REVIEW PAPER

Changing sugar partitioning in FBPase-manipulated plants

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Abstract

This review offers an overview of the current state of our knowledge concerning the role of fructose-1,6-bisphosphatase (FBPase) in sugar partitioning and biosynthesis, through the analysis of genetically manipulated plants. The existence of two well-characterized isoforms is a consequence of the subcellular compartmentalization of photosynthetic eukaryotes, conditioning their respective regulatory mechanisms and their influence over plant metabolism and photosynthesis. Both isoforms are important, as has been deduced from previous work with different plant species, although there is still much to be done in order to gain a definitive vision of this issue. Despite that, alteration of the FBPase content follows a general pattern, there are some differences that could be considered species-specific. Modifications lead to profound changes in the carbohydrate content and carbon allocation, raising questions as to whether flux of some sugars or sugar precursors from one side to the other of the chloroplast envelope occurs to rebalance carbohydrate metabolism or whether other compensatory, though not fully efficient, enzymatic activities come into play. Due to the pleiotropic nature of modifying the core carbon metabolism, an answer to the above questions would require an exhaustive study involving diverse aspects of plant physiology.

Key words: Calvin cycle, carbon partitioning, fructose-1,6-bisphosphatase, genetic engineering, starch, sucrose, thioredoxin.

Introduction

Photosynthesis enables plants to transform sunlight energy into useful biochemical power (NADPH and ATP) which is subsequently used for carbohydrate biosynthesis using atmospheric CO₂ and H₂O, the building blocks of carbon metabolism. The efficiency of this process requires finely coordinated control at multiple levels. In C₃ plants, CO₂ is fixed in the chloroplast during the reductive pentose phosphate pathway or the Calvin cycle (CC), which consists of 13 reaction steps catalysed by 11 different enzymes, leading to the production of triose phosphates (Fig. 1). A fraction of these triose phosphates are used to produce ribulose-1,5-bisphosphate (RuBP) for CC regeneration and the remainder can either be exported to the cytosol for sucrose synthesis or stored as transitory starch inside the chloroplast (Woodrow and Berry, 1988; Geiger and Servaites, 1994). It is crucial to maintain a balance between export and regeneration so that the cycle does not become depleted of intermediates. To establish this balance, the catalytic activities of certain CC enzymes such as fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) are highly regulated (Fridlyand et al., 1999; Raines et al., 1999), and some of them are light dependent.

Chloroplast FBPase (cpFBPase) catalyses the conversion of fructose-1,6-bisphosphate (FBP) to fructose-6-phosphate (F6P) and is a key carbon-metabolism enzyme, the activity of which is modulated by the reduction of disulphide bonds via thioredoxin (TRX) as well as changes in the pH and Mg²⁺ concentration that result from illumination (Anderson et al., 1979; Buchanan et al., 1980). In addition to the above-mentioned chloroplast FBPase, there is another cytosolic isoform (cyFBPase) which is also key in the sucrose-biosynthesis pathway, catalysing the first irreversible reaction in the conversion of triose phosphates to sucrose (Daie, 1993). Aside from specific subcellular
locations, enzymatic activities of cpFBPase and cyFBPase are differentially inhibited by AMP and fructose-2,6-bisphosphate (F2,6BP), a sugar phosphate similar to FBP producing competitive (cpFBPase) or allosteric inhibition (cyFBPase) (Cadet et al., 1987).

Biochemical features of plant FBPases have been intensively studied and redox activation of cpFBPase through thioredoxin interaction has also been widely reported over the last decade (Jacquot et al., 1995, 1997; Hermoso et al., 1996; Chen and Xu, 1996; Jaramillo et al., 1997; Chiadmi et al., 1999; Wangensteen et al., 2001; Cazalis et al., 2004). Because of the large amount of interest in cpFBPase redox regulation, the scientific literature regarding the biochemistry of both plant isoforms clearly leans towards the chloroplast enzyme.

Regardless of the number and quality of the biochemical data accumulated, we are just starting to know how important FBPases are for plant carbon metabolism. The development of plant genetic-transformation techniques is promoting knowledge concerning the real influence of FBPases on the synthesis and accumulation of plant carbohydrates and photosynthesis. This review focuses on some aspects of this activity regulation and, primarily, on the characterization of mutant plants with modified levels of chloroplast or cytosolic FBPases. The analysis of these mutants offers a qualitative change to the study of the role of FBPases in plant-carbon metabolism and it suggests putative biotechnological applications to improve crop production and quality.

Structural features and differential regulation of plant FBPase isoforms

Evolution has selected redox control as a way of regulating enzymatic activity in chloroplasts whereas the protein-kinase/protein phosphatase system is the main one used for controlling the activity of cytosolic enzymes. Redox modulation in chloroplasts is closely linked to photosynthesis through the ferredoxin-thioredoxin system (Lemaire et al., 2007). This system transfers the redox power from the ferredoxin (Fd), which takes electrons coming from PSI to
a chloroplast thioredoxin (TRX f or m) through ferredoxin thioredoxin reductase (FTR). Together with cpFBPase, many other CC enzymes have been established as putative or proven thioredoxin targets, such as sedoheptulose-1,7-bisphosphatase (SBPase), NADP-glyceraldehyde-3-phosphate dehydrogenase (NADP-G3P dehydrogenase), ribulose-5-phosphate kinase (Ru5P kinase), and RUBISCO activase (Lemaire et al., 2007). Although TRX-mediated control is common in CC enzymes, the regulation mechanisms vary among them and always involve conformational changes in target proteins upon disulphide reduction.

Chloroplast FBPase probably originated through duplication of an ancient cytosolic isoform (Martin et al., 1996) and the insertion of 20–30 amino acids (called ‘loop 170’) rich in negative charges (Villaret et al., 1995). This loop contains three Cys able to form a regulatory disulphide (Chiadmi et al., 1999; Chueca et al., 2002) and confers exclusive redox features to the chloroplast isoform. The oxidation of the loop by a disulphide bridge formation between Cys153 and Cys173 has an allosteric effect by displacing Glu105, disrupting the catalytic binding site of the Mg²⁺ and, consequently, inactivating the enzyme (Chiadmi et al., 1999; Dai et al., 2000). The reduction of the disulphide bond by a reduced TRX f restores the catalytic activity of the cpFBPase upon illumination. However, other photosynthetic organisms, such as the red alga *Galdieria sulphuraria*, bear a similar insertion (in position and size) with two conserved Cys but conferring poor redox regulation (Reichert et al., 2000, 2003). This FBPase could be a transitional form to the fully redox-regulated enzymes of chloroplasts.

Recently, it has been reported that 2-Cys peroxiredoxin (2-Cys PRX) can also modulate cpFBPase by a non-reductive way in the presence of both Ca²⁺ and FBP (Caporaletti et al., 2007). The 2-Cys PRX-mediated FBPase activation is dependent on the presence of the three conserved cysteines (Cys153, 174, and 179) of the redox regulatory loop. Nonetheless, FBPase modulation by 2-Cys PRX is not as high as by TRX f. It bears mentioning that Caporaletti et al. (2007) discuss two possible activation mechanisms under normal (TRX-mediated) and oxidative stress (2-Cys peroxiredoxin-mediated) situations; while further experiments are necessary to determine the physiological meaning of this non-reductive activation.

In addition to the already-known plant FBPases, a new chloroplast isoform is being characterized from *Fragaria x ananassa* (strawberry) which lacks ‘loop 170’ and is not activated by TRX f (AJ Serrato, unpublished data). This enzyme seems to be exclusive of land plants and would add more complexity to the plant carbon-metabolism network.

The redox dependence of cpFBPase (and other CC enzymes) is imperative to the co-ordination of C partitioning in plants in order to avoid a futile cycle. In photosynthetic organisms, light is both the source of energy and the signal directly regulating the core carbon metabolism. Due to the close relationship between light uptake and C fixation, nature has chosen redox signalling as a way of sensing the quality and quantity of light and hence the photosynthetic activity. Outside the chloroplast, this redox-mediated enzymatic regulation becomes less strong and favours other ways of control, as occurs with some C-metabolism isoforms such as glucose-6-phosphate dehydrogenases (Fickenscher and Scheibe, 1986) and FBPases. Apart from the already-known allosteric inhibition of cpFBPase by F2,6BP and AMP (Daie, 1993), no redox activation/deactivation mechanism has been described so far.

### How biomass, photosynthesis, and carbohydrate content and allocation can vary in plants with altered levels of cpFBPase

#### Repressing cpFBPase levels

To date, potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) have been the only crops subjected to genetic manipulation in order to repress cpFBPase levels (Knöllmann et al., 1994; Obiadalla-Ali et al., 2004).

Antisense potato plants expressing reduced levels of cpFBPase were created to answer several questions referring to photosynthesis, plant development, sugar partitioning, and biomass yield (Knöllmann et al., 1994). A few plants were selected to express different cpFBPase amounts. In the most highly inhibited plants (15% activity), photosynthesis was greatly impaired and FBP was accumulated 8-fold with respect to WT plants (Fig. 2A). Analysis of CC metabolites revealed an accumulation of triose phosphates and a decrease in the primary product of CO₂ fixation (PGA), which activates ADP-glucose pyrophosphorylase (AGPase), key in starch synthesis (Cross et al., 2004). The scarce F6P is preferentially used for CC regeneration rather than as a precursor for starch synthesis and, consequently, lower accumulations in F6P led to a dramatic decrease in starch content and, to a lesser extent, to a lower soluble-sugar accumulation. It bears noting that leaves with decreased cpFBPase activity had an increased ability to direct exogenously supplied carbon into starch in darkness. Cross et al. (2004) hypothesized that these plants could have a higher expression of the hexose transporter. Nevertheless, an *Arabidopsis* mutant in the plastid isoenzyme of phosphoglucosomerase has been reported to contain substantial amounts of starch in non-green tissues, whereas photosynthetically active tissues were starch-free (Yu et al., 2000). This result indicated that mesophyll chloroplasts do not have significant G6P transport capacity that could bypass the interrupted link between CC and starch biosynthesis. However, due to the high number (around 150) of presumed metabolite transporters (Ferro et al., 2002; Koo and Ohlrogge, 2002; Schwacke et al., 2003) a feasible involvement of as yet uncharacterized transporters introducing other sugars or sugar-precursors into the chloroplast cannot be ruled out. It is notable that the reduction of cpFBPase in potato plants did not affect
the expression of other genes involved in carbon assimilation and partitioning (Koßmann et al., 1994).

Some years later, a similar experimental approach was carried out by our group. In this case, *Arabidopsis thaliana* transgenic plants were obtained by transformation with an antisense pea *cpFBPase* construct (Sahrawy et al., 2004). Surprisingly, after analysing the starch and sugar content of the transgenic plants, it was found that a moderate decrease in foliar *cpFBPase* activity (71–80% of the WT) induced an unexpected rise in both starch and sucrose content together.

![Diagram](image-url)
with bolstered growth and photosynthesis (Fig. 2B, C). A cpFBPase activity of around 60% of WT plants prompted a slight decline in hexose concentration (glucose and fructose) while, surprisingly, the starch and sucrose content remained at WT values. The explanation of the discrepancies between the results of this work and those reported in Koßmann et al. (1994) could lie in the degree of cpFBPase repression reached for each species. When these two works are taken into account, it is tempting to conclude that a low cpFBPase decrease (less than 20%) improves photosynthesis and carbon assimilation, while a high cpFBPase repression (more than 80%) impairs them both. However, this hypothesis needs to be supported by other experimental data and much work would be necessary to confirm this theory.

Obiadalla-Ali et al. (2004) reported on the specific reduction of the cpFBPase activity in tomato fruits. These organs can supply 10–15% of the carbon skeletons in the green stages of fruit development (Tanaka et al., 1974), combining aspects of both autotrophic and heterotrophic nutrition. The authors studied the influence of cpFBPase in fruits for the first time. Taking advantage of the potato and tomato phylogenetic proximity, these researchers created antisense tomato plants using the potato gene (Koßmann et al., 1992) under the control of the promoter patatin B33, which confines transcription to the fruit (Frommer et al., 1994). Although the cpFBPase activity rose 50% with respect to WT plants, the Western blot analyses detected no protein in these transgenic lines, attributing the residual activity to the fruit cyFBPase. Starch and soluble contents were determined in the pericarp between 25 DAF and 65 DAF. Surprisingly, unlike antisense potato plants, the starch contents did not significantly differ in comparison with the control. Glucose and fructose concentrations significantly increased in the transgenic lines but only between 25 DAF and 35 DAF, showing a similar content at the end of ripening. As with the starch content, no significant differences appeared in the sucrose concentrations between the transgenic lines and controls. The amount of PGA increased in all lines, contrary to the decrease observed in potato leaves (Koßmann et al., 1994). This increase did not impede the normal rate of starch synthesis but even acted as an AGPase activator. Antisense plants produced fruits with weight reduced by 15–20%. Remarkably, this percentage of reduction is similar to the photosynthesis fruit contribution calculated by Tanaka et al. (1974). The conclusion drawn from this work was that the role of cpFBPase in sink organs is trivial compared with that played in source organs (leaves). In a similar way, when the FBPase content in strawberry fruits was analysed, the signal corresponding to the cytosolic isoform was visualized whereas cpFBPase was not detected by using Western blotting (AJ Serrato, unpublished data).

**Increasing cpFBPase content**

The chloroplast FBPase is not present in several starch-storing tissues (Entwistle and ap Rees, 1990). Potato tubers import photosynthetic source organs such as hexoses (Hatzfeld and Stitt, 1990; Viola et al., 1991), and triose phosphates are not precursors for starch biosynthesis in isolated amyloplasts (Naem et al., 1997). Thorbjørnsen et al. (2002) activated an alternative pathway for starch biosynthesis in tubers by restraining cpFBPase expression. When intact amyloplasts were provided with [U-14C]DHAP, these researchers noted, in the transformant expressing the highest cpFBPase activity, an increase of up to 7-fold in triose phosphate incorporation, and there is a good correlation with the cpFBPase activity level in these organs. Despite this noteworthy result, no changes were detected in the content of starch, soluble sugars, and hexose-phosphates in mature tubers of transgenic plants.

The expression of a cyanobacterial fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase (FBP/SBPase) in plants has been proven to enhance biomass production in *Nicotiana tabacum* (Miyagawa et al., 2001). This gene, whose protein is more resistant to H2O2 inactivation than plant cpFBPase (Tamoi et al., 1996), was placed under the control of the *rbcS* promoter and the corresponding enzyme was targeted to the chloroplast through the addition of the *rbcS* transit peptide. The authors stated that one of the reasons for the success of this approach might be the avoidance of gene silencing because of the low sequence identity with plants FBPase and SBPase. Measurements of CC metabolites showed an increase in the concentrations of RuBP, PGA, DHAP, F6P, and G6P. Moreover, as might be expected for more active CC and photosynthesis, the hexose, sucrose, and starch content was higher than in WT plant leaves (2.5-, 2.0-, and 1.6-fold, respectively, in the highest overexpressing transormant). Notably, in contrast to the invariant CC metabolite concentration found in some antisense plants, increasing the FBPase activity in *N. tabacum* augmented intermediate metabolites. Nevertheless, in this work, it was not possible to discern the relative contribution of the FBPase and SBPase activities to these results.

**Plants with reduced levels of cyFBPase**

Different ways of concentrating CO2 have evolved in plants which are referred to as C3, C3–C4, and C4 plants, depending on the strategy employed. *Flaveria linearis* is one member of the C3-C4 intermediate species. Sharkey et al. (1988) reported a natural F. *linearis* line, called 84-9, which lacks most of the cyFBPase activity and is less sensitive to O2 that WT plants (called 85-1). It is known that the two key enzymes for sucrose synthesis are cyFBPase and sucrose phosphate synthase (SPS). Surprisingly, 84-9 accumulated more sucrose in the vacuole than did the WT. Nonetheless, in this mutant, partitioning to sucrose was diminished (2.5-fold) and carbon flux was balanced towards starch biosynthesis (Sharkey et al., 1992). The authors hypothesized that the cyFBPase mutant plants could survive, thanks to partitioning more C to starch than to sucrose during the day and remobilizing the excess at night. The analysis of F1 and F2 generations resulting from crossing 84-9 with 85-1
indicated that cyFBPase activity in *Flaveria* was controlled by one nuclear gene and showed the existence of codominance between the two alleles of the corresponding lines (Micallef and Sharkey, 1996). The determination of total biomass and CO₂ assimilation pointed to a direct effect of cyFBPase activity reduction over the growth rate and photosynthesis (Micallef et al., 1996) in *F. linearis*.

A decade later, Leonardos et al. (2006) carried out a study of the diel (i.e. day and night) patterns of C export to leaves in the lines 84-9 and 85-1. This work was the first to quantitatively assess the fate of fixed C during a day and night cycle in the leaves of a plant with reduced cyFBPase activity. According to the results reported by Sharkey et al. (1992), starch accumulation was higher in the mutant 84-9 than in WT plants; however, there are certain discrepancies with respect to the sucrose content, which was approximately the same throughout the complete cycle, although higher at the beginning of the daytime (Leonardos et al., 2006). Nonetheless, the above discordance might be explained by the units handled in each work (rating sucrose concentrations with respect to either fresh weight or leaf area). Whereas glucose and fructose concentration was relatively constant in WT plants, in the reduced FBPase line the sugar concentrations in leaves were much higher at the beginning of the daytime period as well as at the end of the night period. Furthermore, other dissimilarities were evident between the two lines, such as the pattern of allocation to sink organs and stem growth, which was reduced in the cyFBPase mutant. In any case, the unexpected sucrose concentration in the mutant line led the authors to highlight the possible role that pyrophosphate:fructose-6-phosphate-1-phosphotransferase (PPi-PFP) could develop by replacing cyFBPase, which can hydrolyse FBP when it is accumulated in the mutant line (Stitt et al., 1987; Yaunn et al., 1988). Finally, the authors suggested that unidentified genetic variations could be responsible for the results and that additional work consisting of complementing the mutant line with a WT cyFBPase would be necessary.

In addition to the above studies showing the control of cpFBPase in potato carbohydrate biosynthesis and allocation (Koßmann et al., 1994; Thorbjornsen et al., 2002), another work reported the effects of a reduction of the cytosolic isoform (ranging from 9% to 55% of WT plants) over sucrose biosynthesis in potato (Zrenner et al., 1996). By means of antisense transformation, several lines were generated which did not show any obvious difference in phenotype and growth with respect to WT plants. Nevertheless, in the antisense plants, the FBP, PGA, and triose phosphates contents were higher than in WT plants (up to 6-, 3- and 2.5-fold, respectively). On the contrary, levels of F6P, G6P, and UDPG remained constant. The determination of the CO₂ fixation rate showed limitation at light-saturation intensities, in agreement with the results reported by Micallef et al. (1996) in *F. linearis*. This photosynthesis limitation is in accordance with the changes observed in the CC metabolite pool. Despite the reduced cyFBPase activity in the most inhibited line (9% activity), soluble sugars (hexoses and sucrose) were not significantly altered, as has been recently reported by Leonardos et al. (2006) in the *Flaveria* cyFBPase mutant line; nevertheless, as occurred in *Flaveria*, the starch content increased up to 3-fold after 14 h of illumination compared with WT plants.

The importance of cyFBPase and SPS in sucrose biosynthesis led Strand et al. (2000) to generate antisense *A. thaliana* plants with repressed expression for each protein (Fig. 3). Several selected antisense lines contained 12–60% of cyFBPase and 23–42% of SPS with respect to WT plants. As expected, both transformants showed reduced shoot growth (37–57%) and a lower fresh (10–17%) and dry (15–20%) weight compared with WT plants. In addition, photosynthesis was impaired in both cyFBPase and SPS transformants. One consequence of a decrease in the cyFBPase activity (Fig. 3A) was a lower sucrose content, accumulation of phosphorylated intermediates as PGA (which activates AGPase), reduction of the triose phosphate pool, Pi-limitation of photosynthesis, and higher synthesis of starch. Strikingly, plants with decreased SPS activity (Fig. 3B) also showed an inhibition of sucrose synthesis but no accumulation of phosphorylated intermediates and, unlike cyFBPase mutants revised up to now, carbon partitioning was not re-directed to starch. Other sugar phosphates, such as G6P, G1P, and FBP, increased in the antisense cyFBPase plants, whereas the reducing sugars, glucose and fructose, dramatically diminished. These differences suggest another compensatory mechanism in the SPS mutants.

**Conclusions**

Thanks to a key metabolic position, some enzymes might become potential targets for biotechnological manipulation in order to generate plants with enhanced starch or sucrose contents. Among these enzymes, the chloroplast and the cytosolic isoforms of FBPase are good candidates for this purpose, as reflected in previous works. Up to now, the generation of plants with altered levels of FBPase has been limited to a small number of species, and only three crops. Modifications have either been directed to an increase or to a decrease in the corresponding FBPase activity. The generation of antisense plants has been used to lower the cyFBPase activity and only in *F. linearis* have studies been based on the analysis of a naturally occurring mutant. The antisense approach is useful because it provides a set of plants with different degrees of inhibition and thus correlates the effects on photosynthesis, biomass yield, and carbohydrate content with gradually decreasing activities. However, it is striking to realize the paucity of studies on knock-out *Arabidopsis* plants with no cpFBPase or cyFBPase activity. This would help to gain some new insights into the role of these key metabolic enzymes.

Based on the works mentioned in this review, it seems clear that altering FBPase levels provokes dramatic effects in the manipulated plants. These effects consist, when cpFBPase is drastically depressed, of lower starch and sucrose contents and of CC and photosynthesis disorders derived from phosphorylated metabolite accumulation and
the consequent Pi limitation. A similar reduction in cyFBPase leads to low sucrose levels and photosynthetic rate, whereas starch content is higher than in WT plants. Accordingly, in our laboratory, a major starch accumulation in Arabidopsis cyFBPase knock-out plants with a WT phenotype was noted (AJ Serrato, unpublished data). In summary, the overall changes are much more notable in the cpFBPase mutants because of the impact over the CC, which is the core of plant–carbon metabolism. Nonetheless, it is worth mentioning that cyFBPase can be important in non-photosynthetic organs. In fact, it was observed that cyFBPase is the only FBPase isoform present in developing strawberry fruits (AJ Serrato, unpublished data).

A useful approach by Miyagawa et al. (2001) was the introduction into N. tabacum of a cyanobacterial gene encoding an FBPase with different characteristics to the ones found in plants. In this sense, the knowledge of the biochemical features of FBPases could be of interest in the near future in order to produce, by using directed mutagenesis, new enzymes suitable for plant transformation in order to enhance certain aspects of plant physiology and metabolism as, for example, are photosynthesis, plant biomass, and starch or sucrose content in important crops.

Starch and sucrose are basic for animal and human nutrition. Therefore, scientific efforts may be directed to a better understanding of plant mechanisms related to carbohydrate biosynthesis in order to help improve the quantity and quality of plant products that are fundamental for sustaining an exponentially increasing population.

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