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Molecular Manipulation of Source-Sink Interactions in Crop Plants

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The world will have to produce about 50% more food by 2025 to feed an estimated population of about 8 billions. Furthermore, this increased demand will have to be met from less land with less water, less labour and less use of chemicals. Moreover, a large number of biotic and abiotic stresses and unfavourable post harvest conditions particularly, in Asian and African subcontinents take a serious toll of crop production. Therefore, crop varieties with higher yield potential and yield stability are needed to meet the goal of increasing crop productivity. In the last 60 years of plant breeding, the total plant dry weight harvested per hectare has not increased significantly. Rather, the increase in crop yield had been mainly due to the improvement in harvest index. Therefore, the best strategy to increase crop yield would be to design the architecture of the crop plants in such a way that source-sink interactions are maximized. The molecular approaches to increase crop yield have therefore, remained restricted either to increasing carbon export in the photosynthetic active organs or to optimizing carbon utilization in storage organs. The approaches exploited maximally included overexpression of endogenous or heterologous enzymes in tissues or subcellular compartments where they donot normally occur, and underexpression of endogenous enzymes using antisense technology. Both these approaches have been successfully employed in manipulating different components of source-sink interactions such as primary photosynthetic processes, synthesis of transitory starch in chloroplast, export of carbon out of chloroplast, synthesis of sucrose, export of sucrose (phloem loading), phloem unloading, hydrolysis of sucrose, transport of metabolites into plastids and synthesis of seed reserves. In each of these cases, transgenic plants have been produced where the effect of over and/or under expression of specific enzymes involved in these processes have been studied in relation to partitioning of photoassimilates for better crop production. The present review covers the current knowledge in the area of molecular genetics of source-sink interactions in crop plants.

Key Words: Source-sink interactions, Molecular approaches, Transgenics, Physiological and Biochemical analysis

Introduction

The world population which is estimated to be 5.8 billions at present is likely to go upto 8.0 billions in 2025. Accordingly, we will have to produce fifty percent more food compared to the present day level. Regionwise, Africa will have to increase almost 400 percent, Latin America 200 percent and Asia 50 percent. In past, increase in potential crop yield by traditional breeding had been achieved mainly through improvement of harvest index (harvest index is the ratio of the dry weight of the harvested organ to the total plant dry weight). Increase in cropped area and use of chemicals alongwith assured irrigation etc were few other factors which contributed towards increased

production. However, the future scenario will be different and it will not be possible to bring more area under crops. Rather, in most of the Asian countries, cultivated area is declining due to continuous increase in population and also due to pressures of urbanization and industrialization. Environmentalists are showing greater concerns about the harmful effects of chemicals being used in agriculture. Moreover, industry has started competing with agriculture for water and labour etc. Thus, we will have to produce more food from less land, with less chemicals, less labour and less water. Under these circumstances, there is no other alternative but to increase the crop productivity *per se* to feed the world population in 21st century. However, plant breeders find it difficult to increase the crop productivity further as yield plateaus have already reached in most of the food crops. Moreover, in the last 60 years of plant breeding, the total plant dry weight harvested per hectare has not increased significantly. The increase in crop yield had been mainly due to better allocation of dry matter to the harvestable plant parts, suggesting that the increase in yield was achieved mainly through an optimization of source-sink interactions. Therefore, the best strategy to increase crop yield under the present circumstances would be to manipulate the architecture of the plant in such a way so that the source-sink interactions are optimized towards more yield. The major components of source sink interactions which need investigations include: (i) primary photosynthetic processes including light and dark reactions, (ii) synthesis of transitory starch in the chloroplast, (iii) export of carbon out of chloroplast, (iv) interconversion of assimilates to sucrose, (v) export of sucrose into the conductive tissues (phloem loading), (vi) phloem unloading, (vii) hydrolysis of sucrose, (vii) transport of metabolites into plastids etc for the synthesis of reserves, and (ix) conversion of metabolites into seed reserves.

In the past about two decades, the molecular techniques have been exploited to manipulate source-sink interactions in essentially two ways (Sonnewald & Willmitzer 1992, Sonnewald et al. 1994, Frommer & Sonnewald 1995, Kossmann et al. 1996). The first is through over expression of endogenous or heterologous enzymes, sometimes in tissues or subcellular compartments, where they do not normally occur (ectopic expression); the second is to downregulate the expression of endogenous enzymes mainly through antisense technique. A brief discussion of results obtained by use of these techniques is presented here in the following sections.

Manipulation of Primary Photosynthetic Processes

Primary photosynthetic processes include light dependent and light independent reactions. In light reactions of photosynthesis, ATP and NADPH are produced which are then utilized for assimilation of CO_2 through reductive pentose phosphate pathway (Calvin cycle). In this pathway, ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco) catalyses primary carbon fixation, in which ribulose-1,5-bisphosphate (RuBP) and CO_2 are converted to two molecules of 3-carbon compound, 3-phosphoglycerate (3-PGA). In subsequent reactions, 3-PGA is phosphorylated and reduced by the products of light reactions of photosynthesis to produce triose phosphates, which ultimately serve as the source of carbon for sucrose synthesis in cytosol and starch synthesis in chloroplast. The cycle (figure 1) comprising of thirteen reactions catalyzed by eleven enzymes and located in chloroplasts of C_3 plants has four principal features which include carboxylation, reduction, regeneration and autocatalysis (Singh 1999, Sheoran & Singh 1999). Recent advances in plant molecular biology have allowed scientists to generate stable transgenic plants with altered activity of one or the other enzyme of the above cycle. Some of the major results of these studies are given below phasewise.

Carboxylation Phase

A large number of studies conducted with transgenic tobacco plants have revealed that Rubisco protein can easily be reduced to 50% without having effect on the rate of photosynthesis (Quick et al. 1991a, 1991b, Quick 1994, Stitt & Schulze 1994, Stitt & Sonnewald 1995). The plants with reduced Rubisco protein were able to compensate by fully activating the remaining

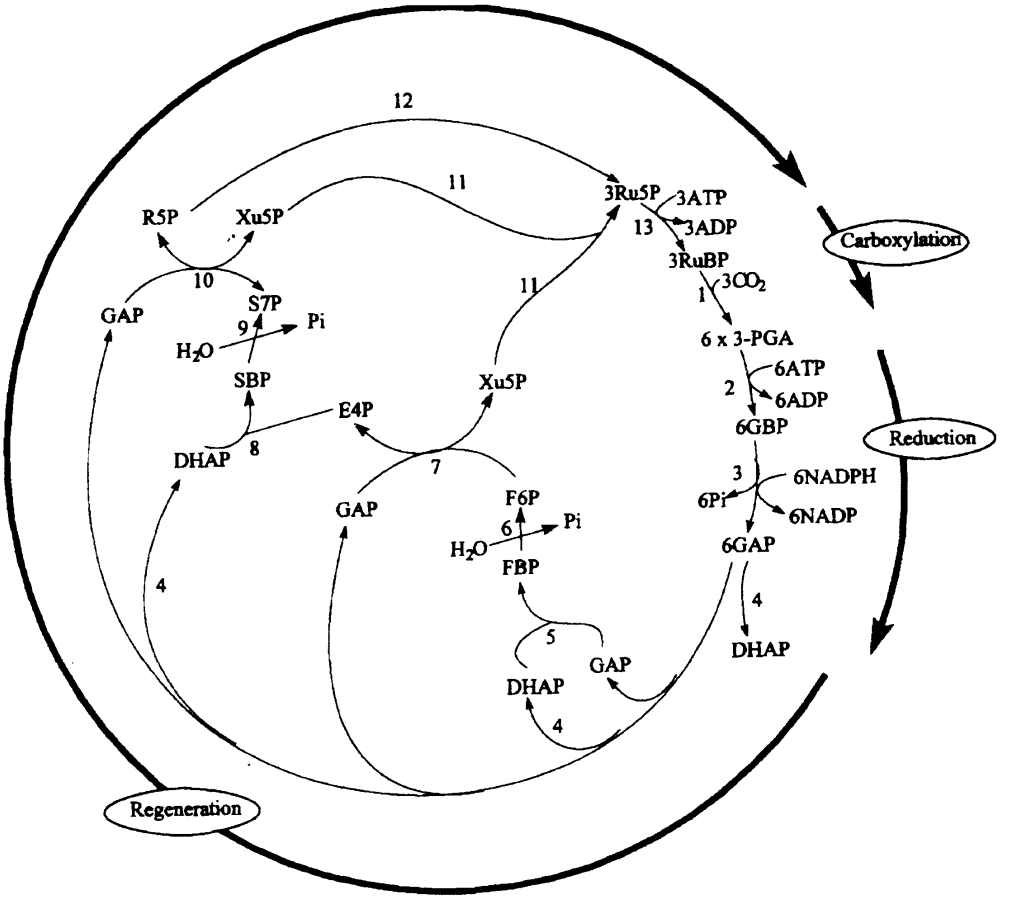

Figure 1 The autocatalytic Calvin cycle showing the three distinct phases of the Cycle: Carboxylation, reduction and regeneration. **1,** Rubisco; **2,** 3-PGA kinase; **3,** Glyceraldehyde-3-P dehydrogenase; **4,** Triose phosphate isomerase; **5,** Aldolase; **6,** FBPase; **7,** Transketolase; **8,** Aldolase; **9,** SBPase; **10,** Transketolase; **11,** Ribulose-5-P 3-epimerase; **12,** Ribose-5-P isomerase; **13,** Phosphoribulokinase

enzymes. These studies were conducted under 300 mol m^{-2} sec^{-1} irradiance and a temperature of 20°C. However, when the experimental conditions were altered in short term studies, it was found that the control exerted by Rubisco on photosynthesis was not constant e.g. under conditions of high light intensity, Rubisco was the major factor determining the rate of photosynthesis. Similarly, when CO_2 concentration was reduced from 450 to 250 µbar, Rubisco exerted an increased control (Stitt et al. 1991), indicating that the control exerted by Rubisco was influenced by the environmental growth conditions. Growth of plants in low nitrogen also leads to a selective loss of Rubisco protein as shown by Fichtner et al. (1993) using antisense tobacco plants. This again led to increased control of Rubisco, confirming that the high concentration of Rubisco protein in leaves is necessary to allow the plant to respond to changing environmental conditions and to achieve higher growth rate. Hence in such type of studies, one must always specify the growth conditions under which the plants were grown.

Rubisco activase plays an important role in the regulation of Rubisco. However, the significance of this enzyme in maintaining active Rubisco and hence photosynthetic rates still remains uncertain. Somerville et al. (1992) identified activase null mutants of *Arabidopsis*, which could not grow under atmospheric CO_2 concentration, suggesting an important role for activase in the control of Calvin cycle. Transgenic tobacco plants expressing an antisense construct to the *rca* mRNA encoding activase with less than 25% of wild type activase activity required growth at elevated CO_2 for survival (Mate et al. 1993). In these plants, the rate of activation of

Rubisco during a dark to light transition was also reduced to 25% of the wild type. This proved that activase plays an important role in the rapid adjustment of Rubisco activity in environments where rate of photosynthesis fluctuates. In another study, where Rubisco activase was reduced less severely (upto 50% of the wild type), the enzyme became limiting for photosynthesis only when its activity was reduced below 60% of wild type (Jiang et al. 1994). However, the light saturated rate of photosynthesis was sensitive even to minor reductions in activase. In a recent study, activase has been shown to have a flux control coefficient of unity with respect to Rubisco activation (Hammond et al. 1998). However, steady state photosynthesis remained largely unaffected by activase concentration until it was reduced below approximately 15% of the wild type level. These results thus proved that a regulatory enzyme not directly involved in the metabolic pathway can also exert control on the rate of photosynthesis.

Reduction Phase

Phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase are the two enzymes involved in the reductive phase of Calvin cycle. Price et al. (1995) recently produced a series of transgenic plants with reduced chloroplastic glyceraldehyde-3-phosphate dehydrogenase. In these plants, the enzyme activity could be reduced to 35% of wild type activity before any effect on the rate of photosynthesis was observed, suggesting that this enzyme exerts very little control on the rate of photosynthesis in tobacco. However, there was a drop in the size of RuBP pool, indicating that the immediate limitation was on the regeneration reactions of the Calvin cycle.

Regeneration Phase

Kossmann et al. (1994) produced transgenic tobacco plants by expressing an antisense cDNA construct for FBPase. These plants had FBPase activity between 12 to 36% of wild type. Plants with 36% wild type activity had almost similar growth, tuber yield or phenotype as that of wild type plants, confirming that this enzyme again exerts little control on the rate of photosynthesis. However, plants with further reduction in FBPase activity showed reduced photosynthetic capacity, plant growth and tuber yield. The concentration of triose phosphates was increased, whereas that of hexose phosphates remained unchanged, suggesting increased partitioning of photosynthates toward cytosolic sucrose synthesis with concomitant decline in the amount of starch.

Harrison et al. (1998) produced a range of tobacco plants by expression of an antisense cDNA construct to the chloroplastic SBPase under the control of a 35S promoter. Plants with 40% reduction in SBPase protein had chlorotic leaves and reduced growth. Furthermore, the leaves had reduced starch content, suggesting that starch rather than sucrose synthesis was inhibited in transgenic plants. It was further shown that control exerted by SBPase varied depending upon the rate of photosynthesis. At light saturated rate of photosynthesis, the control exerted by SBPase was much stronger than when rate of photosynthesis was reduced by light intensity. In plants with reduced SBPase activity, the partitioning of labelled CO_2 into starch was also found to be reduced in transgenic plants, indicating the importance of this enzyme in the regulation of partitioning of photosynthates in starch and sucrose. To date much of the analysis of photosynthetic capacity in antisense Calvin cycle plants has focussed on mature fully expanded leaves. Analysis of the antisense SBPase plants has been extended to include younger developing leaves. The results obtained from these studies showed significant developmental effects on the control of photosynthesis by SBPase. Flux control coefficients for photosynthesis increased from very low values, 0.15 in young expanding leaves to values of between 0.55 to 0.7 in fully expanded mature leaves (Raines et al. 1999). These results provide further evidence that the regulatory capacity of Calvin cycle enzymes *in vivo* is not constant and varies during development and in response to environmental conditions.

Phosphoribulokinase (PRK) is another regulatory enzyme, which catalyzes the synthesis of RuBP from Ru-5-P and ATP. Its activity could

very well be reduced by more than 80% without having any effect on rate of photosynthesis and growth etc in transgenic tobacco plants produced through expression of a PRK antisense cDNA construct (Knight & Gray 1994, Paul et al. 1995). However, low N in addition to a 94% decrease in PRK by antisense reduced the activity of PRK sufficient to diminish photosynthesis, which limits biomass production under conditions normally considered sink limited (Banks et al. 1999).

Aldolase is another enzyme, whose role in the control of photosynthesis has been investigated by use of transgenic plants. Sonnewald et al. (1994) cloned plastidic isoform of aldolase from potato and the cDNA sequence used to produce transgenic potato plants that expressed an antisense RNA under the control of CaMV 35S constitutive promoter. The rate of photosynthesis and growth in these plants were not affected until the enzyme activity was reduced below 60% of the wild type. Analysis of carbohydrates revealed starch synthesis to be inhibited more than sucrose synthesis; a 70% reduction in aldolase activity led to 74% reduction in the amount of starch and yet had no effect on the leaf sucrose concentration. In a later study (Haake et al. 1998), the decrease in aldolase activity in transformed potato plants was shown to be due to inhibition of plastid aldolase. These plants also showed a small increase of Rubisco activity, a small decrease of PRK activity, and larger and subproportional decreases of SBPase and plastid FBPase activity. The transformants contained increased triose phosphates, and very low RuBP and 3-PGA. These results confirmed that though plastid aldolase catalyzes a readily reversible reaction, possesses no known regulatory properties and appears irrelevant for the control of metabolism and growth, small changes in its activity have marked consequences for photosynthesis, carbon partitioning and growth.

The above information on Calvin cycle enzymes has revealed that the control is shared among several enzymes of the pathway and that the distribution of the control changes with changes in growth conditions and experimental conditions. Moreover, the control does not necessarily reside in highly regulated enzymes that catalyze essentially irreversible reactions. Enzymes catalyzing freely reversible reactions could also exert control when their activity is reduced below 50% of wild type.

C_3/C_4 Mechanism of Photosynthesis

Based on the differences in the mechanism of CO_2 assimilation, the land plants have been divided into three major photosynthetic types namely C_3, C_4 and Crasulacean acid metabolism (CAM) plants. Contrary to C_3 photosynthesis, which occurs in mesophyll cells, C_4 photosynthesis requires the coordination of two photosynthetic cell types, the mesophyll cells and bundle sheath cells. In this type of photosynthesis, CO_2 from the atmosphere is fixed in mesophyll cells by the reaction catalyzed by the enzyme PEP carboxylase producing a 4-carbon acid, oxaloacetate, which is rapidly converted to the stable 4-carbon acids, malate or aspartate (Hatch 1987, 1992, 1997). Malate/aspartate thus produced is then transported from the mesophyll cells to bundle sheath cells and decarboxylated. C_4 plants have further been classified as NADP-ME, NAD-ME and PEPCK types, according to the major decarboxylase involved in the decarboxylation of C_4 acids in the bundle sheath cells (Hatch et al. 1975). The CO_2 released is assimilated in the bundle sheath cells via Rubisco in the reductive pentose phosphate (C_3) cycle. Thus C_4 plants require two carboxylation reactions catalyzed by PEP carboxylase and Rubisco for assimilation of one molecule of CO_2. In the process, CO_2 concentration increases in bundle sheath cells of C_4 species by 5 to 10 times compared to atmospheric CO_2 (Hatch 1987). Though more than 90% of land plants, including many agronomically important crop species assimilate atmospheric CO_2 through C_3 pathway of photosynthesis, they suffer from the low affinity of Rubisco towards atmospheric CO_2. Rubisco besides catalyzing carboxylation reaction also catalyzes the oxygenation reaction in which O_2 and RuBP are the substrates and PGA and phosphoglycolate are the products. This is the first reaction in the process of photorespiration and under these conditions, O_2 competes with

CO_2 for reaction with RuBP. This competition combined with loss of CO_2 in photorespiration, reduces photosynthesis by about 30% in C_3 plants (Bjorkman 1976). C_4 plants by developing the above mechanism of photosynthesis have overcome the limitation of low CO_2 and photorespiration as this pathway serving as a CO_2 pump concentrates CO_2 at the site of Rubisco, suppressing oxygenase activity and photorespiration (Hatch 1987, Dai et al. 1993, Furbank & Taylor 1995, Leegood 1997, Singh 1999). These differences in C_3 and C_4 metabolism have resulted in several differences in CO_2 exchange parameters of leaf. C_4 species usually attain higher photosynthetic rates at atmospheric levels of CO_2, high irradiance and optimum temperature. The CO_2 compensation point at 30°C and atmospheric CO_2 level is very low (5µl/l) in C_4 plants, but in C_3 plants, the CO_2 compensation point is 50-60 µl/l. Furthermore, in C_3 plants, compensation point increases with O_2 concentration, whereas in C_4 plants O_2 concentration has no effect on compensation point or on photosynthesis. Photosynthesis in C_3 plants, decreases above 25 to 30°C partly because of the fact that photorespiration increases at higher temperature. Thus the higher photosynthetic efficiency of C_4 plants under tropical conditions results in higher growth rate and dry matter production in these crops (dry matter production in C_4 species nearly averages twice than in C_3 species). C_4 plants are also more efficient in water use compared to C_3 plants (Orsenigo et al. 1997). C_4 plants are also about twice efficient as C_3 plants in the use of N. This has been attributed in part at least to substantially lower level of Rubisco in C_4 leaves (Sage et al. 1987). Oakes (1994) in a recent review has again held photorespiration in C_3 plants to be responsible for reduced nitrogen-use efficiency. She has demonstrated that high levels of NO_3^- seen in barley and wheat (C_3 plants) relative to maize and sorghum are related to a carbon deficiency caused by the inhibition of the mitochondrial pyruvate dehydrogenase complex by monovalent cations, in particular by the NH_4^+ produced by photorespiration in C_3 plants (Schuller & Randall 1989, Gamel & Randall 1992). NH_4^+ production is lower in C_4 than in C_3 cereals.

The advantages of C_4 photosynthesis in crop production have prompted much of the biochemical research on C_4 pathway of CO_2 assimilation. However, very little is known about genetic manipulation to attain/improve C_4 photosynthesis in crop plants. In earlier studies, much emphasis was given to search of mutants among C_3 crop species with reduced photorespiration (Chollet & Ogren 1975, Brown & Bouton 1993). However, all attempts made in this direction proved futile and most research aimed at reducing photorespiration in C_3 species indicated little possibility of increasing net photosynthesis. Similarly, a large number of chemicals employed for decreasing photorespiration failed to increase dry matter yield in C_3 plants. Even the goal of interspecific hybridization to manipulate C_4 photosynthesis for improved crop productivity is not yet realized. However, the variability in metabolism and crossability of plants differing in degree of C_4 may help us in manipulating C_4 photosynthesis (Brown & Bouton 1993). Hybridization studies have shown that C_4 photosynthesis is a combination of independently inherited traits and in such cases, converting C_3 species into functional C_4 types may require too many changes to be incorporated through a systematic mutation breeding or even a genetic engineering approach. Hence, introduction of important features of C_4 photosynthesis into C_3 crop species with current knowledge and technology appears unlikely in the absence of close C_4 relatives.

Considerable efforts have also been made in producing a better Rubisco to increase photosynthetic rate and productivity. Improving the CO_2/O_2 specificity of Rubisco without reducing the catalytic activity of the enzyme is one of the ways to increase photosynthetic efficiency. This idea has stimulated considerable studies of the enzyme and its various aspects, including structure, mechanism, molecular biology and physiology etc. The ultimate aim of these investigations was to obtain more efficient Rubisco using genetic engineering approaches. Although there have been major advances in these areas, the possibility of designing more efficient enzyme still remains a dream. Moreover, large scale efforts to select mutants with

improved CO_2/O_2 specificity have also failed. No eukaryotic Rubisco has been expressed successfully in any foreign host perhaps because of mismatches between the foreign Rubisco and the host chaperone system (Gutteridge & Gatenby 1995, Roy & Andrews 1999). This has restricted mutagenic analysis of the structure and function of eukaryotic Rubisco to organisms (green algae), where well developed method exists for mutagenesis and transformation of the chloroplast genome (Spreitzer 1998). Development of a method for transforming the chloroplast genome of tobacco provides a means for testing of the wealth of information that exists about Rubisco's structure and function (Andersson 1996). Chloroplast transformation strategy developed by Whitney et al. (1999) surmounts previous obstacles to mutagenesis of higher plant Rubisco and allows the consequences of mutagenesis for leaf photosynthesis to be assessed. This has given us a hope to genetically manipulate Rubisco with improved CO_2/O_2 specificity.

Manipulation of Transitory Starch Biosynthesis in Photosynthetic Tissues

Photoassimilates produced in the photosynthetically active leaf tissues through Calvin cycle are partitioned into either sucrose or starch, which are synthesized in cytosol and chloroplast, respectively. One can, therefore attempt to shift this partitioning towards sucrose by down regulating the synthesis of transitory starch. In Calvin cycle operating in chloroplast, fru-6-P is the metabolite which is channeled into starch biosynthesis through the concerted action of the plastidic isoforms of phosphoglucoisomerase (PGI), phosphoglucomutase (PGM) and ADP-glucose pyrophosphorylase (ADPGPPase) (figure 2). These enzymes convert fru-6-P to ADP-glucose, which is the substrate for starch synthesis (Sangwan & Singh 1987a). ADP-glucose pyrophosphorylase is the key regulatory enzyme of this pathway both in leaf and reserve tissues (Preiss 1982, 1988, 1991, 1997, Preiss & Sivak 1996, Sivak & Preiss 1995). The enzyme has been characterized in detail from both these sources. The activity of this enzyme is regulated by the ratio of 3-PGA (activator) and inorganic phosphate (Pi, inhibitor). The enzyme is tetrameric with two types of sub-units with differing molecular weights (50-56 kD for small sub-unit, and 51-60 for the large subunit). Molecular studies conducted in the recent years have led to the identification of cDNA and genomic clones encoding the various sub-units (Olive et al. 1989, Bae et al. 1990, Bhave et al. 1990, Muller-Rober et al. 1990, Villand et al. 1992, 1993, LaCognata et al. 1995). Experiments have also been conducted, where starch synthesis in leaf tissues was specifically reduced through the expression of an antisense RNA targeted to the ADPGPPase driven by the leaf specific promoter (Stockhause et al. 1989, Muller-Rober et al. 1992). In these experiments, though the starch level in leaves was reduced by 50% to 80%, depending on the degree of inhibition and time of sample collection, no change in photosynthesis was observed when measured under ambient conditions. Similarly, the levels of soluble sugars, malate and amino acids etc. remained almost unaffected in transgenic plants. However, compared to wild-type plants which in the dark usually export carbon at a rate which is 75% of the day rate (Heineke et al. 1994, Leidreiter et al. 1995), ADPGPPase antisense plants utilized larger portion of the carbon assimilated during the light period itself. In these plants, the utilization during the dark was about one third of the day rate (Leidreiter et al. 1995). This specific inhibition of starch synthesis in leaves of transgenic plants which changed the export characteristics of potato leaves did not specifically increase the tuber yield.

Manipulation of Carbon Transport Across the Chloroplast Envelope

As discussed above, the carbon which is not retained within the plastidic compartment, is exported into the cytosol for biosynthesis of sucrose (figure 2). The carbon mainly leaves the chloroplast in the form of triose phosphate which is exported out in exchange of inorganic phosphate (Pi) through triose phosphate translocator (TPT) (Flugge & Heldt 1991). This translocator is located in the inner membrane of chloroplast envelope and has been purified and the corresponding cDNA isolated from spinach

(Flugge et al. 1989). Transgenic potato plants have been produced through expression of a fragment of cDNA clone for potato TPT in reverse orientation under the control of the constitutive CaMV 35S promoter (Schulz et al. 1993, Riesmeier et al. 1993). In these transgenic plants, a 30% decrease in Pi transport activity resulted in 50% decrease of the maximal photosynthetic activity. These plants accumulated up to 5 times more transitory starch in the leaves during the day, indicating blockage of the export of photoassimilates from the chloroplast into the cytosol. Reduction of TPT activity corresponded to a four fold increase in 3-PGA and decrease in Pi in the stromal compartment (Heineke et al. 1994). In experiments, where both TPT and ADPGPPase were inhibited by creating transgenic potato plants, starch accumulation was reduced in all lines of these plants (Hattenbach et al. 1997). In one line with a 50% reduction of ADPGPPase activity, the rate of CO_2 assimilation was unaltered. In these plants, the stromal level of triose phosphate was increased, enabling a high rate of triose phosphate export inspite of the reduction of the TPT protein by antisense repression. In a second line with 95% reduction of ADPGPPase activity, the amount of chlorophyll was significantly reduced as a consequence of the lowered triose phosphate utilization capacity. From these results, it was concluded that export of carbohydrates from the chloroplast into the cytosol is rate limiting for photosynthesis. TPT thus represents a possible target for increasing the flow of carbon by overexpression of the transporter. Even in these plants grown under green house conditions, no significant changes were observed in tuber yield. However, it would be interesting if one could increase the exchange rate by introducing TPTs with different characteristics which may increase photosynthetic activity and crop productivity under high light conditions. This seems possible in view of the fact where maize mesophyll chloroplast TPT has been shown to have five fold lower K_m for Pi and

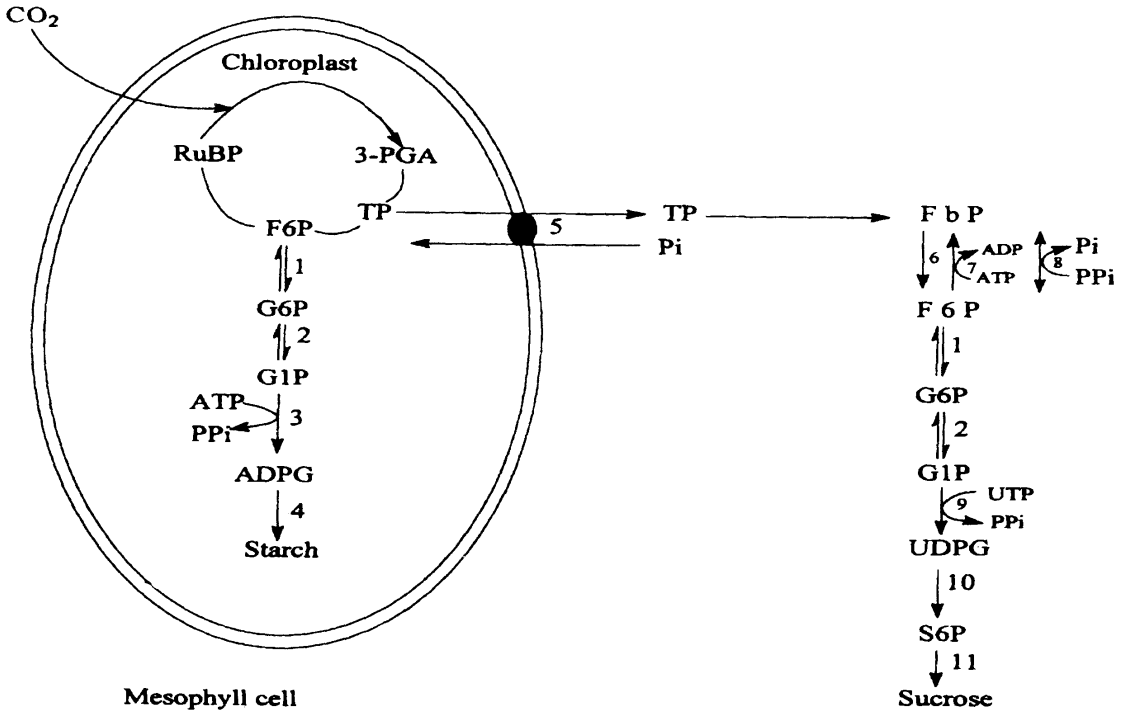

Figure 2 Partitioning of photoassimilates between chloroplast and cytosol of mesophyll cells of source tissues. 1, Phosphohexose isomerase; **2,** Phosphogluco mutase; **3,** ADP-glucose pyrophosphorylase; **4,** Starch synthase; **5,** Triose-P translocator; **6,** FBPase; **7,** Phosphofructo kinase; **8,** Pyrophosphate: fructose-6-P-1-phospho transferase; **9,** UDP-glucose pyrophosphorylase; **10,** Sucrose phosphate synthase; **11,** Sucrose phosphate phosphatase

two to three fold lower K_i values for DHAP and 3-PGA (Heldt et al. 1991). A TPT cDNA clone has recently been isolated from maize (Fischer et al. 1994). The ectopic expression of this protein in transgenic plants may demonstrate the potential for increasing export from chloroplast.

Manipulation of Sucrose Biosynthesis

Sucrose is the major form in which carbon is translocated in most plants and is usually the first product of photosynthetic cells. In C_3 plants, it is synthesized in the cytosol of mesophyll cells from triose-P that is generated within the chloroplast and released into the cytosol by operation of Pi translocator (figure 2). In this pathway of sucrose biosynthesis, the regulatory steps are supposed to be the interconversion of fru-1,6-P_2 and fru-6-P and the formation of sucrose-6-P from UDP-glucose and fru-6-P (Sonnewald 1997). Fructose-1,6-bisphosphatase (FBPase), phosphofructokinase (PFK) and PPi: fru-6-P-1-phosphotransferase (PFP) are the enzymes involved in the interconversion of fru-1,6-P_2 and fru-6-P (Sangwan & Singh 1988, Mahajan & Singh 1989, Kumar & Singh 1984). Sucrose phosphate synthase (SPS) catalyzes the formation of sucrose-6-P which is ultimately converted to sucrose via the action of sucrose phosphate phosphatase.

Transgenic tobacco and potato plants expressing either a pyrophosphatase from *E. coli* (Geigenberger et al. 1996, Jellito et al. 1992, Sonnewald 1992), or a rat liver 6-phosphofructo 2-kinase (Scott et al. 1995) or by antisense inhibition of PFP (Hajirezaei et al. 1994, Paul et al. 1995) and FBPase (Zrenner et al. 1996) have been used to analyze the interconversion of fru-1,6-P_2 and fru-6-P. The activities of FBPase and PFP are allosterically controlled by a signal metabolite fru-2,6-P_2 (Singh 1988, Stitt 1990), which stimulates PFP and inhibits FBPase. The level of this metabolite *in vivo* is determined by the relative activities of 6-phosphofructo-2-kinase and fru-2,6-bisphosphatase. In transgenic tobacco plants expressing only phosphofructo-2-kinase activity (Scott & Kruger 1995, Scott et al. 1995) and consequently having elevated levels (2-3 fold) of fru-2,6-P_2, the flux of assimilates towards sucrose was inhibited and towards starch was stimulated.

A series of antisense tobacco (Paul et al. 1995) and potato (Hajirezaei et al. 1994) plants with reduced PFP have also been produced. The growth performance of these plants was comparable with that of the wild type. Furthermore, the rate of photosynthesis and partitioning of starch and sucrose were also unaffected. In leaves of transgenic plants, the level of hexose-P was higher than that of 3-PGA and PEP was lower compared to wild type plants (Paul et al. 1995). Similar effects were observed in potato plants. These results proved that PFP does not play an essential role in regulating normal vegetative plant growth nor does it affect the adaptation to low nitrogen and phosphate and temperature.

In case of PFP catalyzed synthesis of fru-1,6-P_2, the reaction could be shifted towards the formation of fru-6-P by removing the PPi formed (Mahajan & Singh 1989, 1990). Though chloroplasts contain very high activities of pyrophosphatase, the only known activity in the cytosol is that of the tonoplast-bound enzyme (Maeshima & Yoshida 1989). One could, therefore, shift the interconversion between fru-1,6-P_2 and fru-6-P in the direction of fru-6-P by introducing an alien gene coding for a cytosolic inorganic pyrophosphatase. Sonnewald (1992) achieved the above objective by introducing *ppa* gene from *E. coli* linked to the CaMV 35S promoter into potato plants. Transgenic plants expressing *ppa* gene had reduced PPi content and a 2-4 fold increased ratio of soluble to insoluble carbohydrates (Sonnewald 1992, Jellito et al. 1992). This increase was mainly due to a decrease in the starch content and a partial increase in the sucrose content. However, the plants showed stunted growth and reduced root formation, suggesting that in addition to increased sucrose synthesis in leaf mesophyll cells, the export of photoassimilates might have also been affected. Geigenberger et al. (1998) in a later study investigated the consequences of overexpression of inorganic pyrophosphatase from *E. coli* in the cytosol of plants on sucrose-starch interconversions in growing potato tubers. Over-expression of PPase resulted in an accumulation

of sucrose and UDP-glucose, and decreased concentrations of hexose phosphates and glycerate-3-P in growing *ppa1* tubers. Unexpectedly, the rate of degradation of [^{14}C] sucrose was increased by up to 30%, the rate of starch synthesis was increased, and the starch content was increased by 20-30% in *ppa1* tubers compared to wild-type tubers. The transformed tubers contained increased activities of several enzymes required for sucrose-starch interconversions, suggesting that PPi has a wider significance than just being an energy donor for specific reactions in the cytosol. It is thus possible to change the partitioning between soluble sugars and starch in source leaves by taking advantage of the ability of PFP to catalyze the interconversion between fru-1,6-P_2 and fru-6-P in both directions and driving it into the direction of fru-6-P formation by removal of PPi.

Transgenic potato plants expressing an antisense cytosolic FBPase gene showed decreased sucrose biosynthetic capacity, decreased growth rates and increased shoot root ratio (Zrenner et al. 1996). Detailed analysis of transgenic potato plants with 9-55% of the wild type cytosolic FBPase activity revealed that 45% reduction of the activity did not cause any measurable change in metabolite concentration, growth behaviour or photosynthetic rate. Inhibition of activity below 20% of the wild type led to an accumulation of 3-PGA, triose-P and fru-1,6-P_2 in source leaves, resulting in a reduced light saturated rates of assimilation and photosynthesis. Consequently, there was 53-65% reduction in sucrose synthesis and 18-24% in starch. However, steady state sucrose concentration was not affected in source leaves, whereas starch accumulated by more than a factor of 3 compared to wild type leaves. This provided strong evidence for the hypothesis that hexoses and/or hexose phosphates are exported out of the chloroplasts. This circumvents the limitations of sucrose biosynthesis caused by the inhibition of cytosolic FBPase in the dark. This may be the reason why plant growth and potato tuber yield remained unaffected. From these observations, it could safely be concluded that reduced photosynthetic sucrose biosynthetic capacity could very well be compensated without any reduction in crop yield by changing strategy for carbon export. This shows that there may be alternative translocator activity for assimilate export out of the chloroplasts in the dark (Trethewey & apRees 1994, Quick et al. 1995).

cDNA clones encoding cytosolic FBPase have also been isolated from *Beta vulgaris, Brassica napus, Spinacia oleracia* and *Solanum tuberosum* (Harn & Daie 1992, Hur et al. 1992, Laroche et al. 1995). The cytosolic FBPase from spinach has been expressed in transgenic tobacco plants under the control of CaMV 35S promoter. The fully developed leaves from progeny of self pollinated primary transformants showed a 4-6 fold increase in FBPase activity without having any dramatic changes in growth and development of the plants (Juan & Vasconcelos 1994). However, these plants exhibited slight decrease in starch content.

SPS is the key regulatory enzyme in the pathway of sucrose biosynthesis. Changes in its activity have often been correlated with changes in sucrose synthesis and export (Rocher et al. 1989, Stitt et al. 1988). The enzyme is modulated by metabolites (activator, glu-6-P ; inhibitor, Pi), by changes in the amount of protein (Cheikh et al. 1992, Cheikh & Brenner 1992) and by post-translational modification via reversible protein phosphorylation-dephosphorylation (Huber & Huber 1996). cDNA clones encoding SPS have been isolated from various plants including maize, spinach and sugarbeet (Hesse et al. 1995, Klein et al. 1993, Sonnewald et al. 1993, Worrell et al. 1991). Worrell et al. (1991) produced transgenic tomato plants with about 12 fold higher SPS activity by transferring maize SPS cDNA clone under the control of the constitutive 35S CaMV or the leaf specific Rubisco *SSU* promoter. In these and subsequent studies conducted by Galtier et al. (1993, 1995) with transgenic tomato, the source leaves had reduced starch content and increased sucrose content, indicating stimulation of carbon partitioning towards sucrose. These results proved that SPS plays an important role in carbon partitioning in higher plants (Galtier et al. 1993, Micallef et al. 1995, Laporte et al. 1997, Lunn & Hatch 1997). The overall growth of transgenic plants remained

unaltered relative to that of control plants. However, root shoot ratio changed, with a tendency to increase shoot dry matter production and decrease root production. No difference was observed in the rate of CO_2 assimilation between control and transgenic plants at low irradiances. However, at high irradiances, the assimilation rate was slightly higher in transgenic plants. Similarly, under saturating CO_2 conditions, the photosynthetic capacity was 15-20% higher in transgenic plants (Micallef et al. 1993). The amount of Rubisco was also found to be doubled and Rubisco carbamylation was increased by 37%. Shoot growth rate as well as development of flowers and fruits was also accelerated in transformed tomato plants. Under normal CO_2 concentration, fruit production was 75% higher in control plants even when there was no change in photosynthesis.

Geigenberger et al. (1995) and Krause (1994) studied the effect of decreased SPS activity in transgenic potato plants carrying either a chimeric antisense or sense (cosuppression SPS gene). In these plants, SPS activity decreased by about 60 to 70%. However, there was no effect on rate of photosynthesis. The ratio between starch and sucrose was altered in favour of starch. Accumulation of soluble sugars was also decreased which was mainly due to decreased carbon partitioning towards sucrose. From these results, it was concluded that SPS is important but is not the only factor regulating the rate of sucrose synthesis. In plants with reduced SPS activity, there was no visible phenotypic difference as well as tuber yield remained unaffected. The above studies on over and under expression of SPS have clearly revealed that SPS has important role to play in carbon partitioning. However, it remains questionable whether the actual rate of photosynthetic sucrose formation does determine final crop yield.

Manipulation of Export of Sucrose

Once sucrose is synthesized in mesophyll cells of source leaves, it has to be translocated to sink tissues. This transport occurs through phloem which contains elongated cells joined by sieve plates, consisting of diagonal cell walls perforated by pores (figure 3). The single cells called sieve elements are surrounded by companion cells. Sieve elements and companion cells in turn are connected to each other by many plasmodesmata. Photoassimilates generated in the mesophyll cells diffuse via plasmodesmata to the phloem parenchyma cells. The further transport of assimilates from here to sieve tubes can occur either symplastically via plasmodesmata without involving translocators (Frommer & Sonnewald 1995) or apoplastically in which photoassimilates are just transported from the source cells via the phloem parenchyma cells to the extracellular compartment, the apoplast. This export does not require any energy as the concentration of sucrose is much higher in source cells than in the apoplast. The apoplastic transport has recently been confirmed by measuring flux of ^{14}C from CO_2 to the potato tuber (Sweetlove et al. 1998). In these experiments, flux and apoplastic sucrose concentration were varied either by changing the light intensity or using transgenic manipulations that specifically affected the source or sink blocks. The elasticity coefficients were used to calculate the flux control coefficients of the source and sink blocks, which were 0.8 and 0.2, respectively, suggesting that the best strategy for manipulation of tuber yield in potato will involve increase in photosynthetic capacity, rather than sink metabolism. Further transport of sucrose from apoplasts to companion cells proceeds via proton symport, which is driven by a proton gradient between the apoplast and the interior of the sieve tube. This gradient is generated by proton pumping ATPase present in the plasma membrane (Bouche-Pillon et al. 1994). The H^+-sucrose translocator involved in phloem loading has recently been identified and characterized in several plants (Frommer & Sonnewald 1995, Grusak et al. 1996). Schulz et al. (1998) inhibited sucrose-proton/cotransporter activity of potato by anti-sense repression of *StSUT1* under control of either a ubiquitously active (CaMV 35S) or a companion cell-specific (*rolC*) promoter in transgenic plants. The transformed plants had reduced levels of the sucrose-transporter mRNA and showed a dramatic reduction in root and tuber growth. Regardless of the promoter used,

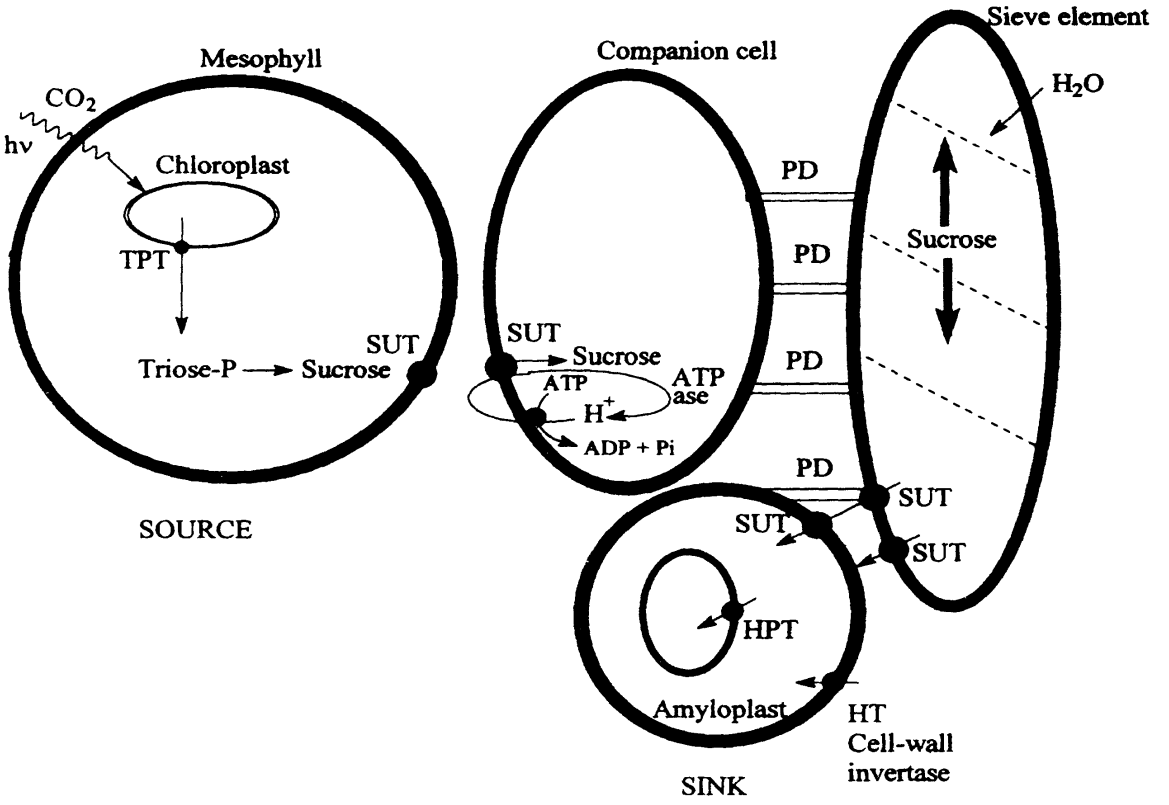

Figure 3 Schematic representation of the membrane proteins involved in the translocation of photoassimilates from the chloroplasts of source leaves via the phloem to the amyloplasts of sink organs. *TPT*, Triose-P translocator; *SUT*, Sucrose translocator; *PD*, Plasmodesmata; *HT*, Hexose translocator; *HPT*, Hexose-P translocator.

source leaves from transformants showed an altered leaf phenotype and a permanent accumulation of assimilates (Kuhn et al. 1996, Burkle et al. 1998, Schulz et al. 1998).

To gain an insight in to the process of sucrose loading, a number of workers (VonSchaewen et al. 1990, Dickinson et al. 1991, Heineke et al. 1992) have used the expression of yeast invertase (*Suc-2*) in the apoplastic space in transgenic tobacco, potato, *Arabidopsis* and tomato plants. In all these cases, significant phenotypic changes were observed e.g. transformed tobacco plants showed stunted growth, suppressed root formation and development of pale and necrotic regions in older leaves. These leaves also accumulated high levels of carbohydrates and starch. From these results, the above workers concluded that expression of invertase in the apoplast intrupts the export of photoassimilates. This caused the phenotypic differences observed in these studies. In symplastic loading, sucrose does not enter into apoplastic space and is not accessible to the invertase. In these cases no phenotypic or physiological change would be expected in transgenic plants. Since major phenotypic changes were observed in transgenic plants, it favours strongly the transport of sucrose via apoplastic mechanism. The isolation of cDNA clones encoding plant sucrose transporters has further helped the investigation for the importance of this transport protein in phloem loading (Riesmeier et al. 1992, 1993). Transporter genes from spinach (*SoSUT1*), potato (*StSUT1*), tomato (*LeSUT1*), and tobacco (*NtSUT1*) have been shown to encode highly hydrophobic proteins having molecular mass of 47 kDa. These proteins structurally belong to the class of metabolite transporters consisting of two sets of six membrane spanning regions, separated by a large cytoplasmic loop (Henderson 1990). Frommer et al. (1994) have further shown these polypeptides to be specifically expressed in

source leaves of sugarbeet. It is also possible that the different sucrose transporters are localized in different cell types, allowing a division of labour, or a phloem loading mechanism which occurs in several steps in various cells (Harrington et al. 1997a, Weber et al. 1997, Tegeder et al. 1999, Kuhn et al. 1999).

In another series of experiments, Riesmeier et al. (1994) transformed potato plants with an antisense construct. The growth of these plants was strongly retarded, mature leaves were curled, bleached and accumulated anthocyanins at the rims, whereas sink leaves did not exhibit any phenotypic changes. Furthermore, flow of sucrose was reduced in antisense plants as measured by the export of soluble sugars through the petiole. This in turn strongly affected the supply of carbohydrates to sink tissues. Tubers were also not formed in these plants showing highest reduction of mRNA for sucrose transport. On quantitative analysis, the soluble carbohydrate content was found to be increased up to 20 fold and the starch up to 10 fold in mature leaves. The rate of photosynthesis was decreased to 50% compared to that of wild type plants. These data are consistent with the assumption that an apoplastic transport step mediated by the sucrose transporter is necessary for the sucrose loading of the phloem. However, the question of involvement of sucrose transporter protein in phloem unloading still remains unresolved. It therefore, seems feasible to manipulate photoassimilate translocation from source to sink tissues by increasing the capacity of phloem loading and unloading. This could be achieved either by overexpression of plant sucrose transporter cDNA under the control of tissue or cell type specific promoters particularly in situations of high irradiance etc., or by heterologous expression of sucrose transporter proteins from different organisms with novel enzymatic characteristics. An attempt has already been made in this direction by Bockmann et al. (1992), who have isolated and analyzed sucrose/H^+ symporter gene from *E.coli*. This transporter was found to have five fold lower K_m for sucrose as compared to plant proteins suggesting that this protein can be an interesting target for overexpression studies in crops. However, major limitation of this approach is the lack of information about the heterologous expression of prokaryotic membrane proteins and their targeting to the plasma membrane. Since active loading of sucrose is driven by the *p*H gradient between the apoplast and the companion cells, increased ATPase activity might result in a higher loading rate, as has been observed in yeast expressing potato sucrose transporter (Riesmeier 1993) where sucrose transporter was found to be two fold higher at *p*H4.5 than at *p*H 5.5.

Phloem cells contain high activity of sucrose synthase (Martin et al. 1993, Nolte & Koch 1993, Yang & Russell 1990, Geigenberger et al. 1993), which breaks sucrose leading to the formation of fructose and UDP-glucose. UDP-glucose is further converted to glu-1-P via the action of UDPG pyrophosphorylase in the presence of Pi. Thus removing PPi would limit the utilization of UDP-glu. This would result in reduced energy gains and inhibition of sucrose loading into the phloem system. To investigate the role of above phenomenon, Lerchl et al. (1995) constructed a chimeric gene using the phloem specific *rolC* promoter of *Agrobacterium rhizogene* to drive the expression of *ppa gene*. Removal of cytosolic PPi in these cells led to an accumulation of photoassimilates in source leaves. Furthermore, there was loss of chlorophyll and reduced plant growth, suggesting that respiratory metabolism is required to maintain phloem function along the transport path and for initial phloem loading (Geigenberger et al. 1996). This sucrose hydrolysis via sucrose synthase is essential for photosynthate partitioning. Lerchl et al. (1995) produced transgenic plants expressing the yeast *suc-2* gene encoding cytosolic invertase in their phloem cells. This was done to bypass the PPi dependent sucrose synthase step. They further made crosses between invertase and pyrophosphatase transgenic plants to combine the two traits. Analysis of offsprings from these crosses revealed that the invertase could complement the phenotypic effects caused by the removal of PPi in phloem cells. The approaches discussed above have made us hopeful to increase crop yield through the manipulation of photoassimilate translocation.

Manipulation of Phloem Unloading

There are again two possibilities for phloem unloading. In symplastic unloading, sucrose reaches the cells of the sink organ directly from the sieve elements via plasmodesmata, whereas, in apoplastic unloading, the sucrose is first transported from the sieve tubes to the extracellular compartment and is then taken up in the cells of the sink organ. Investigations of the plasmodesmatal frequency carried out with electron microscope have indicated that in vegetative tissues, phloem unloading occurs symplastically, whereas in storage tissues, unloading is often apoplastic. In later case unloading may occur by two alternative pathways. In one case sucrose is taken up from the apoplast into the storage cells via a sucrose carrier and converted there through sucrose synthase (Anand & Singh 1986) and UDP-glucose pyrophosphorylase (Kumar et al. 1995) to fructose and glu-1-P, whereas, in the second case, the enzyme invertase hydrolyzes sucrose in the apoplast into glucose and fructose and then transported into the cells via hexose transport systems, where fructokinase and hexokinase convert them into corresponding hexose phosphates. Respective hexose transporter genes have already been isolated from *Arabidopsis* and tobacco, some of which are specifically expressed in sink tissues (Sauer et al. 1990, Sauer & Stadler 1993). Sonnewald et al. (1994) produced transgenic potato plants in which a neutral invertase derived from yeast was expressed in the apoplastic space in a tuber specific manner. This resulted in an increase of up to three fold in the individual tuber fresh weight, decrease in the tuber number per plant and up to 30% increase in total tuber yield. However, there was an accumulation of reducing sugars and a decrease in starch content. When the yeast invertase was expressed in the apoplast in a constitutive manner, the plants hardly grew as they were unable to export their assimilates from the source tissues. This is how the use of organ specific promoters can result in completely different phenotype. Therefore to increase productivity, the enzyme must be expressed only in tubers.

The role of sucrose synthase in determining sink strength has also been investigated by generating transgenic potato plants expressing sucrose synthase antisense RNA corresponding to *sus-4* isoform (Zrenner et al. 1994). Although they used constitutive 35S CaMV promoter to drive the expression of antisense RNA, inhibition of sucrose synthase activity was tuber specific. This inhibition of sucrose synthase had no effect on sucrose content but the level of reducing sugars increased and that of starch decreased in developing potato tubers. These changes were accompanied by a decrease in total tuber weight and a reduction in soluble tuber proteins. These data are thus consistent with the assumption that sucrose synthase is also the major determinant of potato tuber sink strength. Antisense repression of even vacuolar and cell-wall invertase in transgenic carrot affected sucrose partitioning (Tang et al. 1999). In another case, Sonnewald et al. (1997) produced transgenic potato plants over-expressing yeast invertase specifically in the cytosol of tubers. They expected that the irreversible cleavage of sucrose by a heterologous enzyme would lead to an increased sink strength and starch accumulation. Unexpectedly, there was a 10-15% reduction in the starch content of mature tubers of these lines. Biochemical analysis showed that the tubers contained essentially no sucrose, were unchanged in fructose contents, but accumulated large quantities of glucose. This led to the hypothesis that the capacity to metabolize glucose in these lines was not sufficient to keep pace with its production. They, therefore, further introduced a second transgene, encoding a glucokinase from *Zymomonas mobilis* into an invertase-expressing transgenic line, with the intention of bringing the glucose into metabolism (Trethewey et al. 1998, 1999). Transgenic lines thus obtained had up to three fold more glucokinase activity than in parent invertase line. However, there was a further dramatic reduction in starch content, down to 35% of wild-type levels. Biochemical analysis of growing tubers revealed large increases in the metabolic intermediates of glycolysis, organic acids and amino acids, two to three fold increases in the maximum catalytic

activities of key enzymes of respiratory pathways, and three to five fold increses in CO_2 production. Based on these results, the authors concluded that the expression of invertase in potato tubers leads to an increased flux through the glycolytic pathway at the expense of starch synthesis and that heterologous over-expression of glucokinase enhances this change in partitioning. They further demonstrated a significant stimulation of sucrose synthesis, leading to a rapid cycle of sucrose degradation and resynthesis (Trethewey et al. 1999). The labelling pattern indicated that SPS was responsible for the enhanced recycling of the label into sucrose. Based on these results, the authors proposed that the activation of SPS is promoted by the elevated glu-6-P levels in the transgenic tubers. These results indicate the pitfalls of metabolic engineering without a full appreciation of the metabolic system and regulatory circuits present in the tissue under investigation.

Manipulation of Sucrose Metabolism in Sink Tissues

Many a times metabolism/cleavage of sucrose may be a limiting factor in storage tissues and therefore a target for manipulation in order to increase crop yield. As discussed above, there are two enzymes, invertase and sucrose synthase, which are responsible for cleavage of sucrose in storage tissues. Their manipulation in transgenic plants with subsequent effects on plant phenotype and tuber yield etc has been explained above. Hexosyl transferases are the other enzymes which hydrolyse sucrose but are normally not present in potato tubers. On cleavage of sucrose by these enzymes, one part of the molecule is polymerized either to fructans or to glucans depending upon the enzyme. Van Der Meer et al. (1994) produced transgenic potato plants in which fructosyl transferases from *Bacillus subtilis* and *Streptococcus mutans* were overexpressed in the vacoular compartment. There were dramatic losses in the dry matter contents of transgenic tubers expressing these enzymes. However, significant proportions of starch were substituted with fructans in the transgenic tubers.

As stated earlier, products of sucrose synthase activity, UDP-glucose and fructose are to be metabolized further to hexose phosphates which are then translocated into amyloplasts for starch synthesis. These reactions are catalyzed by the enzymes fructokinase, UDP-glucose pyrophosphorylase, phosphogluco isomerase and phosphoglucomutase. Zrenner et al. (1993) reduced the amount of UDP-glucose pyrophosphorylase down to 4% of wild type in potato plants. This did not have any effect on carbohydrate metabolism in tubers indicating that the residual enzyme activities are sufficient for maintaining normal carbohydrate fluxes.

Manipulation of Transport of Metabolites into Plastids

The form in which carbon crosses the amyloplast membrane is still a matter of debate. It is generally accepted that in storage tissues such as the cotyledons or endosperm, hexose phosphates are imported into the amyloplast (Wang et al. 1998). In cereals, glu-1-P is the important substrate (Singh 1992). However, in barley and maize, it has been reported that ADP-glucose pyrophosphorylase activity is located outside the plastid (Smith et al. 1997) and ADP-glucose is specifically transported into the plastid by a protein encoded at the *brittle 1* locus in maize (Kleczkowski 1996, Cao et al. 1995). Elimination of this protein resulted in a five fold decrease in the rate of starch synthesis and a twelve fold increase in the amount of ADP-glucose in the endosperm (Shannon et al. 1996), suggesting that ADP-glucose is synthesized in the cytosol and is translocated in the amyloplast by the product of *BT-1* gene. In pea, carbon enters in the amyloplast in the form of glucose-6-P (Hill & Smith 1991). Similar is the situation in developing seeds of *Brassica napus* (Kang & Rawsthorne 1994, Singh 1998). This needs detailed biochemical and molecular analysis to elucidate the importance of carbon import into amyloplasts for the starch yield in crops (Romero et al. 1999). Unfortunately, neither a hexose-P translocator nor a corresponding gene has so far been isolated from crop plants. Since *brittle 1* (*bt1*) mutant with reduced starch content was known in maize,

corresponding cDNA for the *bt1* locus has been isolated and analyzed (Sullivan et al. 1991). The predicted *bt1* protein has been shown to be hydrophobic with N-terminus sequence characteristic of a plastid transit peptide. The protein showed some homology with mitochondrial transport proteins and yeast ATP/ADP translocator, suggesting that *bt1* might be encoding an amyloplast metabolite transporter. Under *in vivo* conditions, the *bt1* protein was imported into the inner envelope of purified plastids (Li et al. 1992). However, this needs further investigations. Again, the transgenic plants which either overexpress *bt1* or have reduced mRNA levels for *bt1* will help us in understanding the importance of this protein for metabolite transport into amyloplasts.

Manipulation of Starch Biosynthesis in Storage Tissues

Starch is the major form of carbon reserves in plants. It consists of two different polymers-amylose and amylopectin arranged into a three dimensional, semicrystalline structure- the starch granule (Singh 1989, 1990). Amylose is a linear polymer of $\alpha 1 \rightarrow 4$ linked glucose residues with molecular weight of about 10^4-10^5 and makes up about 30% of starch. This proportion, however, varies depending upon plant species/varieties, plant organ, the developmental age of that organ and also the growth conditions of the plant. Amylopectin, on the other hand, consists of highly branched glucan chains and makes up about 70% of starch. Amylopectin with molecular weight of about 10^7, consists of short chains of $\alpha 1 \rightarrow$ - D-glucopyranose units primarily linked by $\alpha 1 \rightarrow 4$ bonds with $\alpha 1 \rightarrow 6$ branches.

Starch biosynthesis in sink tissues involves three committed enzymes, namely ADP-glucose pyrophosphorylase (ADPGPPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21) and starch branching enzyme (SBE; EC 2.4.1.28). Both amylose and amylopectin are synthesized from ADP-glucose which in turn arises from glu-1-P and ATP in a reaction catalyzed by ADPGPPase. In non-photosynthetic sink tissues, glu-1-P is either imported directly from cytosol or synthesized in the amyloplast (site for starch synthesis in storage organs) from glu-6-P (figure 4) via the action of plastidial phosphoglucomutase. The other reactant ATP is also imported from cytosol probably by ADP/ATP translocator (Martin & Smith 1995, Smith et al. 1997, Preiss 1997, Wang et al. 1998). Pyrophosphate produced in the reaction catalyzed by ADPGPPase is hydrolyzed by alkaline pyrophosphatase (Kumar & Singh 1983), displacing the equilibrium of the reaction in favour of ADP-glucose synthesis. In the next step of starch synthesis, SS catalyzes the synthesis of $\alpha 1 \rightarrow 4$ linkage between the nonreducing end of a pre-existing glucan chain and glucosyl moiety of ADP-gluose releasing ADP. The $\alpha 1 \rightarrow 6$ branches in starch polymers are made by SBE. This enzyme hydrolyzes the $\alpha 1 \rightarrow 4$ linkage within the chain and then catalyzes the formation of $\alpha 1 \rightarrow 6$ linkage between the reducing end of the "cut" glucan chain and 6th carbon of a glucose residue in the other $\alpha 1 \rightarrow 4$ linked glucan chain. Branches are not created randomly but show an average periodicity of 20 glucose residues.

A number of genetic and biochemical studies conducted with mutants of maize have proved that the ADP-glucose pathway as stated above is the important pathway for starch synthesis. Shrunken-2 and brittle-2 mutants of maize deficient in ADPGPPase were also found deficient in starch (Dickinson & Preiss 1969a, 1969b). Similarly, a pea line having recessive *rb* gene (gene controlling the level of ADPGPPase activity), had only 38 to 70% of the starch found in normal pea line (Smith et al. 1989). In *Arabidopsis thaliana*, mutants containing 2% of the ADPGPPase activity compared to normal strain also had less than 2% of the starch (Lin et al. 1988a).

ADPGPPase is the best studied committed enzyme of the starch synthesis. Different properties of both plant and bacterial ADPGPPase such as subunit structure, substrate and allosteric kinetics, allosteric and substrate binding sites and regulation etc have extensively been studied and reviewed (Preiss 1982, 1984, 1991, Preiss & Romeo 1994, Preiss & Sivak 1996,

Sivak & Preiss 1995, Smith et al. 1997, Preiss 1997). The enzyme has been purified from several photosynthetic and non-photosynthetic sources, its kinetic properties studied and the genes that encode it have been cloned from several sources. The enzyme supplies ADP-glucose for starch biosynthesis and under some conditions, its activity is the most important factor in determining the rate of starch accumulation. This has been established unequivocally in leaves of *Arabidopsis*, where a mutation in a gene encoding one of the two subunits of enzyme reduced ADPGPPase activity to 7% and the starch accumulation to 26% of the normal values in leaves (Lin et al. 1988b). The enzyme is strongly regulated by 3-PGA and Pi; 3-PGA being the activator and Pi being the inhibitor. These allosteric properties of enzyme have gained great importance in determining its role in controlling the rate of starch synthesis in all orders of the plants (Plaxton & Preiss 1987, Preiss 1991, 1996, Preiss et al. 1991, Okita 1992, Singh 1992, Martin & Smith 1995).

Antisense RNA technique has been used extensively for down regulation of expression levels of this enzyme in both leaves and tubers of potato plant (Muller-Rober et al. 1992). In these experiments, the leaf ADPGPPase decreased to 5-30% of the wild type activity, whereas an even stronger reduction (down to about 2%) was

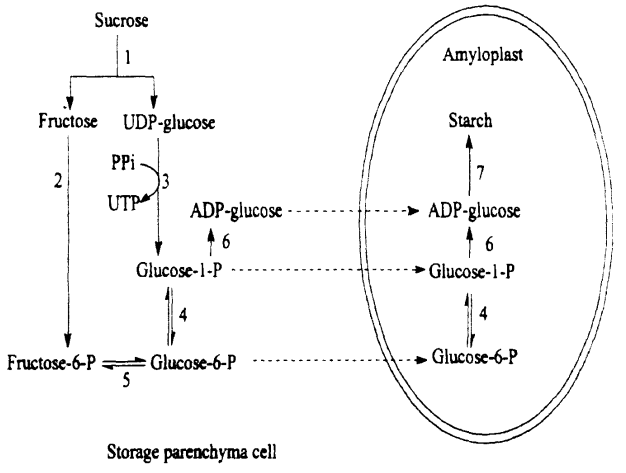

Figure 4 Carbon metabolism in sink organs of crop plants. **1,** Sucrose synthase; **2,** Hexokinase; **3,** UDP-glucose-pyrophos-phorylase; **4,** Phosphoglucomutase; **5,**Phosphohexose isomerase; **6,**ADP-glucose pyrophosphorylase; **7,** Starch synthase

observed in developing tubers. Depending upon the growth conditions, starch level in leaves was about 10 to 50% of the wild type level, whereas, only about 5-35% of the wild type starch was detected in developing tubers. The loss of starch in transgenic tubers was partially compensated for by an increase in the level of soluble sugars mainly sucrose and glucose. The total tuber dry weight was reduced by around 30-40% as compared to controls, proving that constitutive inhibition of starch synthesis results in decreased yield of potato tubers. However, it was not clear from these experiments whether the reduction in tuber yield was induced by the reduced capacity of the tubers to form starch or by the combined inhibition in the leaves and tubers. This has been clarified by inhibiting ADPGPPase activity in a tuber specific manner using the patenting *B-33* promoter. In these plants as well, tubers accumulated lower levels of starch and increased amounts of soluble sugars. This was reflected in reduced total tuber yield in these plants.

A major breakthrough with respect to starch content has been reported by Stark and coworkers (Stark et al. 1992). They introduced *glgC-16* gene from *E. coli* encoding a mutated form of ADPGPPase into potato tubers. In transgenic potato, this gene fused to a transit peptide of small subunit of ribulose-1,5-bisphosphate carboxylase gene from *Arabidopsis* and controlled by a tuber specific patenting promoter resulted in 35% more starch than in controls and amounted to an increased starch content of nearly 60% in the best lines. Detailed experiments with different lines of transgenic potato further revealed that increased starch synthesis in transformed tubers was obtained mainly due to non- allosteric nature of the bacterial enzyme introduced in transgenic plants. Similar results were obtained in tobacco calli where *glgC* gene increased starch levels over the controls lacking this gene by about 1.7 to 8.7 fold. The increase in starch yield owing to the overexpression of the *glgC-16* ADPGPPase and the formation of sugars due to antisense inhibition of resident ADPGPPase proves the key regulatory function of this enzyme in starch metabolism. These experiments further demonstrate that over

expression of a single enzyme can help us to increase the production of an important compound resulting into higher yield.

Conclusions and Future Outlook

From the information contained in this review, it is evident that most of the molecular approaches undertaken to increase crop yield are either related to increasing carbon export to photosynthetic active organs or to optimizing carbon utilization in storage organs. Though agronomists have long realized that yield is not related to maximal photosynthetic rates yet a large number of earlier investigations concentrated on improving the photosynthetic efficiency of carbon assimilation. The discovery of C_4 pathway of photosynthesis in late 60's in crop species possessing higher photosynthetic rates and little or no photorespiration gave us a hope that perhaps photosynthetic efficiency in C_3 crops could be improved by incorporating some important features of C_4 pathway of carbon assimilation. Earlier studies in this direction concentrated mainly on finding mutants among C_3 crop species with reduced photorespiration. However, all attempts made in this direction proved futile and most research aimed at reducing photorespiration in C_3 species indicated little possibility of improving net photosynthesis. Similarly, a large number of chemicals employed for inhibition of one or the other steps of photorespiratory cycle failed to increase dry matter yield in C_3 plants. Even the goal of interspecific hybridization to manipulate C_4 photosynthesis for improved crop productivity could not be achieved. However, the variability in both metabolism and crossability in plants differing in degree of C_4 has improved the prospects of understanding the genetics of C_4 photosynthesis and its use in improving photosynthetic productivity. This area, therefore, needs more research efforts in this millennium. Efforts made to produce a better Rubisco with improved CO_2/O_2 specificity without reducing the catalytic activity of the enzyme did not yield expected results. Though the structure, mechanism, molecular biology and physiology of Rubisco are better understood today, these studies did not help us to manipulate Rubisco through genetic engineering approaches. However, recent development of a method for transforming chloroplastic genome of crop plants has opened new chapter in the mutagenic studies of Rubisco for designing an enzyme with altered active site for better CO_2/O_2 specificity. Such a change in the structure of the enzyme may help us in engineering a better Rubisco which could enhance photosynthetic efficiency by suppression of photorespiration.

Genetic engineering has successfully been employed to produce transgenic plants in which various components of source-sink interactions have been manipulated. This has allowed us to assess the *in vivo* function and contribution (fluxes) of individual steps to overall synthesis and partitioning of assimilates. Control analysis of the various enzymes involved in different pathways has revealed many interesting features of carbon metabolism in higher plants. Some of these are: (a) Control is shared among several enzymes of the pathway and that the distribution of control changes with changes either in short term experimental conditions or plant growth conditions. Moreover, the changes in one step are compensated by changes elsewhere in the system indicating that plant metabolism is highly flexible and alternative pathways exist to compensate changes made at one particular step, (b) Enzymes that catlyze irreversible reaction and possess regulatory properties have low flux control coefficients suggesting that control does not necessarily reside with highly regulated enzymes. These enzymes generally operate at a fraction of their catalytic activity. In the wild types, the fine regulation allows their activity to respond to flux changes that have been initiated elsewhere in the pathway, (c) Enzymes catalyzing freely reversible reactions and achieving very high unidirectional rates of catalysis such as phosphoglucomutase, phosphogluco isomerase, aldolase etc. can also exert control when their activity is reduced below 50% of their wild type, (d) Coordination of fluxes into various pathways would be impossible if each is controlled by a quasi-independent enzyme with a very high flux control coefficient and no regulatory interactions with other pathways. These factors have

complicated the overall process of carbon allocation and it is difficult to test directly the importance of an enzyme or a process which have direct bearing on plant productivity. This has amply been demonstrated when biomass production was found to be unrelated to antisense FBPase and antisense *rbcS* in potato and tomato plants, respectively. Even photosynthate partitioning can be altered without having drastic effects on growth in many conditions. Hence the probability of obtaining a desired change diminishes as the number of steps between the manipulation and the goal increases. However, the continued use of transgenic technology would certainly lead to further significant advances in the understanding of various steps of pathways involved in source-sink interactions for better plant productivity. Furthermore, the whole process needs detailed investigations under field conditions before the impact of these changes on crop yield could uneqivocally be established. However, increase in knowledge that has become available through the application of molecular approaches to source-sink interactions in crop plants has laid a strong basis for further progress possibly through combination of several constructs within one plant. Hence, the future work will build on these earlier experiments as the day is not far off when the scientists engaged in crop improvement will be able to break the yield barriers through the use of molecular approaches complemented with conventional breeding techniques.

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