO_2 (1,350 μmol mol^{-1}) were measured under a PFD of 1,600 μmol m^{-2} s^{-1} for all nine genotypes. Leaf temperatures were maintained at 26 ± 0.5°C during all the measurements.

Leaf sampling and analysis of non-structural carbohydrates

Leaf samples were taken at sunset (dusk) and predawn, immediately frozen in liquid nitrogen, freeze-dried, and ground to pass 1 mm screen. Soluble sugars were extracted from 50 mg samples (with xylitol added as an internal standard) with 80% ethanol three times at 80°C, and then passed through ion exchanges columns (Cheng and Fuchigami 2002). Sorbitol, sucrose, glucose, and fructose were separated and quantified by a Dionex DX-500 series chromatograph system with a pulsed amperometric detector (Dionex, Sunnyvale, Calif.) as previously described (Cheng and Fuchigami 2002). The tissue residue after soluble sugar extraction was dried, then digested with amyloglucosidase (EC 3.2.1.3) in an acetate buffer (pH 4.5) at 55°C overnight to convert starch to glucose. The concentration of glucose was quantified by using the Dionex.

Steady state pulse-chase labeling of $^{14}CO_2$

Mature leaves attached to plants were pulsed with $^{14}CO_2$ (2,500 MBq mol^{-1}) for 10 min at steady state photosynthesis, then the assimilated ^{14}C was chased with $^{12}CO_2$ for 15 min, under a PFD of 1,000 μmol m^{-2} s^{-1} and air temperature of 25°C as described in Sharkey et al. (1985). The leaves were exposed to CO_2 concentrations of 360 μmol mol^{-1} before, during, and after the labeling. CO_2 assimilation was monitored during the entire process to ensure that the labeling was done at a steady state. Less than 1% of the total radioactivity found in the leaf was detected in the petiole by the end of the 15 min chase period, indicating that ^{14}C export was minimal. At the end of the chase period, leaves were immediately frozen in liquid nitrogen and stored at −80°C.

After being freeze-dried, 100 mg leaf tissue was extracted for soluble ^{14}C-assimilates with 80% ethanol at 80°C three times. The soluble fractions were combined and dried. After being re-dissolved in 2 ml water, 0.5 ml solution was passed through a Dowex $50H^+$ column and then a Dowex $1Cl^-$ column. Soluble sugars were eluted with water and the total volume was brought to 2.5 ml. A 50 μl sample was injected into a Shimadzu

HPLC equipped with an RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The ^{14}C-soluble sugars were separated on a Bio-Rad HPX-87C (Bio-Rad, Richmond, Calif.) carbohydrate column (300×7.8 mm) as described by Wang and Stutte (1992). Fractions containing sucrose, glucose, fructose and sorbitol were collected separately and counted for radioactivity. Organic cations were eluted from the Dowex $50H^+$ column with 5 M NH_4OH whereas organic anions were eluted from the Dowex $1Cl^-$ column with 2 M HCl for radioactivity counting. The tissue residue after extracting soluble ^{14}C-assimilates was dried, then digested with amyloglucosidase (EC 3.2.1.3) in an acetate buffer (pH 4.5) at 55°C overnight to convert starch to glucose. An aliquot (0.5 ml) was passed through Dowex $50H^+$ and Dowex $1Cl^-$ columns before determining the radioactivity.

Assay of photosynthetic enzymes

Leaf discs (1 cm^2 in size) were taken between 11:00 am and noon under a PFD of 1,600 μmol m^{-2} s^{-1}, frozen in liquid N_2, and stored at −80°C until assayed.

Aldose 6-phosphate reductase was extracted according to Negm and Loescher (1981) with some modifications. Three frozen leaf discs were ground with a pre-cooled mortar and pestle in 1.5 ml extraction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 0.3%(v/v) Triton X-100, 5% insoluble polyvinylpolypyrrolidone (PVPP), and 6% (v/v) glycerol. The extract was then centrifuged at 13,000 *g* for 5 min in an Eppendorf microcentrifuge, and the supernatant was used immediately for the assay. A6PR was assayed in 1 ml reaction mixture containing 0.1 M Tris-HCl (pH 9.0), 0.11 mM NADPH, 50 mM G6P and 25 μl leaf extract.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), both stromal and cytosolic fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), and sucrose-phosphate synthase (SPS, EC 2.4.1.14) were extracted according to Chen and Cheng (2003). Two frozen leaf discs were ground with a pre-cooled mortar and pestle in 1.5 ml extraction buffer containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM DDT, 1% (v/v) Triton X-100, 5% (w/v) insoluble PVPP, 1% (w/v) bovine serum albumin, and 10% (v/v) glycerol. The extract was centrifuged at 13,000 *g* for 5 min in an Eppendorf microcentrifuge at 2°C, and the supernatant was used immediately for enzyme assays.

Total Rubisco activity was measured after incubating the leaf extract in the assay solution for 15 min at room temperature as described previously (Cheng and Fuchigami 2000).

Stromal FBPase was assayed in a mixture (1 ml) of 50 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM fructose 1,6-bisphosphate (FBP), 0.5 mM NADP, 4 units (U) phosphoglucoisomerase (PGI, EC 5.3.1.9) and 2 U glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). The reaction was initiated by adding the enzyme extract (Holaday et al. 1992; Leegood 1990).

Cytosolic FBPase was assayed according to Holaday et al. (1992) with some modifications. The enzyme was assayed in a 1 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 2 mM $MgCl_2$, 0.1 mM FBP, 0.5 mM NADP, 4 U PGI and 2 U G6PDH. The reaction was initiated by adding the enzyme extract.

SPS was assayed according to Grof et al. (1998) with some modifications. Enzyme extract (60 μl) was incubated for 15 min at 30°C with 100 mM Hepes-KOH (pH 7.5), 100 mM KCl, 6 mM EDTA, 30 mM uridine 5′-diphosphoglucose (UDPG), 10 mM F6P, and 40 mM G6P in a total volume of 100 μl. At the end of the incubation period, the reaction was stopped by adding 100 μl ice-cold 1.2 *N* $HClO_4$ and held on ice for another 15 min. The reaction mixture was neutralized by adding 60 μl 2 M $KHCO_3$, held on ice for 15 min, then centrifuged at 13,000 *g* for 1 min. An aliquot (130 μl) of the supernatant was assayed for uridine 5′-diphosphate (UDP) by coupling to oxidation of NADH with lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40). The reaction mixture (1 ml) contained 50 mM Hepes-NaOH (pH 7.0), 5 mM $MgCl_2$, 0.3 mM NADH, 0.8 mM phosphoenolpyruvate, 14 U lactate dehydrogenase, and 4 U pyruvate kinase. The reaction was initiated by adding pyruvate kinase (Stitt et al. 1988). Controls without F6P and G6P were carried out for all the samples.

ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) was extracted and assayed according to Chen and Cheng (2003) without including reduced glutathione (GSH) in the extraction buffer.

Metabolite analysis

Leaf samples for metabolite analysis were taken between 11:00 am and noon under a PFD of 1,600 μmol m^{-2} s^{-1}, frozen immediately in liquid nitrogen, and stored at −80°C.

G6P, F6P, and PGA were extracted and measured essentially as in Chen and Cheng (2003). F2,6BP was extracted and assayed according to Trevanion (2000). Frozen leaf tissue (1 g) without the midrib was pulverized in liquid nitrogen in a pre-cooled mortar and pestle and homogenized in 10 ml 50 mM KOH with 5% PVPP on ice. The homogenate was kept at 80°C for 10 min and then cooled on ice. Activated charcoal was added to10 mg ml^{-1} and the mixture was incubated on ice for another 10 min. After centrifugation at 13,200 *g* for 5 min, the supernatant was stored at 4°C for 24 h before measuring F2,6BP. More than 80% recovery was obtained with this procedure. F2,6BP was determined in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.1 mM NADH, 4.8 mM F6P, 0.6 U aldolase, 6 U triose phosphate isomerase, 0.4 U

glycerol-3-phosphate dehydrogenase, and 0.05 U pyrophosphate-dependent fructose-6-phosphate phosphotransferase, which was purified from potato tuber according to van Schaftingen et al. (1982).

Results

Steady state transcript level and activity of A6PR

RNA gel blot showed that the transcript level of A6PR in mature leaves was significantly decreased in the lines transformed with the antisense construct of A6PR (Fig. 1). Antisense line A27 had about 30% of the A6PR activity in the untransformed control; all the other antisense lines had about 15% of the control A6PR activity (Fig. 2).

CO_2 assimilation

There was no significant difference in the response of CO_2 assimilation to incident PFD or intercellular CO_2 concentration in mature leaves of antisense line A27, A04, A10 and the control (Fig. 3a, b). The rates of CO_2 assimilation of all the antisense lines, both at ambient and saturating CO_2, did not differ significantly from those of the control (data not shown).

Non-structural carbohydrates

Sorbitol concentration in mature leaves of antisense line A27 was decreased to about 60% of that in the untransformed control whereas all the other lines had about 30% of the sorbitol concentration in the untransformed control at both dusk and predawn (Fig. 4a). Both sucrose and starch concentrations were higher in the antisense lines than in the control at both dusk and predawn (Fig. 4b, c). The difference in starch concentration between dusk and predawn was larger in the antisense lines compared with the control (Fig. 4c). Concentrations of glucose and fructose were higher in the antisense lines than in the control at dusk, but were similar at predawn (Fig. 4d, e).

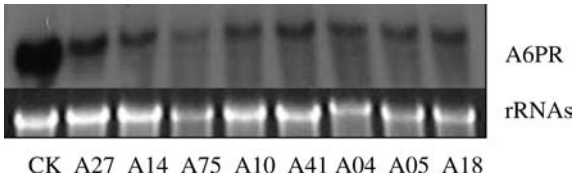

Fig. 1 Steady state transcript level of aldose 6-phosphate reductase (A6PR) in mature leaves of the untransformed control (*CK*) and the antisense lines of 'Greensleeves' determined by RNA gel blotting. Total RNA (20 μg) was used in each lane for formaldehyde-agarose gel electrophoresis

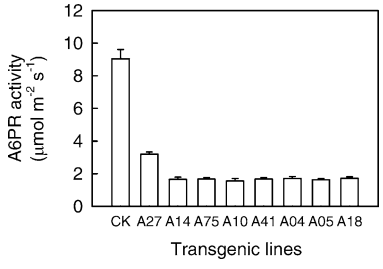

Fig. 2 A6PR activity in mature leaves of the untransformed control (*CK*) and the antisense lines of 'Greensleeves'. *Bars* Mean of five replicates with standard error

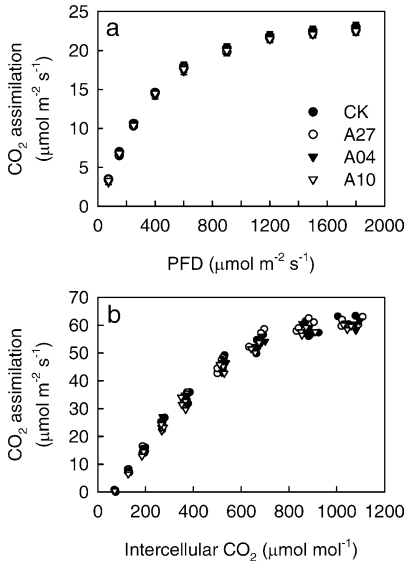

Fig. 3 CO_2 assimilation of mature 'Greensleeves' leaves in response to incident PFD at ambient CO_2 (360 μmol mol^{-1}) (**a**) or intercellular CO_2 concentration under saturating PFD (1,600 μmol m^{-2} s^{-1}) (**b**). Four mature leaves were measured for each antisense line and the control. *CK* Untransformed control; *A27* antisense line with 30% of the control A6PR activity; *A04*, *A10* antisense lines with about 15% of the control A6PR activity. Leaf temperature was 26 ± 0.5°C during the measurements

Partitioning of the newly fixed ^{14}C

Compared with the untransformed control, ^{14}C partitioning to sorbitol was decreased significantly in antisense lines A27, A04, and A10 (Fig. 5a); ^{14}C partitioning to sucrose remained unchanged (Fig. 5b) whereas that to starch was significantly increased (Fig. 5c). In antisense line A10, where ^{14}C partitioning to sorbitol was decreased to one-quarter of the control, partitioning of ^{14}C to starch was increased by about four times. Partitioning of ^{14}C to glucose and fructose was also increased in antisense lines A27, A04, and A10 (Fig. 5d, e). There was no difference in ^{14}C partitioning to the ionic fractions between the antisense lines and the control, with only 2–3% of the total ^{14}C found in the ionic fractions at the end of the 15 min chase period.

Activities of key enzymes in photosynthesis

In the antisense lines with decreased A6PR activity (Fig. 6a), SPS activity, AGPase activity and Rubisco

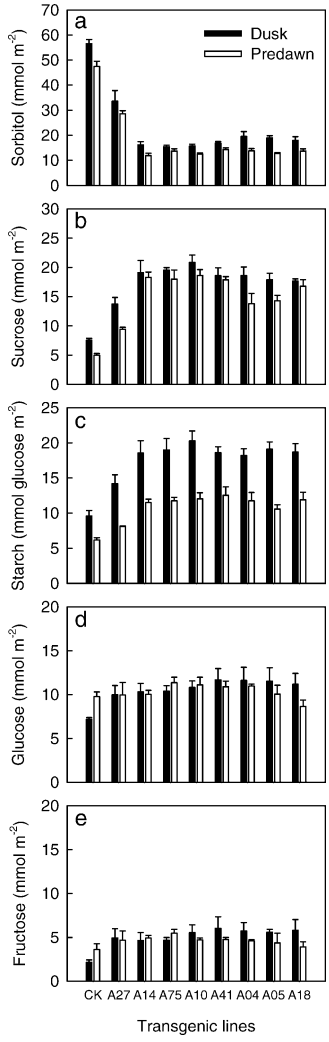

Fig. 4 Concentrations of sorbitol (**a**), sucrose (**b**), starch (**c**), glucose (**d**), and fructose (**e**) in mature 'Greensleeves' leaves at dusk and predawn. *Bars* Mean of five replicates with standard error. *CK* Untransformed control, *A27* antisense line with 30% of the control A6PR activity; all the other lines have about 15% of the control A6PR activity

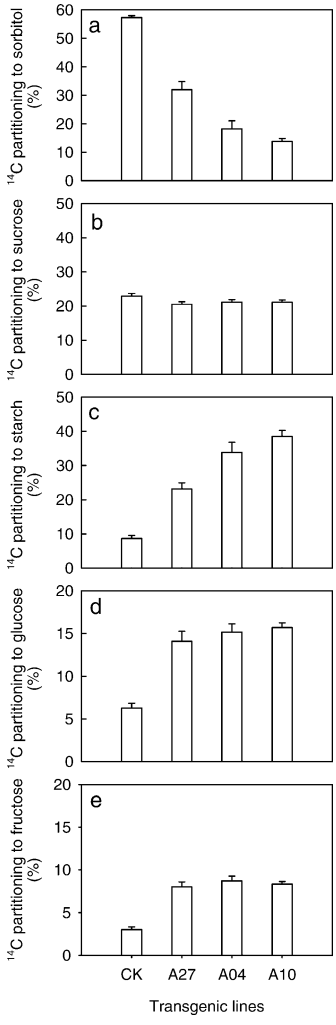

Fig. 5 Partitioning of the newly fixed carbon (% of the total incorporated ^{14}C) to sorbitol (**a**), sucrose (**b**), starch (**c**), glucose (**d**) and fructose (**e**) in mature 'Greensleeves' leaves at the end of a 15 min chase period. *Bars* Mean of five replicate leaves with standard error. *CK* Untransformed control; *A27* antisense line with 30% of the control A6PR activity; *A04*, *A10* antisense lines with about 15% of the control A6PR activity. Each leaf was pulsed with $^{14}CO_2$ (2,500 MBq mol^{-1}) for 10 min, then chased with $^{12}CO_2$ for 15 min. The total ^{14}C incorporated into 100 mg dry weight leaf tissue at the end of the 15 min chase period was 322,376 ± 7,693 dpm, and there was no difference between CK, A27, A04 and A10. The recovery of ^{14}C-soluble sugars from the ion exchange fractionation steps was approximately 94%. The partitioning (%) of ^{14}C to organic cations and anions fractions pooled together was 1.90 ± 0.14, 2.29 ± 0.22, 3.08 ± 0.28, and 2.60 ± 0.13 for CK, A27, A04, and A10, respectively. During the pulse-chase period, CO_2 assimilation was 21.25 ± 0.3, 22.77 ± 0.43, 22.33 ± 0.54, and 22.63 ± 0.37 for CK, A27, A04, and A10, respectively

activity were not significantly altered (Fig. 6c, d, f). Both cytosolic FBPase and stromal FBPase activities were increased in the antisense lines with 15% of the control A6PR activity (Fig. 6b, e).

Metabolite levels

Concentrations of both G6P and F6P were significantly higher in the antisense lines with decreased A6PR activity (Fig. 7a, b). F2,6BP concentration was significantly increased in antisense line A04 and A10 (Fig. 7c). There was no significant difference in PGA concentration between CK and A27 whereas A04 and A10 had lower concentrations of PGA (Fig. 7d).

Plant growth

No significant difference was found between the antisense lines and the untransformed control in terms of plant height, stem diameter, leaf number or average leaf area during the first year after grafting (Table 1). The visual phenotype of the transgenic plants was indistinguishable from the untransformed control.

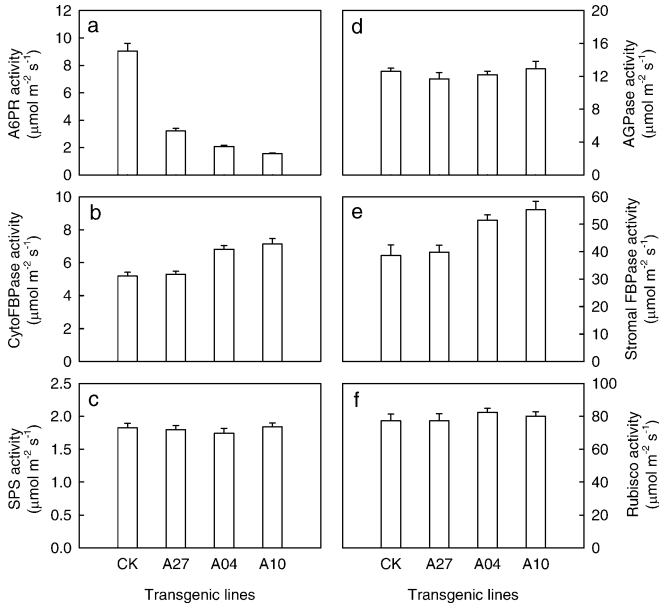

Fig. 6 Activities of **a** A6PR, **b** cytosolic fructose-1,6-bisphosphatase (FBPase), **c** sucrose-phosphate synthase (SPS), **d** ADP-glucose pyrophosphorylase (AGPase), **e** stromal FBPase, and **f** ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in mature 'Greensleeves' leaves. *Bars* Mean with standard error of five replicates. *CK* Untransformed control; *A27* antisense line with 30% of the control A6PR activity; *A04*, *A10* antisense lines with about 15% of the control A6PR activity

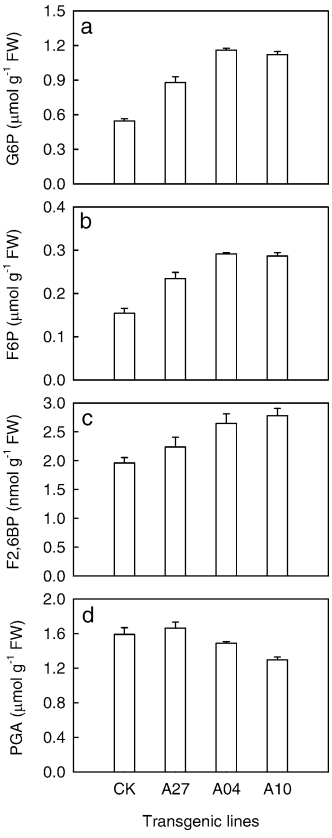

Fig. 7 Concentrations of **a** glucose 6-phosphate (G6P), **b** fructose 6-phosphate (F6P), **c** fructose 2,6-bisphosphate (F2,6BP) and **d** 3-phosphoglycerate (PGA) in mature 'Greensleeves' leaves. *Bars* Mean with standard error of five replicates. *CK* Untransformed control; *A27* antisense line with 30% of the control A6PR activity; *A04*, *A10* antisense lines with about 15% of the control A6PR activity

Discussion

Our data showed that antisense inhibition of A6PR expression significantly decreased A6PR activity and sorbitol synthesis (Figs. 2, 5a). Transgenic plants with decreased A6PR activity accumulated lower concentrations of sorbitol but higher concentrations of sucrose and starch at both dusk and predawn (Fig. 4a–c). Partitioning of the newly fixed carbon to starch was significantly increased whereas that to sucrose remained unchanged in the transgenic plants (Fig. 5b, c). The up-regulation of starch synthesis completely compensated for the decrease in triose phosphate utilization caused by reduced sorbitol synthesis so that CO_2 assimilation was sustained at both ambient and saturating CO_2 (Fig. 3). The response of starch synthesis to a decrease in sorbitol synthesis was similar to that observed when sucrose synthesis was decreased in transgenic plants or mutants with reduced activity of TPT (Häusler et al. 1998, 2000; Heineke et al. 1994; Riesmeier et al. 1993; Schneider et al. 2002), cytoFBPase (Sharkey et al. 1992; Strand et al. 2000; Zrenner et al. 1996) or SPS (Krause 1994). However, the increase in starch synthesis in sucrose synthesizing species was not sufficient to sustain CO_2 assimilation at saturating CO_2, although it fully compensated for the decrease in triose phosphate utilization caused by decreased sucrose synthesis at ambient CO_2 (Häusler et al. 1998, 2000; Riesmeier et al. 1993; Schneider et al. 2002; Sharkey et al. 1992). Mature leaves of the untransformed apple trees partitioned about 60% of the newly fixed carbon to sorbitol and less than 10% to starch, yet still maintained a high potential for starch

Table 1 Growth of the untransformed control and antisense 'Greensleeves' apple trees with decreased aldose 6-phosphate reductase (A6PR) activity during the first year after being grafted onto M. 26 rootstock. Each number is the mean ± standard error of ten plants. Leaf number and leaf area were determined after cessation of stem elongation and leaf expansion. Four mature leaves in the middle were measured for leaf area on each plant. After leaf fall, plant height was measured for scion growth only and stem diameter was measured at 5 cm above the graft union

Line number	Plant height (cm)	Stem diameter (mm)	Leaf number	Leaf area (cm^2/leaf)
CK	90.8 ± 2.1	10.33 ± 0.29	39.0 ± 0.8	37.7 ± 0.72
A27	91.8 ± 2.1	10.49 ± 0.43	39.3 ± 0.9	39.4 ± 0.89
A14	89.8 ± 2.1	10.29 ± 0.28	40.4 ± 0.7	40.3 ± 1.01
A75	94.6 ± 1.4	9.98 ± 0.25	41.6 ± 0.7	40.1 ± 0.97
A10	95.5 ± 2.1	10.16 ± 0.22	42.7 ± 0.9	38.9 ± 0.86
A41	93.8 ± 2.0	9.95 ± 0.32	40.1 ± 0.7	38.0 ± 0.93
A04	94.6 ± 2.0	9.59 ± 0.20	38.7 ± 0.9	38.8 ± 0.89
A05	93.9 ± 2.1	9.98 ± 0.26	40.9 ± 0.8	37.3 ± 0.81
A18	92.9 ± 2.7	10.04 ± 0.35	38.8 ± 1.0	39.3 ± 1.00

synthesis (Fig. 5). It appears that having an additional branch in the end-product synthesis in sorbitol-synthesizing species brings extra plasticity to the photosynthetic system.

It is surprising to find that partitioning of the newly fixed carbon to sucrose remained unchanged in the transgenic plants (Fig. 5b) considering that sucrose synthesis shares a common hexose phosphate pool with sorbitol synthesis in the cytosol and the total hexose phosphate pool increased significantly when sorbitol synthesis was decreased (Fig. 7a, b). Sucrose-phosphate synthase differs between plant species in allosteric regulation by G6P and Pi and covalent modification via reversible protein phosphorylation (Huber and Huber 1992; Huber et al. 1989). Compared with SPS from maize (Huber and Huber 1991) and spinach (Doehlert and Huber 1983), apple leaf SPS is not inhibited by Pi and only weakly activated by G6P (Zhou et al. 2002). This may explain why sucrose synthesis did not respond to a decrease in sorbitol synthesis although the G6P/Pi ratio was expected to rise in the transgenic plants as a result of accumulation of G6P at the expense of Pi in the cytosol. Alternatively, sucrose synthesis in apple leaves may have already been operating at its maximum capacity.

Although partitioning of the newly fixed carbon to sucrose remained unchanged (Fig. 5b), the transgenic plants had higher steady state concentrations of sucrose at both dusk and predawn (Fig. 4b). This is similar to the result recently reported by Kanamaru et al. (2004) on transgenic 'Orin' apple plants with decreased sorbitol synthesis. Higher concentrations of sucrose were also observed where partitioning of the newly fixed carbon to sucrose was significantly decreased in a *Flaveria linearis* mutant deficient in cytosolic FBPase activity (Sharkey et al. 1992). It remains unclear why transgenic apple leaves accumulate higher concentrations of sucrose. Even though partitioning of the newly fixed carbon to sucrose remained unchanged, the total carbon flux to sucrose is likely to be greater in the transgenic apple plants than in the untransformed control during a 24 h period. This is because starch is degraded and the resulting maltose and glucose are exported from chloroplasts to the cytosol for sucrose synthesis during the night (Niittylä et al. 2004; Schleucher et al. 1998; Weise et al. 2004), and it appears that more starch was degraded at night in the transgenic plants than in the control (Fig. 4c). The higher sucrose concentration may result from the imbalance between its synthesis and export. However, the total carbon export from source leaves did not seem to decrease in the transgenic plants as the growth of these plants was not significantly different from the control (Table 1). Accumulation of a higher background level of sucrose may facilitate phloem loading and transport of sucrose in the transgenic plants. Alternatively, a higher sucrose concentration may help to compensate for the reduction in osmotic potential caused by decreased sorbitol concentration.

The fact that the partitioning of the newly fixed carbon to the cytosol was significantly decreased in the transgenic plants with decreased A6PR activity (Fig. 5a, b) indicates that cytoFBPase activity in vivo was down-regulated in the transgenic plants. Both G6P and F6P concentrations were higher in the transgenic plants than in the untransformed control (Fig. 7a, b). In sucrose-synthesizing species, F2,6BP level increases as a result of activation of fructose 6-phosphate 2-kinase and inhibition of F2,6BP phosphatase in response to accumulation of F6P, leading to a decrease in cytoFBPase activity (Scott et al. 1995; Stitt 1990; Stitt et al. 1984a, 1984b). The concentration of F2,6BP increased in the leaves of transgenic apple plants (Fig. 7c), but it did not increase to the extent expected from the increase in F6P comparing sucrose synthesizing species (Stitt et al. 1984a, 1984b). Characterization of cytoFBPase from apple leaves showed that F2,6BP inhibits apple leaf cytoFBPase activity and F2,6BP interacts with F6P synergistically to regulate cytoFBPase activity (Zhou and Cheng 2004). We suggest that the down-regulation of cytoFBPase in the transgenic apple leaves was achieved by the interaction between F6P and F2,6BP.

The significant increase in the partitioning of the newly fixed carbon to starch (Fig. 5c) indicates that AGPase activity in vivo was up-regulated although the total AGPase activity remained unchanged in the transgenic plants. The exact mechanism of up-regulation of AGPase, however, remains unclear. Accumulation of extra hexose phosphates in the cytosol could lead to a rise in PGA/Pi in the chloroplast (Häusler et al. 1998; Heineke et al. 1994; Neuhaus et al. 1989; Zrenner et al. 1996), which activates AGPase (Preiss 1988; Sowokinos 1981). However, the total PGA concentration was lower in the transgenic plants with 15% of the control A6PR activity (Fig. 7d). Alternatively, considering AGPase activity is also regulated by post-translational redox modification in response to sucrose levels in both potato tubers (Tiessen et al. 2002) and leaves (Hendriks et al. 2003), the higher concentrations of sucrose in the transgenic leaves could activate AGPase via redox modulation without involving alteration of PGA/Pi. Determining the distribution of PGA between chloroplasts and cytosol and redox status of AGPase is needed to clarify which mechanism operates in up-regulating starch synthesis in the transgenic plants.

It is interesting to note that the total activity of cytosolic FBPase increased in the transgenic plants with 15% of the control A6PR activity (Fig. 6b) although its in vivo activity was down-regulated as indicated by the ^{14}C-partitioning data (Fig. 5a, b). An increase in total cytoFBPase activity was also observed in transgenic tobacco plants with elevated levels of F2,6BP due to expression of a modified mammalian fructose-6-phosphate, 2-kinase (Scott et al. 1995). When Rubisco activase expression was reduced by antisense inhibition in transgenic tobacco plants, total Rubisco protein increased to partially offset the decreased Rubisco carbamylation (Mate et al. 1993). Therefore, it is likely that in vivo down-regulation of cytosolic FBPase activity in the transgenic apple plants has led to increased expres-

sion of cytosolic FBPase, which may help to keep the carbon flux to cytosol at a certain level. The increase in stromal FBPase in the transgenic plants (Fig. 6e) is likely a response to the need for increased carbon flux through stromal FBPase, thereby providing more carbon to starch synthesis.

In conclusion, the photosynthetic system in sorbitol-synthesizing species has considerable plasticity. When sorbitol synthesis is decreased by antisense inhibition of A6PR expression, cytosolic FBPase activity in vivo is down-regulated to reduce the carbon flux to cytosol and starch synthesis in the chloroplast is consequently up-regulated. As a result, CO_2 assimilation is sustained at both ambient and saturating CO_2.

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