



RESEARCH PAPER

Increased sucrose level and altered nitrogen metabolism in *Arabidopsis thaliana* transgenic plants expressing antisense chloroplastic fructose-1,6-bisphosphatase*

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Abstract

The pea chloroplastic fructose-1,6-bisphosphatase (FBPase) antisense construct reduced the endogenous level of expression of the corresponding *Arabidopsis thaliana* gene. The reduction of foliar FBPase activity in the transformants T₂ and T₃ generation ranged from 20% to 42%, and correlated with lower levels of FBPase protein. FBPase antisense plants displayed different phenotypes with a clear increase in leaf fresh weight. Measurements of photosynthesis revealed a higher carbon-assimilation rate. Decreased FBPase activity boosted the foliar carbohydrate contents, with a shift in the sucrose:starch ratio, which reached a maximum of 0.99 when the activity loss was 41%. Nitrate reductase activity decreased simultaneously with an increase in glutamine synthetase activity, which could be explained in terms of ammonium assimilation regulation by sugar content. These results suggest the role of FBPase as a key enzyme in CO₂ assimilation, and also in co-ordinating carbon and nitrogen metabolism.

Key words: Antisense, *Arabidopsis*, fructose-1,6-bisphosphatase, nitrogen, sucrose, transgenic.

Introduction

Carbohydrates are produced in higher plants by the fixation of atmospheric CO₂ via the reductive pentose phosphate (Calvin) pathway. This process takes place in the chloro-

plast, and the synthesized sugar can be kept in the stromal compartment as starch, or may be exported to the cytosol for sucrose biosynthesis. During photosynthesis, the recently synthesized carbohydrate is balanced in one form or another depending on the needs of the plant and the environmental conditions. The Calvin cycle is a complex pathway of 13 reactions catalysed by up to 11 enzymes, some directly involved in the maintenance of a correct sucrose:starch ratio in the plant.

One of these enzymes is the chloroplastic fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), which catalyses the breakdown of fructose-1,6-bisphosphate to fructose-6-phosphate and Pi, one of the regeneration steps of the cycle to recover the CO₂ acceptor, ribulose-1,5-bisphosphate. This reaction is irreversible, playing a regulatory role in the pentose phosphate pathway (Bassham and Krause, 1969). However, fructose-6-phosphate can also be channelled to starch synthesis in the chloroplast. In addition, triose phosphate (TP), the precursor forms of fructose-1,6-bisphosphate, can be exported to the cytosol through a Pi/TP exchange for sucrose synthesis along a set of enzymatic reactions. The stoichiometry of these three alternatives is different and, among other factors, is regulated by modulation of FBPase activity. This increases in the light because of a change in pH and Mg²⁺ concentration in the chloroplast stroma, and because of the reduction of a disulphide bridge in the protein via a reductive interaction with thioredoxin *f* (Buchanan, 1980; Scheibe, 1990). In addition to chloroplast FBPase, a cytosolic isoform is involved in sucrose synthesis.

* The authors dedicate this publication to the memory of their colleague and co-author, Dr Julio López Gorgé, who died on 7 June 2004.

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Abbreviations: FBPase, fructose-1,6-bisphosphatase; GS, glutamine synthetase; NR, nitrate reductase; *rbcS*, small Rubisco subunit gene; *ws*, *Arabidopsis thaliana* ecotype Wassilewskija.

It displays regulatory conditions similar to those of the gluconeogenic enzyme: it is inhibited by excess substrate, and displays an allosteric inhibition by AMP and fructose-2,6-bisphosphate (Cséke *et al.*, 1984).

Both FBPase isoforms are tetramers of about 160 kDa molecular mass, comprising four identical subunits (Ke *et al.*, 1991). Eight genes encoding the chloroplastic FBPase have been isolated from different species (Chueca *et al.*, 2002). Even though cytosolic FBPase shows 50% homology with the chloroplastic type, both display similar tertiary structures with the exception of an extra sequence of 20–30 amino acids in the regulatory domain of the chloroplast enzyme (called 'loop 170'), which holds three cysteines (C153, 173, and 178 for the pea enzyme), two of these forming the disulphide bridge involved in the light-mediated reductive modulation (Chiadmy *et al.*, 1999).

Photosynthesis provides not only the primary source of energy and carbon compounds for carbohydrate formation, but also the carbon skeletons and the energy for amino-acid biosynthesis. A co-ordination of N and C metabolism is required since the N assimilation demands C skeletons in the form of 2-oxoglutarate, ATP, and reductant (Lancien *et al.*, 2000). As a consequence, certain metabolites such as sucrose and amino acids play an important role in the regulation of enzymes involved in nitrogen assimilation that is going to be of fundamental importance to the metabolism (Sheen, 1990). In this pathway, glutamine synthetase (GS) and nitrate reductase (NR) are key enzymes. GS catalyses the ATP-dependent assimilation of ammonium into glutamine, using glutamate as a substrate. NR reduces nitrate to nitrite and is considered the major metabolic control point, limiting the rate of primary N assimilation in roots and leaves. Studies on the regulation of NR activity have established that the NR gene is induced by NO_3^- , light, and sugars (such as sucrose and glucose), but inhibited by some amino acids, especially glutamine (Morcuende *et al.*, 1998; Vincentz *et al.*, 1993). This type of regulation suggests co-ordinated regulation of nitrate reductase by carbon and nitrogen metabolism (Stitt and Krapp, 1999).

The key position of chloroplastic FBPase in the Calvin cycle suggests a role for this enzyme in sugar partitioning. Many attempts have been made to direct carbon metabolism in one direction or another by modifying the expression of different enzymes of the cycle (Stitt and Schulze, 1994). In this way, antisense technology has been used to develop transgenic plants with altered levels of several enzymes of the pentose phosphate pathway, as a biotechnological tool for possible agricultural application. Early studies with transgenic potato plants expressing less than 15% FBPase activity (Kobmann *et al.*, 1994) has shown a decrease in carbohydrate content as well as a lower tuber yield in the transformed plants. Since that system has a different partitioning pattern than that for *Arabidopsis* plants, it was decided to conduct a set of experiments to analyse how

a controlled decrease of FBPase activity is affecting the sucrose content in the photosynthetic organ of herbaceous plants. In the present work, the involvement of chloroplastic FBPase in the allocation of sucrose and starch during carbohydrate biosynthesis was analysed. The use of transgenic *Arabidopsis* plants expressing one antisense construct of the pea chloroplastic FBPase, allowed the sucrose content to be increased and the biomass production of the plant to be improved. Because of metabolic crosstalk and the reciprocal regulatory mechanisms between the carbon and nitrogen metabolisms present in plants, the activities of the key enzymes GS and NR as putative targets of simultaneous regulation in that system have also been determined.

Materials and methods

Plant material

Arabidopsis thaliana ecotype Wassilewskija (ws) and transgenic seeds were germinated in compost (2/3 compost plus 1/3 vermiculite) under controlled environmental conditions: 140 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PAR; 21 °C temperature; 8/16 h light/dark photoperiod, and daily watering with an appropriate nutrient solution. For *in vitro* germination, the MS medium described by Murashige and Skoog (1962) was used, supplemented with vitamins and the appropriate antibiotic (kanamycin) for the transgenic lines. All the measurements were made in the whole leaf rosette and were collected 3 h after the beginning of the light period.

Plant transformation

The *Agrobacterium tumefaciens* strain C58C1 was used for *Arabidopsis* transformation. Bacteria were cultivated using standard procedures (Sambrook *et al.*, 1989). A cDNA construct encoding pea chloroplastic FBPase was ligated in an antisense orientation between the two copies of the 35S CaMV promoter and the nopaline synthase polyadenylation signal into the binary vector pBinJit (Fig. 1), a modification of the original, pBin (pBI120) (Jefferson *et al.*, 1987). *A. thaliana* was transformed by infiltration with *Agrobacterium*, incorporating the antisense construction pBinJitFBPOF (Bechtold *et al.*, 1993). Seeds from infiltrated plants were sterilized for 1 min with 70% ethanol and 5 min with 5% sodium hypochlorite, 0.25% Tween 20, and then rinsed three times with sterile water and germinated on the MS medium with the addition of 50 $\mu\text{g ml}^{-1}$ kanamycin. Seedlings were grown as described above, and the kanamycin-resistant plantlets were transplanted individually into sterile compost. The primary transformants were selfed and the homozygous seeds from the second or third generations (T_2 , T_3) were used for later experiments after cultivation under similar conditions. After 4 weeks of growth the whole leaf rosette (8–9 leaves) was harvested 3 h after the beginning of the light period. Samples were immediately frozen in liquid nitrogen and stored individually at -80 °C until assayed.

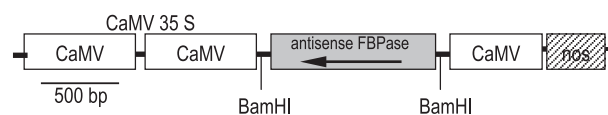


Fig. 1. Diagram of the fructose-1,6-bisphosphatase antisense gene construct. A cDNA fragment of 1.1 kb containing the coding sequence for chloroplast FBPase (dark area) was subcloned in reverse orientation between the 35S promoter of CaMV and the nopaline synthase polyadenylation signal termination sequences, in the vector pBinJit.

Protein extraction and western blotting

The frozen rosette corresponding to transformed and untransformed seeds were homogenized with 25 mM TRIS-HCl pH 7.5, and 5 mM MgCl₂ (1:3 w/v). After 15 min centrifugation at 12 000 g, the supernatant was removed and the protein concentration determined (Bradford, 1976). After electrophoresis in polyacrylamide gel according to Laemmli (1970), proteins were transferred electrophoretically to a nitrocellulose membrane, and western blotting was performed as in Towbin *et al.* (1979), using polyclonal antibodies against pea-leaf chloroplastic FBPase. The FBPase protein content were quantified using Quantity One software (Bio-Rad).

Determination of enzyme activities

FBPase activity was determined at 28 °C by measuring the increase in absorbance at 340 nm, in 100 mM TRIS-HCl pH 8.8, containing 0.6 mM fructose-1,6-bisphosphate, 0.5 mM Na-EDTA, 10 mM MgCl₂, 0.3 mM NADP⁺, 0.6 units of glucose-6-P-dehydrogenase, 1.24 units of phosphoglucose isomerase, and leaf extract (Lázaro *et al.*, 1974). One unit is the enzyme activity which produces 1 μmol of NADPH min⁻¹ under the experimental conditions. Results were quoted with reference to fresh weight.

For the determination of glutamine synthetase and nitrate reductase activities, plant rosettes were homogenized in 25 mM K-phosphate buffer pH 8.8, 1 mM EDTA, 20 mM cysteine, and 1 mM mercaptoethanol. After centrifugation at 12 000 g for 30 min, GS activity was determined in the supernatant by measuring the increase in absorbance at 550 nm after incubation at 37 °C in 90.6 mM imidazol pH 7, 20 mM Na arsenate, 3 mM MnCl₂, 60 mM hydroxylamine, 0.4 mM ADP, and 120 mM glutamine, as described by Cánovas *et al.* (1984). NR was determined by incubation for 30 min at room temperature in 25 mM K-phosphate buffer pH 7.5, 10 mM KNO₃ and 0.4 mM NADH; 0.01% naphthol and 0.5% sulphanylamide were added and the activity was measured at 550 nm after 30 min (de la Haba *et al.*, 2001).

Carbohydrate analysis

Carbohydrates were extracted twice from frozen leaf rosettes with 80% ethanol at 80 °C for 30 min, followed by further washing with 50% ethanol at 80 °C for 15 min. After centrifugation, sucrose, glucose, and fructose were measured enzymatically in the supernatant to determine the reduction of NADP at 340 nm after successive additions of the coupling enzymes glucose-6-P-dehydrogenase, hexokinase, phosphoglucose-isomerase, and invertase (Sekin, 1978). Starch was measured as glucose from the extracted pellet, following incubation at 60 °C for 3 h with α-amylase and amyloglucosidase.

Other determinations

Chlorophyll was determined according to Arnon (1949). Measurements of CO₂ assimilation were made on individual leaves of the rosette in an open gas-exchange system (ADC3, Herts, UK) under 500 μmol photons m⁻² s⁻¹ PAR. All photosynthesis measurements were made on at least five leaves of the same transgenic line.

Reverse transcription polymerase chain reaction analysis (RT-PCR)

Total mRNA was obtained from extracts of 100 mg wild-type and transgenic *A. thaliana* rosettes by binding on a biotin-labelled oligo-dT probe fixed on streptavidin magnetic particles, which were then later isolated with a magnetic device. After mRNA elution with a low-ionic-strength solution, one-tenth of the eluted mRNA was subjected to reverse transcription and DNA amplification in the presence of specific primers (Ara2, Ara3) spanning the 500 bp region towards the 3' end of the *Arabidopsis* FBPase gene. The constitu-

tively expressed UBQ10 mRNA (Sun and Callis, 1997) was used as an internal control for uniform loading, and amplified as a 220 bp UBQ10 fragment. The specific primers used were as follows: Ara3/Ara2: 5'-GTACTCGAGCTCGGTCATCTTCGTTCTTA-3' and 5'-AGCCAAGTACTTCTCCAACCTTCTACTTC-3' for FBPase; UBQ10 5'/3': 5'-GGACCAGCAGCGTCTCATCTTCGCT-3' and 5'-CTTATTCATCAGGG ATTATACAAGGCC-3' for ubiquitin.

After previous assays at 10, 20, 30, and 40 cycles, the following experimental conditions were chosen for the RT-PCR amplification of the template: one cycle of 48 °C for 45 min, and 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 58 °C for 2 min, and 68 °C for 3 min; and finally one cycle of 68 °C for 10 min. Amplified DNA was electrophoresed in 2% agarose and visualized by staining with ethidium bromide. Identical results were found when the deoxynucleotide mixture of the kit was supplemented with 5% (w/v) of digoxigenin-labelled dUTP for DNA synthesis. After transfer of the PCR products to a positively charged nylon membrane, these were developed with alkaline phosphatase-labelled antidigoxigenin. The signals were quantified using Quantity One software (Bio-Rad) and normalized using ubiquitin expression. The linearity between mRNA concentration and the densitometric optical reading of the ethidium bromide or alkaline phosphatase reaction was verified by using increasing amounts of isolated total mRNAs (Pagano *et al.*, 2000).

Results

Antisense constructs and molecular characterization of transformants

A full-length pea chloroplastic FBPase cDNA was ligated into the *Bam*HI site of the binary vector pBinJit in an antisense orientation (Fig. 1). The pea nucleotide sequence shows 75% identity with that of *Arabidopsis thaliana* FBPase, which represents 86% identity when referred to the amino-acid sequence. This construction was transferred into *A. thaliana* ecotype Wassilevskija plants by vacuum infiltration via *Agrobacterium tumefaciens*. After a primary selection on MS medium containing 50 μg ml⁻¹ kanamycin, the presence of the pea FBPase cDNA was verified in the transformants by PCR (data not shown). Thirteen individual lines containing the insert of 1.1 kb were selected for further characterization. The endogenous *A. thaliana* chloroplastic FBPase gene displayed a 1.3 kb band in the PCR reaction (data not shown). T₂ seeds from each transformant were sown separately to produce T₃ generation seeds, and the homozygous transformants from T₂ or T₃ generation were collected and used for further studies.

RT-PCR analysis

The amount of endogenous chloroplastic *A. thaliana* FBPase transcript was determined in the transgenic lines using RT-PCR. Ubiquitin (UBQ) was amplified as an internal control. Lines with less than 80% of FBPase activity relative to untransformed plants, displayed a decrease in the amount of their transcripts (Fig. 2), as much as 55% for the a4c line, that was in perfect agreement with the decrease of FBPase activity detected, which showed 58% enzyme activity with respect to control plants.

Chemical and biochemical measurements

Among the transgenic lines displaying the cDNA insert, only the homozygous lines, a3a, a5e, a3b, and a4c were selected for further characterization. A decrease of specific chloroplastic FBPase activity was determined, 80, 71, 59, and 58% related to the wild type (Table 1). The loss of activity was in perfect correlation with the FBPase protein level. Western-blot analysis was performed using a pea anti-FBPase serum. Lines a3a, a5e, a3b, and a4c contained 70, 68, 67, and 61% of FBPase protein relative to the untransformed plants (Table 1). There was a clear inhibition of FBPase expression, even if there was not an exact correlation between enzyme level and activity. In the experimental conditions used, the cytosolic FBPase is inactive at 0.6 mM of FBP and therefore the activity determination is specific for the chloroplastic enzyme (Zimmermann *et al.*, 1978). In addition, both FBPases did not show cross reaction in the western-blot when specific antibodies were used (Fonolla *et al.*, 1994).

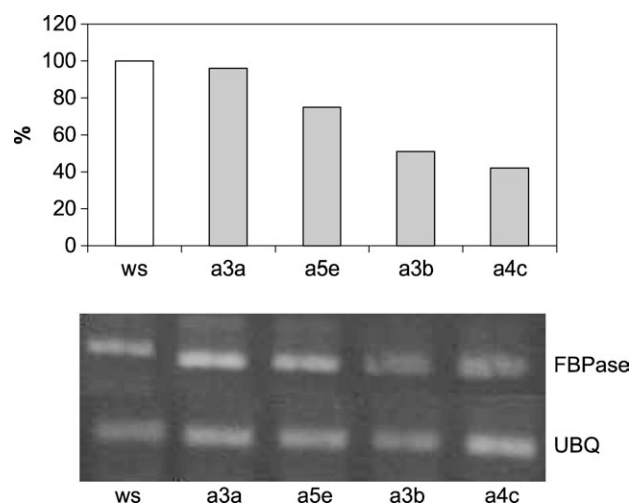


Fig. 2. FBPase expression in wild type (ws) and *A. thaliana* transformants. Expression was determined by RT-PCR (bottom panel) and the signals were quantified and normalized using UBQ10 expression as a control (top panel, arbitrary units). PCR products were visualized in the agarose gel by ethidium bromide staining. Results are the means of five individual plants.

Table 1. FBPase activity and FBPase protein level in transformed and control *A. thaliana* plants

Samples were harvested 3 h after the beginning of a light period. Results are means of five different plants.

Line	FBPase activity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	% WT activity	FBPase (% protein)
WT	13.4 \pm 0.9	100	100
a3a	10.7 \pm 2.0	80	70
a5e	9.5 \pm 0.7	71	68
a3b	7.9 \pm 0.8	59	67
a4c	7.8 \pm 0.8	58	61

Total leaf protein content decreased by 15–31% in the anti-FBPase lines (Table 2). Rosette fresh weight displayed an increase for lines a3a, a5e, a3b, and a4c (120, 163, 125, and 140%, respectively, of the wild type) (Table 2). Leaf area of the transformants increased more than 1.5-fold over control values (Table 2). All these parameter changes are consistent with the phenotypic changes observed in the lines under study. In fact, there was a larger size of leaves and rosettes, and a higher number of leaflets relative to the wild type (Fig. 3). Net photosynthesis was measured on single leaves using an open gas-exchange system (ADC3, Herts-England) under 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR. Photosynthesis of the different transgenic lines rose 2–3-fold compared with the control plants (Table 2). A loss of FBPase activity does not appear to interfere with the net CO_2 assimilation. Equally, the chlorophyll (Chl) content in the transgenic lines was not affected, ranging between 1.16 and 1.88 mg g^{-1} FW (Table 2). However, lines a3b and a4c seem to have a lower chlorophyll content which did not visibly affect leaf colour.

Carbohydrate content

Sucrose, glucose, fructose, and starch were measured in the rosette of the wild type and transformed lines plants grown for 4 weeks under 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR (Table 3) in order to establish if the repression of FBPase could be responsible for the increase in sucrose synthesis at the expense of starch. As expected, the sucrose content increased in the lines, ranging between 113% (line a4c) and 226% (line a3a) relative to the wild-type value (Fig. 4). The levels of glucose and fructose were similar among the transgenic and the control plants, whereas a surprising starch accumulation was recorded in lines a3a and a5e, being exceptionally high in the former. It is emphasized that uncontrolled sucrose hydrolysis cannot take place in the experimental conditions used in this work, and the amount of monosaccharides that were determined is the result of the *in vitro* enzymatic hydrolysis. As an indicator of photo-assimilate allocation, the foliar sucrose/starch ratio was also determined. Control plants showed a 0.62 ratio, a similar value being determined for lines a5e and a4c, however, a higher quotient was exhibited by lines a3a and a3b (0.79 and 0.99, respectively) (Fig. 4). These data show that the suppression of FBPase expression could raise the sucrose content in the transgenic lines from 13% to 125% of the wild-type value.

Glutamine synthetase and nitrate reductase activity

Since the pathways of carbon and nitrogen metabolism are highly co-ordinated in higher plants, it is likely that crosstalk signals derived from carbon and nitrogen metabolism could affect both. It is well known in plants that supplementation of growth media with sucrose and organic nitrogen can cause changes in the expression of some nitrogen-assimilatory genes, such as those encoding the enzymes nitrate reductase

Table 2. Total protein, chlorophyll, net photosynthesis, leaf fresh weight (LFW), and leaf area (LA) for wild type and five different transgenic lines

 Each value represents the mean (\pm SE) of at least five different plants.

Line	Total protein (mg g ⁻¹ FW)	Chl (mg g ⁻¹ FW)	Photosynthesis (μ mol CO ₂ m ⁻² s ⁻¹)	LFW (mg)	LA (cm ²)
WT	72 \pm 11	1.52 \pm 0.11	0.92 \pm 0.09	0.081 \pm 0.015	1.21 \pm 0.08
a3a	61 \pm 3	1.88 \pm 0.06	2.17 \pm 0.81	0.097 \pm 0.024	1.74 \pm 0.38
a5e	50 \pm 11	1.63 \pm 0.11	2.26 \pm 1.06	0.132 \pm 0.030	1.76 \pm 0.39
a3b	55 \pm 10	1.16 \pm 0.13	2.60 \pm 0.58	0.101 \pm 0.017	1.73 \pm 0.14
a4c	52 \pm 4	1.38 \pm 0.15	1.80 \pm 0.59	0.113 \pm 0.020	1.63 \pm 0.13

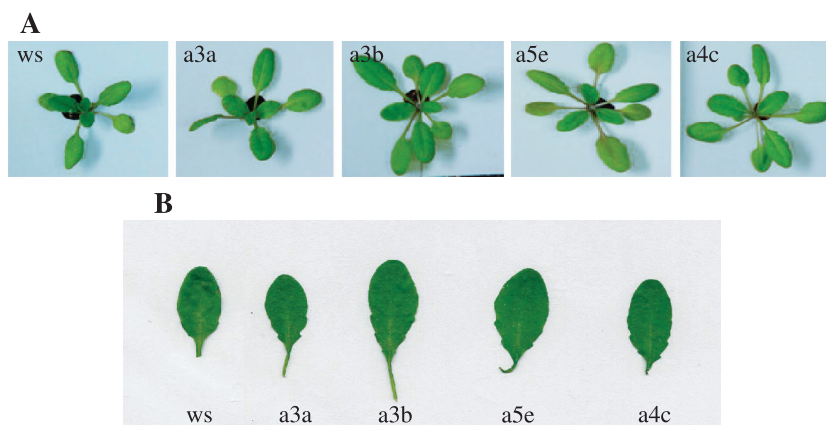

Fig. 3. Study of FBPase antisense expression over the phenotype of transgenic *A. thaliana* plants. (A) Comparison of rosette ws, a3a, a3b, a5e, and a4c. (B) Comparison of individual leaves of the corresponding rosettes.

Table 3. Carbohydrate content of the whole rosette of untransformed control and anti-FBPase plants grown in air

Results are the means of five different plants.

Line	Total sugars (mg g ⁻¹ FW)	Sucrose (mg g ⁻¹ FW)	Glucose (mg g ⁻¹ FW)	Fructose (mg g ⁻¹ FW)	Starch (mg g ⁻¹ FW)
WT	981 \pm 33	124 \pm 65	350 \pm 41	306 \pm 16	201 \pm 11
a3a	1317 \pm 25	280 \pm 40	354 \pm 37	329 \pm 13	354 \pm 10
a5e	1108 \pm 23	176 \pm 13	337 \pm 32	325 \pm 27	270 \pm 18
a3b	863 \pm 39	166 \pm 55	270 \pm 59	259 \pm 28	168 \pm 13
a4c	918 \pm 21	140 \pm 36	292 \pm 13	275 \pm 21	211 \pm 12

and glutamine synthetase. Determination of glutamine synthetase activity showed a significant increase, ranging from 111% to 170% in the different transgenic lines with respect to the control (Table 4). By contrast, nitrate reductase displayed a loss of activity between 11% and 56% (Table 4). These data suggest that the expression of both proteins are inversely affected by the sucrose level.

Discussion

Carbohydrates may be one of the products which can be modified to increase plant biomass, crop productivity or food quality either in the whole plant or in some specific organs (tuber, fruit, seed, etc). In addition to classical breeding methods, plant genetic engineering has, in recent

years, been seeking new technologies to reach this objective. It has been shown that about 60% of Rubisco can be removed in transgenic tobacco plants with an antisense *rbcS* gene before a significant inhibition of photosynthesis occurs, since the Rubisco protein is produced in excess within the plant (Quick *et al.*, 1991a, b). In the Benson–Calvin cycle, several key enzymes have been genetically modified in transgenic plants resulting in different responses related to carbohydrate content. Thus, a reduction of sedoheptulose-1,7-bisphosphatase (SBPase) lowers photosynthetic capacity as well as the carbohydrate content in transgenic tobacco leaves (Harrison *et al.*, 1998). The overexpression of a maize cDNA coding for the sucrose phosphate synthase (SPS) in *Arabidopsis thaliana* (Columbia) plants results in increased foliar

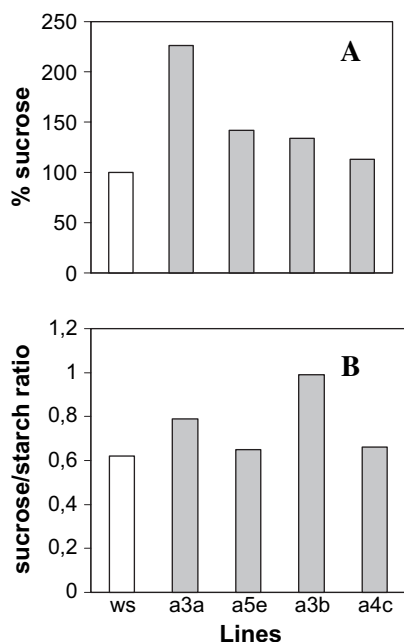


Fig. 4. Carbohydrate content in whole *Arabidopsis thaliana* rosette. Levels of sucrose (A) and ratio sucrose/starch (B) were determined 3 h after the beginning of the light period. Results are means of five individual plants.

Table 4. Activities of GS and NR in transformed and control *A. thaliana* plants

Samples were harvested 3 h after the beginning of a light period. Results are the means of five different plants.

Line	GS activity ($\mu\text{mol min}^{-1}$ g^{-1} FW)	% WT activity	NR activity ($\mu\text{mol min}^{-1}$ g^{-1} FW) $\times 10^5$	%WT activity
WT	14.6 \pm 0.42	100	379 \pm 10.2	100
a3a	17.9 \pm 1.98	123	337 \pm 4.8	89
a5e	16.3 \pm 0.24	111	168 \pm 4.8	44
a3b	24.9 \pm 0.48	170	301 \pm 11.4	79
a4c	17.1 \pm 0.84	117	337 \pm 10.2	89

sucrose:starch ratios, however, growing this plant for long periods in a CO_2 -enriched atmosphere favoured a lower foliar carbohydrate content (Signora *et al.*, 1998). Similarly, when a cyanobacterial fructose-1,6/sedoheptulose-1,7-bisphosphatase is overexpressed in tobacco plants, net photosynthesis is enhanced, resulting in an increase in soluble sugars and starch content (Miyagawa *et al.*, 2001). In any case, the Benson–Calvin pathway is modulated by the interaction between different regulatory enzymes. Gardemann *et al.* (1983) hypothesized that phosphoribulokinase-reduced activity is counterbalanced by a drop of FBPase activity, probably by an excess of fructose-6-P. In this way, the plant maintains a sufficient level of triose phosphate necessary for proper sucrose synthesis, provided by a suitable ATP:ADP ratio.

Because of the key position in this cycle, chloroplast FBPase can be a good candidate to modify the sucrose versus the starch biosynthetic pathways. In the present work, the role of chloroplastic FBPase in carbohydrate metabolism has been studied in transgenic *Arabidopsis thaliana* plants that express the enzyme in antisense. All the transgenic lines with a reduced level of FBPase activity/protein showed a higher photosynthetic rate when measured in an open gas-exchange system. This increase in photosynthetic activity is reflected in higher vegetative growth of the rosette, as shown in Fig. 3, which was confirmed with data for leaf size and fresh weight for the whole aerial part of the *Arabidopsis* plants (Table 2). In addition, transgenic plants show a normal light-green colour, with minimal changes in the chlorophyll content in lines a3b and a4c. However, FBPase activity and content appeared to be reduced at different levels in the different transgenic lines (Tables 1, 2). As discussed below, this is presumably due to the inhibition of FBPase synthesis, but also to a lower ratio of protein:fresh weight. The 75% identity between the nucleotide sequences of pea and *A. thaliana* chloroplastic FBPases probed to be sufficient to reduce the endogenous mRNA expression when an antisense pea cDNA was expressed into the plants. However, it is necessary to reduce the FBPase activity by more than 20% to achieve an appreciable inhibition at the transcriptional level. In any case, when FBPase expression is normalized with the ubiquitin transcript content, the values of the different transgenic lines appear clearly down-regulated. This inhibition was more evident in the case of lines a3b and a4c (Fig. 2).

In most FBPase-transformed plants the content of total leaf carbohydrate was higher than in wild-type *Arabidopsis* plants, indicating that lower FBPase activity boosted sucrose synthesis (Table 3). Nevertheless, it should be noted that higher levels of soluble sugars did not lead to a lower starch accumulation in all cases. In any case, the sucrose:starch ratio was invariably positively balanced towards sucrose content, line a3b displaying an increase of 60% in relation to the wild-type control (Fig. 4). With the exception of lines a3b and a4c, those with lower FBPase activity showed no significant changes with relation to the glucose and fructose levels. These results strengthen the possibility of modifying the carbon partitioning towards soluble sugars by changing the activity of one of the key enzymes of carbohydrate biosynthesis. Earlier results showed that sugar accumulation in the source leaves inhibits photosynthesis and lowers the levels of Calvin cycle enzymes (Stütt *et al.*, 1990). However, in this case, the higher sugar content bolstered photosynthetic activity.

Figure 3 shows that lines a3a, a5e, and a3b produced a highly developed rosette resulting in a greater fresh weight and leaf area compared with the wild-type plants. These phenotypic changes became discernible after one month of growth, indicating a clear correlation between the

sucrose level and biomass accumulation. It can therefore be concluded that a controlled reduction of FBPAse activity increased sucrose synthesis, inducing greater growth and higher photosynthetic activity in transgenic *Arabidopsis* plants. By contrast, lower levels of FBPAse activity in transgenic potato plants reduced photosynthetic activity, and plant growth, as well as tuber yield (Kobmann *et al.*, 1994). However, after the potato seedlings were transferred from tissue culture to soil, the transformed plants reached the same size as control ones after two months of growth. Thus, it could be considered that cultivation conditions and the size of the pots are limiting factors for plant growth, and could be responsible for the differences found between the transgenic lines and controls (Kobmann *et al.*, 1994). In this case, seeds were directly germinated in pots containing compost, phenotypic differences being evident between transgenic *Arabidopsis* and controls after one month of growth (Fig. 3). Moreover, the discrepancies between the results of this work and those reported for transformed potato plants may be linked to the differences in carbon storage among organs between the two species: the tuber is a sink organ for the potato while rosette leaves constitute a source for *Arabidopsis*.

In addition to the complexity of the Calvin cycle, the data obtained from experimental work indicate that the regulation of CO₂ assimilation is fine-tuned, and that a co-ordinated manipulation of carbon and nitrogen metabolism is necessary for modifying the sugar content in the proposed sense. In principle, there is some type of competition between C and N assimilation concerned with the use of the ATP and reduced pyridine nucleotides formed in photosynthesis. In addition to supplying carbon skeletons for amino acid synthesis, C assimilation is essential for building up organic acids which act as counter ions for nitrate uptake. On the other hand, N assimilation must be fine-tuned to prevent the accumulation of toxic ammonium derived either from primary assimilation or from the catabolic processes that take place in plants. To maintain a correct carbon-to-nitrogen ratio in transgenic plants, increased sucrose may induce a parallel stimulation of GS activity. Some authors have pointed out that, in addition to light-induced changes in sucrose levels, an induction of GS mRNA expression is partially light-mediated via phytochrome (Oliveira and Coruzzi, 1999), but in part is also mediated by light-induced changes in sucrose levels. Nitrate reductase, the first enzyme in nitrate assimilation, is located at the crossroads of two energy-consuming pathways: nitrate assimilation and carbon fixation. It has been shown that sucrose can replace light in eliciting an increase of nitrate reductase mRNA accumulation in dark-adapted green *Arabidopsis* plants. It has also been shown that sucrose alone is sufficient for the full expression of nitrate reductase genes in etiolated *Arabidopsis* plants (Cheng *et al.*, 1992). In accordance with these experimental data (Table 4), there is an inverse relationship between

the foliar-glutamine concentration and foliar-NR activity. This is in accordance with the results reported by Deng *et al.* (1991) and Vincentz *et al.* (1993), who found a repression of NR transcription by glutamine or other closely related metabolites. However, recent results suggest that the negative effect of glutamine on NR transcription was offset by high α -ketoglutarate, the relative amounts of α -ketoglutarate and glutamine being more important in controlling NR gene transcription than was the concentration of either metabolite alone (Ferrario-Méry *et al.*, 2001). If the foliar-glutamine concentration is inversely related to NR activity in leaves, co-ordinated regulatory mechanisms of GS and NR allow the regulation of the nitrogen-assimilation pathway in plants. An induction of NR activity by nitrate (Lin *et al.*, 1994) and sugars (Vincentz *et al.*, 1993) has also been reported. In addition, faster post-transcriptional modulation of NR activity appears superimposed on the enzyme gene expression. A dephosphorylation-dependent light activation, and a concomitant phosphorylation-induced dark inactivation of NR has been found by Huber *et al.* (1996). These changes in NR activity must be related to a modification of sugar availability rather than to a light-dependent change in photosynthetic activity, since a similar activity can be obtained 'in vitro' after sugar addition in the dark (Kaiser and Brendle-Behnisch 1991), whereas the enzyme becomes inactive in light when CO₂ fixation is prevented (Kaiser and Spill, 1991).

In conclusion, the increased allocation of soluble sugar towards the cytosol opens the possibility of selecting plants with reduced FBPAse activity, in which net photosynthesis is scarcely affected, but with an increased net content in soluble sugars. The improvement of plant-yield potential by genetic engineering can complement classical breeding programmes, the modification of FBPAse expression being a tool for controlling the carbon economy of the leaf, including assimilation, production, and availability.

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