Role and significance of sucrose-6-phosphate phosphatase in regulating sucrose biosynthesis and carbon partitioning in photosynthetic and non-photosynthetic tissues

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Contents

1 Introduction

Photosynthesis is a process whereby plants, algae and some prokaryotes directly use light energy to synthesize organic compounds. This makes it the most important biological process on earth because it provides the biomass which is not only used as food either directly or through herbivorous animals but also is the principle component of a lot of industrial materials and fossil fuels. Beyond that it produces as a by-product the oxygen which is required for aerobic respiration activity by all multicellular and many unicellular organisms. In higher plants, carbon assimilation during photosynthesis is followed by partitioning of newly fixed carbon within the cells and resource allocation between tissues. The process of carbon assimilation and partitioning is believed to be a major determinant of crop yield (Gifford *et al*., 1984). Dispite intensive research in this area the regulation of these processes is not sufficiently understood. Modern molecular biology provides a powerful tool to dissect and understand the driving forces behind regulation of assimilation and partitioning of carbon and nitrogen. A better understanding of these processes might in turn help to devise new breeding strategies to increase crop yield.

1.1 *Sink and source concept*

In higher plants carbon fixed during photosynthesis is either directly metabolised to provide energy and carbon skeletons for the cell's own respiration and growth, or it is exported, mainly in form of sucrose, to other organs to support their growth and development and/or to provide assimilates for the synthesis of storage compounds. Therefore, the organs in higher plants can be generally divided into source and sink organs (Turgeon, 1989). Source organs that are usually photosynthetically active are defined as net exporters of photoassimilates, represented mainly by mature leaves, and sink organs that are photosynthetically inactive are referred to as net importers of fixed carbon. Sinks can be further divided into at least two different classes: utilization and storage sinks. Utilization sinks are highly metabolically active, rapidly growing tissues such as meristems and immature leaves, while storage sinks are the organs like tubers, seeds and roots, where the imported carbohydrates are deposited in the form of storage compounds (*e.g.* starch, sucrose, fatty acids, or proteins) (Sonnewald and Willmitzer, 1992). The storage sinks are usually specialized for other essential processes, such as mineral acquisition (roots) or reproduction (seeds, fruits and potato tubers). Metabolic sink or source status of a particular organ is under developmental control. For example, immature

leaves are metabolic sinks while after maturation leaves become photosynthetically active sources. Growing potato tubers are storage sinks, however during sprouting they turn into source organs where the stored compounds are mobilised to provide transportable organic nutrients for the growth of the buds. The evolution of sink and source organs in higher plants generates the need of resource allocation between sink and source organs, which is a major determinant of plant growth and productivity (Gifford *et al*., 1984). Accordingly, two general strategies can be envisaged to improve crop yields. One strategy is to increase source capacity namely the rate of carbon export. Potential targets to improve source capacity have been proposed including: (a) the rate of photosynthesis, (b) the rate of carbon translocation via specific carrier proteins, (c) partitioning of photoassimilates between anabolism and catabolism, and (d) the rate of sucrose synthesis (Sonnewald and Willmitzer, 1992). The other strategy is to increase sink strength which has been considered as a product of sink size and sink activity (Sonnewald and Willmitzer, 1992). Sink size is genetically determined in some plants *e.g.* several wheat varieties (Ho, 1988) while it is more flexible in case of tuber- or storage root-forming plants like potato or sugar beet (Sonnewald and Willmitzer, 1992). With respect to sink activity, several strategies to create a chemical gradient as well as an osmotic gradient between the sinks and sieve elements to increase the amount of photoassimilates imported into the sink tissue, have been discussed. For instance accelarated utilization of the imported sucrose for production of storage compounds like starch (Stark *et al*., 1992), or conversion of sucrose into a compound no longer in equilibrium with sucrose (e.g. hexose) (Sonnewald *et al*., 1997), or compartmentation of the sucrose within symplastic or apoplastic pools (Sonnewald and Willmitzer, 1992).

1.2 *Carbon partitioning between starch- and sucrose-synthesis in source leaves*

Besides the above mentioned assimilate allocation between sink and source organs, carbon fixed during photosynthesis has also to be partitioned between starch synthesis in the chloroplast for temporary storage and sucrose synthesis in the cytosol for export within the mesophyll cell (Stitt *et al*., 1987; Stitt, 1990b; Stitt, 1996). The principal biochemical pathway of photoassimilate partitioning in source leaves is given in Fig. 1-1.

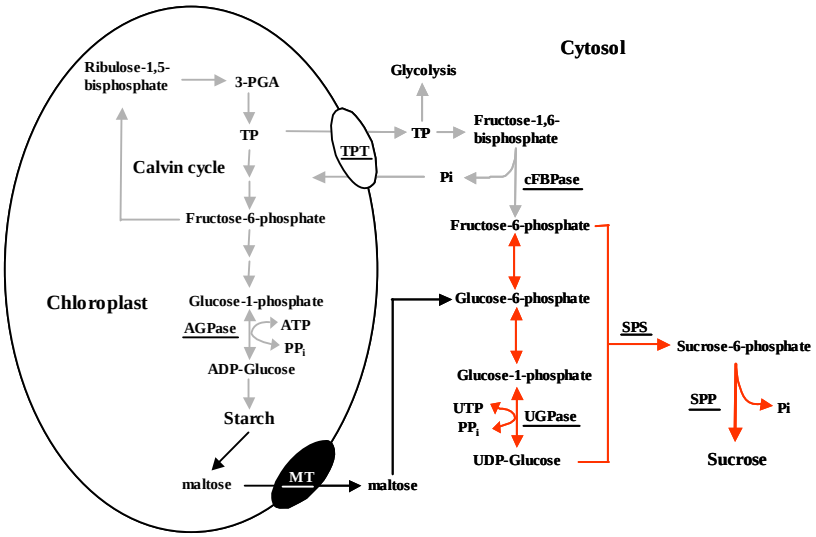


Fig. 1-1: Schematic representation of sucrose and starch synthesis in a photosynthetically active mesophyll cell. 3-PGA: 3-Phosphoglycerate; AGPase: ADP-Glucose pyrophosphorylase; cFBPase: cytosolic fructose-1,6-bisphosphatase; MT: maltose transporter; Pi: inorganic phosphate; PPi: inorganic pyrophosphate; SPS: sucrose-phosphate synthase; SPP: sucrose-phosphate phosphatase; TP: triose-phosphate; TPT: triose-phosphate translocator; UGPase: UDP-Glucose pyrophosphorylase. The grey arrows represent reactions occuring in the light, the black arrows represent the reactions in the dark and the red arrows represent the reactions in both the light and dark. The enzymes are underlined.

Triose-phosphates synthesized in the chloroplast during photosynthesis can either be transported into the cytosol by the inner chloroplast membrane localized triose-phosphate translocator (TPT) to mainly feed into sucrose synthesis, or they can be retained within the chloroplast and used for starch synthesis. These two pathways are highly coordinated in source leaves. During the course of photosynthesis rising sucrose levels lead to posttranscriptional inactivation of sucrose-phosphate synthase (SPS), one of the regulatory enzymes in the pathway of sucrose synthesis (Stitt *et al*., 1988). When sucrose accumulates during the day, feedback control of SPS in turn leads to the inhibition of sucrose synthesis and consequently results in the accumulation of phosphorylated intermediates and depletion of Pi. ADP-Glucose pyrophosphorylase (AGPase) catalyzes the first committed step in the pathway of starch synthesis. The enzyme from higher plants is a heterotetramer composed of two “regulatory”, (AGPS, 51 kD) and two slightly smaller “catalytic” (AGPB, 50 kD) subunits, respectively (Morell *et al*., 1987; Okita *et al*., 1990). AGPase activity is under allosteric control with 3-PGA as an activator and Pi as an inhibitor (Sowokinos, 1981; Sowokinos and Preiss, 1982; Preiss, 1988). The rising 3-PGA to Pi ratio due to the feedback inhibition of

sucrose synthesis eventually activates AGPase, leading to a stimulation of starch synthesis (Stitt *et al.*, 1987). Down-regulation of TPT and cFBPase in transgenic plants leads to the inhibition of sucrose synthesis and concomitantly to a stimulation of starch synthesis via accumulation of 3-PGA (Heineke *et al.*, 1994; Zrenner *et al.*, 1996; Strand *et al.*, 2000), providing additional evidences in support of this biochemical model. However, other studies demonstrated that starch synthesis could also be uncoupled from the changes in the content of 3-PGA in leaves. For example, starch synthesis was stimulated without an increase in 3-PGA level when sugars were fed to detached spinach leaves (Krapp *et al.*, 1991) or when spinach leaves were cold-girdled to decrease export (Krapp and Stitt, 1995). On the other hand, the augmented levels of 3-PGA in the transgenic potato plants with antisense repression of cytosolic phosphoglycerate mutase did not lead to any increase of starch in their leaves (Westram *et al.*, 2002). Recent studies revealed another mechanism involving the posttranslational regulation of AGPase in both photosynthetic and non-photosynthetic tissue in a range of plant species (Fu *et al.*, 1998; Tiessen *et al.*, 2002; Hendriks *et al.*, 2003). This mechanism involves the redox regulated, reversible formation of an intermolecular Cys bridge between the AGPB subunits in the AGPase heterotetramer. Monomerization in response to light or rising sugar levels leads to activation of the enzyme, which allows starch synthesis to be increased without any necessary changes in the levels of 3-PGA and Pi (Hendriks *et al.*, 2003). The presence of this redox regulatory mechanism increases the flexibility of the regulatory network involving the control of photosynthetic carbon partitioning. It is assumed that the main significance of the allosteric regulation of AGPase is more likely to rapidly increase Pi recycling in the stroma in case of transient imbalance between photosynthesis and triose phosphate export, although it may in some conditions be part of a regulatory sequence that links sugar accumulation to an increase of starch synthesis (Hendriks *et al.*, 2003). The redox regulation of AGPase seems more likely to participate in the direct light activation of starch synthesis and allows starch synthesis to be stimulated upon the accumulation of sugars in leaves (Hendriks *et al.*, 2003).

1.3 *Sucrose synthesis in source leaves*

In most higher plants, sucrose is the main form of carbohydrates transported from source to sink tissues. As mentioned above, during photosynthesis triose-phosphates are exported into the cytosol and subsequently converted into sucrose (Fig. 1-1). Photosynthetic sucrose

synthesis in source leaves is assumed to be regulated at the two enzymatic steps catalyzed by cytosolic FBPase (cFBPase) and sucrose-phosphate synthase (SPS), respectively. cFBPase, representing the first regulatory step in photosynthetic sucrose synthesis, converts Fruc-1,6-bisP into Fruc6P (Khayat *et al*., 1993; Daie, 1993). This enzyme is subject to allosteric control by the signal metabolite Fructose-2,6-bisphosphate (Fruc-2,6-bisP) which acts as a potent inhibitor of enzymatic activity (Stitt, 1990a). Early studies in transgenic potato and *Arabidopsis* plants with antisense repression of cFBPase or a mutant line of *Flaveria linearis*, respectively, showed that a reduction of this enzyme strongly impaired the photosynthetic sucrose synthesis, thus demonstrating its importance in the control of the pathway (Zrenner *et al*., 1996; Strand *et al*., 2000; Sharkey *et al*., 1988). The formation of sucrose-6-phosphate (Suc6P) from UDP-Gluc and Fruc6P catalyzed by SPS represents the second regulatory step in the pathway of photosynthetic sucrose synthesis (Huber *et al*., 1985; Stitt and Quick, 1989). SPS is known to be highly regulated by a hierarchy of several interacting mechanisms. Firstly, transcription is under developmental control or responds to changes in irradiance (Klein, *et al*., 1993). Secondly, SPS activation is modulated by reversible protein phosphorylation at multiple sites (Huber and Huber, 1996). In spinach leaves phosphorylation of two distinct seryl residues, Ser-158 and Ser-424, is required for inactivation of the enzyme in the dark (Huber and Huber, 1992) and activation by water stress (Toroser and Huber, 1997), respectively. Thirdly, SPS activity is subject to allosteric modulation by metabolic effectors with Gluc6P as an activator and Pi as an inhibitor (Doehlert and Huber, 1984). Moreover, Gluc6P and Pi can also inhibit SPS-kinase and SPS protein phosphatase, respectively, thus affecting the regulation of SPS by phosphorylation/dephosphorylation (McMichael *et al*., 1995; Weiner *et al*., 1993). Lastly, SPS can interact with 14-3-3 proteins, a group of small acidic regulatory proteins, in a phosphorylation-dependent way although the biochemical implication of this protein-protein interaction is not clear yet as both inactivation and activation of the enzyme have been observed (Toroser *et al*., 1998; Moorhead *et al*., 1999).

Sucrose-6-phosphate phosphatase (SPP; EC 3.1.3.24) catalyzes the final step in the pathway of sucrose synthesis, hydrolyzing Suc6P to sucrose and Pi. The reaction catalyzed by SPP is essentially irreversible and displaces the reversible SPS reaction from equilibrium into the direction of net sucrose synthesis (Stitt *et al*., 1987). Early reports suggested that SPP activity *in vivo* is substantially higher than SPS activity, indicating that SPP is unlikely to contribute to control of flux through the pathway of sucrose synthesis in plants (Hawker and Smith, 1984). Consequently, less attention has been paid to this enzyme. However, recent studies showed that SPS activity was enhanced in the presence of SPP and migration of the

two proteins on nondenaturing gels was altered in the presence of the other, indicating that SPS and SPP might associate to form a complex in vivo (Salerno *et al*., 1996; Echeverria *et al*., 1997). This promotes the re-examination of the role of SPP in control of sucrose synthesis and also opens the possibility that SPP might play a role in metabolite channeling between the two enzymes. It was not until recently that the enzyme has been purified to homogeneity from rice leaves, and the gene cloned (Lunn *et al*., 2000). Biochemical characterization revealed that the rice leaf enzyme exists as a dimer of 50 kDa subunits and is highly specific for Suc6P (Lunn *et al*., 2000). Genes encoding SPP have as yet been identified from several plant species, including *Arabidopsis*, maize, tomato, rice, barley and wheat and it turns out that multiple isoforms of SPP are present in each species, e.g. four members in *Arabidopsis* and three in rice, respectively (Lunn, 2003). N-terminal region of plant SPP shows significant similarity to proteins from the haloacid dehalogenase (HAD) superfamily of phosphatases/hydrolases (Aravind *et al*., 1998; Lunn *et al*., 2000). Three conserved motifs that are associated with the active site are found in the members of this family. The first motif contains the sequence DXDX(T/V), in which the first Asp residue is assumed to form a phosphoacyl intermediate during catalysis (Collet *et al*., 1998; Lunn, 2003). SPP genes have also been isolated from cyanobacteria (Lunn *et al*., 2000; Cumino *et al*., 2001). However, prokaryotic SPP proteins only contain the approximately 260-aa HAD phosphatase domain and lack the approximately 160-aa C-terminal domain of plant SPPs (Lunn and MacRae, 2003). Given the fact that plant SPPs are dimeric while cyanobacterial SPPs are monomeric, a hypothesis has been put forward that the C-terminal domain of plant SPPs might be involved in dimerisation of the enzyme (Lunn and MacRae, 2003). Amino acid comparison revealed that the catalytic domain of SPP has some similarity (about 35 %) to the C-terminal region of SPS and it has been suggested that the SPP-like domain of SPS might be involved in SPS-SPP complex formation, comparable to the trehalose-synthesizing complex in yeast which is, among others, composed of trehalose-phosphate synthase (TPS) and trehalose-phosphate phosphatase (TPP) (Lunn *et al*., 2000).

1.4 *Phloem loading and long-distance transport of sucrose*

The evolution of sink and source tissue systems generated a need for long-distance transport between these specialized tissues, which is carried out by the phloem cells of the plant's vascular system. To accomplish long-distance transport from source to sink, sucrose has to be loaded into the phloem in source tissues. In most plant species such as *Arabidopsis*, tobacco

and potato, compelling evidence suggest sucrose to be loaded into the phloem via an apoplastic route (Gottwald *et al*., 2000; Kühn *et al*., 1997; Riesmeier *et al*., 1992; Riesmeier *et al*., 1994; Stadler *et al*., 1995; von Schaewen *et al*., 1990). In the apoplastic phloem loading route, sucrose is released into the extracellular space somewhere in the proximity of the phloem, where it diffuses through the cell wall milieu. After reaching the sieve element and companion cell (SE/CC) complex, sucrose is then loaded against a concentration gradient into the phloem via the well-characterized plasmalemma-bound sucrose/proton symporter (Riesmeier *et al*., 1992; Riesmeier *et al*., 1994; Stadler *et al*., 1995; Kühn *et al*., 1997; Gottwald *et al*., 2000). However, in some species like *Coleus blumei* and *Cucumis melo* (melon), evidences suggest sucrose loading into the phloem might be symplastic (Turgeon and Wimmers, 1988; van Bel, 1987; Ayre et al., 2003). In symplastic phloem loaders, sucrose is supposed to be transported from cell to cell via plasmodesmata – nanopores lined by plasma membrane that bridge the cytoplasm of most plant cells to their neighbors, and eventually released into the SE/CC. In both loading routes, once sucrose is loaded into sieve tubes in the source organs, water is drawn in osmotically across the cell membranes, leading to a building up of physical pressure. The hydrostatic pressure then drives mass flow transport to less pressurized regions of the plant (sink organs) following the sieve tubes (Münch, 1930).

1.5 *Sucrose unloading and metabolism in sink organs*

After reaching the sink tissues, water and sucrose move out from the phloem to the surrounding cells, where sucrose is utilized. Evidences from the use of transport inhibitors (Schmalstig and Geiger, 1985), anoxia treatment (Turgeon, 1987), feeding experiments (Schmalstig and Geiger, 1987) and the analysis of plasmodesmal “size exclusion limit” in sink leaves (Imlau et al., 1999; Oparka *et al*., 1999) suggest that symplastic unloading of sucrose from the phloem to heterotrophic sink tissues through plasmodesmata connections constitutes the principal unloading route. Although the symplastic unloading route is predominant, apoplastic route is inevitably required in some cases e.g. unloading to the filial tissue in developing seeds that has no symplastic connection with the maternal tissue (Patrick, 1997). In the apoplastic unloading pathway, sucrose released into the apoplast can either directly enter the heterotrophic cells via a plasmamembrane-bound sucrose transporter or be hydrolyzed into glucose and fructose by cell wall-bound invertase (cw-Inv) that are subsequently imported into the cells via a hexose transporter located in the plasmamembrane (Bush, 1999). In some organs, sucrose phloem unloading mode is also under developmental

control. For example, in potato tubers, during the early stages of tuberization apoplastic unloading of sucrose into swelling stolons is predominant, followed by cleavage of sucrose into glucose and fructose with the action of cell wall invertase (Appeldoorn *et al*., 1997; Viola *et al*., 2001). After tuberization, sucrose imported from the leaves is delivered into growing tubers essentially via symplastic phloem unloading (Viola *et al*., 2001). The different phloem unloading routes are illustrated in Fig. 1-2.

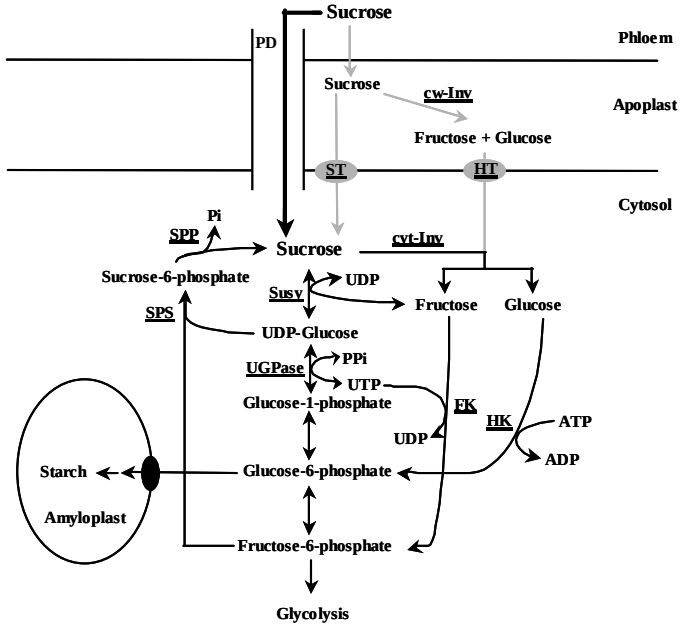


Fig. 1-2: Possible routes for sucrose unloading and pathways of sucrose metabolism in sink tissues. cw-Inv: cell-wall invertase; cyt-Inv: cytosolic invertase; FK: fructokinase; HK: hexokinase; HT: hexose transporter; Pi: inorganic phosphate; PPi: inorganic pyrophosphate; SPP: sucrose-phosphate phosphatase; SPS: sucrose-phosphate synthase; ST: sucrose transporter; Susy: sucrose synthase; UGPase: UDP-glucose pyrophosphorylase. The black arrows represent the major flux of phloem unloading in sink tissues and the grey arrows represent the minor flux. The enzymes are underlined.

Once sucrose or hexoses, respectively, enter heterotrophic cells, they are further metabolised to provide energy and carbon skeletons for development and growth of the sink organs. In storage sinks such as potato tubers, a large proportion of the imported sucrose is metabolised and converted to storage compounds *e.g.* starch. Inevitably, sucrose metabolism in the sink organs plays an important role in determining sink strength. Potato tubers are ideal storage sinks to study sink strength and hence have been subject to intensive studies for a long time. As mentioned above, apoplastic unloading of sucrose is predominant in swelling stolons

and the sucrose is then cleaved into glucose and fructose by the action of cell wall invertase (Appeldoorn *et al*., 1997; Viola *et al*., 2001). In the growing tuber, sucrose is mainly unloaded from the phloem via plasmodesmata and subsequently cleaved within the cytosol mainly by sucrose synthase (Susy) leading to the formation of UDP-Gluc and fructose, respectively (Geigenberger and Stitt, 1993). Accordingly, Susy activity markedly increases during tuberization whereas cell wall invertase levels dramatically decrease (Appeldoorn *et al*., 1997). UDP-Gluc can be further converted into Gluc1P via UGPase and fructose is readily phosphorylated into Fruc6P by fructokinase. The resulting hexose phosphates are equilibrated by the action of cytosolic isoforms of phosphoglucose isomerase and phosphoglucomutase, and Gluc6P is subsequently transported into amyloplast and used for starch synthesis (Kammerer *et al*., 1998; Tauberger *et al*., 2000). Antisense repression of Susy in transgenic potato tubers led to a reduced starch content and a decrease in dry weight of total tuber per plant, suggesting that sucrose synthase plays an important role in determining the sink strength in potato tubers (Zrenner *et al*., 1995). Although the net flux in the growing tubers is towards sucrose degradation, sucrose (re)synthesis is concomitantly active with a considerable rate within this tissue (Geigenberger and Stitt, 1993; Geigenberger *et al*., 1997). Two different pathways can contribute to sucrose (re)synthesis within the growing tubers either via the reversible reaction catalyzed by Susy, or via the sequential actions of SPS and SPP using Fruc6P and UDP-Gluc as substrates (Geigenberger and Stitt, 1993). Consequently, a futile cycle between sucrose degradation and (re)synthesis is formed in the growing tubers, which has also been observed in other plant tissues (Dancer *et al*., 1990a; Geigenberger and Stitt, 1991; Wendler *et al*., 1990; Hill and ap Rees, 1994). Although the existence of sucrose (re)synthesis is well known, its function in the growing tubers is not yet fully understood. An idea has been put forward in that simultaneous presence of sucrose (re)synthesis and sucrose degradation in growing tubers could facilitate sensitive regulation of sucrose mobilisation in response to changes in supply and demand of sucrose (Geigenberger *et al*., 1995). Down-regulation of Susy with an antisense approach resulted in a reduced starch content in the tubers (Zrenner *et al*., 1995). However, this result can be well interpreted by the role of Susy in sucrose degradation rather than that in sucrose (re)synthesis. The function of sucrose (re)synthesis via SPS and SPP was also investigated in the transgenic tubers with reduced SPS activity, which proves to be dispensable under normal growth conditions and has only a minor influence on starch metabolism (Geigenberger *et al*., 1999). However, this pathway turns out to be important for the ability of growing tubers to cope with water deficits (Geigenberger *et al*., 1997; Geigenberger *et al*., 1999). It is well known that changes in carbon metabolism in

leaves or growing tubers are induced in response to water deficiency, including increased sucrose synthesis and decreased starch synthesis (Morgan, 1984; Quick *et al*., 1989; Zrenner and Stitt, 1991; Oparka and Wright, 1988; Geigenberger *et al*., 1997). Moderate water stress stimulates sucrose synthesis in growing tubers by activating SPS, which in turn leads to a decrease in metabolite levels, in particular 3-PGA. The decrease in 3-PGA level probably results in inhibition of AGPase and consequently decreases the rate of starch synthesis (Geigenberger *et al*., 1997; Geigenberger *et al*., 1999).

In stored potato tubers, sucrose metabolism can also be strongly induced under certain environmental conditions. For example, low temperature (4-6°C), widely used to prevent potato tubers from sprouting during storage, leads to induction of starch degradation and stimulation of sucrose synthesis (Lorberth *et al*., 1998; Krause *et al*., 1998;). The resulting sucrose is transported into vacuole where it is further cleaved into glucose and fructose by a vacuolar invertase (VI), leading to accumulation of hexose in the cold stored tubers (Zrenner *et al*., 1996b). This phenomenon is well known as cold-induced sweetening (Müller-Thurgau, 1882) and it makes tubers unsuitable for industrial processing, e.g. frying, because of the Maillard reaction occurring between the reducing sugars and free amino acids under high frying temperature. In accordance with these biochemical changes in cold stored potato tubers, the expression of a number of genes involved in sucrose metabolism, e.g. UGPase, SPS and vacuolar invertase genes are induced by low temperature (4-6°C) (Zrenner *et al*., 1993; Reimholz *et al*., 1997; Zrenner *et al*., 1996b).

1.6 *Sink regulation of photosynthesis and sugar signalling*

Photosynthesis is regulated as a two-way process. Light exerts feedforward control of photosynthesis by regulating the expression of genes involved in photosynthesis and the activity of their products. On the other hand, utilisation rate of end products down-stream of the Calvin cycle can feedback control photosynthetic activity and photosynthetic gene expression (Paul and Pellny, 2003). For example, end-product synthesis can exert short-term metabolic feedback control of photosynthesis through Pi recycling. During photosynthesis, triose phosphate is exported into the cytosol and subsequently converted to sucrose, releasing Pi then returns into the chloroplast to support photosynthesis. If sucrose synthesis rate is too slow, inorganic phosphate (Pi) will be sequestered into phosphorylated intermediates, thus impairing the recycling of Pi back to the reactions of photosynthesis, in particular, into

photophosphorylation that is very sensitive to Pi concentration. Eventually these changes will lead to the inhibition of photosynthesis (Stitt *et al*., 1987). The importance of sucrose synthesis in recycling Pi to support photosynthesis is further illustrated in transgenic plants with either decreased or increased sucrose synthetic capacity (Zrenner *et al*., 1996; Strand *et al*., 2000; Galtier *et al*., 1993; Baxter *et al*., 2003). Beyond this short-term metabolic feedback control through Pi recycling, carbohydrate accumulation in source leaves due to low sink demand can also lead to decreased expression of photosynthetic genes. This feedback regulation, which is referred to as sink regulation of photosynthesis, plays an important role in coordinating growth process at the whole plant level (Paul and Foyer, 2001). Despite its importance, the mechanisms of sink regulation of photosynthesis are not yet fully understood. However, it is most likely that sugar signalling pathways are involved in this process. In earlier work, Sheen (1990) showed that glucose, sucrose or acetate applied to maize protoplasts led to the repression of seven photosynthetic genes. Later on, carbohydrate regulation of photosynthetic gene expression was demonstrated in plants fed with glucose through the transpiration stream (Krapp *et al*., 1993). Based on this pioneering work, the idea has been put forward that sugars act not only as fuels for plant respiration and growth but also as signalling molecules regulating gene expression. Although discovered while studying the regulation of photosynthesis, sugar signalling is clearly involved in regulating various vital processes such as metabolism, growth and development (Bush, 2003). An ever-growing body of evidence suggests a number of different carbon-based regulatory pathways to be active in plants, e.g. the existence of independent signalling pathways for glucose (Jang *et al*., 1997; Jang and Sheen, 1997; Rolland *et al*., 2002; Moore *et al*., 2003), sucrose (Chiou and Bush, 1998; Rook *et al*., 1998; Vaughan *et al*., 2002; Wiese *et al*., 2004) and trehalose-6-phosphate (Eastmond and Graham, 2003; Schluepmann *et al*., 2003; Schluepmann *et al*., 2004). In the past few years, the most advances in sugar signalling have come from glucose signalling that is intermeshed with hormone signalling networks, regulating many vital processes in plants (Moore *et al*., 2003; Rolland *et al*., 2002; Xiao *et al*., 2000). Compelling evidence suggest that hexokinase is a glucose sensor whose signalling function is not dependent on its catalytic activity (Jang *et al*., 1997; Rolland *et al*., 2002; Moore *et al*., 2003). In yeast, the SNF1 (sucrose nonfermenting 1) complex is involved in glucose signalling, responsible for the derepression of glucose repressed genes. SNF1 homologs in plants are the SNF1-related protein kinase (SnRK), and evidences suggest that plant SnRK may be also an important player in the activation of gene expression in response to sucrose and glucose (Bhalerao *et al*., 1999; Purcell *et al*., 1998). Besides regulating gene expression in response to sugars, plant

SnRK also controls the activity of certain metabolically important enzymes such as nitrate reductase and SPS by direct phosphorylation of the enzymes (Ikeda *et al*., 2000; Toroser *et al*., 2000). With respect to the role of sucrose in signalling, it is difficult to elucidate sucrose-specific signalling effects in plant cells because sucrose is readily hydrolyzed to glucose and fructose by the action of invertase. However, recent evidences indicate the presence of such sucrose-specific signalling pathways in plants. For example, sucrose can specifically repress the expression of a sucrose symporter gene in sugar beet (Chiou and Bush, 1998; Vaughan *et al*., 2002), and it also can mediate the repression of ATB2 mRNA translation via a conserved upstream open reading frame in the mRNA leader sequence (Rook *et al*., 1998; Wiese *et al*., 2004). Until now the nature of the sucrose sensors is still not clear although certain sucrose transporters have been proposed to be sensors of sucrose in plants (Lalonde *et al*., 1999; Barker *et al*., 2000). Another recently emerging carbon-based signaling molecule is trehalose-6-phosphate (T6P), an intermediate of trehalose synthesis. T6P is synthesized from Gluc6P and UDP-Gluc by the action of trehalose-phosphate synthase (TPS) and subsequently dephosphorylated into trehalose by trehalose-6-phosphate phosphatase (TPP). In yeast, T6P is not only an intermediate of trehalose metabolism, but also a signal molecule regulating glucose influx into metabolism through interaction with hexokinase (Blazquez *et al*., 1993; Thevelein and Hohmann, 1995). Initially, trehalose metabolism was thought to be physiologically important only in resurrection plants, protecting these plants from desiccation by accumulation of trehalose to high levels (Wingler, 2002). Although in nonresurrection plant species trehalose does not appear to accumulate to readily detectable levels, the discovery of genes for trehalose synthesis in *Arabidopsis* and in a range of crop plants suggests that this pathway is probably widely present in the plant kingdom (Blazquez *et al*. 1998; Goddijin and van Dun, 1999; Wingler, 2002). It has recently been demonstrated that TPS1 in Arabidopsis is essential for embryo maturation, normal vegetative growth and transition to flowering (Eastmond *et al*., 2002; van Dijken *et al*., 2004), indicating trehalose synthesis pathway plays an important role in plants. Remarkably, recent evidences also suggest that T6P possess important signaling functions in plants, regulating carbohydrate utilization, photosynthesis performance and plant growth (Pellny *et al*., 2004; Schluepmann *et al*., 2003; Schluepmann *et al*., 2004). The amounts of T6P correlate with expression of *AKIN11,* a SnRK in Arabidopsis, which links T6P to a kinase-controlling signal transduction response to sugars (Schluepmann *et al*., 2004). Based on the biosynthetical and structural similarities between Suc6P and T6P, a similar function in signaling for Suc6P has also been proposed (Lunn *et al*., 2000; Paul *et al*., 2001; Lunn and MacRae, 2003). In sugar starved

Arabidopsis cells, SPS was proteolytically cleaved and the truncated SPS retained catalytic activity (Cotelle et al., 2000). It was proposed that the truncated SPS might lose its SPP-like domain and thus its ability to interact with SPP (Lunn and MacRae, 2003). Therefore, the proteolytic cleavage of SPS might result in dissociation of the potential SPS-SPP complex, disrupt the channeling of Suc6P from SPS to SPP and consequently increase the free concentration of Suc6P, which might signal sugar starvation (Lunn and MacRae, 2003). However, until now no experimental evidence exists for such a proposal.

1.7 *Reversed genetics approaches for the identification of metabolic control steps*

The control of metabolic flux through a pathwy is often shared between several enzymes (Stitt 1994). The identification of such metabolic control steps has unarguable impacts on the design of strategies for the manipulation of metabolism by reversed genetics and for maker-based breeding. Over the past two decades, reverse genetics approaches have proven to be powerful in the identification of metabolic control steps. The key element is generation and analysis of a set of plants with progressively decreased expression of the enzyme in question. This can be achieved by using T-DNA or transposon insertion or chemical-induced mutants to generate a set of plants where the number of functional gene copies is varied. This approach has been applied to analyse the distribution of control between the enzymes of the starch synthesis pathway in leaves (Kruckeberg *et al*., 1989; Neuhaus *et al*., 1989; Neuhaus and Stitt, 1990). The accurate analysis of flux control requires a range of plants with a relative small change in activity. A large inhibition of expression usually leads to an overestimation of the control coefficient of the enzyme. However, it is difficult to obtain a wide range of dosage mutants. In most cases, only two kind of dosage mutants can be generated, hemizygous mutants with 50% reduction of the enzyme in question and homozygous mutants with 100% inhibition. Therefore the use of the mutant approach in analysing metabolic regulation is limited. An alternative reverse genetic approach is to down-regulate the gene of interest using antisense or co-suppression technologies (van der Krol *et al*., 1988; Smith *et al*., 1988; Napoli *et al*., 1990; van der Krol *et al*., 1990), or the recently developed RNA-interference (RNAi) technology (Fire *et al*., 1998; Waterhouse *et al*., 1998; Chuang *et al*., 2000). These approaches have been widely applied to study plant carbon metabolism (Frommer and Sonnewald, 1995; Stitt and Sonnewald, 1995) and are extremely useful to identify metabolic

control steps (Geigenberger *et al.*, 2004). Antisense, co-suppression and RNAi based gene silencing phenomena are collectively referred to as post-transcriptional gene silencing or RNA silencing, which describes a nucleotide sequence-specific RNA degradation process naturally providing a defense mechanism against invasive nucleic acids such as viruses, transposons and transgenes (Baulcombe, 2002; Matzke *et al.*, 2001; Vance and Vaucheret, 2001; Voinnet, 2001). According to current models of RNA silencing (Fig. 1-3), double-stranded RNA (dsRNA), an intermediate formed in all the three methods, is further processed into 21-25nt RNA fragments of both polarities by an RNaseIII-type endonuclease named Dicer (Hamilton and Baulcombe, 1999). These short interfering RNAs (siRNA) are subsequently incorporated into an RNA-induced silencing complex (RISC) to guide cycles of specific RNA degradation (Hammond *et al.*, 2000; Tang *et al.*, 2003; Zamore *et al.*, 2000).

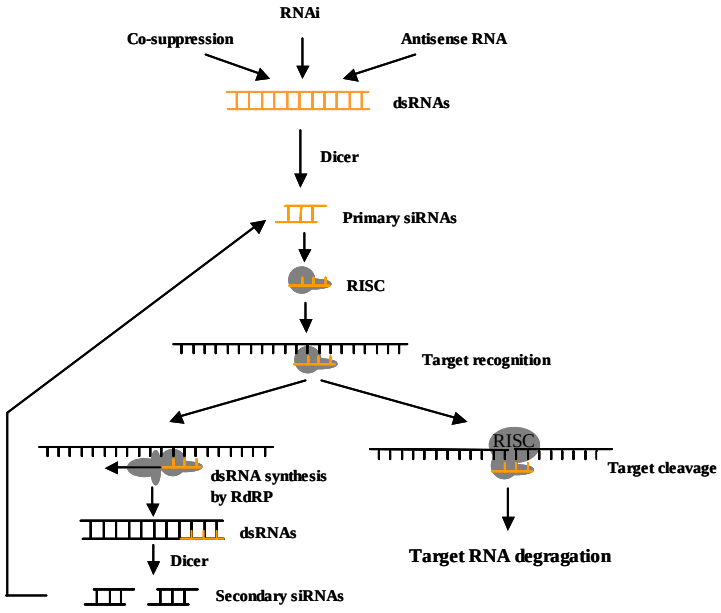


Fig. 1-3: Schematic representation of current models of RNA silencing. RNAi: RNA interference; dsRNAs: double-stranded RNAs; siRNAs: short interfering RNAs; RISC: RNA-induced silencing complex; RdRP: RNA-dependent RNA polymerase. Modified from Plasterk, 2002.

RNA silencing approach has several advantages in studying the metabolic regulation as compared to the dosage mutant strategy. For example, it is much easier to obtain a range of transgenic plants with various levels of the enzyme in question, thus leading to a more accurate estimation. As RNA silencing is a homology-dependent process, it is also versatile in studying gene families. A specific gene family member can be silenced when a unique region is targeted, or multiple members of a gene family can be simultaneously knocked down by

targeting highly conserved sequence domains. Therefore, gene families with redundant functions can be easily tackled and it is also possible to study the contribution of the individual member to the flux control.

1.8 *Chemical-inducible expression of transgenes to study plant metabolism*

Transgenic plants with up-regulation or down-regulation of the gene of interest have been extensively used to elucidate gene function and the molecular and cellular aspects of carbon metabolism (Frommer and Sonnewald, 1995; Stitt and Sonnewald, 1995). The most widely utilised promoter to control the expression of transgenes is cauliflower mosaic virus (CaMV) 35S promoter, which drives strong constitutive expression in almost all plant tissues (Franck *et al.*, 1980). However, the constitutive expression of transgenes consistently entails pleiotropic effects which might superimpose the primary impact of the manipulation of the target gene and thus mask true gene function. Particularly when metabolic processes are under scrutiny, the observed phenotype could rather reflect a physiological adaptation to the manipulation of a metabolic function than a primary response to the latter, and usually it is difficult to dissect the sequence of the consequences in the transgenic plants. Moreover, if the gene under manipulation is essential to plant regeneration from tissue culture, viable plants might not be recovered. Therefore, the introduction of extrinsic gene switches into transgenic system has long been of interest for controlling target gene expression in a ligand-inducible fashion (Gatz, 1997). To this end, several inducible systems have been developed in plants during the last decade such as GVG, TVG and XVE systems (Aoyama *et al*., 1997; Bohner *et al.*, 1999; Zuo *et al*., 2000). However, due to the hormone nature of the inducers these systems might not be readily applicable to soil-grown plants and thus their use in physiological studies is limited. In the past few years, an ethanol inducible system based on the well-characterized *alc* regulator of the ascomycete fungus *Aspergillus nidulans* has been developed in plants with the potential for broad utility (Caddick *et al*., 1998; Salter *et al.*, 1998). In plants, the system basically consists of two modules: a modified *alc*A promoter that controls the gene of interest and the *alc*R transcriptional regulator that can bind to *alc*A promoter and activate the transcription from *alc*A promoter at the presence of ethanol (Fig. 1-4). The *alc* system has been successfully applied to tightly control gene expression in a wide range of plant species including *Arabidopsis, Brassica napus, Nicotiana tabacum* and detached potato tubers (Caddick *et al*. 1998; Junker *et al*., 2003; Roslan *et al*., 2001; Salter *et*

al., 1998; Sweetman *et al*., 2002). Several ways have been developed to effectively induce this system in plants, including root drenching (Caddick *et al*., 1998; Salter *et al*., 1998), leaf spraying (Salter *et al*., 1998) or exposing the plants to ethanol vapor (Sweetman *et al*., 2002). Alternatively, the *alc* gene switch can also be activated by other related chemicals. A recent study demonstrated the *alc* system could be switched on more rapidly with the application of acetaldehyde, the physiological inducer of the *alc* regulon in *A. indulans*, than that of ethanol (Junker *et al*., 2003). Since ethanol and acetaldehyde in concentrations necessary for induction are physiologically affable and exert only minimal physiological side effects, the *alc* system has been used to study carbon metabolism by over expressing a yeast derived invertase in an inducible fashion (Caddick *et al*., 1998; Qu, 2001; Junker *et al*., 2003). Although the *alc* system has been successfully applied to control overexpression of transgenes in transgenic plants, the potential of its application in control of gene silencing in transgenic plants has not been investigated until now.

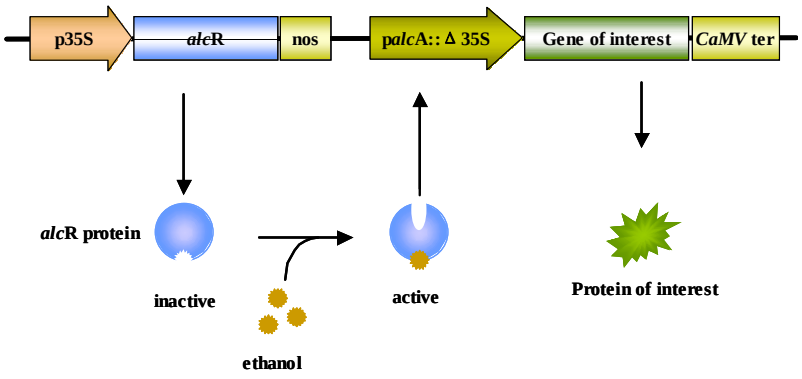


Fig. 1-4: Schematic representation of the ethanol inducible system (the *alc* system). CaMV 35S promoter (p35S) is used to drive the *alc*R gene that encods the *alc*R regulatory protein. The expression of the gene of interest is under the control of the chimeric *alc*A promoter which contains the *alc*R protein recognition site (*alc*A) and CaMV 35S minimal promoter elements (Δ 35S). In the presence of inducers such as ethanol, the *alc*R protein becomes activated, binds to the *alc*A promoter and activates the expression of the gene of interest.

1.9 *Scientific aims of this work*

The experiments described in this thesis are, on one hand, to study the role and significance of sucrose-6-phosphate phosphatase (SPP) in regulating sucrose synthesis and carbon partitioning in photosynthetic and non-photosynthetic tissues; on the other hand, to study the potential signaling function of sucrose-6-phosphate (Suc6P) in regulating plant metabolisms. This includes:

1) Molecular cloning of SPP from the leaves of *Nicotiana tobacum* and surveying its *in planta* role. RNA interference technology shall be applied to down-regulate the expression of SPP in transgenic tobacco plants. The contribution of SPP to the control of sucrose synthesis pathway shall be investigated in the SPP silenced transgenic tobacco plants. The transgenic plants also shall be analyzed with respect to the effects on photosynthesis, sucrose synthesizing capacity and carbohydrate partitioning. The potential impact of Suc6P on plant metabolisms shall be investigated in the transgenic tobacco leaves.
2) Molecular cloning of SPP from *Solanum tuberosum* and investigating its contribution to sucrose (re)synthesis in potato tubers. Constitutive or tuber-specific inhibition of SPP shall be achieved in the transgenic potato plants using *CaMV* 35S promoter or patatin B33 promoter, respectively, to control the expression of the SPP-RNAi cassette. Sucrose metabolism shall be analyzed in the transgenic tubers at different developmental stages and under various environmental conditions. The potential impact of Suc6P on plant metabolisms shall be investigated in the transgenic potato tubers.
3) Establishment of an inducible silencing system and its potential use in studying carbon metabolism. Two strategies, inducible expression of antisense fragments or inducible expression of RNAi cassettes using the *alc* system, shall be applied to achieve gene silencing in an inducible fashion. Different genes from various pathways shall be targeted to test these strategies and the detailed characterisation of this system shall be performed. In order to dissect the possible primary and secondary effects associated with constitutive reduction of SPP activity, SPP expression shall be down-regulated in an inducible way using the inducible RNAi system.

2 Materials and Methods

2.1 *Chemicals, enzymes and other consumables*

All the chemicals, enzymes and other consumables with appropriate purity were purchased from specialised companies including: Amersham Pharmacia (Braunschweig), Applichem (Darmstadt), Boehringer Mannheim (Mannheim), Bio-Rad (München), Fluka (Buchs, Switzerland), New England Biolabs (Beverly, MA, USA), Merck (Darmstadt), Pierce (Rockford, IL, USA), Qiagen (Hilden), Roth (Karlsruhe), Roche (Mannheim), Sera (Heidelberg), Stratagene (Amsterdam, Netherland), Sigma Aldrich (Steinheim), and Whatman (Maidstone, England).

2.2 *Plant materials and growth conditions*

2.2.1 *Nicotiana tabacum*

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were obtained from Vereinigte Saatzuchten eG (Ebsdorf, Germany) and grown in tissue culture under a 16h light/8 h dark regime (irradiance 150 µmol quanta m^{-2} s^{-1}) at 50% humidity on Murashige Skoog medium (Sigma, St. Louis, MO, USA) containing 2% (w/v) sucrose. Plants in the greenhouse were kept in soil under a light/dark regime of 16 h light (ca. 250 µmol m^{-2} s^{-1}; 25 °C) and 8 h dark (20 °C).

2.2.2 *Solanum tuberosum*

Solanum tuberosum L. var. Solara (potato) was obtained from Vereinigte Saatzuchten eG (Ebstorf, Germany) and maintained in tissue culture under a 16-h-light/8-h-dark period (150 μM m^{-2} s^{-1} light, 21°C) at 50% relative humidity on Murashige and Skoog medium (Sigma, St. Louis) containing 2% (w/v) sucrose. Plants from the selected lines were clonally propagated from stem cuttings and then grown in soil either under controlled conditions (70% humidity, 16-h day/8-h night regime, 19°C/15°C, 500 μmol m^{-2} s^{-1} light) in a growth chamber or in the greenhouse with supplementary light (250 μmol m^{-2} s^{-1} light). Tubers were harvested from the senescent plants and stored in the dark at room temperature (22°C) or transferred to the cold (4°C) one week after harvest.

2.3 *DNA cloning procedures*

Standard cloning procedures such as amplification of DNA fragments with polymerase chain reaction (PCR), ligation of a DNA fragment into plasmid vectors, transformation of *Escherichia coli* (*E. coli*) cells, preparation of plasmid DNA, restriction digest and agarose gel electrophoresis were carried out as described (Sambrook *et al*., 1989).

2.4 *Oligonucleotides and DNA Sequencing*

Standard sequencing primers (M13 forward, M14 reverse, T7, SK, KS) were purchased from Stratagene (Heidelberg, Germany). Custom sequencing and PCR oligonucleotides were purchased from Metabion (Martinsried, Germany) (listed in Appen. II). DNA sequencing was done at the IPK service facility.

2.5 E. coli *strains and plasmids*

E. coli strain XL1-blue and TOP 10F´ were used for plasmid amplification, and *E. coli* strain M15 was used for the expression of recombinant protein. The genotypes and source of bacterial strains are summarized in Tab. 2-1. The vectors used in this work are listed in Tab. 2-2.

Tab. 2-1: E.coli *strains*

Strain	Genotype or relevant characteristic	Source/reference
E.coli XL1 Blue	*RecA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F_, *proAB*, *lac1*$_q$*Z_M15*, *Tn10* (*tet_*)]	Bullock *et al*. 1987 Stratagene
E.coli TOP10F´	F´ {*lac*Iq Tn*10* (TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
E.coli M15 [pREP4]	*nal*^{s} *str*^{s} *rif*^{s} *thi*$^-$ *lac*$^-$ *ara*$^+$ *gal*$^+$ *mtl*$^-$ *f recA*$^+$ *uvr*$^+$ *lon*$^+$	Qiagen

Tab. 2-2: *Vectors*

Vector	Application/resistance	Source/reference
pBinAR	Binary vector, Kan^r	Höfgen and Willmitzer, 1990
pBinB33	Binary vector, Kan^r	Rocha-Sosa *et al*., 1989
p35S-*alc*R	Binary vector, *alc* system, Kan^r	Caddick *et al*., 1998
pUC-*alc*A	Cloning vector, *alc* system, Amp^r	Caddick *et al*., 1998
pUC-RNAi	Cloning vector, hpRNA construct with *St*GA20-oxidase intron, Amp^r	Chen *et al*., 2003
pBluescript SK-	Cloning vector, Amp^r	Stratagene
pCR-Blunt	Cloning vector, Kan^r	Invitrogen
pCR2.1	Cloning vector, Kan^r	Invitrogen
pUC18, pUC19	Cloning vector, Amp^r	Yanisch-Perron *et al*., 1985
pMal-c2	*E.coli* expression vector, Amp^r	New England Biolabs

2.6 *Expression of recombinant protein in* E. coli

A cDNA fragment comprising the entire coding region of NtSPP2 was ligated into the vector pMal-c2 to create a translational fusion between the maltose-binding protein of *E. coli* and NtSPP2. The maltose-binding protein allows for the affinity purification of the recombinant protein on an amylose matrix. The resulting plasmid pMal-NtSPP2 was transformed into *E. coli* strain M15 (Qiagen, Hilden, Germany) and the protein expressed as outlined in the instructions accompanying the pMal-c2 vector (New England Biolabs, Beverly, MA). 20 ml of overnight culture was inoculated into 1 liter of fresh LB-medium supplemented with 50 mg/L Kanamycin, 100mg/L Ampicillin and 0.2% (w/v) glucose. The fresh culture was shaken at 37°C until the OD_{600} reached about 0.6. The expression of recombinant protein was induced by adding 0.5 mM IPTG and the culture was further shaken for 4 h before collecting the cells by centrifugation. Bacteria were lysed by sonication in a buffer containing 20 mM Tris-Cl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 µM PMSF and 1 mM DTT. After centrifugation, the MBP-NtSPP2 fusion protein was purified from the extract using amylose-agarose resin and eluted using maltose. Eluted protein was analyzed by SDS-PAGE.

2.7 *Plant transformation*

The binary plasmids were used for direct transformation of *Agrobacterium tumefaciens* (*A. tumefaciens*) as described by Höfgen and Willmitzer (1988). Plant transformation was carried out via *Agrobacterium*-mediated gene transfer using *A. tumefaciens* strain C58C1:pGV2260

(Deblaire *et al*., 1985). Tobacco plants were transformed as described by Rosahl *et al*. (1987) and transformation of potato plants was carried out according to Rocha-Sosa *et al*. (1989).

2.8 *Ethanol induction*

Plants (42 days old) cultivated in the greenhouse in 2.5-l pots were induced with 100ml of 1% (v/v) ethanol solution via root drenching. Normal watering was resumed the day after application of ethanol solution. Samples for RNA, chl, carbohydrates, metabolites, protein and enzyme activity analysis were taken at various time points indicated in the section under Results. If not otherwise stated, young leaves, being approximately 5 cm at the time point of induction, were followed over the time course of the experiment. For spatial induction, an individual leaf was enclosed in a 15 cm x10 cm transparent plastic bag with 3 ml of 4% (v/v) ethanol as described previously by Sweetman *et al*. (2002). The bag was removed after 48 h and phenotype development was monitored by eye.

2.9 *Antibody preparation and immunodetection*

Antibodies against SPP from tobacco were manufactured by a commercial organization (Eurogentec, Seraing, Belgium) by immunizing rabbits with affinity purified MBP-NtSPP2 fusion protein. Antibodies against FNR and SPS were described by Hajirezaei *et al*. (2002) and Reimholz *et al*. (1994), respectively. Antibodies against GSA were kindly provided by Prof. Bernhard Grimm (Humboldt University, Berlin).

Protein extracts were prepared by homogenization of leaf material in a buffer containing 25 mM HEPES pH 7.0, 12 mM MgCl2, 0,5 mM EDTA, 8 mM DTT, 10 µM PMSF, 0.1% Triton and 10% glycerol. Protein content was determined according to Bradford (1976) with bovine γ-globulin as the standard. After heat denaturation, 30-50 µg total protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane (Porablot, Macherey und Nagel, Düren, Germany). Immunodetection was carried out using the ECL kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer, using the corresponding primary antibody and peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA).

2.10 *RNA isolation and northern blot*

Total RNA was extracted from tobacco leaf material as described by Logemann *et al*., (1987), and 30 µg per sample were separated on a 1.5% (w/v) formaldehyde-agarose gel using conditions described by Sambrook *et al*., (1989). After electrophoresis, RNA was transferred to a nitrocellulose membrane (GeneScreen, NEN Lifescience Products, Boston, USA) and fixed by UV-crosslinking. Radioactive labeling of respective cDNA fragments was performed using the High Prime-kit (Roche, Mannheim, Germany) and [α-^{32}P]-dCTP. Hybridization was carried out as described previously (Herbers *et al*., 1994) and signals were detected by exposure to Kodak X-ray films (Sigma).

2.11 *Semi-quantitative RT-PCR*

For RT-PCR experiments, 2,5 µg of DNase-treated total RNA was reverse transcribed into cDNA with oligo(dT) (30-mer) using M-MLV[H$^-$] Reverse Transcriptase (Promega). A fraction (about 1/20) of the first strand cDNAs was used as a template for PCR with gene specific primers in a volume of 100 µl with 1 U of Taq-polymerase (Takara, Shouzo, Japan), 20 µM each dNTP, and 0.25 µM of each primer. An initial denaturation step for 5 min at 95°C was followed by 25 – 35 cycles of 5 s at 95°C; 45 s at 55°C and 1 min at 72°C. PCR products were separated on 1% (w/v) agarose gels containing ethidium bromide and visualized by UV light. Amplification of actin using primers CS40 and CS41 served as an internal control.

2.12 *Enzyme activities*

Snap-frozen plant material from different tissues was extracted as described above and used for analysis of enzyme activities. SPP activity was determined according to Lunn *et al*., (2000). The crude extracts were desalted with Sephadex G25 and the SPP activity was measured in 25 mM Hepes-K$^+$ buffer (pH 7.0) containing 1.25 mM Suc6P and 8 mM $MgCl_2$ in a total volume of 300 µl. The residual activity was determined omitting $MgCl_2$ but including 40 mM EDTA in the assay buffer. The reactions were incubated at 30°C, if not stated otherwise, for 45 min and stoped by adding 30 µl of 2 M trichloroacetic acid. The released Pi was quantified using the ascorbic acid-ammonium molybdate reagent as described by Ames (1966). In order to determine the temperature coefficients (Q_{10}) for SPP, its activity was measured at 30°C, 15°C and 4°C respectively. Q_{10} was calculated using the equation of T_2-T_1=10*ln(k_2/k_1)/ln(Q_{10}) which is derived from the Arrhenius equation (Freitas Jr. 1999). k_1

and k_2 in the equation are the SPP activities measured at temperature T_1 and T_2. SPS activity was assayed by quantifying the fructosyl moiety of sucrose using the anthrone test as described by Baxter *et al*., (2003). The protein extracts were desalted and incubated for 20 min at 25°C in the assay buffer (50 mM Hepes-K^+, pH 7.5, 20 mM KCl, 4 mM $MgCl_2$) in a total volume of 200 µl containing: (a) V_{max} assay; 12 m MUDP-Gluc, 10 mM Fruc6P and 40 mM Gluc6P, or (b) V_{sel} assay; 4 mM UDP-Gluc, 2 mM Fruc6P, 8 mM Gluc6P and 5 mM KH_2PO_4. The reaction was stopped by incubation at 95°C for 5 min and 100 µl of centrifuged supernatant was further incubated with the same volume of 5 M KOH at 95°C for 10 min. After adding 800 µl of anthrone reagent, absorbance was measured at 620 nm and used for quantification of the resulted sucrose or Suc6P. The determination of AGPase activity was carried out at 25°C in a final volume of 800 µl according to Müller-Röber *et al*., (1992). The composition of the reaction mixture was 80 mM Hepes-K^+ (pH 8.0), 5 mM $MgCl_2$, 1 mM ADP-Gluc, 5 mM 3-PGA, 2 mM DTT, 0.5 mM NADP, 10 µM Gluc-1,6-bisP, 0.8 U/ml Phosphoglucomutase and 0.7 U/ml Gluc6P dehydrogenase. The reaction was started with 2 mM sodium pyrophosphate. The formation of Gluc1P from ADP-Gluc and PPi was monitored. cFBPase activity was assayed at 25°C in a final volume of 800 µl as described by Zrenner *et al*. (1996). The reaction was carried out in the imidazol buffer (pH 7.1) containing 5 mM $MgCl_2$, 0.25 mM NADP, 1 U/ml Gluc6P dehydrogenase and 1 U/ml phosphoglucoisomerase. The reaction was started with 1.25 mM Fruc-1,6-bisP final concentration. Sucrose synthase, vacuolar acid invertase and cell wall acid invertase activities were determined as detailed by Hajirezaei *et al*., (1994). Susy activity was determined in the direction of sucrose cleavage and the resulting UDP-Gluc was quantified using the IC-MS method as described below. Vacuolar acid invertase activity was determined in 50 mM sodiumacetate buffer (pH5.2) containing 0.1 M sucrose in a final volume of 100 µl using the desated protein extracts. The reaction mixture was neutralized by adding 10 µl of 1 M Tris-HCl (pH 8.0) and stopped at 95°C for 5 min. The resulting glucose was quantified using a Spectrophotometer. The pellets during protein extraction were washed, resuspended in the extraction buffer and used for determination of cell wall acid invertase. The reaction was carried out in the way as described for vacuolar acid invertase. Starch phosphorylase activity was measured as described in Steup (1990). The reaction mixture contained 25 mM Imidazol (pH6.9), 5 m$MgCl_2$, 1 mM EDTA, 5 mM Na_2MoO_4, 0.6 mM NAD, 2.5 µM Gluc-1,6-bisP, 0.4 U/ml Phosphoglucomutase, 0.4 U/ml Gluc6P dehydrogenase and 0.1% soluble starch. The reaction was started with 2 mM NaH_2PO_4 final concentration.

2.13 *Chlorophyll determination*

Chlorophyll was measured in ethanol extracts and concentrations were determined as described by Lichtenthaler (1987).

2.14 *CO_2 exchange and carbon partitioning*

Net CO_2 uptake rates were measured with a portable photosynthesis system LI-6400 (LI-COR, Lincoln, NE). For determination of light response curves of CO_2 gas exchange, photosynthetic photon flux density (PPFD) was varied between 0 and 2000 µmol photons m^{-2} s^{-1}. CO_2 concentration of the air entering the chamber was adjusted to 400 µmol mol^{-1} and leaf temperature was maintained at 25°C. The resulting data for the relationship between *A* and *I* (light response curves) were fitted by the equation of a non-rectangular hyperbola as described by Hajirezaei *et al*., (2002). A – c_i response curves at PPFD = 2000 µmol m^{-2} s^{-1} were obtained by measuring net CO_2 uptake rates at three different ambient CO_2 concentrations (approximately 200, 380, and 940 µmol mol^{-1}). The incorporation of $^{14}CO_2$ into tobacco leaf discs was performed as described by Quick *et al*., (1989). Leaf discs were incubated in an oxygen electrode (LD-2, Hansatech, Kings Lynn, UK) under CO_2-saturated atmosphere, which was generated through the addition of 400 µl of 2 M K_2CO_3/$KHCO_3$ (pH 9.3) solution enriched with $NaH^{14}CO_3$ (specific activity 0.14 MBq $mmol^{-1}$). Samples were illuminated for 20 min using the beam of a slide-projector and immediately frozen in liquid nitrogen.

2.15 *Measurements of respiration rates in potato tubers*

Respiration rates in intact potato tubers were measured as described by Hajirezaei *et al*., (2003), using an infrared gas analysis in an open system (Compact minicuvette System CMS-400, Walz GmbH, Effeltrich, Germany). Whole tubers were enclosed in a standard chamber MK-022/A and the release of CO_2 was monitored continuously. Chamber temperature and dew point temperature of the air entering the chamber were adjusted to 20 °C and 13 °C, respectively. Measurements were done at a gas flow rate of 1500 cm^3 s^{-1} and ambient CO_2 concentration of about 100 µmol mol^{-1}. CO_2 evolution rate referred to tuber fresh weight is given as nmol CO_2 g^{-1} s^{-1}.

2.16 *Determination of chlorophyll fluorescence*

Chlorophyll fluorescence was measured using a PAM-2000 portable fluorometer (Walz, Effeltrich, Germany) as described in Hajirezaei *et al*., (2002).

2.17 *Starch, soluble sugars and metabolites*

Soluble sugars and starch levels were determined in leaf samples extracted with 80% (v/v) enthanol/20 mM HEPES, pH 7.5 as described (Stitt *et al*., 1989). Maltose was measured from the same extracts with HPLC using the conditions exactly as described by Börnke *et al*., (2001). 3-PGA and hexose-phosphates were measured spectrophotometrically in neutralized trichloroacetic acid (TCA) as described (Stitt *et al*., 1989). From the same extracts fructose-1,6-bisphosphate, UDP-glucose, sucrose-6-phosphate and adenylates were determined using ion chromatography coupled to mass spectrometry (IC-MS). Alternatively, the leaf or tuber materials were homogenized in liquid nitrogen and followed by extracting with methanol/chloroform (1:1, v/v). After adding water, the water/methanol phase was further separated from the chloroform phase by centrifugation. The water/methanol phase was dried out using a Speedvac and the pellets were resuspended in H_2O. From these extracts, sucrose-6-phosphate, 3-PGA, UDPG, Glucose-6-phosphate and Fructose-6-phosphate and adenylates were determined using ion chromatography coupled to mass spectrometry (IC-MS). Fruc-2,6-bisP was extracted from frozen leaf material using ice-cold 50 mM NaOH as described (van Schaftingen, 1984) and measured using IC-MS.

The IC-MS instrumentation consisted of a Dionex HPLC system (Dionex, Idstein, Germany) with a MSQ mass detector (Dionex). Anionic compounds were separated on a 250 x 2 mm AS11-HC column (Dionex) connected to a 10 x 2 mm AG 11-HC guard column (Dionex) and an ATC-1 anion trap column which is placed between the eluents and separation columns to remove anions present in the solutions. The gradient was accomplished with H_2O (buffer A; HPLC-grade, Millipore) and 100 mM NaOH (buffer B). The column was equilibrated with a mixture of buffer A (96%) and buffer B (4%) at a flow rate of 0.3 ml min^{-1} and heated to 30°C during the whole measurement. The gradient was produced by changes of buffer B as follows: 0 – 8 min, 4%; 8 – 28 min, 30%; 28 – 38 min, 60%; 38 – 51 min, 100%; 51 – 64 min, 4%.

Detection of metabolites was achieved by atmospheric pressure ionization-electrospray mass detection in the negative ion mode. The drying/carrier gas was nitrogen heated to 350°C

with a flow of 400 L h^{-1} at a pressure of 60 psi. The capillary was set to 3,500 V with a fragmentator voltage of 50 V and dwell time of 1 second. Ions in the range *m/z* 100 to 600 were scanned. Single ion mode (SIM) was performed in time windows of 5 min during total run time (64 min), i.e. up to five SIMs were run in parallel to enhance sensitivity. Sample injection volume varied from 5 to 10 µL.

Recovery experiments were undertaken in order to confirm the validity of the measurements. A defined portion of the metabolite of interest was added to the leaf discs before extraction and determined exactly as described above. The recovery rates were between 85 and 110% of the amount added.

2.18 *Determination of amino acids*

Total amino acids were determined in leaf samples extracted with 80% (v/v) ethanol. The ethanol extracts were evaporated to dryness, redissolved in purest H_2O, and separated on a reversed-phase HPLC system (Waters Associates, Milford, MA) after derivatization using the AccQ-Tag method as described (Rolletschek *et al*., 2002). Amino acids were identified by cochromatography with authentic standards and quantified by comparison with internal standards.

2.19 *Determination of total phosphate*

Determination of inorganic phosphate on a total cell basis was carried out using the ascorbic acid – ammonium molybdate reagent as described (Ames, 1966).

2.20 *Visualization of starch in tobacco leaves*

Following 12h illumination plants were kept in complete darkness for 24h. Subsequently, leaves were destained with 80% ethanol at 80°C and then stained with Lugol's solution to visualize the starch content.

3 Results

3.1 *Constitutively decreased SPP level in transgenic tobacco inhibits photosynthesis, alters carbohydrate partitioning and reduces growth*

3.1.1 Cloning and characterization of sucrose-6-phosphate phosphatase encoding cDNAs from *Nicotiana tabacum*

The GenBank EST database was searched with the deduced amino acid sequence of the *Arabidopsis AtSPP1* gene (AF283565) using the TBLASTN algorithm, to find cDNA clones from *Solanaceaous* species that might encode SPP. Several SPP-like ESTs were identified from potato and tomato, respectively. Two ESTs from tomato (BI933832 and BG125217) contained the putative translational start and stop site, respectively, and hence were used to deduce oligonucleotides (FB223 and FB225) flanking the potential SPP coding region. These were used to amplify SPP sequences from cDNA derived from tobacco source leaves by RT-PCR. Subsequent cloning and sequence analysis of the PCR fragments revealed that they fell into two different classes sharing 88 % identity on the nucleotide level to each other. Both clones showed around 67% identity with *AtSPP1* gene. The structural analysis of the proteins deduced from the two tobacco clones revealed that both contained the ~260 aa HAD phosphatase domain at the N-terminal region. The two different clones were designated as *NtSPP1* and *NtSPP2* (GenBank Acc. No. AY729655 and AY729656, respectively). Both contained a contiguous open reading frame (ORF) of 1278 bp encoding a protein with a predicted molecular mass of 48 kDa. To further confirm that these clones indeed were SPP genes, the putative *NtSPP2* was expressed as an MBP-NtSPP2 fusion protein in *E. coli,* and the recombinant protein was purified using amylose-agarose resin (Fig. 3-1) and used for determination of SPP activity. The recombinant protein showed phosphatase activity of 260 nmol min^{-1} mg^{-1} protein when Suc6P was used as the substrate and exhibited no phosphatase activity when Fruc6P used as the substrate in presence of Mg^{2+} (Tab. 3-1). These data showed that *NtSPP2* encodes a functional SPP enzyme. The tobacco SPP requires the presence of Mg^{2+} to exert its phosphatase activity, and the removal of Mg^{2+} and the presence of EDTA totally inhibted the SPP activity, which is in accordance with the earlier observation that SPP has an absolute requirement for Mg^{2+} (Lunn *et al*., 2000).

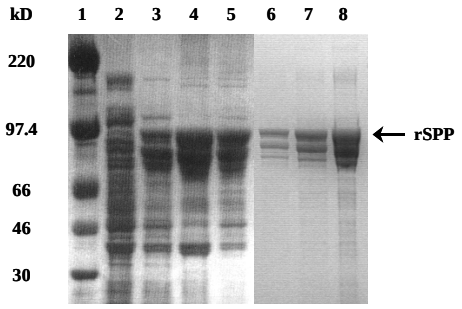


Fig. 3-1: Heterologous overexpression of recombinant NtSPP2 in *E. coli*.

Recombinant NtSPP2 was overexpressed *E. coli* as a fusion to the maltose-binding protein. The expression of recombinant SPP (rSPP) was induced with IPTG for 4 hours. The cells were collected before and after IPTG-induction and lysed by sonication. The rSPP was partially purified using amylose resin. The crude protein extracts and purified rSPP were analyzed by SDS-PAGE. Lane 1, protein marker, RPN756; lane2, protein extracts from the whole cells before induction; lane3, protein extracts from the whole cells 4 hrs after IPTG-induction; lane4, protein extracts from the pellets after sonication 4 hrs after IPTG-induction; lane5, protein extracts from the supernatants after sonication 4 hrs after IPTG-induction; lane6-8, 1, 2.5 and 5 µg column purified rSPP, respectively. The arrow indicates the position of rSPP.

Tab. 3-1: *Enzyme activity of recombinant NtSPP2*

Recombinant NtSPP2 was overexpressed *E. coli* as a fusion to the maltose-binding protein and partially purified on amylose resin. Phosphatase activity was measured using Suc6P or Fru6P, respectively, as substrates either in the absences (SPP activity) or presence of 20 mM EDTA (residual activity). n.d., not detectable.

	Phosphatase activity, nmol $min^{-1}mg^{-1}$ protein	
Substrate	SPP activity	Residual activity
Suc6P	260	n.d.
Fruc6P	n.d.	12

3.1.2 Generation of transgenic tobacco plants with constitutively decreased expression of SPP

In order to study the *in planta* role of SPP, transgenic tobacco plants with decreased SPP activity were constructed using an RNA interference (RNAi) strategy. To this end, a fragment containing the first 660 bp of the *NtSPP*2 ORF was amplified by PCR using primers FB228 and FB229 which introduced *Bam*HI and *Sal*I recognition sites, respectively, into the sequence. The resulting DNA fragment was digested with *Bam*HI and *Sal*I before it was ligated in sense orientation into pUC-RNAi, a vector containing the first intron of the gibberellin 20 oxidase gene from potato flanked by a short polylinker sequence. The same fragment was inserted in antisense orientation into the *Bgl*II/*Xho*I sites of pUC-RNAi already

carrying the sense fragment. The resulting SPP-RNAi fragment was excised by *Pst*I and cloned between the 35S CaMV promoter and the OCS terminator of the binary vector pBinAR (Höfgen und Willmitzer, 1990) using a compatible *Sbf*I site, producing the plasmid pBin-SPP-RNAi. The sequence identity in the selected region was more than 90% between *NtSPP1* and *NtSPP2* and thus assumed to be sufficiently high to target both transcripts for RNAi. The RNAi-cassette containing gene-specific fragments in sense and antisense orientation interspersed by a short intron was driven by 35S *CaMV* promoter with antisense fragment directly downstream of the promoter (Fig. 3-2). The resulting RNAi construct was subsequently transformed into tobacco plants via *Agrobacterium*-mediated gene transfer. Eighty primary transformants were transferred into the greenhouse and screened according to phenotypical alterations. Ten of the primary transformants exhibited various degrees of growth retardation, had jagged pale-green leaves, and developed necrotic lesions in the old leaves. Detection of the endogenous SPP transcript with northern blot analysis failed most likely due to its low expression level. However, degradation products from transgene and endogenous gene could be found in these ten transgenics with strong smear hybridisation signal as characteristics. Reduction of SPP mRNA level in the transgenics was confirmed with semi-quantitative RT-PCR using gene-specific primers targeting both isoforms. Sucrose level in these 10 primary transformants was reduced as compared to the control plants. At an early stage a decision was taken to analyse the progeny of these transformants to avoid potential complication arising from regeneration in tissue culture. To this end, three strongly repressed lines (SPPi17, 18 and 36) and one moderately affected line (SPPi31) were chosen for a detailed analysis in T1 generation. To keep growth conditions between transgenic and control plants comparable, ME-1, a transgenic line containing the GUS gene driven by a cytosolic FBPase (cFBPase) promoter from potato (Ebneth, 1996), was used as control instead of wild type plants. Seeds from the afore mentioned lines were germinated on kanamycin containing medium, resistant seedlings were transferred to the greenhouse, and detailed analyses were carried out 6-7 weeks after the plants were transferred to the greenhouse. T1 plants of each line had a range of phenotypes, indicating inhibition of

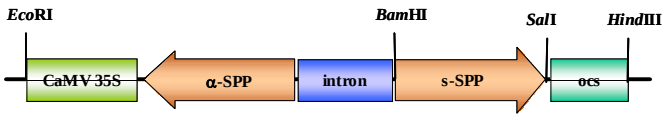


Fig. 3-2: Schematic representation of pBinSPPi construct for RNAi-mediated SPP silencing.
A fragment of NtSPP2 (nucleotides 1 to 660) was amplified and used to create pBinSPPi construct. α-SPP: SPP fragment in antisense orientation; s-SPP: SPP fragment in sense orientation.

SPP expression was not inherited uniformly in T1 generation probably due to either hemizygous state of the transgene locus or segregation of different transgene loci. Progeny from line SPPi36 displayed a particular wide range of phenotypes with individuals varying from wild-like appearance to plants strongly retarded in growth. This line was chosen to estimate the flux control coefficient for SPP in sucrose synthesis pathway (see below). Offspring of the other three lines was selected according to the phenotype development comparable to that of the parent plants and these lines were chosen for a detailed physiological analysis. Fig. 3-3A illustrates the growth retardation of the selected transgenic lines versus control plants.

3.1.3 SPP transcript, protein and enzyme activity in the selected transgenic lines

In order to assess the degree of inhibition of SPP expression in the selected transgenic lines, detection of SPP transcript and protein, respectively, and measurements of enzyme activity were carried out on fully expanded leaves. Since initial attempts to detect SPP transcript with Northern blot analysis failed, semi-quantitative RT-PCR with gene specific primers targeting both isoforms was applied using actin as an internal control. As illustrated in Fig. 3-3B, the steady state level of SPP transcript in all the three lines was strongly reduced. In accordance, SPP protein was virtually eliminated from the transgenics as revealed by Western blot analysis (Fig. 3-3C). SPP activity was measured to accurately quantify the inhibition degree of SPP repression. The results showed that SPP activity was reduced to 4.3% (SPPi17), 4.6% (SPPi18) and 8.2% (SPPi31), respectively, of that of control plants when calculated on a leaf area basis (Fig. 3-3D).

3.1.4 Basic characterisation of the selected transgenic lines

Shoot growth was reduced by 23-77% in the SPPi transformants (Tab. 3-2) compared to control plants. Fresh weight per leaf area was slightly increased while dry weight decreased by 14-22%, indicating that the transgenic plants contained more water (Tab. 3-2). Consistent with the pale green phenotype chlorophyll (chl) was decreased by 24-43% (Tab. 3-2). Protein decreased by 17-50% on a leaf area basis (Tab. 3-2). When comparing transgenic plants to wild-type of the same age (6 weeks), leaf number in SPPi plants was reduced as compared to the control (Tab. 3-2). However, RNAi plants germinated at the same time after sowing as the

wild-type plants, and the first true leaves of the transgenic plants emerged at the same time as in the wild type plants. Flowering was delayed 16 – 25 d in SPPi plants, however, total leaf number at the time of flowering was increased by 6 – 13, which is indicative for a prolongation of senescence (Tab. 3-2).

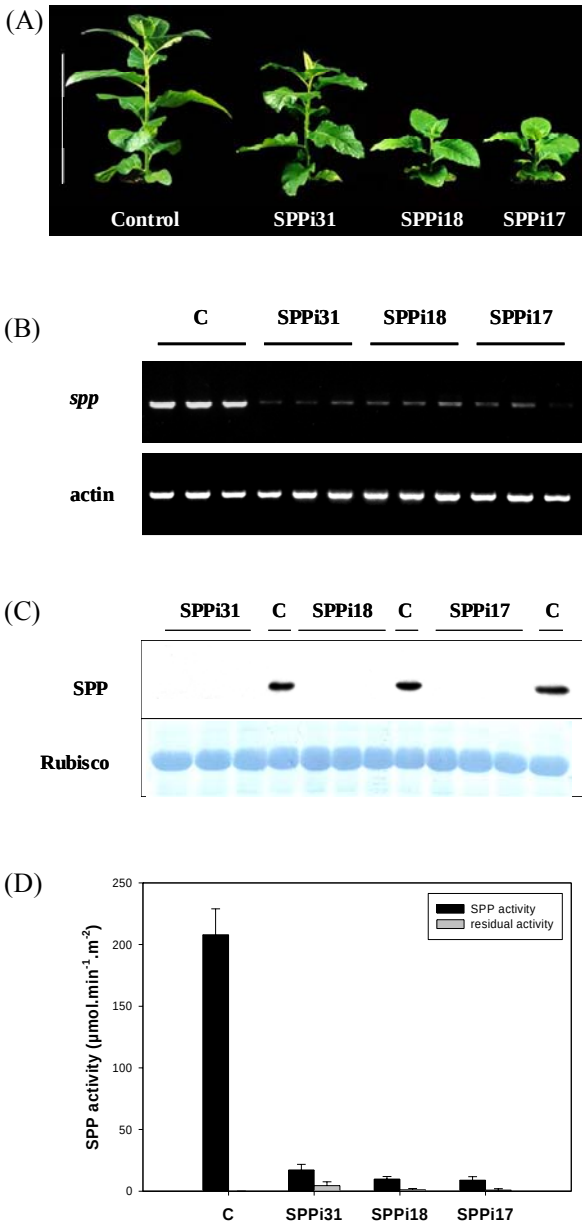


Fig. 3-3: Basic characterization of tobacco plants inhibited in SPP expression. A, phenotype of SPPi tobacco plants. Transgenic lines showed strong growth retardation with respect to the control plant. The picture was taken six weeks after transfer of the plants to the greenhouse. The white bar is equivalent to 40 cm. B, RT-PCR analysis of SPP expression in SPPi tobacco plants. Total RNA was isolated from mature leaves and used for RT-PCR as described in Materials and methods applying 35 amplification cycles. Equal amounts of cDNA were controlled by amplification of a constitutively expressed *actin* gene (30 cycles). C, Western-blot analysis of SPP expression. Fifty µg of protein was separated by SDS-PAGE and immunodecorated using a polyclonal SPP antibody. Amidoblack staining of the Rubisco band was taken as a control for equal loading. D, SPP activity in mature leaves. Enzyme activity was determined in mature leaves from six individual plants per line. Black bars indicate phosphatase activity in the absence of 20 mM EDTA whereas grey bars stand for the residual activity in the presence of 20 mM EDTA.

Tab. 3-2: *Basic characterization of* Nt*SPPi transformants.*

Shoot length, specific leaf fresh weight (SLFW) and dry weight (SLDW), respectively, chlorophyll, total protein and leaf number were determined six weeks after transfer of the plants into the greenhouse. Flowering time was recorded from the time the plants were soiled till the first blooming flower appeared. Total leaf number was counted again when the plants were flowering. Each value represents the mean (±SD) of six different plants.

Line	Shoot length cm	SLFW mg FW.cm^{-2}	SLDW mg DW.cm^{-2}	Chl µg.cm^{-2}
Control	42,9 ± 4,1	16,1 ± 0,7	2,19 ± 0,08	45,2 ± 3,6
SPPi31	32,8 ± 3,1	17,2 ± 0,8	1,89 ± 0,08	34,5 ± 3,2
SPPi18	10,8 ± 1,2	20,8 ± 1,3	1,88 ± 0,12	31,2 ± 1,9
SPPi17	9,7 ± 1,5	18,4 ± 1,3	1,72 ± 0,23	25,6 ± 2,9

Line	Total protein µg.cm^{-2}	Flowering time days	Leaf number 6-week-old plant	Leaf number flowering plant
Control	440,9 ± 37,2	69 ± 0,8	23 ± 0,5	30 ± 1,0
SPPi31	364,1 ± 24,0	85 ± 1,2	20 ± 0,4	36 ± 2,2
SPPi18	288,6 ± 28,8	92 ± 1,7	18 ± 0,8	38 ± 2,9
SPPi17	218,0 ± 32,7	94 ± 0,8	18 ± 0,8	43 ± 2,6

3.1.5 Influences of decreased expression of SPP on photosynthesis in transgenic tobacco plants

Retarded development and the reduced amount of total protein and chlorophyll, respectively, indicated that photosynthesis was affected in the SPPi transformants. Light-dependent photosynthesis at 400 µmol mol^{-1} CO_2 was measured on attached, fully expanded leaves using an open gas-exchange system. Under such conditions, light-saturated photosynthesis was inhibited by 52, 62 and 67% in SPPi31, SPPi18 and SPPi17, respectively (Fig. 3-4A). Maximum quantum yield (calculated as the initial slope of light response curves) was only slightly decreased in SPP silenced transgenic plants as compared to the control plants (Fig 3-4B), indicating that the efficiency of the light reactions, including light harvesting and electron transport, was only marginally affected in SPPi plants. Other factors are involved in the reduction of maximum net CO_2 uptake. These could include limitation of the rate of diffusion CO_2 to the site of carboxylation and/or the amount and the catalytic activity of Rubisco (Long *et al*., 1993). In an attempt to separate these possible co-limiting factors A/C_i response curves were measured for a PPFD value of 2000 µmol quanta m^{-2} s^{-1} for two plants of SPPi line 17 and one control plant (Fig. 3-4C). The initial slope of the A/C_i response

curves, i.e. the carboxylation efficiency, was distinctly higher in the control plant than in the transgenic plants indicating a correspondingly higher Rubisco activity in controls. This is in keeping with the reduced protein content of SPPi plants which also affects Rubisco content. The inhibition of photosynthesis in SPPi transformants was accompanied by a decrease of F_v/F_m (from 0.83 ± 0.004 in control plants to 0.74 ± 0.02, 0.76 ± 0.019 and 0.77 ± 0.008 in lines 17, 18 and 31, respectively), indicative of a damage to these leaves from photoinhibition.

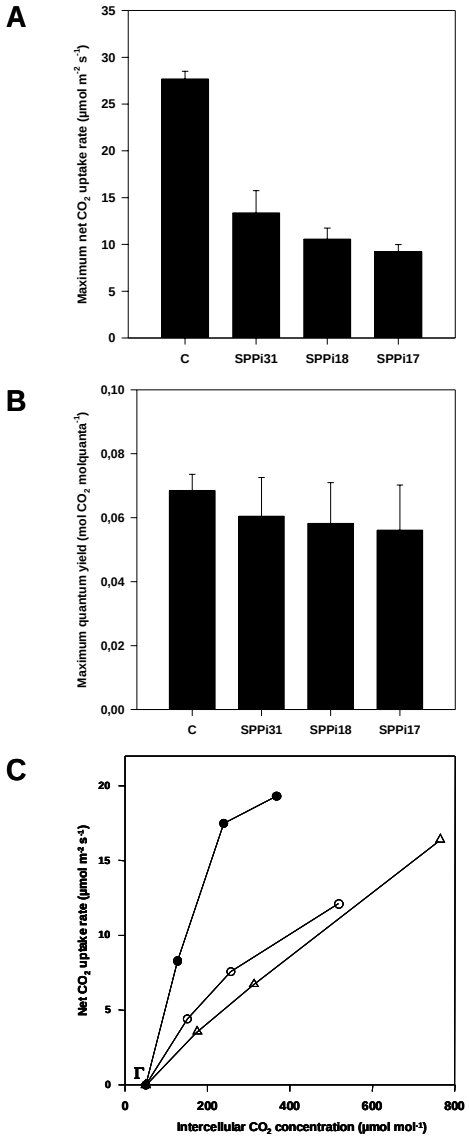


Fig. 3-4: CO_2 exchange in SPPi tobacco plants versus control plants. CO_2 uptake rates were measured on attached mature leaves using a portable photosynthesis system. Photosynthetic capacity parameters, maximum net CO_2 uptake rate (A) and maximum quantum yield of CO_2 fixation (B) were derived from the light response curves of gas exchange. C, A – c_i response curves at PFFD = 2000 µmol m^{-2} s^{-1} were obtained by measuring light response curves at three different ambient CO_2 concentrations (approximately 200, 380, and 940 µmol mol^{-1}) in source leaves of two transgenic tobacco plants from line SPP17 (open circles and triangles) and a control plant (closed circles), respectively. Leaf No. 8 as counted from the top was used for the measurement. Values represent the mean (±SD) of six measurements.

3.1.6 Repression of SPP strongly impairs leaf carbohydrate metabolism

Analysis of assimilate accumulation provided strong evidence that the decreased SPP content compromised the ability of SPPi plants to synthesize sucrose. As shown in Tab. 3-3 sucrose levels determined in source leave 5 h into the light period were reduced by 59 – 73% in the transgenics as compared to the control. These data provided strong evidence that loss of SPP activity compromised the ability of SPPi plants to synthesize sucrose. Moreover, the levels of glucose and fructose were also decreased by 65 – 85% in those plants (Tab. 3-3), as would be expected if they were produced primarily by a breakdown of sucrose. Taken together, the levels of total soluble sugars were reduced by 63-80% in SPPi plants compared to the control (Tab. 3-3). Starch in leaves represents another important carbohydrate pool for temporary storage of photosynthetic assimilates, whose synthesis is usually balanced with photosynthetic sucrose synthesis. In contrast to the reduced sucrose level, starch content was dramatically increased by a factor of 3 to 5 in the transgenics (Tab. 3-3). Consequently, the ratio of soluble sugars to starch was strongly reduced in the transgenic plants as compared to the control (Tab. 3-3). This indicated that photosynthetic carbon partitioning was altered in favour of starch.

In wild-type tobacco plants, starch built up during the day is remobilized during the subsequent night to provide precursors for sucrose synthesis that also requires SPS and SPP activity. Therefore, a loss in SPP activity would also impaired sucrose synthesis during starch remobilization at night. In agreement with this postulation, sucrose levels at the end of dark period were also decreased by 38-71% in the transgenics as compared to the control (Tab. 3-4). Moreover, Suc6P levels remained extremely high in the transgenics (Tab. 3-4). To investigate whether the impaired sucrose synthesis due to the reduction of SPP activity had any influence on starch degradation, SPPi plants along with control plants were subjected to a prolonged dark period and subsequently starch in the mature leaves was visualized by iodine staining. In leaves from control plants starch was totally remobilized after 24 h of dark treatment, whereas the leaves from SPPi plants exhibited a starch-excess phenotype that was well correlated with SPP expression level (Fig. 3-5). Consistent with the starch-excess phenotype revealed by iodine staining, starch levels determined at the end of night remained distinctly higher in the transgenics as compared to the control (Tab. 3-4). Taken together, these data indicated that SPPi plants were unable to mobilize excess starch accumulated in the previous day.

Recent evidences suggest that maltose exported from the chloroplast to the cytosol during starch mobilization at night serves as a major intermediate in the conversion of starch to sucrose (Niityllä *et al*., 2004; Chia *et al*., 2004). Interestingly, a considerable accumulation of maltose was observed in the transgenics at the end of night as compared to the control (Tab.

3-4). In this experiment the different compartments of a plant cell cannot be distinguished, therefore, it is not clear at the moment whether the export of maltose from chloroplast to cytosol or its metabolism in the cytosol was affected in SPPi plants. Nevertheless, these data indicated that starch breakdown *per se* is operating in SPPi plants but primary products of this process cannot be efficiently further metabolized.

Tab. 3-3: *Metabolite levels in mature leaves of 6-week-old SPPi transformants compared to control plants under light*

Samples were harvested from mature leaves 5 h into the photoperiod (except for maltose where sampling took place at the end of the dark period). Concentrations are given in µmol m^{-2} except that of starch which is presented as mmol m^{-2} hexose equivalent. The level of soluble sugars is given as µmol m^{-2} hexose equivalent. 3-PGA/Pi and phosphoeseter/Pi ratios are given as percentage. Values represent the mean (±SD) from at least five different plants per line.

	Control	SPPi31	SPPi18	SPPi17
Carbohydrates				
Sucrose	1416 ± 200	576 ± 171	524 ± 109	388 ± 146
Glucose	1640 ± 410	511 ± 143	686 ± 154	231 ± 142
Fructose	1652 ± 354	585 ± 243	482 ± 167	257 ± 151
Soluble sugars	6124±979	2247±660	2216±321	1262±465
Starch	3,45 ± 1,25	11,14 ± 2,24	15,77 ± 5,05	17,96± 4,62
Soluble sugars/ starch	1,98±0,79	0,20±0,05	0,15±0,05	0,07±0,02
maltose	15,25 ± 6,09	21,09 ± 15,67	76,34 ± 35,68	290,77± 67,69
Nucleotides and Nucleotide sugar				
ATP	17,14 ± 1,55	12,02 ±3,72	8,92 ± 2,17	7,74 ± 2,24
ADP	17,50 ± 5,99	6,92 ±1,27	8,59 ± 2,17	11,65 ± 4,61
AMP	2,09 ± 0,34	2,50 ± 0,47	3,36 ± 0,58	2,23 ± 1,13
ATP/ADP	0,98	1,74	1,04	0,66
UDP-Gluc	18,13 ± 2,90	16,71 ± 3,27	15,77 ± 3,14	12,62 ± 6,53
Phosphorylated intermediates				
Suc6P	0,203 ± 0,093	172,8 ± 50,1	233,5 ± 01,7	214,5 ± 59,0
3-PGA	8,69 ± 3,36	7,42 ± 2,19	7,37 ± 2,31	7,56 ± 2,24
Fru-1,6-bisP	1,01 ± 0,18	0,68 ± 0,28	0,60 ± 0,32	0,48 ± 0,20
Glc6P	36,77 ± 6,61	26,40 ± 9,44	23,75 ± 4,28	22,13 ± 2,54
Fru6P	11,85 ± 2,13	9,59 ± 3,33	9,04 ± 1,69	8,47 ± 0,91
Glc1P	2,49 ± 0,55	2,01 ± 0,74	1,41 ± 0,18	1,41 ± 0,30
Pi	875,9 ± 188,1	3141,1 ± 523,1	2765,7 ± 386,6	2885,2± 558,7
3-PGA/Pi	1,028 ± 0,456	0,241 ± 0,073	0,268 ± 0,080	0,268 ± 0,085
Phosphoester/Pi	6.96	6.97	9.97	8.82

Tab. 3-4: *Metabolite levels in mature leaves of 6-week-old SPPi transformants compared to control plants in the dark*

Samples were harvested from mature leaves at the end of the dark period. Concentrations are given in µmol m^{-2} except that of starch which is presented as mmol m^{-2} hexose equivalents. Values represent the mean (±SD) from at least five different plants per line. The metabolites which were not measured are marked with "–".

	Control	SPPi31	SPPi18	SPPi17
Carbohydrates				
Sucrose	781±148	227±85	482±63	447±77
Glucose	2120±686	399±168	476±218	487±111
Fructose	2435±698	712±186	534±116	546±112
Starch	19,61±8,95	37,51±4,12	40,08±6,91	41,68±5,04
Metabolic intermediates				
Suc6P	0,5±0,1	–	1253,3±85,0	1280,8±202,7
UDP-Gluc	24,4±3,1	–	25,4±3,3	31,0±5,1
Gluc6P	37,7±3,2	–	39,8±4,6	38,1±5,0
3-PGA	36,6±9,3	–	9,9±3,1	11,4±2,2
PEP	11,2±2,7	–	2,3±1,5	2,7±0,7
pyruvate	6,5±2,0	–	17,6±7,5	12,8±5,1

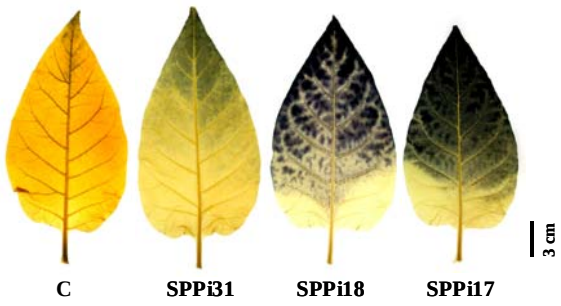


Fig. 3-5: Visualization of starch accumulation in mature leaves from SPPi plants as compared to the control. After 12 h of illumination plants kept in complete darkness for 24 h. Source leaf blades were de-colored with hot 80 % ethanol and subsequently stained with Lugol's solution. The 10th leave (as counted from the top) is shown in each case.

3.1.7 $^{14}CO_2$ partitioning

The steady-state level of assimilates in a given plant cell reflects the balance between its rate of synthesis and degradation, respectively. To further investigate whether the increased starch level in the transgenics is cumulative and thus merely due to a reduced starch turn-over rate during the dark period, or whether it is additionally caused by alterations in carbon partitioning during the course of photosynthesis, the fate of recently assimilated $^{14}CO_2$ was investigated. To this end, leaf discs of dark adapted plants were incubated in a leaf disc electrode under saturating light and saturating $^{14}CO_2$ for 20 min. Following ethanol extraction,

radiolabel was determined in the soluble and insoluble fraction. Surprisingly, although steady state photosynthesis under ambient CO_2 conditions was substantially decreased in intact plants (see above) total label incorporated under saturating conditions increased by 30 to 60% in SPPi plants as compared to the control (Tab. 3-5). Additional carbon assimilated was exclusively found in the insoluble fraction and thus fed into starch synthesis while the total incorporation of label into the soluble fraction remained unchanged between SPPi lines and controls. The percentage of ^{14}C incorporated into insoluble fraction (mainly starch) was increased from 48% in the control to 62-66% of the total label incorporated in SPPi plants (Tab. 3-5), indicating carbon partitioning during photosynthesis was redirected in favor of starch synthesis in the transgenics. The soluble fraction was further subdivided into neutrals (soluble sugars), anions (phosphorylated intermediates and organic acids) and cations (amino acids) by ion exchange chromatography. Consistent with a block in sucrose synthesis, less label was incorporated into the neutral fraction in the transgenics (6-9 % of the total incorporation) as compared to the control (38% of the total incorporation). Labeling of the cationic fraction, predominantly consisting of amino acids, was largely unchanged between the transgenic and control plants. A massive 3 to 4-fold increase in the proportion of label incorporated into the anionic fraction was observed in the SPPi plants as compared to the control. Consequently, the soluble fraction in the control plants is predominantly composed of soluble sugars whereas phosphorylated intermediates and organic acids account for the largest proportion of the soluble fraction in the transgenics.

Tab. 3-5: *Incorporation of $^{14}CO_2$ into starch and soluble components under saturating light and CO_2.*
Leaf discs were cut from six separate dark-adapted plants per line and illuminated in an oxygen electrode containing air saturated with $^{14}CO_2$. After 20 min the leaf discs were extracted and fractionated. Data are presented as percentage (±SD) of total incorporation (n = 6). The ratios between the incorporation into insoluble fraction and that into soluble fraction are given in percentage (±SD).

	^{14}C-incorporation			
	Control	SPPi31	SPPi18	SPPi17
Total (DPM)	3001.5±624.5	4004.8±368.7	4331.5±903.4	4792.3±509.0
Insoluble	48.46 ± 5.64	62.27 ± 6.46	67.37 ± 2.74	66.97 ± 1.38
Soluble	51.54 ± 5.64	37.73 ± 6.46	32.63 ± 2.74	33.03 ± 1.38
Insoluble/soluble	95.9 ± 21.8	171.7 ± 48.6	208.3 ± 27.5	203.2 ± 13.0
Neutral	38.12 ± 8.00	9.45 ± 4.19	6.20 ± 0.73	6.07 ± 0.27
Anionic	5.46 ± 1.59	18.82 ± 3.86	19.55 ± 2.41	20.73 ± 1.65
Cationic	7.95 ± 1.04	9.46 ± 1.83	6.88 ± 1.05	6.23 ± 0.38

3.1.8 Metabolic intermediates

The results of the above experiments indicate that profound changes occur in the SPPi plants in the content of metabolites undergoing high fluxes. Therefore, levels of metabolic intermediates were determined in the samples collected from the mature leaves 5 h into light period. In addition to conventional enzymatic assays for the determination of hexose-phosphates and 3-PGA, high-performance ion-exchange chromatography coupled to mass-spectrometry (IC-MS) was used for the determination of a range of other low abundance metabolites (for details see material and methods). The results showed that a tremendous build-up of Suc6P occurred in the mature leaves from SPPi plants with a ~1000-fold increase as compared to that in the control plants (Tab. 3-3), which was in keeping with the reaction catalyzed by the enzyme. In contrast to the massive accumulation of Suc6P, reduced SPP acitivity left UDP-Gluc levels largely unaltered and caused even a decrease in hexose-P levels in the transgenics (Tab. 3-3). This was unanticipated since hexose-P is mainly (Gerhardt *et al*., 1987) and UDP-Gluc is almost exclusively (Dancer *et al*., 1990b) located in the cytosol, upstream of SPP in the sucrose biosynthetic pathway. However, this result may be explained by the increase of SPS activity in SPPi plants (see below). The loss of SPP activity resulted in a lower rate of sucrose synthesis and a massive accumulation of Suc6P, which might severely impair Pi recycling into chloroplast leading to an increase in 3-PGA level and eventually cause Pi-limitation of photosynthesis. Unexpectedly, 3-PGA content was largely unaltered in SPPi plants (Tab. 3-3), indicating that no Pi-limitation of photosynthesis occurred under steady-state conditions. PEP and pyruvate levels remained largely unchanged and thus glycolysis most likely does not substantially contribute to the 3-PGA pool (Tab. 3-3). Although 3-PGA pool was not altered, Fruc-1,6-bisP levels decreased in all SPPi lines (Tab. 3-3). Surprisingly, a marked increase in inorganic phosphate level was observed in SPPi plants regarded on a whole cell level, which actually quenched the increase in phosphoester/phosphate ratio entailed by the increase in Suc6P (Tab. 3-3). The increase in Pi level might contribute to release the cells from Pi limitation during photosynthesis due to the lower rate of sucrose synthesis in SPPi plants.

The total adenylate pool in SPPi lines was reduced to approximately 60% of wild-type level but the ATP:ADP ratio was not consistently changed (Tab. 3-3).

Recent evidences suggested that trehalose-6-P (T6P) might serve as a signaling molecule involved in the regulation of photosynthesis and starch synthesis in leaves (Pellny *et al*., 2004; Peter Geigenberger, personal communication). Interestingly, a 2 to 5-fold increase in T6P

levels was observed in the transgenics as compared to the control (Fig. 3-6), indicating that a signaling transduction chain with T6P as a key component might be stimulated in SPPi plants probably to counteract the decreased photosynthesis or to enhance starch synthesis.

To obtain further insights into sucrose synthesis during starch remobilization in SPPi plants, metabolic intermediates were also determined in the mature leaves at the end of dark period. As shown above, a massive accumulation of Suc6P was observed in SPPi plants at the end of night (Tab. 3-4). The levels of UDP-Gluc and hexose-P, as observed in the leaves 5 h into light period, were unexpectedly unaltered in SPPi plants as compared to the control (Tab. 3-4). The amounts of 3-PGA and PEP were significantly decreased whereas pyruvate levels substantially increased in SPPi plants at the end of dark period (Tab. 3-4).

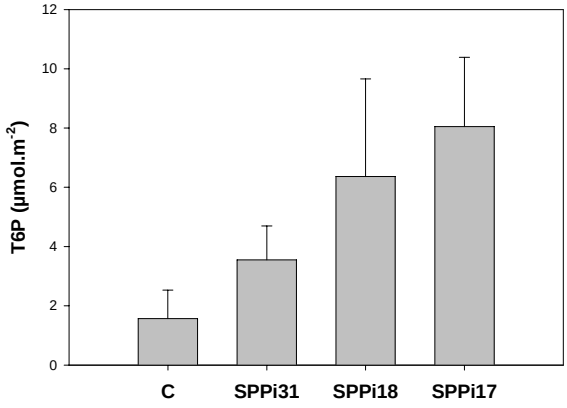


Fig. 3-6: T6P levels in the mature leaves from the control and transgenic tobacco plants. Samples were harvested 5 h into the photoperiod from mature leaves of 5-week-old soil-growing plants and T6P content was determined using IC-MS. Values represent the mean (±SD) from at least five different plants per line.

3.1.9 Measurement of the activities of the key enzymes involved in sucrose and starch synthesis in mature leaves

Next it was investigated whether decreased SPP expression affects the activities of other key enzymes of carbohydrate metabolism. To this end, two key enzymes involved in sucrose synthesis (SPS and cFBPase) and one regulatory enzyme in starch synthesis (AGPase) were investigated in samples taken from mature leaves.

The V_{max} activity of SPS that is measured under the conditions with saturating concentrations of substrates (UDP-Gluc and Fruc6P) and activator (Gluc6P) in the absence of inhibitor (Pi) represents the maximal catalytic capacity of the enzyme. In SPPi plants SPS V_{max} activity was increased slightly by 10%, 13% and 23% in lines 17, 18 and 31, respectively, when calculated on a leaf area basis (Tab. 3-6) but more appreciable when calculated on a total protein basis (53%, 63% and 54%, respectively; data not shown, compare tables 2 and 5). To investigate whether a regulation of the enzyme at the point of transcription eventually causes SPS protein

amounts to increase, northern analyses were carried out with samples from the mature leaves. As shown in Fig. 3-7, the results revealed an up-regulation of SPS mRNA steady state level in the SPPi lines. In plants, activation of SPS is modulated by reversible protein phosphorylation, which alters the affinity of the enzyme for substrates and effectors. Dephosphorylated SPS protein is less sensitive to the inhibitor, Pi, exhibits higher affinity to the substrates and activator, and thus more active (Huber *et al*., 1989; Reimholz *et al*., 1994; Siegl *et al*., 1990; Siegl and Stitt, 1990). In order to investigate the activation state of the enzyme, SPS activity (V_{sel} activity) is measured under the selective conditions with limiting concentrations of substrates and activator in the presence of the inhibitor Pi. A higher ratio of V_{sel}/V_{max} reflects a lower phosphorylation state of SPS and thus an enhanced activation state of the enzyme. Under V_{sel} assay conditions SPS activity increased by 127%, 148% and 154% in SPPi-17, -18 and 31, respectively (Tab. 3-6). The ratio between V_{max} and V_{sel} was increased by approximately one third from 20% in control plants to 30% in SPPi lines (Tab. 3-6), indicative for a lower phosphorylation state of the enzyme pool and thus a higher activation state of SPS in SPPi plants. Given the exceptionally high levels of Suc6P in the SPPi plants this metabolite might also act as a phosphate sequester causing SPS phosphorylation state to decrease and thus leading to a rise in its activation state. If this were to be the case, SPS would lose its ability to respond to light/dark transitions. Therefore diurnal changes of SPS enzyme activity were determined. The results clearly demonstrated that the activation state of the enzyme is modulated by light dark transitions in SPPi plants just as in control plants (Fig. 3-8). This implies that the higher activation state of SPS in the transgenics is not merely a secondary effect imposed by phosphate sequestration, but rather a compensatory response by the plant in order to increase its sucrose biosynthetic capacity.

Tab. 3-6: *Enzyme activities in mature leaves of SPPi tobacco transformants.*

Enzyme activities were determined in mature leaves of 6-week-old plants. Samples were taken 5 h into the light period. SPS activity was measured under both optimal (V_{max}) and limiting (V_{sel}) assay conditions. Data are means (±SD) of determinations on six individual plants per line.

	Enzyme activity µmol min^{-1} m^{-2}			
	Control	SPPi31	SPPi18	SPPi17
SPS				
V_{max}	101,7 ± 17,2	125,2 ± 11,8	114,6 ± 17,4	112,3 ± 15,8
V_{sel}	19,7 ± 3,6	40,1 ± 5,7	34,0 ± 7,5	32,5 ± 4,4
V_{sel}/V_{max} (%)	19,6 ± 3,7	32,0 ± 1,7	29,4 ± 2,3	29,0 ± 0,9
AGPase	74,6 ± 4,4	30,1 ± 4,0	31,5 ± 8,7	39,9 ± 5,2
cFBPase	50,5 ± 6,5	41,4 ± 4,1	40,3 ± 2,7	38,3 ± 3,1

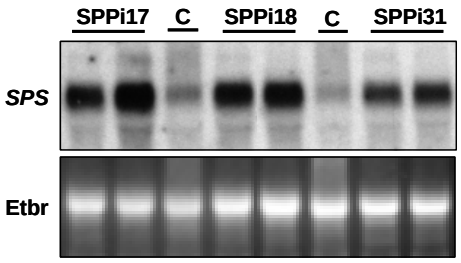


Fig. 3-7: Northern blot analysis of SPS mRNA steady-state level in control and SPPi plants. Samples were harvested 5 h into the light period from mature leaves and total RNA was isolated. Thirty µg of total RNA was subject to gel blot hybridization using ^{32}P-labeled SPS cDNA from tobacco (GenBank Acc. No. AF194022). Ethidium bromide staining of 25S rRNA serves as control for equal loading.

Control of sucrose synthesis is assumed to be shared by SPS and cFBPase and the two enzymes are supposed to be coordinately regulated (Stitt, 1989). However, unlike the increased SPS mRNA level, the steady-state level of cFBPase mRNA remained unaltered in SPPi plants as compared to the control (Fig. 3-9A). *In vitro* assay of cFBPase activity revealed that it dropped by approximately 20% in SPPi lines when calculated on a leaf area basis (Tab. 3-6) which is in the range of the reduction in total protein content observed in these lines. The *in vivo* activity of cFBPase is subject to allosteric control by the signal metabolite fructose-2,6-bisphosphate (Fruc-2,6-bisP) (Stitt, 1990a), which strongly inhibits cFBPase during night. Upon illumination, Fruc-2,6-bisP levels are diminished and thus causing feed-forward stimulation of photosynthetic sucrose biosynthesis. Fruc-2,6-bisP levels determined 5 h into the light period revealed no differences in the level of this metabolite between SPPi plants and the control (Fig. 3-9B), indicating *in vivo* cFBPase activity is unlikely to be higher in SPPi plants than that in the control. Taken together, the data clearly demonstrate that in

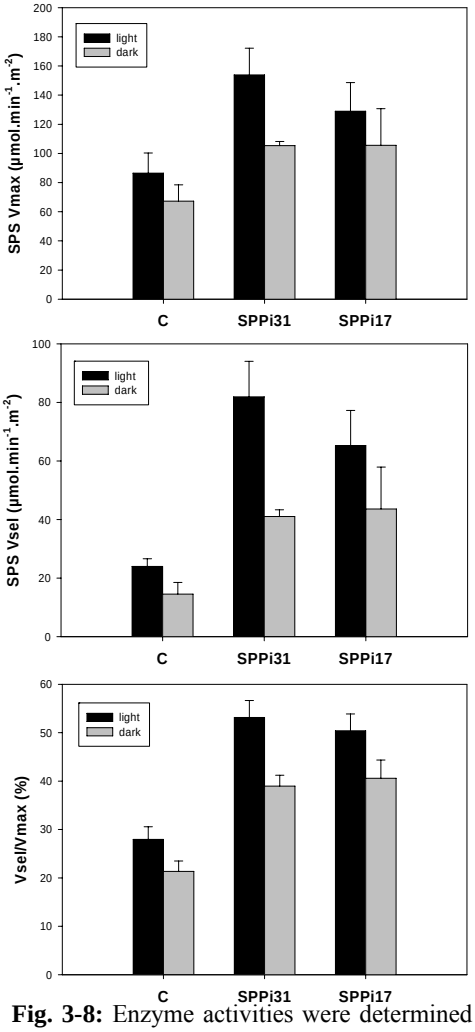


Fig. 3-8: Enzyme activities were determined in mature leaves of 7-week-old plants. Samples were taken 5 h into the light period (light) and at the end of dark period (dark). SPS activity was measured under both optimal (V_{max}) and limiting (V_{sel}) assay conditions. Data are means (±SD) of determinations on six individual plants per line.

SPPi plants sucrose biosynthesis was not up-regulated at the cFBPase step.

AGPase activity, which was chosen as a representative for starch synthesis, decreased in all the SPPi lines (Tab. 3-6) which is in contradiction to the increased partitioning of fixed carbon into starch which has been observed in short-term labeling experiments. However, the recent discovery that AGPase is subject to redox-dependent posttranslational regulation (Hendriks *et al.*, 2003) makes it questionable whether *in vitro* measurements accurately reflect AGPase *in vivo* activity. On the other hand, a decrease in AGPase activity after 5 h into the light period could reflect an acclimation towards the build-up of starch over time.

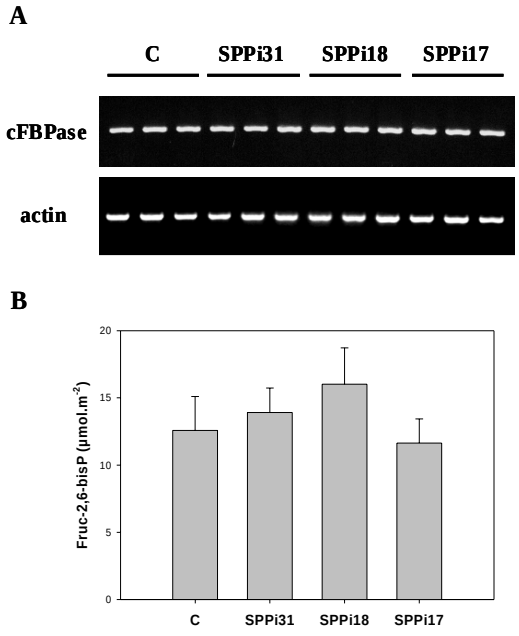


Fig. 3-9: The expression of cFBPase (A) and Fruc-2,6-bisP contents (B).

RT-PCR analysis of cFBPase expression in SPPi tobacco plants (A). Samples were taken 5 h into light period from mature leaves. Total RNA was isolated and used for RT-PCR as described in Materials and methods applying 35 amplification cycles. Equal amounts of cDNA were controlled by amplification of a constitutively expressed *actin* gene (30 cycles).

Levels of Fruc-2,6-bisP in mature leaves of SPPi plants and the control, respectively (B). Samples were taken 5 h into the light period from 6-week-old plants and Fruc-2,6-bisP content was determined using IC-MS. Values represent the mean (±SD) from at least five different plants per line.

3.1.10 Reduction of SPP activity leads to only a moderate increase of Suc6P and less severe changes in carbon metabolism in young leaves

Sucrose synthesis activity in leaves is regulated by leaf development with higher activity in mature leaves and lower activity in young leaves (Baxter *et al.*, 2003). In order to investigate the potential effects of decreased SPP activity on sucrose synthesis and carbon metabolism at different developmental stages, analyses were further conducted in expanding young leaves. As predicted from the constitutive expression pattern of CaMV 35S promoter, SPP activity was also strongly inhibited in the young leaves with a degree similar to that observed in the mature leaves of SPPi plants (Tab. 3-7). However, the strong reduction of SPP activity only led to a moderate increase of Suc6P content (4 to 14 folds) in the young leaves of SPPi plants

(Tab. 3-8A). Furthermore, decreased SPP activity caused less severe changes in carbon metabolism in the young leaves than in the mature leaves. In detail, sucrose levels in the young leaves were reduced 34-62% in SPPi plants as compared to the control whereas a decrease by 61-84% in sucrose levels occurred in the mature leaves (Tab. 3-8B). In both genotypes sucrose content in the young leaves was higher than that in the mature leaves. The levels of glucose and fructose were decreased by 38-49% and 39-68% in the young leaves of SPPi plants, respectively, whereas a reduction of 86-88% and 75-83% in the mature leaves, respectively (Tab. 3-8A, B). Interestingly, the increase in starch content that occurred in the mature leaves was largely eliminated from the young leaves, indicating starch synthesis was not stimulated in the young leaves as it was in the mature leaves (Tab. 3-8A, B). Accordingly, starch excess phenotype after a prolonged dark period was not observed in the young leaves (Fig. 3-10).

Tab. 3-7: *Enzyme activities in expanding young leaves of SPPi tobacco transformants.*

Enzyme activities were determined in expanding young leaves (leaf No.5 as counted from the top) of 6-week-old plants. Samples were taken 5 h into the light period. SPS activity was measured under both optimal (V_{max}) and limiting (V_{sel}) assay conditions. Values are given in means ±SD (n=6).

	Enzyme activity µmol min^{-1} m^{-2}			
	Control	SPPi31	SPPi18	SPPi17
SPP	214,0±27,9	21,4±8,0	13,9±2,6	9,1±3,0
SPS				
V_{max}	102,9±10,9	110,2±14,7	101,4±7,9	92,0±3,8
V_{sel}	18,5±4,7	12,9±1,9	13,9±1,9	12,6±1,6
V_{sel}/V_{max} (%)	16,4±3,4	11,7±1,0	13,7±0,9	14,8±2,5

Tab. 3-8: *Metabolite levels in young leaves of 6-week-old SPPi transformants compared to control plants under light*

Samples were harvested from young leaves (leaf No.5 as counted from the top) and mature leaves (leaf No.9 as counted from the top) 5 h into the photoperiod, respectively. Concentrations are given in µmol m^{-2} except that of starch which is presented as mmol m^{-2} hexose equivalents. Values represent the mean (±SD) from at least four different plants per line. (A), young leaves; (B) mature leaves.

(A)

	Control	SPPi31	SPPi18	SPPi17
Sucrose	4262±647	2817±581	2264±368	1623±354
Glucose	2460±614	1535±706	1282±261	1243±340
Fructose	2503±352	1521±517	1067±131	796±134
Starch	11,22±2,75	8,02±1,65	8,76±2,06	12,81±4,67
Suc6P	0,57±0,46	2,27±0,32	5,47±1,41	8,25±3,83

(B)

	Control	SPPi31	SPPi18	SPPi17
Sucrose	2192±185	851±280	566±163	354±158
Glucose	2425±636	296±198	338±89	293±231
Fructose	2910±666	733±387	648±160	486±301
Starch	21,77±6,13	42,02±3,80	42,59±5,05	43,60±6,24

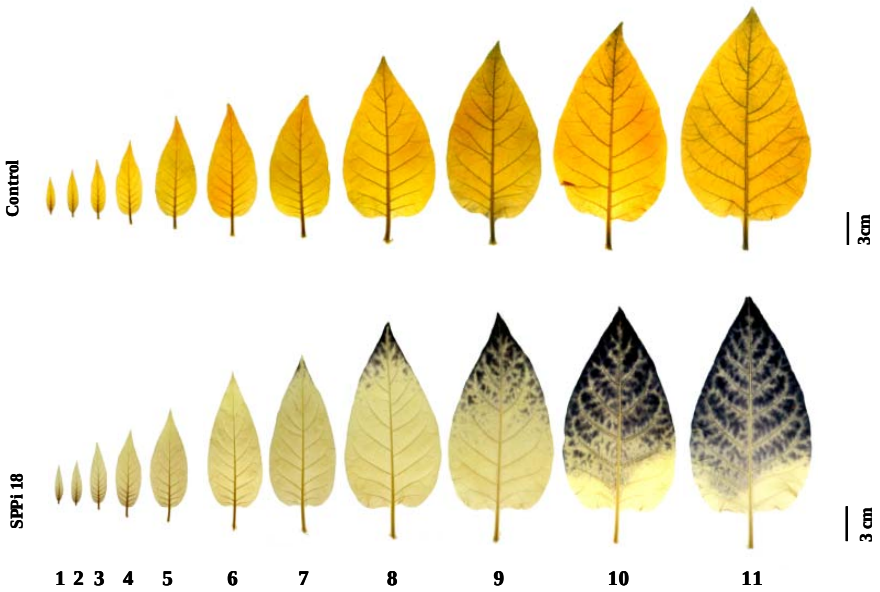


Fig. 3-10: Visualization of starch accumulation in leaves. After 12 h of illumination plants kept in complete darkness for 24 h. Leaf blades were de-colored with hot 80 % ethanol and subsequently stained with Lugol's solution. The leaf numbers are given as counted from the top. The control plant (upper panel) and SPPi transgenic line 18 (lower panel) were shown here. The other two transgenic lines, SPPi31 and SPPi17, exhibited similar pattern of starch accumulation as SPPi18.

3.1.11 Photosynthesis and SPS enzyme activity were largely unaffected in the young leaves of SPP silenced transgenic plants

Photosynthesis capacity was further determined in three different leaves (corresponding to leaf stages 10, 8 and 5 in Fig. 3-10). In the control plants maximum net CO_2 uptake rates in upper and lower mature leaves were dramatically increased as compared to that in the young leaves (Fig. 3-11). However, such increase in photosynthetic capacity in mature leaves was inhibited in SPPi plants, leading to a constant level in maximum net CO_2 uptake rate in all the

three leaves (Fig. 3-11). When comparing the photosynthetic capacity between the control and transgenic plants, a strong decrease in maximum net CO_2 uptake rate occurred in the mature leaves in SPPi plants as observed before while no changes could be observed in the young leaves between the two genotype plants.

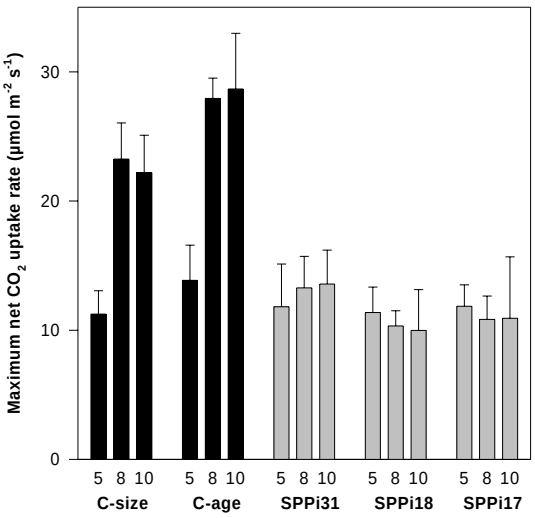


Fig. 3-11: CO_2 exchange in SPPi tobacco plants versus control plants. CO_2 uptake rates were measured on attached leaves using a portable photosynthesis system. Maximum net CO_2 uptake rates were derived from the light response curves of gas exchange. Leaves No. 5, 8 and 10 as counted from the top were used for the measurement. C-size, the control plants that are two-week younger than the transgenic plants but have similar size as the transgenic line SPPi 18. C-age, the control plants that are in the same age as the transgenic plants. Values represent the mean (±SD) of six measurements.

Steady state level of Suc6P in leaves reflects the balance between its synthesis rate catalysed by SPS and the degradation rate catalysed by SPP. Similar degree of inhibition in SPP activity was obtained in young and mature leaves of SPPi plants, however, only a moderate accumulation of Suc6P occurred in the young leaves of SPPi plants whereas a massive accumulation of Suc6P in the mature leaves of SPPi plants. To gain better understanding of this phenomenon, SPS activity was measured in the expanding young leaves. As shown in Tab. 3-8, in contrast to what observed in the mature leaves, SPS activities measured under both V_{max} and V_{sel} conditions and the activation state of SPS indicated by the ratio of V_{sel}/V_{max} were largely unchanged or even slightly decreased in the young leaves of SPPi plants as compared to the control plants. These data indicated signalling pathways involved in up-regulation of SPS at different levels were activated in the mature leaves but not in the young leaves. To obtain further insights into the relationship between Suc6P levels and the activities of SPS and SPP, Suc6P levels in the leaves at different developmental stages were plotted against the ratios of SPS V_{max} activity to SPP activity (SPS V_{max}/SPP) and SPS V_{sel} activity to SPP activity (SPS V_{sel}/SPP), respectively. A clear correlation could be found between the Suc6P level and the ratio of SPS V_{sel}/SPP, whereas no obvious correlation could be found between Suc6P level and the ratio of SPS V_{max}/SPP in the leaves at different

developmental stages in the two genotype plants (Fig. 3-12). These results were in agreement with the idea that SPS V_{sel} activity is a more important factor in the control of sucrose synthesis rate than SPS V_{max} activity. These data also indicated that accumulation of Suc6P in SPPi plants was a consequence of the imbalance between SPS V_{sel} activity and SPP activity. In the control plants, SPP activity was in large excess over SPS V_{sel} activity in both young and mature leaves and Suc6P level was constantly low. In the young leaves of SPPi plants, the ratio of SPS V_{sel} to SPP rose up to about 1:1 due to the inhibition of SPP activity and consequently Suc6P was accumulated to a moderate level (4 to 14-fold as compared to the control). Although the degree of inhibition in SPP activity was similar in the mature leaves of SPPi plants as in the young leaves, the increase in SPS V_{sel} activity in the mature leaves led to a higher ratio of SPS V_{sel}/SPP and thus resulted in a massive accumulation of Suc6P (thousand-fold of that in the control).

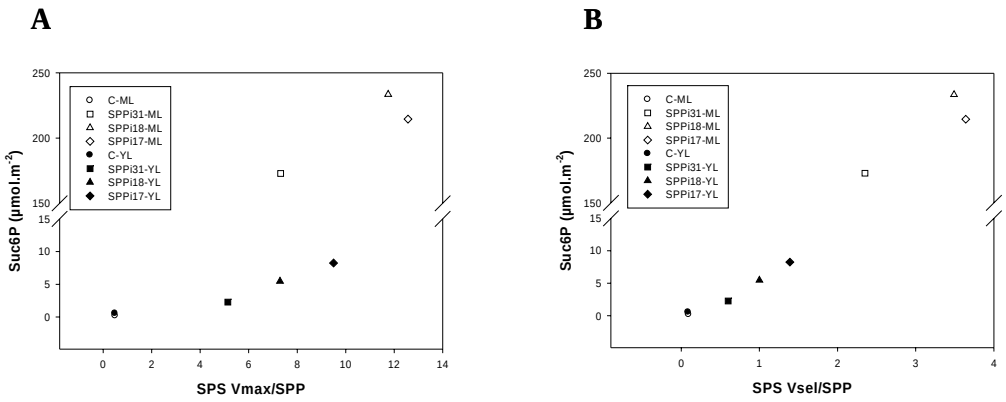


Fig. 3-12: Positive correlation between Suc6P levels and the ratio of SPS V_{sel} activity to SPP activity in the leaves. Suc6P levels were plotted against the ratios of SPS V_{max}/SPP (A) and SPS V_{sel}/SPP (B) in the leaves at different developmental stages from the transgenic and control plants. The mean value from Fig. 3-2, and Tab. 3-3, 3-8 and 3-9 were used for the calculation. ML, mature leaves; YL, young leaves.

3.1.12 Estimation of the flux control coefficient for SPP in the pathway for sucrose synthesis

The relative contribution of an enzyme to the control of flux in a pathway can be experimentally estimated from transgenic plants with a stepwise reduction in the activity of the enzyme. The change in enzyme activity and the resulting change in the flux through the pathway are quantified and plotted against each other to estimate the control coefficient for that enzyme (Kacser and Burns, 1973). If the enzyme under investigation is markedly reduced

in its activity over other enzymes in the pathway the control coefficient will tend to rise and thus will be over estimated. It is therefore important to include plants with a relative small change in activity and to estimate the control coefficients from the slope of the flux-enzyme amount plot in the vicinity of the wild-type value (Stitt, 1989; Geigenberger *et al.*, 2004). T1 plants from the segregating SPPi line 36 were chosen for this experiment since a considerable proportion of those plants were indistinguishable from the control plants. Nevertheless residual SPP activity in these plants ranged from 80 to 20% as compared to the control plants (Fig. 3-13). The control coefficient for SPP over sucrose synthesis estimated from the slope of the plot in that range of residual enzyme activity was found to be close to zero (-0.081 ± 0.095).

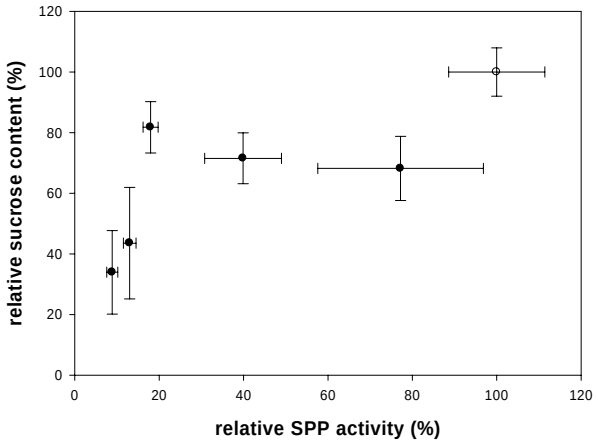


Fig. 3-13: Relation of SPP enzyme activity and sucrose content in a segregating T1 population of line SPPi 36 (close circles) normalized to the control plants (open circle). The control coefficient for SPP for the pathway of sucrose biosynthesis was estimated from plants with 20-80% residual SPP activity. Each value represents the mean of at least 4 independent plants ± SD.

3.2 *Decreased expression of SPP in potato tubers results in impeded sucrose synthesis, reduced induction of vacuolar acid invertase during cold storage and a dramatic decrease in cold-induced hexose accumulation*

Once sucrose is imported from source leaves into growing tubers, it will be cleaved by Susy and the resulting hexose are used for starch synthesis. However, the pathway of sucrose (re)synthesis via SPS and SPP is also present in this organ at the same time. Therefore, a futile cycle is formed between breakdown and resynthesis of sucrose in growing tubers (Geigenberger and Stitt, 1993). The function of this futile cycle is not clearly understood yet although early evidence suggested that it might be involved in coping with water stress (Geigenberger *et al*., 1997; Geigenberger *et al*., 1999). The pathway of sucrose synthesis via SPS and SPP also exists in the stored tubers and is stimulated concomitantly with starch degradation in the cold (4-6°C), a condition to prevent tubers from sprouting during storage. The resulting sucrose is subsequently imported into the vacuole where it is cleaved into glucose and fructose by the action of vacuolar invertase. The accumulation of hexose in potato tubers during cold storage is a phenomenon well known as cold sweetening, which makes tubers unsuitable for processing industry. Since sucrose synthesis connects starch degradation and hexose generation in the cold-stored tubers, it is important to study the regulation of sucrose synthesis in order to gain a better understanding of cold sweetening and in turn to manipulate this economically important trait. So far the studies have mainly focused on SPS, the regulatory enzyme in sucrose synthesis. The contribution of SPP to the control of sucrose synthesis pathway in cold-stored potato tubers is not clear until now. In this work, we applied an RNAi approach to address this question.

3.2.1 Molecular cloning of SPP encoding cDNAs from potato and construction of SPP silenced transgenic potato plants

Two potato EST clones (BG889871 and BM109368) were previously identified to form a contiguous sequence that covers 75% of the expected SPP protein coding region (Lunn, 2003). This SPP gene was subsequently renamed as StSPP1 in this study. The Genbank EST database was blasted using the deduced StSPP1 sequence and another class of ESTs from potato was found which contained 9 members. These nine ESTs form a contiguous sequence

which covers an entire ORF predicted to encode a 47,8 kD SPP protein (StSPP2). StSPP1 and StSPP2 share 88% identity based on the known coding region at the nucleotide level. As mentioned above, two isoforms of NtSPP were identified from tobacco. The phylogenetic analysis showed that these four SPP genes fell into two groups that contained NtSPP1 and StSPP1, and NtSPP2 and StSPP2, respectively. The coding region of the StSPP genes showed over 88% identity to that of the NtSPP genes.

Earlier studies revealed that a degree of identity between two nucleotide sequences above 88% is sufficient to target both sequences for PTGS by the same silencing construct (Holzberg *et al*., 2002; Schweizer *et al*., 2000). Therefore, RNA silencing would not discriminate between the known tobacco and potato SPP genes and the RNAi construct devised for NtSPP2 silencing which was driven by the CaMV35S promoter could be used to reduce SPP gene expression in transgenic potato plants. Twenty-four transgenic potato plants (designated as 35SPPi) were obtained via *Agrobacterium*-mediated gene transfer. Western blot analysis with mature leaves revealed that a dramatic reduction of SPP protein occurred in eight of the transgenic lines (Fig. 3-14A). The reduction of SPP protein was further confirmed in the tubers in these eight transgenic lines (Fig. 3-14B). Two severely affected lines (line 5 and 8) and one moderately affected line (line18) were propagated from stem cuttings and further analysed.

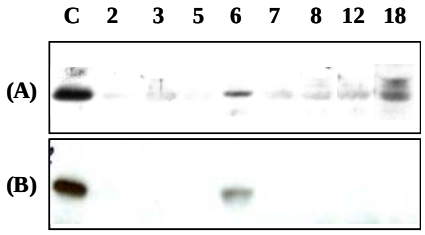


Fig. 3-14: Screening of transgenic potato plants with reduced SPP protein.
Western blot analysis was performed to screen the transgenic potato plants with reduced SPP protein. Thirty µg of total protein from mature leaves (A) and freshly harvested tubers (B) was separated by SDS-PAGE and immunodecorated using a polyclonal SPP antibody raised against *Nt*SPP2. C, solara; 2-18, eight transgenic lines.

3.2.2 Constitutive repression of SPP expression leads to plant growth retardation and reduction of tuber yield

As observed in transgenic tobacco plants, plant growth was also retarded in the 35SPPi transgenic potato plants, concomitant with a prolonged life span. Fig. 3-15A. illustrates the growth retardation in transgenic potato plants. Decreased expression of SPP, as observed in transgenic tobacco plants, led to spontaneous necrotic lesions in the mature leaves of 35SPPi

potato plants, which started from the tips and spread to the basal parts with maturation of the leaves (Fig. 3-15B). Tuber yield, with respect to total tuber number and fresh tuber weight per plant, was dramatically reduced in the severely affected plants (line 5 and 8). In line 18, total tuber number per plant was slightly increased although tuber fresh weight per plant was decreased (Tab. 3-9).

Fig. 3-15: Plant growth retardation and spontaneous necrotic lesion in transgenic potato plants.
The pictures were taken five weeks after the plants were transferred from tissue culture to the growth chamber. A, retardation of plant growth. B, spontaneous necrotic lesion in the mature leaves of transgenic plants. a, solara; b, 35SPPi-18; c, 35SPPi-8; d, 35SPPi-5.

Tab. 3-9: *Tuber yield*

Tubers with diameters larger than 1 cm were harvested from plants grown in the growth chamber when the plants were fully senescent (approximately four months). Tuber yield (including tuber fresh weight and total tuber number per plant) was determined in the freshly harvested tubers. Values are given as mean ± SD (n=15).

line	Tuber fresh weight per plant (g)	Total tuber number per plant
solara	299,6±20,4	11,1±1,5
35SPPi18	191,9±18,9	15±2,8
35SPPi8	110,4±15,9	6,6±1,2
35SPPi5	43,2±13,4	7,3±1,7

3.2.3 Western blot and enzyme activity in the selected transgenic potato plants

In the clonally propagated transgenic plants, SPP protein was virtually eliminated from the freshly harvested tubers (Fig. 3-16A.). Previous evidences indicate that low temperature can reverse the silencing events in various tissues from several plant species including stems from potato plants (Szittya *et al.*, 2003). Therefore, the question arose whether SPP silencing through an RNAi approach is stable in potato tubers during cold storage. If SPP silencing were reversible during cold storage, it would make this approach unsuitable for studying the role of SPP in the control of sucrose synthesis pathway in cold-stored tubers. To this end, western blot analysis was carried out to detect SPP protein in potato tubers during cold storage and the result revealed that the SPP protein remained undetectable in the transgenic tubers 34 days after cold storage (Fig. 3-16B.), indicating that low temperature could not reverse SPP silencing in the cold-stored tubers. This result was in agreement with previous studies where low temperature did not inhibit RNA silencing in cold-stored potato tubers (Zrenner *et al.*, 1996b; Krause *et al.*, 1998). Therefore, the approach applying RNAi technology to knockdown SPP expression can be used to address the initial question.

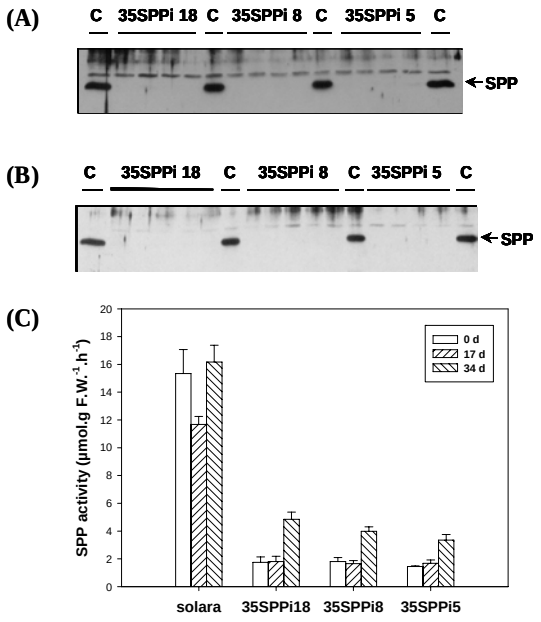


Fig. 3-16: Western blot analysis and SPP activity

Western blot analysis was performed to detect SPP protein in the tubers before cold storage (A) and 34 days after storage (B). Thirty µg of total tuber protein was separated by SDS-PAGE and immunodecorated using a polyclonal SPP antibody raised against *Nt*SPP2. The arrows indicate the signals from SPP protein. C, SPP activity. SPP activity was determined from 4-5 individual tubers per line at the indicated time after transfer of tubers to the cold (4°C). Values are the mean±SD.

Potato SPP activity was measured with varying substrate concentrations (Fig. 3-17), which showed that the enzyme has Michaelis-Menten kinetics with an apparent Km for S6P of 148 µM, which is slightly higher than those reported for rice leaf SPP (65 µM; Lunn *et al.*, 2000)

and spinach leaf SPP (105 µM; Echeverria *et al*., 1997). In order to determine the maximum SPP catalytic capacity in various potato plants, 1250 µM of Suc6P was used for activity determination. In the freshly harvested tubers, SPP activity was decreased by approximately 90% in the transgenic tubers compared to that of control tubers (Fig. 3-16C.). The degree of inhibition of SPP activity was largely unchanged 17 days after cold storage. SPP activity was slightly increased in the transgenics after 34 days in the cold, however it was still drastically decreased in the transgenics compared to that in the control (70-80% decrease in the transgenics).

The above *in vitro* SPP activity was measured at 30°C, however, the cold-stored tubers were kept at 4°C. In order to estimate the catalytic capacity of SPP in the cold-stored tubers based on the *in vitro* SPP activity measured at 30°C, the temperature coefficients (Q10) of potato SPP was determined over a temperature range from 15-30°C and 4-15°C, respectively. Q10 for potato SPP is 2,14±0,024 from 15 to 30°C and 2,23±0,091 from 4 to15°C (calculated from Fig. 3-18). After calculation based on the *in vitro* SPP activity measured at 30°C and the temperature coefficients, the catalytic capacities of SPP in the cold-stored tubers were estimated as 1,55±0,075 µmol/g F.W./h in the controls and 0,22±0,028 to 0,24±0,049 µmol/g F.W./h in the transgenics.

Fig. 3-17: Measurement of Km of SPP from potato tubers

Crude enzyme extracts were prepared from potato growing tubers. SPP activity was measured with varying concentration of Suc6P and plotted against the corresponding concentration of Suc6P. The data were processed with Sigmaplot.

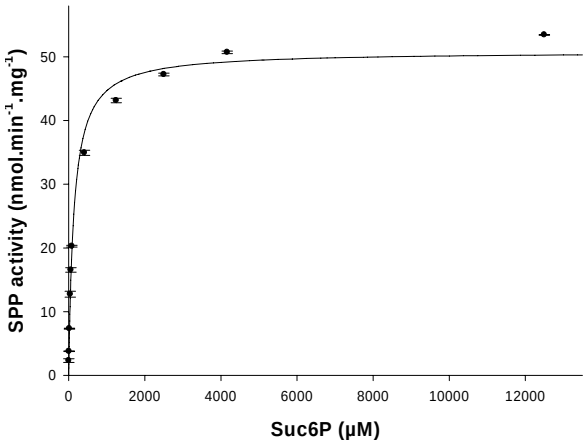
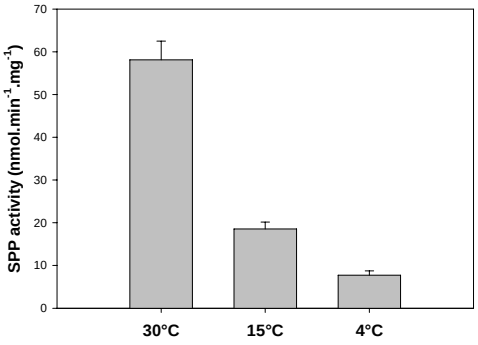


Fig. 3-18: Temperature effects on the SPP activity.

SPP acitivty was measured at 30°C, 15°C and 4°C in crude protein extracts from five individual wild type growing tubers.



3.2.4 Cold-induced sucrose metabolism was strongly altered in the transgenic potato tubers

After harvest, the potato tubers were stored at room temperature for one week and subsequently stored at 4°C. Steady state levels of carbohydrates were determined at the indicated time. As shown in Tab. 3-10, before cold storage the levels of sucrose and hexose were largely unaltered between the control and transgenic tubers. 17 days after cold storage, sucrose levels were substantially increased in both control and transgenic tubers. Surprisingly, the transgenic tubers contained distinctly higher levels of sucrose after cold storage (69%, 35% and 33% more sucrose in 35SPPi-18, 8 and 5, respectively, than that in the controls), whereas the cold-induced hexose accumulation, owing to the increased sucrolytic activity usually observed upon cold treatment, was dramatically decreased by 86-94% in the transgenics (Tab. 3-10). This was unexpected because sucrose biosynthesis was supposed to be blocked in the transgenics. Another enigma was that sucrose in the transgenics seemed to be unmetabolisable in the cold. This phenomenon became much clearer after the tubers were stored at 4°C for 34 days. Sucrose content in the control tubers was decreased by 39% compared to that observed 17 days after cold storage, whereas it steadily increased in the transgenics. Sucrose content in the transgenic tubers was 4 to 5 fold of that observed in the control tubers 34 days after cold storage. However, cold-induced hexose accumulation was still reduced by over 84% in the transgenic tubers as compared to the control tubers (Tab. 3-10). Interestingly, the total carbon presented in hexose and sucrose was largely unchanged between the control and transgenic tubers, indicating that the rate of sucrose synthesis was similar in the two genotype tubers given hexose is mainly derived from a breakdown of sucrose.

Tab. 3-10: *Changes of soluble sugars during cold storage*

Tubers were stored at room temperature for one week before transfer to the cold room (4°C). Samples were taken at the indicated time in the cold and soluble sugar contents were determined. Values represent the mean (± SD) from 4-5 individual tubers.

Line	Gluc (µmol/g F.W.)			Fruc (µmol/g F.W.)			Suc (µmol/g F.W.)		
	0 d	17 d	34 d	0 d	17 d	34 d	0 d	17 d	34 d
solara	0,5±0,2	29,8±16,3	53,7±9,4	0,3±0,2	26,1±12,5	54,0±9,8	6,4±1,7	29,0±12,4	17,8±5,6
35SPPi18	0,6±0,3	3,9±2,3	7,8±4,0	0,9±0,5	3,8±1,8	9,3±5,1	5,4±1,4	49,0±5,9	87,6±11,3
35SPPi 8	0,3±0,1	2,4±1,0	4,2±1,7	0,6±0,2	2,0±1,3	4,6±1,4	4,5±1,8	39,2±7,6	71,5±18,4
35SPPi 5	0,5±0,3	1,9±1,6	3,9±2,9	0,8±0,2	2,4±1,6	5,3±2,9	4,8±1,1	38,5±9,3	70,3±17,0

3.2.5 Suc6P massively accumulated in the transgenic tubers during cold storage

The primary effect exerted by decreased expression of SPP was supposed to be the accumulation of its substrate, sucrose-6-phosphate (Suc6P), and inhibition of sucrose biosynthesis. However, the steady-state level of sucrose in cold-stored tubers did not fit to the predicted effect of inhibition of SPP, therefore the Suc6P level of the tubers was monitored during cold storage. Before cold storage, Suc6P contents in the transgenic tubers was only slightly higher than in control specimens (Tab. 3-11), which was consistent with a low rate of sucrose re-synthesis in growing tubers. Interestingly, Suc6P content was drastically increased in the transgenics 17 days after cold storage, whereas it remained largely constant in the control plants within the same period. The steady state level of Suc6P in the transgenic tubers 17 days after cold storage was over two thousand-fold higher than in the control tubers. These data indicate that an active sucrose biosynthesis via SPS and SPP is induced in the cold-stored tubers. The decreased expression of SPP in the transgenic tubers impeded the conversion of Suc6P to sucrose and led to the massive accumulation of Suc6P in the cold.

Tab. 3-11: *Changes in metabolite contents during cold storage*

Tubers were stored at room temperature for one week before transfer to the cold (4°C). Samples were taken at the indicated time in the cold and metabolite content was measured. The concentration of the metabolites are present in nmol/g F.W. except Pi which is present in µmol/g F.W.. Values are the mean ± SD (n=4-5). The metabolites which were not analysed are marked with “n.a.”.

Line	solara		35SPPi18		35SPPi8		35SPPi5	
	0 d	17 d	0 d	17 d	0 d	17 d	0 d	17 d
Suc6P	0,9±0,4	1,9±1,2	3,2±1,9	4191,4±326,3	5,3±2,7	4426,1±1136,4	37,8±23,0	5348,3±1608,1
Pi	4,34±1,87	6,71±0,99	14,23±1,98	12,38±5,10	12,08±7,55	9,05±3,80	12,58±2,91	10,36±2,20
ATP	n.a.	35,4±20,4	n.a.	36,7±9,8	n.a.	35,6±13,5	n.a.	27,2±8,3
ADP	n.a.	15,1±5,7	n.a.	17,6±9,2	n.a.	11,8±8,8	n.a.	13,6±3,8
3-PGA	237,0±118,0	78,8±32,0	212,6±62,5	31,7±27,1	174,4±8,6	21,2±11,1	214,4±49,6	19,6±8,7
UDPG	131,4±51,4	96,9±24,4	139,6±34,2	45,0±17,6	115,0±45,9	27,5±15,8	93,1±15,6	51,7±18,6
Glu6P	102,8±54,2	83,0±38,9	119,2±71,5	73,1±26,1	84,4±35,2	42,8±25,3	82,6±33,5	61,2±14,5
Fru6P	35,2±6,2	23,6±7,0	38,0±16,5	28,5±14,3	15,3±3,2	19,9±8,0	36,9±9,8	23,0±8,6

Since one molecule of Suc6P covalently binds one phosphate, the massive accumulation of Suc6P led to the suspicion that phosphate would become limiting in the transgenic tubers during cold storage. However, no significant changes in ATP and ADP contents were observed between the transgenics and controls after 17 days of cold storage (Tab. 3-11),

which indicated that there was no phosphate limitation in the transgenics. Interestingly, in the freshly harvested tubers the inorganic phosphate content in the transgenics was about 3-fold of that in the controls (Tab. 3-11). Even 17 days after cold storage, the transgenic tubers still contained much more phosphate than the control (84%, 35% and 54% more in 35SPPi line 18, 8 and 5, respectively). It is proposed that the high inorganic phosphate content in the transgenic tubers might compensate the Pi-sequestrating effects exerted by Suc6P during cold storage. These data also would suggest that Pi-uptake might be stimulated in the transgenics.

3.2.6 The induction of vacuolar acid invertase during cold-storage was strongly impaired in the SPP silenced transgenic tubers

Although Suc6P massively accumulated during cold storage, it was still puzzling why sucrose also accumulated in the transgenics during cold storage. This led to the speculation that sucrose cleavage was impaired in the transgenics. Vacuolar invertase is the main enzyme involved in the conversion of sucrose to hexose in cold-stored potato tubers. It is known that the expression of vacuolar invertase is induced in the potato tubers during cold storage (Zrenner *et al.*, 1996b). Therefore, Northern blot analysis was carried out to investigate whether cold-induction of vacuolar invertase was affected in the transgenics. As shown in Fig. 3-19A, in wild type tubers, vacuolar invertase was strongly induced 17 days after cold storage and the expression level declined after 34 days. This observation was consistent with the previous study that revealed a maximum induction occurring at 7 days in the cold and a strong decline around 42 days after cold storage (Zrenner *et al.*, 1996b). Cold-induction of vacuolar

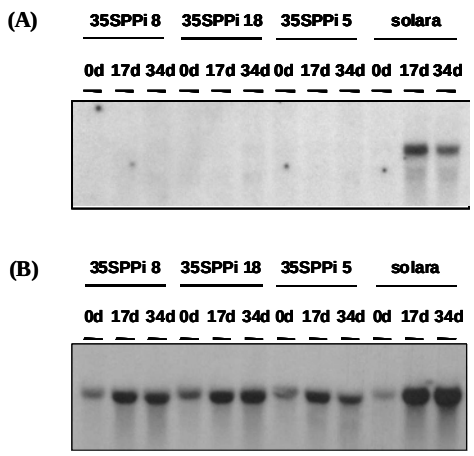


Fig. 3-19: Northern blot analysis of vacuolar invertase and UGPase expression in the transgenic and control tubers during cold storage.
Total RNA was extracted from the tuber at the indicated time after transfer to the cold (4°C) and thirty microgram of total RNA was separated by gel electrophoresis and transferred onto a nitrocellulose membrane. The blots were sequentially hybridized with ^{32}P-labelled vacuolar invertase (GenBank Acc. No. X70368) and UGPase cDNA (GenBank Acc. No. D00667). A, vacuolar invertase; B, UGPase.

invertase in the transgenics, however, was blocked. For transgenic line 5, there was no or only very weak cold-induced expression of vacuolar invertase even after 34 days in the cold, whereas for the other two lines there was also strong induction of vacuolar invertase in several transgenic tubers (2 or 3 out of 5 transgenic tubers in line 8 and 18, respectively) 34 days after cold storage. Interestingly, the cold-induction of vacuolar invertase expression was negatively correlated with the levels of Suc6P in the cold-stored tubers. The expression of vacuolar invertase was induced only when Suc6P levels in the cold-stored tubers were below a certain threshold (Fig. 3-20). The cold-induction of vacuolar invertase expression was not found to correlate with the levels of sucrose, hexose or the other metabolites. Consistent with the expression level, vacuolar invertase activities in the transgenic tubers were also strongly decreased compared to the control tubers after 17 days of cold storage. Although it was still lower in the transgenics than that in the controls, a substantial increase of vacuolar invertase activity occurred in the transgenics 34 days after cold storage (Fig. 3-21). These data indicate that inhibition of cold-induced vacuolar invertase expression could be one reason, but probably not the sole reason, accounting for the accumulation of sucrose in the transgenic tubers during cold storage.

In order to rule out the possibility that the effects were so pleiotropic that cold-induced gene expression was generally inhibited, the expression of UGPase, another well-known cold-inducible gene involved in sucrose metabolism (Zrenner *et al.*, 1993), was analysed in the same samples. The results showed that its induction by low temperature was largely unaffected, indicating that there was no general inhibition of cold-induced gene expression (Fig. 3-19B.).

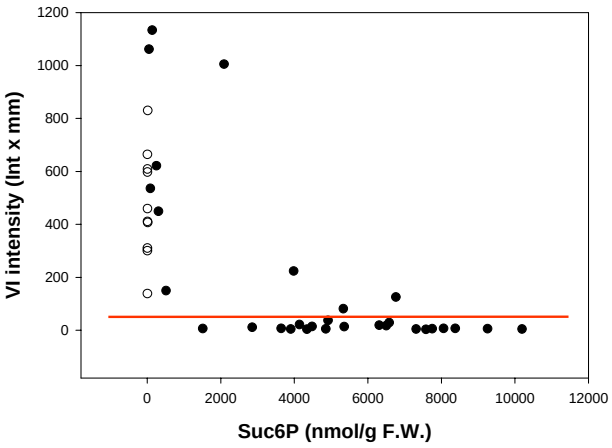
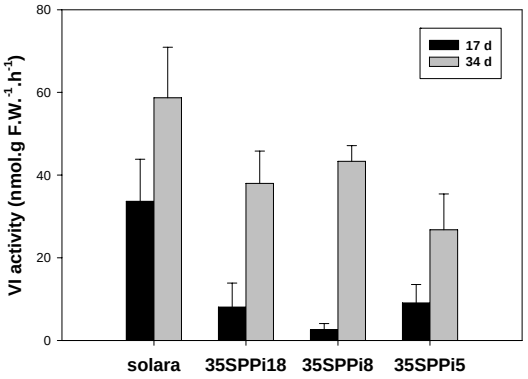


Fig. 3-20: Negative correlation between cold-induction of vacuolar invertase expression and Suc6P contents in the tubers.

The vacuolar invertase signals from Fig. 3-19 were quantified using Quantity One® 1-D analysis software (BIO-RAD) and plotted against Suc6P contents determined in Tab. 3-12. The open circles represent the wild-type control tubers and the closed circles represent the transgenic tubers. The red line indicates the detection limit for vacuolar invertase by Northern blot analysis.

Fig. 3-21: Vacuolar invertase activity during cold storage.

Total protein extract was shaken at 4°C for 30 min before measuring vacuolar acid invertase activity. 4-5 individual tubers per line at the indicated time after transfer of tubers to the cold (4°C) were used for the measurements and the values are presented in the mean±SD.



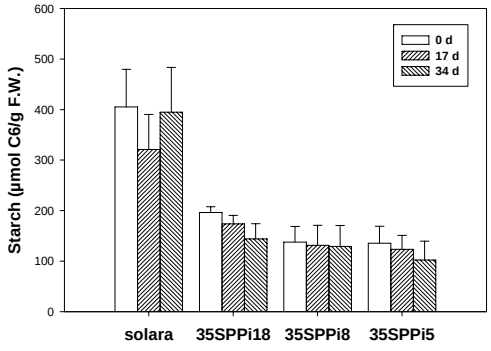
3.2.7 Starch, 3-PGA, UDPGlc and hexose phosphate contents

In the freshly harvested tubers, starch content was decreased by 52-67% in the transgenic tubers as compared to the control tubers probably due to the reduced photosynthesis and photosynthetic sucrose synthesis in the leaves during tuber growth as discussed below. Due to large variations, it was difficult to quantify the amount of starch degraded during cold storage based on the starch contents (Fig. 3-22). Nevertheless, estimate from the sucrose and hexose production in the cold indicated that similar amounts of starch were degraded in the control and transgenic tubers.

As summarized in Tab. 3-11, in the tubers prior to cold storage, the levels of 3-PGA, UDPG and hexose phosphate (Glu6P and Fru6P) were largely unchanged in the transgenic tubers as compared to the control tubers. After 17 days of cold storage, hexose phosphate contents were still largely unchanged whereas 3-PGA and UDPG contents were significantly decreased in the transgenic tubers compared to the control tubers (Tab. 3-11).

Fig. 3-22: Changes in starch content during cold storage.

Tubers were stored at room temperature for one week before transfer to the cold room (4°C). Samples were taken at the indicated time in the cold and starch content was measured. Values are the mean ± SD (n=4-5).



3.2.8 Measurements of the activities of cell wall invertase, sucrose synthase (Susy), SPS and starch phosphorylase

Four enzymes participating in different pathways of carbohydrate metabolism were further analysed in SPP silenced potato tubers (Tab. 3-12). Cell wall invertase (cw-Inv), involved in sucrose degradation, was slightly but significantly decreased in the transgenics as compared to the controls after 34 days in the cold. Sucrose synthase, catalysing a readily reversible reaction, can potentially participate in both sucrose synthesis and degradation. Interestingly, Susy activity in the freshly harvested tubers was decreased by 55-61% in the transgenics as compared to the controls. During cold storage Susy activity was decreased in both control and transgenic tubers with a similar relative rate. After 17 days of cold storage Susy activity was 66-71% lower in the transgenic tubers than that in the control tubers. Previous studies suggested that SPS makes the main contribution to the control of sucrose synthesis in cold-stored potato tubers (Pollock and Rees, 1975; Krause *et al*., 1998). No difference in the maximum SPS activity could be observed between the control and transgenic tubers after 17 days in the cold. Starch phosphorylase is involved in the phosphorolytic starch degradation pathway and its activity was slightly increased in transgenic line 18 and 8 but largely unchanged in line 5 as compared to the controls after 17 days of cold storage.

Tab. 3-12: *Enzyme activities of cw-Inv, Susy, SPS and starch phosphorylase*

Tubers were stored at room temperature for one week before transfer to the cold room (4°C). Samples were taken at the indicated time in the cold and enzyme activities were measured. Susy, SPS and starch phosphorylase activities are present in µmol/g F.W./h. cw-Inv activity is present in nmol/g F.W./h. Values are given as the mean ± SD (n=4-5).

line	cw-Inv	Susy		SPS	Starch phosphorylase
	34 d	0 d	17 d	17 d	17 d
Solara	31,50±4,66	138,9±45,6	84,5±17,7	28,1±2,2	13,5±1,0
35SPPi18	24,62±1,06	73,5±27,0	37,9±12,3	29,7±2,6	18,8±2,9
35SPPi8	24,13±3,85	61,2±9,0	33,0±10,8	26,2±3,2	16,7±2,6
35SPPi5	16,10±2,90	60,4±18,6	36,5±14,3	29,8±1,4	12,0±2,0

3.3 *Tuber-specific silencing of SPP results in a stimulation of respiration and leads to necrotic cell death in potato tubers after harvest*

Inhibition of SPP expression in photosynthetic tissues caused dramatical changes in carbon metabolism, which in turn might have profound effects on metabolisms in sink tissues. Therefore, the use of 35SPPi transgenic potato plants might be limited for functional analysis of sucrose (re)synthesis in growing tubers. In this case, tuber-specific silencing of SPP would be desirable in order to reduce the potential pleiotropic effects from the shoots. Tuber-specific silencing SPP might be difficult to achieve because of the existence of systemic silencing, a phenomenon in which RNA silencing can spread beyond the initiating site. However, the occurrence of systemic silencing is largely dependent on the nature of targets, namely transgenes are much better targets for systemic silencing than endogenous genes (Palauqui *et al*., 1997; Palauqui and Vaucheret, 1998; Voinnet *et al*., 1998). The mechanism behind this phenomenon is not fully clear at the moment. Nevertheless, the fact that endogenous genes are resistant to systemic silencing makes it possible to achieve tissue-specific silencing of endogenous genes.

3.3.1 Generation of SPP silenced transgenic potato tubers using tuber-specific promoter

In order to silence SPP in a tuber-specific manner, the tuber-specific patatin (B33) promoter, was used to drive the same SPP-RNAi cassette as described above. The SPP-RNAi fragment flanked by *Pst*I sites was cloned between the patatin B33 promoter and the OCS terminator of the binary vector pBinB33 (Rocha-Sosa *et al*., 1989) using a compatible *Sbf*I site, producing pBinB33-SPP-RNAi plasmid (depicted in Fig. 3-23A). The resulting construct was transformed into potato plants via *Agrobacterium*-mediated gene transfer. Twenty-six putative transgenic potato plants (designated as BSPPi plants) were obtained, propagated from stem cuttings and transferred into the greenhouse or growth chamber, respectively. When grown under these conditions, the growth behaviour and appearance of BSPPi plants were indistinguishable from those of the control plants and the tubers from BSPPi plants also exhibited a wild-type appearance. Western blot analysis with freshly harvested potato tubers was carried out to screen the transgenic plants with decreased SPP expression in the tubers.

The results showed that SPP protein was virtually eliminated from 9 transgenic lines (Fig. 3-23B). Carbohydrate levels in these nine transgenic lines were not changed in the freshly harvested tubers as compared to the non-transgenic control and internal transgenic control with wild-type level of SPP protein. Interestingly, the tubers from the nine transgenic plants with reduced SPP expression developed strong phenotypical alterations after 4-week storage at room temperature. The parts where the tubers were attached to the mother plants turned brown and shrink, and the cross sections revealed that the vascular and parenchyma tissues became brown and holes appeared around the vascular tissues, indicating that cell death was induced in the transgenic tubers during storage. Three transgenic lines with reduced SPP expression (BSPPi line 3, 11 and 26) were multiplied vegetatively in tissue culture and used for a detailed analysis. The transgenic tubers developed the characteristic phenotypical alterations one week after harvest in transgenic line 11 and about 2 weeks in the other two transgenic lines (BSPPi 3 and 26). The phenotypical alterations in the selected

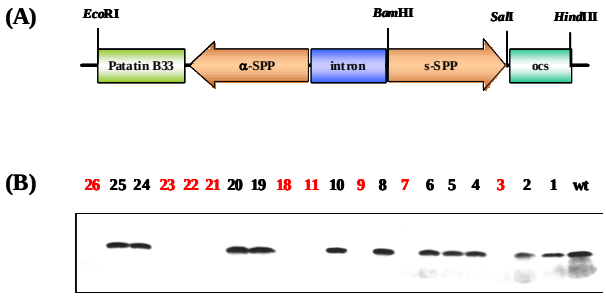


Fig. 3-23: Generation and expression analysis of BSPPi transformants.

(A) Schematic representation of pB33SPPi construct for plant transformation. The same SPP-RNAi cassette as above is driven by a tuber-specific patatin B33 promoter. α-SPP: SPP fragment in antisense orientation; s-SPP: SPP fragment in sense orientation. (B) Western blot analysis of SPP protein in the control and BSPPi tubers. Thirty µg of total tuber protein was separated by SDS-PAGE and detected using a polyclonal SPP antibody raised against *Nt*SPP2. wt, wild-type; 1-26, putative transgenic potato plants. The transgenic plants with virtually eliminated SPP protein were indicated in red numbers.

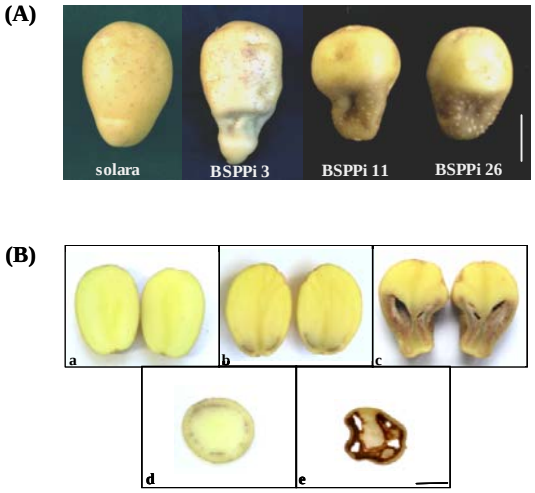


Fig. 3-24: Phenotypical alterations in BSPPi tubers after harvest.

(A): Photos were taken from the control and transgenic tubers 20 dph. The bar indicates 2 cm in length. (B): phenotype development was monitored in the tubers from line BSPPi 11 after harvest. a, 0 dph; b and d, 8 dph; c and e, 20 dph. The bar indicates 2 cm in length.

transgenic tubers were illustrated in Fig. 3-24A, and a time course of phenotype development was followed in BSPPi line 11, which revealed the phenotypical alterations started around the vascular tissues (Fig. 3-24B).

3.3.2 Tissue specificity of inhibition of SPP expression in BSPPi transgenic plants

The phenotype of the transgenic plants either from tissue culture or germinated from tubers was indistinguishable from wild type plants under normal growth conditions, indicating that SPP expression was not influenced in the leaves of transgenic plants. In order to verify the tissue specificity of inhibition of SPP expression, western blot analysis was carried out to determine the levels of SPP protein in mature leaves and growing tubers. The results revealed that SPP expression was unaffected in the mature leaves of BSPPi plants (Fig. 3-25A), whereas it was virtually eliminated from the growing tubers in the transgenics as compared to the controls (Fig. 3-25B), which confirmed the tuber specificity of inhibition of SPP expression in BSPPi plants. SPP activity was determined in the growing tubers, which was reduced about 83% in the transgenics as compared to the controls (Fig. 3-25C).

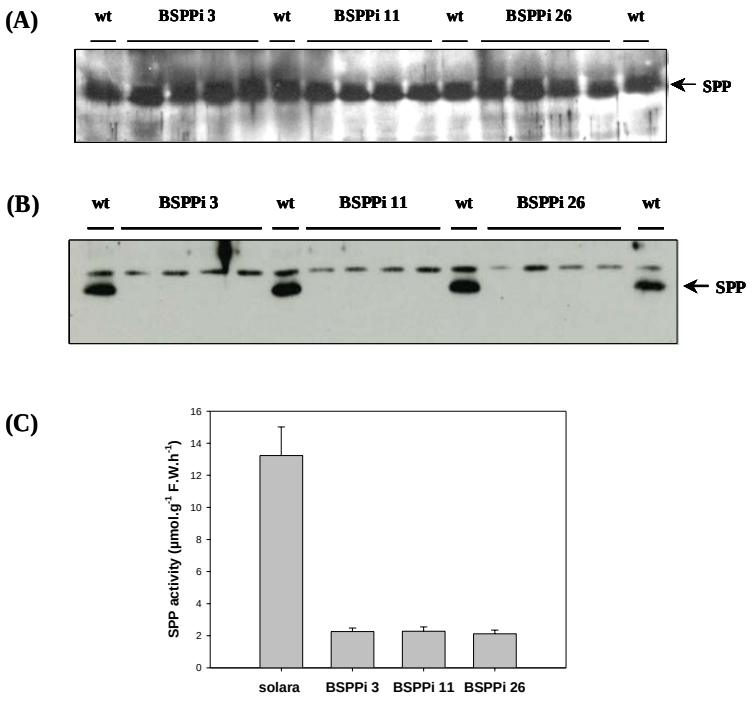


Fig. 3-25: SPP protein and activity in mature leaves and growing tubers from the BSPPi and control plants. Western blot analysis was performed to determine SPP protein in the mature leaves (A) and growing tubers (B). Thirty µg of total protein was separated by SDS-PAGE and detected using a polyclonal SPP antibody raised against *Nt*SPP2. SPP activity was determined in the growing tubers from the control and transgenic plants (C). Values are given as the mean ± SD (n=6). wt, wild-type.

In accordance with SPP expression level, Suc6P content was largely unchanged in the mature leaves of BSPPi plants as compared to the control plants (Fig. 3-26A). In the freshly harvested tubers, Suc6P level was slightly increased (2-6 folds) in the transgenics as compared to the controls (Fig. 3-26B).

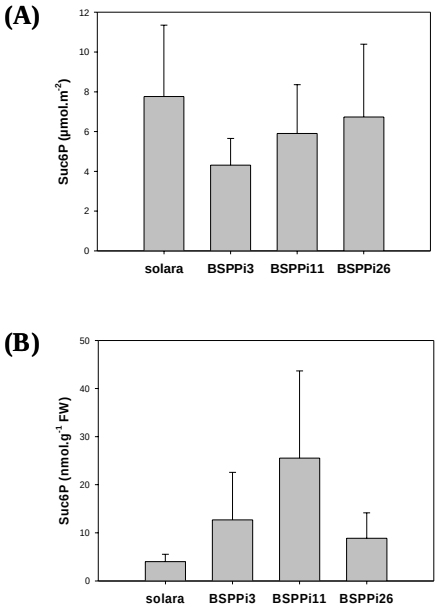


Fig. 3-26: Suc6P levels in the mature leaves and freshly harvested tubers from the transgenic and control plants. Samples were taken from the mature leaves (A) and freshly harvested tubers (B) of the transgenic and control plants grown in the greenhouse. Suc6P levels were determined using IC-MS and the values are given as the mean ± SD (n=5).

3.3.3 Decreased SPP activity in the tuber had no effect on tuber yield

The increased Suc6P level in the freshly harvested transgenic tubers indicated that sucrose (re)synthesis was impaired due to the reduced SPP activity. However, the impaired sucrose synthesis in the tubers had no effect on tuber yield including tuber fresh weight and total tuber number per plant (Tab. 3-13). Protein content in the tubers was also unchanged in the transgenics as compared to the control (Tab. 3-13). The morphology of growing tubers was indistinguishable between the transgenics and controls.

3.3.4 The phenotypical alterations in BSPPi tubers are accompanied by a dramatical increase of respiration rate during storage

The phenotypical alterations indicated that the storage reserves might be burned down and respiration might be induced in the BSPPi tubers during storage. Therefore the rate of respiration in the tubers was monitored within a time range from 0 d to 37 d after harvest. As

shown in Fig. 3-27, in the control tubers the respiration rate was progressively decreased after harvest as was observed in earlier reports (Hajirezaei *et al*., 2003). The decrease in respiration rate also occurred in the tubers from all the transgenic lines during the first 6 days after harvest and the respiration rates in the transgenic tubers were similar to those in the control tubers within this time range. However, the respiration rate steadily went up in the tubers of BSPPi line 11 after nine days post harvest, peaked at around 20 days after harvest and then declined. In the tubers from the other two transgenic lines (BSPPi3 and 26), the respiration rate started to increase at about 15 days after harvest and peaked at around 30 days post harvest. Besides the late occurrence of the peak in respiration rates, the increase in respiration rates was also less pronounced in the transgenic lines BSPPi3 and 26 than that in line BSPPi11. The increase in respiration rate in the transgenic tubers was concomitant with the development of phenotypical alterations.

Plants contain two terminal oxidases, cytochrome c oxidase (COX) and alternative oxidase (AOX), which function in the cytochrome and alternative respiration pathway, respectively. COX is the main oxidase involved in the ATP production during respiration while AOX can bypass the last two sites of energy conservation normally associated with the cytochrome pathway, dissipating the energy in form of heat (Millenaar and Lambers, 2003). The phenotypical alterations and the induced respiration led to the speculation that AOX might be induced in BSPPi tubers during storage helping to dissipate the excess energy. Therefore, the expression of AOX was determined in BSPPi tubers during storage. Interestingly, the expression of AOX was strongly induced in BSPPi tubers 10 days after harvest, but not in the control tubers (Fig. 3-28), indicating that the energy produced by burning-down of the storage reserves during phenotypical alterations in BSPPi tubers might be dissipated in form of heat.

Tab. 3-13: Basic characterisation

The BSPPi and control plants were grown in the greenhouse for 12 weeks before harvest. Tuber yield (including tuber fresh weight and total tuber number per plant), the ratio of tuber dry weight (DW) to fresh weight (FW) and total tuber protein were determined in the freshly harvested tubers. Values represent the mean (± SD) from at least four individual plants per line.

Potato line	Tuber Fresh weight (g) per plant	Total tuber number per plant	Tuber DW/ FW ratio	Total tuber protein (mg/g FW)
solara	120,8±18,2	7,2±1,5	0,219±0,027	4,44±0,22
BSPPi3	102,8±23,6	6,8±1,2	0,177±0,022	4,27±0,32
BSPPi11	146,0±11,1	7,3±0,8	0,200±0,029	4,54±0,50
BSPPi26	116,6±28,5	7,7±1,2	0,214±0,022	4,58±0,19

Fig. 3-27: Influence of reduced SPP activity on respiration rates in the tubers.

Tubers were harvested from 12-week-old control and BSPPi plants, and stored at room temperature. A time course of respiration rate was followed in the tubers of about 15 g fresh weight. Each value represents the mean (±SD) of CO2 evolution rates from six individual tubers.

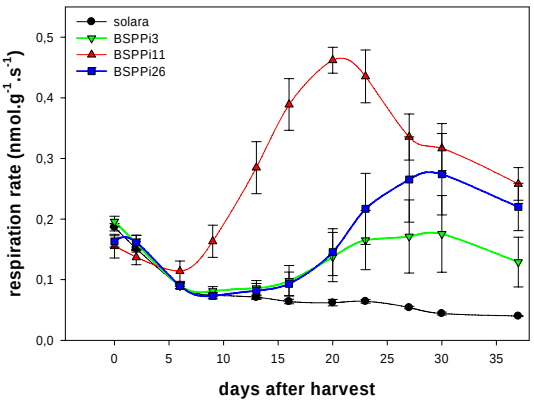
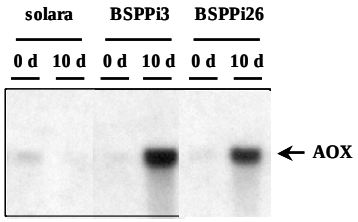


Fig. 3-28: Expression of alternative oxidase in BSPPi and control tubers.

Total RNA was isolated from the freshly harvested (0 dph) and stored tubers (10 dph). Thirty µg of total RNA was electrophoretically separated and subsequently subject to Northern blot analysis. ^{32}P-labeled AOX cDNA from tobacco (GenBank Acc. No. S71335) was used as probe for hybridization.



3.3.5 Carbohydrate and metabolite changes during tuber storage

Carbohydrate levels were determined in the tubers after harvest. As shown in Tab. 3-14, in the freshly harvested tubers, no difference in the levels of soluble sugars and starch could be observed between the transgenic and control lines, indicating that sucrose (re)synthesis plays only a marginal role in carbohydrate metabolisms in growing tubers. During storage at room temperature, the levels of hexose were slightly increased in all the lines and no difference could be observed between the transgenics and controls. Sucrose levels were progressively decreased in the tubers from the control and two transgenic lines (BSPPi-3 and 26) within the first 7 days after harvest, whereas in line BSPPi-11 no decrease in sucrose content occurred within the same time range. Seven days after harvest sucrose levels were slightly higher in the transgenic tubers than that in the control tubers. Starch content was constant in all the lines and no difference could be observed between the transgenic and control tubers. In the experimental set II, carbohydrate content was determined at 0 dph (days post harvest) and 10 dph, respectively. The tendency of the changes in hexose and starch was similar to those observed in the first set (Tab. 3-15). The sucrose levels at 10 dph were significantly higher in the transgenic tubers than those in the control (Tab. 3-15).

Tab. 3-14: *Carbohydrate contents in the freshly harvested and stored tubers from experimental set I.*
Samples were taken at the indicated time from the freshly harvested and stored tubers from the BSPPi and control plants. Carbohydrate levels were determined and concentrations are given in µmol/g FW. Values are the mean ± SD (n=6).

	Glucose			Fructose		
line	0 dph	3 dph	7 dph	0 dph	3 dph	7 dph
Solara	5,2±4,0	2,7±1,8	10,5±7,7	0,54±0,34	1,16±0,57	3,5±1,3
BSPPi3	4,2±2,2	3,0±2,6	12,0±4,3	0,37±0,15	0,79±0,51	3,6±2,1
BSPPi11	4,1±4,5	4,6±2,6	11,6±10,2	0,38±0,26	1,81±0,89	2,3±1,5
BSPPi26	5,5±4,3	2,7±2,5	12,8±9,0	0,51±0,41	0,86±0,65	4,8±2,5

	Sucrose			Starch		
line	0 dph	3 dph	7 dph	0 dph	3 dph	7 dph
Solara	20,1±5,4	12,8±4,2	9,7±4,0	684,3±164,5	685,0±126,8	690,0±184,4
BSPPi3	18,7±5,7	14,2±6,2	13,0±2,7	617,5±79,3	703,9±107,8	659,5±145,4
BSPPi11	17,8±4,1	16,6±3,7	19,2±3,4	724,7±120,6	855,6±182,8	729,9±108,8
BSPPi26	22,7±5,9	13,1±4,9	9,3±2,7	723,8±113,8	676,5±138,4	638,4±121,2

Tab. 3-15: *Carbohydrate contents in the freshly harvested and stored tubers from experimental set II.*
Samples were taken at the indicated time from the freshly harvested and stored tubers from the BSPPi and control plants. Carbohydrate levels were determined and concentrations are given in µmol/g FW. Values are the mean ± SD (n=6).

	Glucose		Fructose		Sucrose		Starch	
Line	0 dph	10 dph	0 dph	10 dph	0 dph	10 dph	0 dph	10 dph
Solara	2,3±1,4	3,9±2,8	1,5±0,6	6,6±3,0	20,1±5,6	8,1±3,8	356,2±81,5	417,8±96,1
BSPPi3	4,5±2,5	8,9±3,1	1,3±0,7	7,0±3,0	23,5±4,4	15,0±7,5	533,8±117,4	359,9±92,3
BSPPi11	6,6±4,2	4,0±1,3	0,6±0,4	3,4±1,6	22,0±5,2	22,7±5,5	510,5±99,6	323,0±88,6
BSPPi26	1,5±0,5	6,0±4,4	1,2±0,6	8,1±4,4	15,3±3,7	24,7±14,1	437,9±94,6	376,7±91,6

In order to obtain a better insight into the changes in BSPPi tubers after harvest, levels of metabolic intermediates were determined in the freshly harvested and stored tubers (3 and 7 days after harvest, respectively) (Tab. 3-16). Interestingly, Suc6P level in the freshly harvested tubers was only slightly increased in the transgenics (2-6 folds) as compared to the controls, whereas a tremendous build-up in Suc6P content occurred in the transgenic tubers 3 days after harvest and a further increase 7 days after harvest. In contrast to that in the transgenic tubers, Suc6P level was constantly low in the control tubers. In the freshly harvested tubers, the levels of pyruvate, PEP, 3-PGA, UDP-Gluc and hexose-P were

comparable in transgenics and controls. Three days after harvest, the levels of metabolites including PEP, 3-PGA, UDP-Gluc and hexose-P were significantly lower in the transgenic tubers than those in the control tubers, whereas pyruvate level was substantially higher in the transgenic tubers than that in the control tubers. Similar changes in metabolite levels were observed in the transgenic tubers 7 days after harvest. Unlike that observed in 35SPPi growing tubers, Pi content in BSPPi growing tubers was similar as that in the control tubers. Due to the massive accumulation of Suc6P, Pi content was decreased by 32% in the tubers from the most strongly affected line BSPPi 11 at 3 dph, and reduced by 52% at 7 dph. In the other moderately affected lines BSPPi-3 and 26, Pi levels in the tubers were only slightly decreased during storage. However, the increased Suc6P levels were much higher than the decreased Pi levels in the transgenic tubers, indicating that certain phosphorus pools other than Pi pool were remobilized and released Pi during storage.

Tab. 3-16: *Metabolite levels in the freshly harvested and stored tubers.*

Samples were taken at the indicated time from the freshly harvested and stored tubers from the BSPPi and control plants. Metabolite levels were determined using IC-MS except Pi contents that were determined with the colormetric method. Concentrations are given in nmol/g FW and values are the mean ± SD (n=6). (A): metabolite levels at 0 dph; (B): 3 dph; (C): 7dph. Hexose-P is composed of Gluc6P and Fruc6P.

(A)

line	solara	BSPPi 3	BSPPi 11	BSPPi 26
Suc6P	4,0±1,5	12,7±9,9	25,5±18,2	8,9±5,3
Pyruvate	14,1±3,2	19,4±7,0	17,8±6,3	15,4±9,1
PEP	16,2±2,0	26,8±19,3	25,5±9,6	16,5±8,4
3PGA	44,0±9,3	56,7±32,8	48,2±15,4	44,9±19,1
UDPGlc	37,0±12,1	31,0±13,0	43,7±16,8	41,3±20,6
hexose-P	59,5±9,0	61,8±25,4	64,9±22,8	55,0±12,8
Pi	5374±1055	5436±560	5434±875	4662±720

(B)

line	solara	BSPPi 3	BSPPi 11	BSPPi 26
Suc6P	7,5±1,8	372±410	4554±2567	1337±1027
Pyruvate	15,7±2,2	30,6±7,7	21,7±9,5	35,6±9,9
PEP	52,8±9,3	13,3±0,9	7,7±5,1	32,3±16,8
3-PGA	134,2±22,6	38,5±5,4	21,8±14,4	90,5±46,7
UDPGlc	232,9±54,1	45,8±18,7	159,7±24,8	151,9±49,1
hexose-P	288,4±41,5	140,1±14,6	104,2±48,8	193,2±59,7
Pi	5375±865	5701±639	3676±690	5104±696

(C)

line	solara	BSPPi 3	BSPPi 11	BSPPi 26
Suc6P	7,7±3,6	4188±2213	10194±4781	5454±3488
Pyruvate	11,4±1,9	23,2±5,5	18,1±6,0	22,2±7,4
PEP	30,5±6,2	27,9±4,6	4,4±0,4	21,9±5,1
3-PGA	62,2±4,9	48,8±10,4	3,6±1,9	34,2±18,3
UDPGlc	99,9±32,7	88,6±16,4	55,3±20,0	90,6±19,3
hexose-P	202,5±16,9	132,5±27,3	51,2±13,8	119,3±38,0
Pi	5586±763	5322±1089	2604±579	4373±629

3.4 *Temporal and spatial control of gene silencing in transgenic plants using ethanol inducible RNAi system*

Constitutive silencing of SPP in transgenic tobacco plants brought about profound metabolic changes, which made it difficult to distinguish the possible primary and secondary effects associated with reduction of SPP activity in those plants. In order to dissect the potential primary and secondary effects arising from constitutive silencing of SPP in transgenic tobacco plants, a system for transient silencing of SPP would be highly desirable. In the past few years, several transient silencing systems have been established in plants, such as virus-induced gene silencing (VIGS) systems (Baulcombe, 1999; Liu *et al*., 2002; Ratcliff *et al*., 2001; Constantin *et al*., 2004) and Agro-infiltration mediated gene silencing system (Schob *et al*., 1997; Johansen and Carrington 2001). However, their use in physiological studies is limited because the silencing phenotype is often accompanied by at least mild disease symptoms due to the introduction of pathogenic organisms into plants, which cause significant biochemical perturbations not directly linked to the suppression of the target genes. In order to overcome such limitations, it was proposed to develop an inducible silencing system in transgenic plants. To this end, the ethanol inducible *alc* system was used to control the expression of silencing constructs and several genes involved in various metabolic pathways were targeted in transgenic plants to test the feasibility of different strategies.

3.4.1 Construction of inducible silencing vectors and generation of transgenic plants

To evaluate the suitability of the *alc* system for inducible gene silencing, two nuclear genes were selected which have previously been described to yield a readily discernable phenotype in conventional antisense experiments. The first target gene, *chl*I, encodes the subunit I of magnesium chelatase (Mg-chelatase) that is a heteromeric enzyme complex composed of three-subunits (designated CHL I, CHL H, and CHL D) catalyzing the incorporation of Mg^{2+} into protophorphyrin IX which represents the first committed step in chlorophyll biosynthesis. Antisense suppression of *chl*I in transgenic tobacco led to a strongly reduced green pigmentation owing to the decreased chlorophyll biosynthetic capacity (Papenbrock *et al*., 2000). A key regulatory step in tetrapyrrole biosynthesis in higher plants, providing the precursors for chlorophyll and heme synthesis, is the formation of 5-aminolevulinate

catalyzed by the activity of glutamate 1- semialdehyde aminotransferase (GSA). Expression of *gsa* antisense RNA in tobacco plants resulted in a decline in chlorophyll content apparently leading to pale leaves (Höfgen *et al*., 1994). Therefore this gene was chosen as the second target.

Initially, two strategies were applied to achieve ethanol inducible gene silencing, inducible RNAi approach and inducible antisense approach, respectively. In order to achieve inducible RNAi using the *alc* system, constructs for plant transformation were cloned into p35S:*alcR*, a derivative of pBin19 (Bevan, 1984), carrying the *alcR* gene from *A. nidulans* between the CaMV 35S promoter and the *nos* terminator (Caddick *et al*., 1998). Fragments containing portions of the respective target gene in sense and antisense orientation separated by an intron were initially assembled into the pUC-RNAi vector mentioned above (Chen *et al*., 2003). A 655 bp fragment of the *N. tabacum chl*I gene (GenBank Acc. No. U67064) comprising nucleotides 454 to 1108 was amplified by PCR from tobacco cDNA and inserted as a *Bam*HI/*Sal*I fragment in sense orientation downstream of the GA20 intron into *Bam*HI/*Sal*I-cut pUC-RNAi vector. The same fragment was inserted in antisense orientation into the *Bgl*II/*Xho*I sites of pUC-RNAi already carrying the *chl*I sense fragment. Subsequently, the entire fragment comprising sense and antisense fragments of *chl*I interspersed by the potato GA20 oxidase intron, was excised from pUC-RNAi using the flanking *Pst*I restriction sites and inserted into a pUC-based plasmid between a chimeric *alcA* promoter and a *nos* terminator sequence (Caddick *et al*., 1998). The resulting *alcA* expression cassette was subsequently inserted into the *Hin*dIII site of p35S: *alcR* yielding the construct *alc-chl*Ii. An 804-bp fragment of *N. tabacum gsa* (GenBank Acc. No. X65974) comprising nucleotides

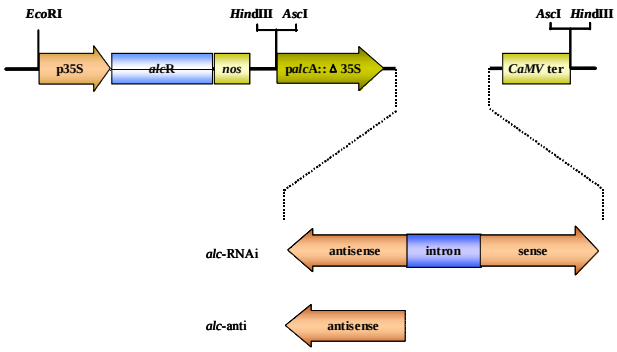


Fig. 3-29: Schematic representation of (not to scale) of *alc*-system-derived silencing constructs.
The *alc*R cDNA is under control of the *CaMV* 35S (p35S) promoter and positioned upstream of the *nos* terminator (*nos*). The *alc*-RNAi construct contains either a *chl*I or *gsa* fragment in antisense and sense orientation separated by intron 1 of potato GA20 oxidase (200 bp) in splicable orientation. The *alc*-anti construct comprises simply the respective antisense fragment. In both constructs, all fragments were inserted downstream of the chimeric *alc*A promoter (p*alc*A::Δ35S), which consists of the *CaMV* 35S minimal promoter (-31 to +5) fused at the TATA-box to upstream promoter sequences of *alc*A (Caddick et al., 1998).

298–1101 of the respective cDNA clone, was amplified by PCR and manipulated as described above to form plasmid *alc-gsa*i.

To obtain constructs for ethanol inducible antisense RNA expression, the same fragments as used before were linked in reverse orientation to the chimeric *alc*A promoter and the entire cassette was subsequently ligated into p35S:*alcR* as above.

The constructs for ethanol inducible RNAi approach (*alc*-RNAi) and ethanol inducible antisense approach (*alc*-anti) are depicted in Fig. 3-29.

3.4.2 Generation and screening of transgenic plants

After *Agrobacterium*-mediated gene transfer (Rosahl *et al*., 1987), approximately 80 primary transformants for each of the above mentioned constructs were transferred to the greenhouse. Prior to the application of ethanol all transgenic plants were indistinguishable from wild type plants. However, after ethanol induction a portion of the transgenics containing the *alc*-RNAi gene cassettes (26 plants for *alc-chl*Ii and 10 plants for *alc-gsa*i) displayed the characteristic phenotype previously described from constitutively silenced plants. Specific silencing of the target gene in phenotypically affected plants was further confirmed by Northern blotting using gene specific probes. In contrast, none of the plants solely expressing the antisense fragment targeted against *chl*I and *gsa*, respectively, displayed any visible phenotypical changes upon induction. Northern blotting was applied to screen for individuals expressing the respective antisense transgenes. Several plants expressing considerable amounts of the antisense transcript could be identified for each construct. However, the expression of the respective endogenous gene was not affected by the presence of the corresponding antisence transcript. From these data, we conclude that ethanol-inducible RNAi system but not inducible antisense approach can effectively achieve inducible silencing of endogenous genes in transgenic tobacco plants, which is comparable to that in constitutively silenced plants.

Three *alc-chl*Ii lines (14, 16, and 45), and two *alc-gsa*i lines (60 and 70) were chosen for a detailed analysis according to their strong inducible phenotype observed in the T0 generation. The selected primary transformants for each construct were selfed and the T1 generation was subject to a detailed analysis.

3.4.3 Rapid and reversible phenotypical changes in plants containing *alc*-RNAi expression cassettes upon application of ethanol

Seeds from the selected primary transformants were germinated on kanamycin containing medium and resistant seedlings were analyzed four weeks after transfer to soil. Prior to ethanol induction, the transgenic tobacco plants displayed wild-type appearance as has been observed in the T0 generation. Application of 1% ethanol by root drenching led to the development of the characteristic phenotype featured by the loss of chlorophyll. The phenotype started to develop approximately 36 h post induction (hpi) for *alc*-c*hl*Ii lines and approximately 48 hpi for *alc*-*gsa*i lines and was first visible in the top leaves (Fig. 3-30). The bleached patches expanded as the leaves grew, and also appeared in nascent leaves over a period of 7 to 9 days. After that, newly emerging leaves looked like wild type again while those initially affected never recovered. Whether this was due to the developmental control of chlorophyll-synthesizing enzymes (Härtel *et al*., 1997; He *et al*., 1994) or due to stable silencing is currently unknown. Leaves that were mature prior to induction always remained green.

In some cases the silencing effect caused by a single induction might be too transient to reveal the full sequence of consequences of reduced gene expression. In order to investigate whether extended periods of gene silencing could be achieved by repeated ethanol treatments, *alc*-*chl*Ii plants were root-drenched with 1 % ethanol every two days for 15 days in total. Phenotypic changes became apparent 2 dpi but, in contrast to the single-induction experiments, were maintained over the entire period of ethanol treatment (Fig. 3-31b.). Therefore, using re-iterated treatments extended periods of gene silencing can be maintained.

3.4.4 Time course of target mRNA degradation, dsRNA accumulation, target protein degradation and changes in chlorophyll contents upon induction of gene silencing

In order to investigate the kinetics of target transcript reduction in *alc*-RNAi transgenic plants, leaves being ca. 5 cm in size at the time point of induction and expected to become phenotypically affected were followed until 96 h post induction. After 9 dpi, newly grown leaves being ca. 5 cm in size were taken for the analysis.

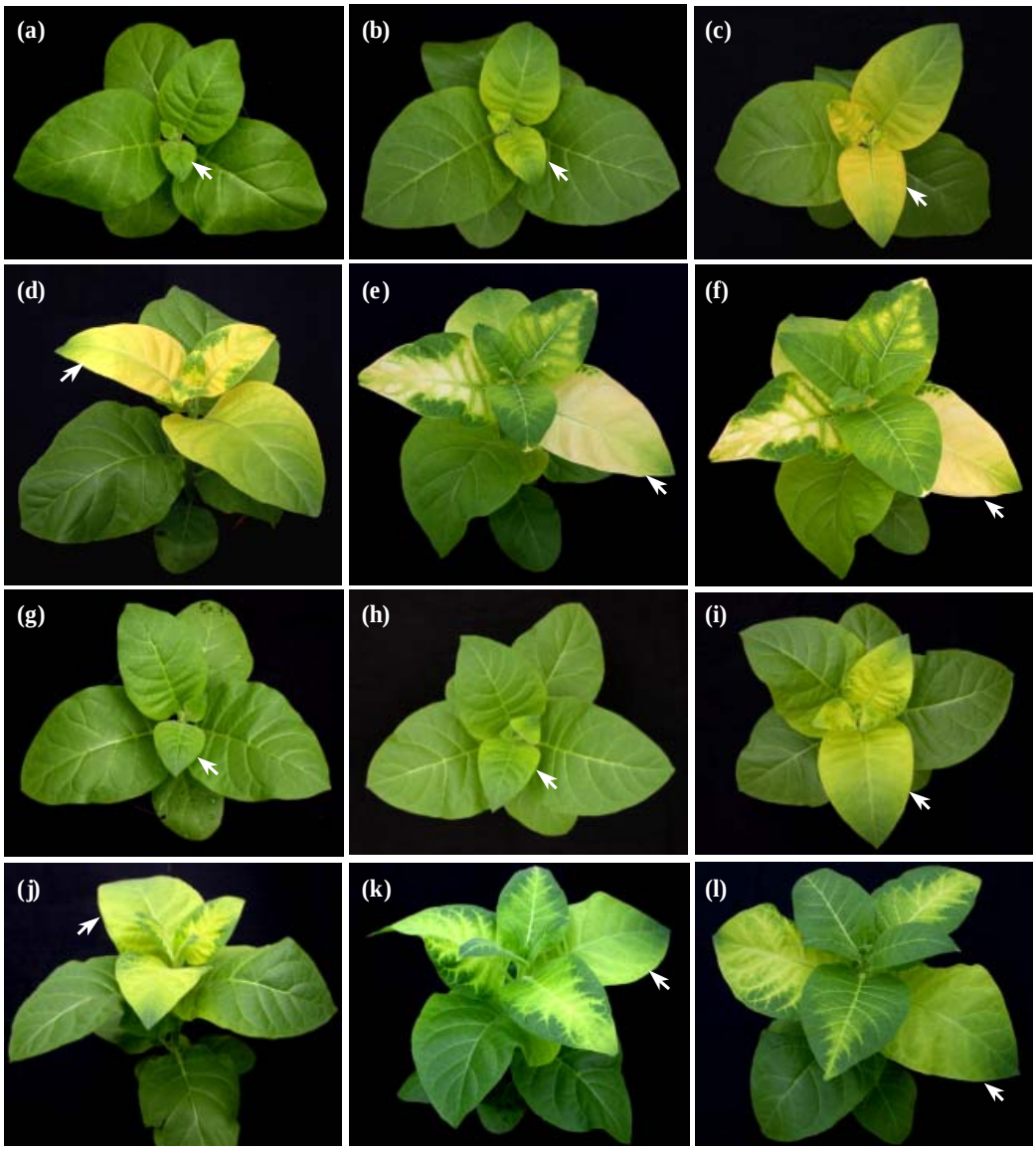


Fig. 3-30: time course of phenotype development in alc-RNAi lines after aplication of ethanol.
Plants (42 days old) were induced via root drenching with 100 ml of 1% ethanol and photographs were taken at different time point. (a-f): *alc-chl*Ii line 16, before and 2, 4, 5, 9 and 12 dpi, respectively. (g-l): *alc-gsa*i line 60, before and 2, 4, 5, 7 and 9 dpi, respectively. The arrows indicate the type of leaf that has been followed for molecular analysis.

To compensate for plant-to-plant variation, at least 3 plants were probed at each time point and samples from the phenotypically affected plants were pooled after preparation of total RNA. Northern blot analysis revealed that the *chl*I mRNA was significantly reduced as early as 8 h after ethanol induction in *alc-chl*Ii transgenic plants. Transcript levels further declined to undetectable levels at 48 hpi so as to rise again from 72 hpi onwards. The initial amount

was reached again at approximately 9 days post induction (dpi) in newly emerging leaves which were phenotypically normal (Fig. 3-32A). *chl*I transcript levels in ethanol treated wild type plants remained stable over the time course of the experiment (Fig. 3-33A). Similar kinetics of target transcript decay and duration of mRNA down regulation was observed in the *alc-gsa*i plants (Fig. 3-34A). In the line under investigation (line 60), an additional band migrating above the endogenous *GSA* transcript, appeared upon induction and was detectable up to 48 hpi. Since PTGS has been shown to affect RNA processing (Mishra and Hanada, 1998), RT-PCR was performed to investigate whether splicing of the endogenous *GSA* messenger was impaired in these plants. No PCR product which could possibly represent an incompletely spliced RNA was detected. However, if primers specific for the intron included in the transgene construct were used, a weak but specific band was amplified. Therefore, the additional band on the *GSA* northern blot was tentatively assigned to the unspliced intron-containing inverted-repeat fragment. This band was never observed in any other *alc-gsa*i line, however, line 60 had the strongest phenotype and high expression of the transgene might interfere with its correct splicing due to sub-optimal splice-sites.

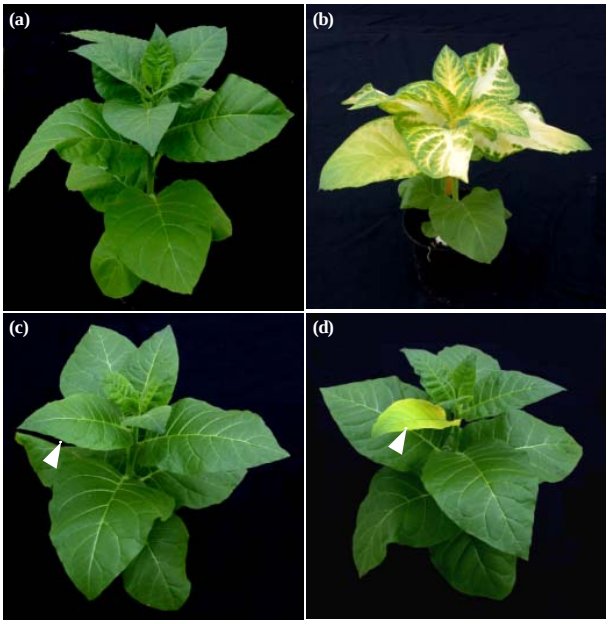


Fig. 3-31: Temporal and spatial control of *chl*I gene silencing. (a, b) Wild-type (a) and *alc-chl*Ii (b) plants (line 16) were root-drenched with 1% ethanol solution every 2 days. The pictures were taken at 15 dpi. (c, d) Local *chl*I silencing. The leaf indicated by an arrow was enclosed with 3 ml of 4% (v/v) ethanol for 48 h. (c), wild-type control plant; (d), *alc-chl*Ii plant from line 16.

In order to monitor the activity of *alc* promoter, time course of double-stranded RNA (dsRNA) production was followed in the *alc*-RNAi plants. To this end, the above mentioned RNA samples were treated with RNase I to remove the single-stranded endogenous transcripts and subsequently subjected to northern blot analysis (Fig. 3-32B; Fig. 3-34B). In both *alc-chl*Ii and *alc-gsa*i plants, dsRNAs expressed from the transgene locus started to be present as early as 4 hpi, reached the maximal expression level at 12 hpi and then declined.

After 72 hpi, dsRNAs were not detectable any more in both *alc-chl*Ii and *alc-gsa*i plants, respectively, indicating the *alc* promoter was switched off. These data were in consistence with the activity of *alc* promoter determined in other studies (Qu, 2001; Hofius, 2003).

In order to follow the silencing process in *alc-gsa*i lines on the protein level, samples were taken from the same leaves as before and subjected to a Western analysis using GSA specific antibodies (kindly provided by Dr. B. Grimm, Humboldt University, Berlin, Germany). Within 48 hpi a considerable reduction of the GSA amount occurred in the transgenics (Fig. 3-34C), which is in good accordance with phenotype development in these plants. No detectable protein was observed 72 – 96 hpi. When newly developing leaves were probed for GSA 9 dpi, the amounts were comparable to those before induction.

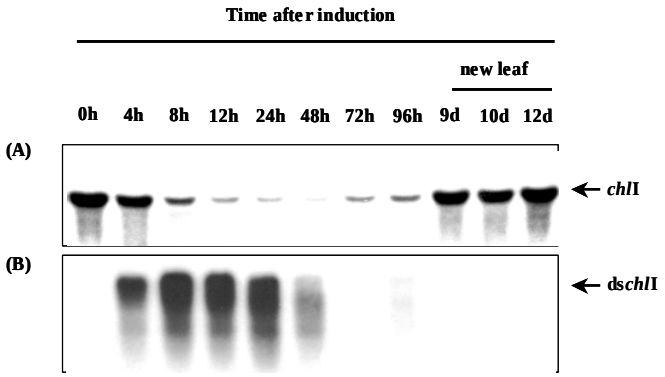


Fig. 3-32: Time course of the endogenous *chl*I transcript degradation and the transgene ds*chl*I transcript production in *alc-chl*Ii plants after application of ethanol.
(A) Time course of the endogenous *chl*I transcript degradation.
(B) Time course of the transgene ds*chl*I transcript production.
Transgenic tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. From 9 to 12 dpi, newly grown leaves were taken for the analysis. Total RNA was isolated from the leaf samples harvested at the indicated periods of time. Thirty µg of total RNA was subject to gel blot hybridization using ^{32}P-labeled *chl*I cDNA fragment to detect endogenous *chl*I transcripts (A). 100 µg of toal RNA was digested with RNase I and subsequently subject to Northern blot hybridization with ^{32}P-labeled *chl*I cDNA fragment to detect the transgene ds*chl*I transcripts (B).

In order to investigate the degree of gene silencing which could be achieved with the ethanol inducible system compared to plants constitutively silenced for *gsa* and c*hl*I (Höfgen *et al*., 1994; Papenbrock *et al*., 2000), the decrease in chlorophyll (chl) content in phenotypically affected leaves over time was taken as an indirect measure for chlorophyll biosynthetic capacity. Therefore, leaves were followed which were approximately 5 cm in length at the time point of induction. Samples were taken at different time points and analyzed for their chlorophyll content. To compensate for local variations within a plant, two samples were taken from each leaf. To compensate for plant-to-plant variation, at least 5 plants were harvested at each time point for each construct tested. After application of ethanol, a gradual

decline in chl content was observed in both *alc*-RNAi transgenic plants (Fig. 3-35), whereas chl content in ethanol treated control plants increased over the time course of the experiment owing to the developmental control of chl biosynthesis. In case of *alc-chl*Ii plants, the progressive loss of chl became apparent as early as 24 hpi, while ethanol treated *alc-gsa*i plants lost their chl more slowly. This is in good accordance with the temporal differences in phenotype development of the two transgenics. 7 dpi, chl content in *alc*-*chl*Ii plants was only approximately 3% of that before treatment, whereas chl content in phenotypically affected leaves of *alc-gsa*i was reduced to levels of about 20% of those before treatment.

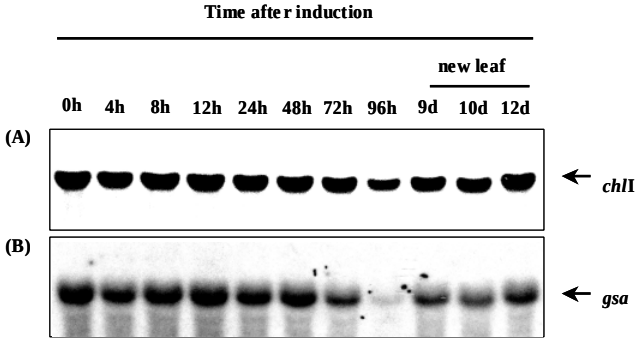


Fig. 3-33: Time course of the expression of *chl*I and *gsa* in wild-type control plants after application of ethanol.
(A) Time course of the expression of *chl*I.
(B) Time course of the expression of *gsa*.
Wild-type tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. From 9 to 12 dpi, newly grown leaves were taken for the analysis. Thirty µg of total RNA was subject to sequential gel blot hybridization using ^{32}P-labeled respective cDNA probe (GenBank Acc. No. U67064).

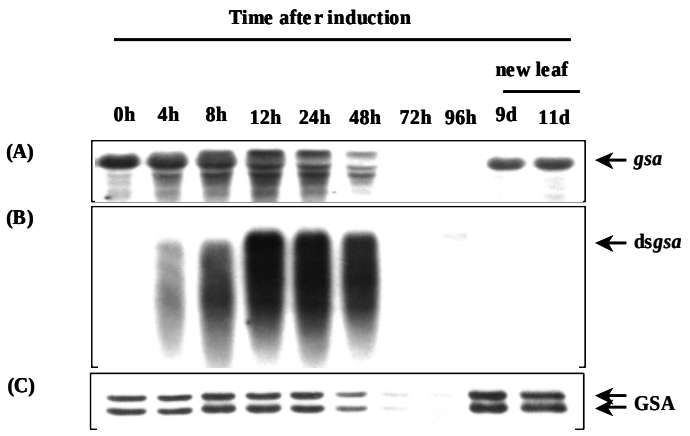


Fig. 3-34: Time course of the endogenous *gsa* transcript degradation, the transgene ds*gsa* transcript production and GSA protein degradation in *alc-gsa*i plants after application of ethanol.
(A) Time course of the endogenous *chl*I transcript degradation.
(B) Time course of the transgene ds*chl*I transcript production.
(C) Time course of GSA protein degradation.
Transgenic tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. At 9 and at 11 dpi, newly grown leaves were taken for the analysis. Total RNA and protein were isolated from the leaf samples harvested at the indicated periods of time. Thirty µg of total RNA was subject to gel blot hybridization using ^{32}P-labeled *gsa* cDNA (GenBank Acc. No. X65974) to detect endogenous *gsa* transcripts (A). 100 µg of toal RNA was digested with RNase I and subsequently subject to Northern blot hybridization with ^{32}P-labeled *gsa* cDNA fragment to detect the transgene ds*gsa* transcripts (B). Thirty µg of total protein was separated by SDS-PAGE and detected using an anti-GSA antibody (C).

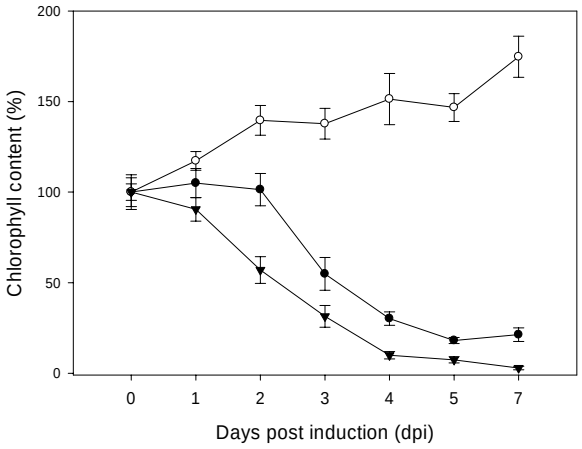


Fig. 3-35: Changes in chl content in alc-RNAi transgenic plants after ethanol induction.
Control plants (○), *alc-gsa*i line 60 (●), and *alc-chl*Ii line 16 (▼) were induced with 100 ml of 1% (v/v) ethanol via root drenching, and chl content was measured at the indicated time. Samples were taken from leaves being ca. 5 cm in size at the time of induction until 7 dpi. The chl content is expressed as a value relative to that at day 0. Bars show the SD of five replicates.

3.4.5 Maintenance of gene silencing in the initially silenced leaves

Due to the fact that the phenotype in the initially silenced leaves never recovered in *alc-chl*Ii and *alc-gsa*i plants the question arose whether gene silencing was maintained in these leaves despite the absence of the initial trigger. However, the situation was complicated by the fact that chlorophyll-synthesizing enzymes are under the developmental control (Härtel *et al*., 1997; He *et al*., 1994). In order to investigate whether gene silencing can be maintained in the initially silenced leaves, another two transgenic tobacco plants were constructed harboring *alc-fnr*i and *alc*-SPSi expression cassettes, respectively. In case of *alc-fnr*i, the target gene encoding ferredoxin-$NADP^+$ oxidoreductase (FNR), a key component in photosynthetic electron transport chain, is highly expressed in both young and mature leaves. SPS encodes sucrose-phosphate synthase, a regulatory enzyme in sucrose synthesis, which is expressed in both young and mature leaves yet with a relative low expression level. Fragments corresponding to *N. tabacum fnr* (GenBank Acc. No. Y14032 ; comprising nt 174–871) and SPS (GenBank Acc. No. AF194022; comprising nt 2565-3165) were amplified by PCR and used to make ethanol inducible RNAi constructs. The plasmids *alc-fnr*i and *alc*-SPSi were constructed in a similar way described above except that an *Asc*I instead of a *Hin*dIII site was used for the cloning of *alc*A expression cassette into p35S: *alc*R vector in case of *alc-fnr*i. After *Agrobacterium*-mediated gene transfer, approximately 80 primary transformants for each construct above mentioned were transferred to the greenhouse. Upon ethanol induction, eleven *alc-fnr*i primary transformants displayed phenotypic alterations with leaves bleached after 72 hpi and the reduction of *fnr* transcript and protein was confirmed by Northern and Western blot analysis, respectively. With respect to *alc*-SPSi plants, no phenotypic change

was observed after ethanol induction. Therefore, western blot analysis was applied to screen the transformants and eight *alc-SPS*i primary transformants were found with various levels of reduction in SPS protein after ethanol induction. T1 seeds from three lines of positive primary transformants for each construct were germinated on kanamycin containing medium and resistant plants were further analyzed in the greenhouse. After ethanol induction, the *alc-fnr*i plants developed phenotypic alterations (Fig. 3-36) that were also never recovered as observed in *alc-chl*Ii and *alc-gsa*i plants. Northern and western analyses were carried out

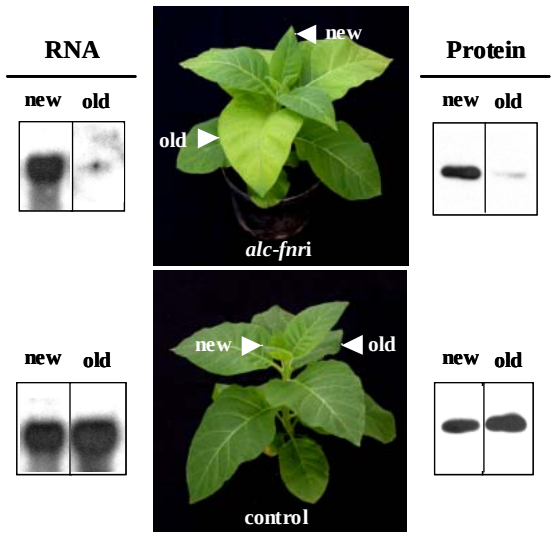


Fig. 3-36: Maintenance of *fnr* silencing in initially silenced leaves from *alc-fnr*i plants.
Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. At 10 dpi, total RNA and protein were isolated from the initially silenced leaves (old; indicated by an arrow) and newly grown leaves (new; indicated by an arrow). Thirty µg of total RNA was subject to gel blot hybridization using ^{32}P-labeled *fnr* cDNA from tobacco (GenBank Acc. No. Y14032). Thirty µg of total protein was separated by SDS-PAGE and detected using an anti-FNR antibody. *alc-fnr*i line 24 was shown here.

in the initially silenced leaves after the new growth displayed wild-type like appearance. In contrast to the high expression level in the control, the expression of *fnr* at both mRNA and protein levels was drastically inhibited 10 dpi in the initially silenced leaves from *alc-fnr*i plants (Fig. 3-36). To rule out the possibility that the maintenance of repression in *fnr* expression in *alc-fnr*i plants is merely a pleiotropic effect due to the strong phenotypic alterations, *alc*-SPSi plants that displayed no phenotypic alteration were investigated 12 dpi. As shown in Fig. 3-37, SPS expression at both transcript and protein levels was still strongly reduced in the initially silenced leaves from *alc*-SPSi plants 12 dpi compared to the control plants. Taken together, these data indicated that silencing was maintained in the initially silenced leaves from the *alc*-RNAi plants.

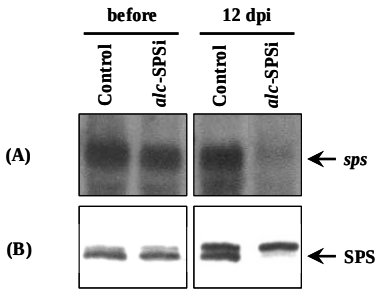


Fig. 3-37: Maintenance of *sps* silencing in initially silenced leaves from *alc*-SPSi plants.

(A) Northern blot analysis of *sps* transcripts. (B) Western blot analysis of SPS protein.

Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. Total RNA and protein were isolated from these leaves before and 12 days after ethanol induction. Thirty µg of total RNA was subject to gel blot hybridization using ^{32}P-labeled *sps* cDNA from tobacco (GenBank Acc. No. AF194022) (A). Thirty µg of total protein was separated by SDS-PAGE and detected using an anti-SPS antibody (B). *alc*-SPSi line 23 was shown here.

3.4.6 Spatial control of gene silencing using the alc system

Previous studies demonstrated that spatial induction of *alc*-reporter gene constructs could be achieved by exposing a single leaf to ethanol vapor (Sweetman *et al*., 2002). In order to test whether this was also possible for inducible gene silencing, an individual leaf of an alc-*chl*Ii plant was exposed to ethanol vapor using a similar "bagging" experiment as described before (Sweetman *et al*., 2002). As shown in Fig. 3-31d, the respective leaf displayed the typical symptoms of *chl*I down regulation observed in whole plant induction experiments. The phenotype was restricted to the treated leaf only, indicating that neither transport of ethanol into the adjacent part of the plant, nor spreading of silencing occurred. Thus the data shown here demonstrate that confined vapor treatment allows for spatial control of gene silencing using the *alc* system.

3.5 *Conditional silencing of SPP in transgenic tobacco plants using inducible RNAi system*

In order to dissect the possible primary and secondary effects associated with constitutive silencing of SPP in the afore-mentioned transgenic tobacco plants, the ethanol inducible RNAi system was applied to down-regulate SPP expression.

3.5.1 Generation of transgenic tobacco plants containing *alc*-SPPi expression cassette

In order to use ethanol inducible RNAi system for the down-regulation of SPP expression, the SPP-RNAi cassette described above was put under the control of *alc*A promoter and followed by 35S polyA signal, and the *alc*A-SPPi expression cassette was further cloned into the binary vector p35S:*alc*R. The resulting *alc*-SPPi plasmid (depicted in Fig. 3-38) was transformed into tobacco plants via *Agrobacterium*-mediated gene transfer.

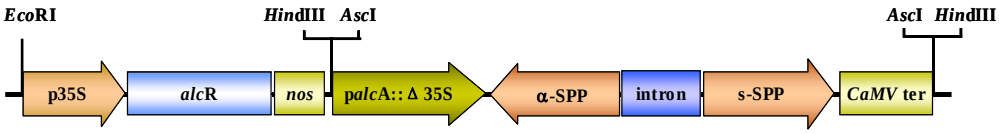


Fig. 3-38: Diagrammatic representation of (not to scale) of *alc*-SPPi constructs.
The *alc*R cDNA is under control of the *CaMV* 35S (p35S) promoter and positioned upstream of the *nos* terminator (*nos*). The SPP-RNAi cassette was inserted downstream of the chimeric *alc*A promoter (p*alc*A::Δ35S), which consists of the *CaMV* 35S minimal promoter (-31 to +5) fused at the TATA-box to upstream promoter sequences of *alc*A (Caddick et al., 1998).

Eighty primary transformants were transferred into the greenhouse. Prior to ethanol induction they were indistinguishable from the control plants. Four weeks after transfer into the greenhouse, ethanol induction was carried out with one additional boost 2 days after the first induction. Eight of the primary transformants exhibited various degree of phenotypical alterations in the young leaves displaying bleached regions along the veins 3 dpi. The phenotypical alterations persisted in the initially silenced leaves and the new growth exhibited wild-type appearance 6 dpi. The phenotypical alterations from three of these lines at 3 dpi were shown in Fig. 3-39. Western blot analysis revealed various degree of inhibition of SPP

expression in these eight transgenic lines at 3 dpi (Fig. 3-40), which correlated with the phenotypical alterations. Three transgenic lines were selected for a detailed analysis, one weakly affected line (*alc*-SPPi-74) and two strongly affected lines (*alc*-SPPi-79 and 81). The primary transformants of the selected lines were propagated in tissue culture by stem-cuttings and the clonal plants were subject to a detailed analysis.

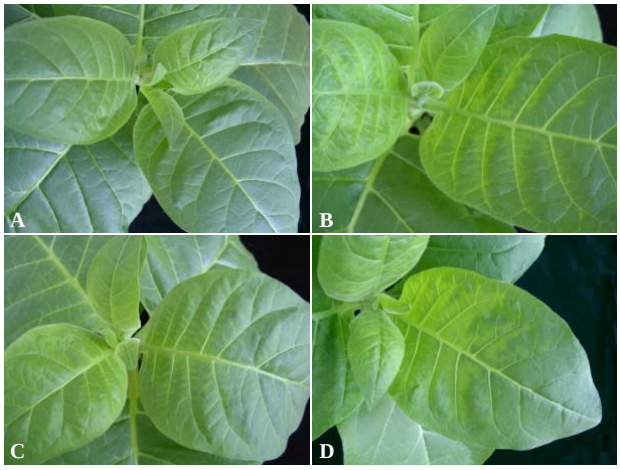


Fig. 3-39: Phenotypical alterations in *alc*-SPPi tobacco plants after ethanol inuction.
Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Pictures were taken at 3 dpi. A, wild-type control; B, *alc*-SPPi line 35; C, *alc*-SPPi line 79; D, *alc*-SPPi line 81.

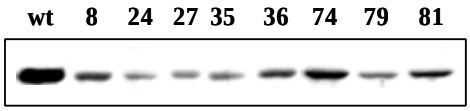


Fig. 3-40: Efficient downregulation of SPP using ethanol inducible RNAi system.
Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. Total protein was isolated from these leaves at 3dpi, separated by SDS-PAGE and detected using an anti-SPP antibody.

3.5.2 Time course of SPP activity, Suc6P accumulation and sucrose contents upon ethanol induction

Four weeks after the clonal plants from the selected lines were transferred into the greenhouse, ethanol induction was carried out with one additional boost 2 days after the first induction. In order to investigate the kinetics of reduction in SPP activity, changes in sucrose levels and accumulation of Suc6P in *alc*-SPPi transgenic plants, leaves being ca. 5 cm in size at the time point of induction and expected to become phenotypically affected, were followed until 6 dpi. Prior to ethanol induction, SPP activity was similar in the transgenic and control plants. After ethanol induction, SPP activities were largely unchanged in the control plants

and the weakly affected transgenic line (*alc*-SPPi-74) as compared to those prior to ethanol induction. However, SPP activities were dramatically reduced by 59-74% in the strongly affected transgenic lines (*alc*-SPPi-79 and 81) at 3 dpi and were further decreased 6 dpi with a reduction of 72-85% as compared to the control plants (Fig. 3-41). Consistent with the changes in SPP activity, Suc6P level in *alc*-SPPi-74 plants was unaltered after ethanol induction as compared to the control, whereas a slight increase in Suc6P level occurred in *alc*-SPPi-79 and 81 plants 3 dpi (29% and 56%, respectively). Suc6P level was further elevated 6 dpi in the two strongly affected transgenic lines, with a 2-fold increase in *alc*-SPPi-79 plants and over 3000-fold in *alc*-SPPi-81 plants compared to the control plants (Fig. 3-42A). In parallel, a time course of sucrose level was also followed. The results showed that sucrose level was largely unchanged in the transgenics as compared to the control plants, irrespective of ethanol induction (Fig. 3-42B).

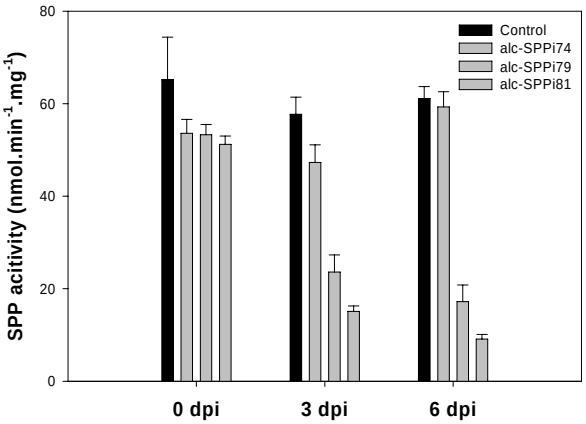


Fig. 3-41: Time course of SPP activity in the control and selected *alc*-SPPi transgenic lines after ethanol induction.
Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. Total protein was isolated from these leaves and SPP activity was determined at the indicated time. Bars show the SD of six individual plants.

Taken together, the above data demonstrated that SPP expression can be down regulated in an inducible way using the ethanol inducible RNAi system in transgenic tobacco plants, which might be of use in dissecting the possible primary and secondary effects associated with constitutive reduction of SPP activity. However, due to the time constraint, it is not possible to perform detailed analyses in this project. This work should be carried out in the future.

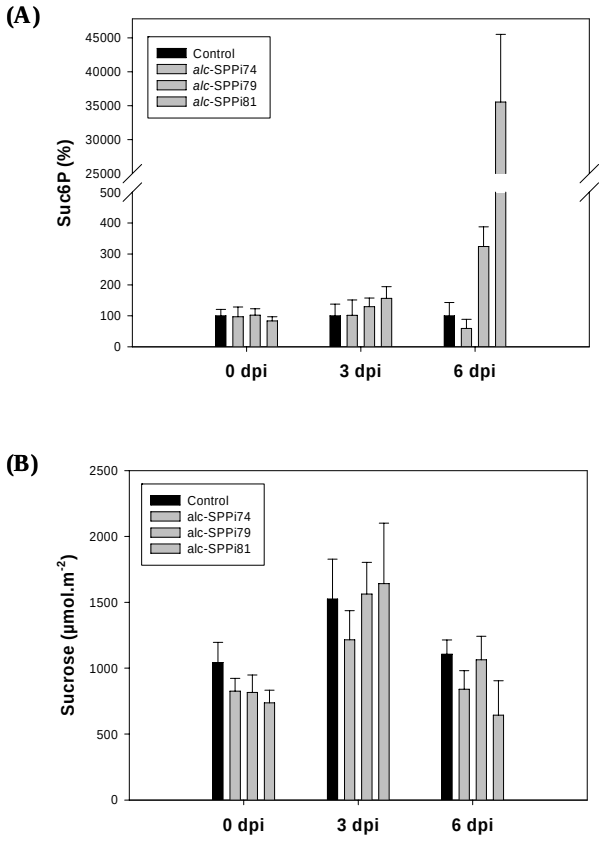


Fig. 3-42: Time course of Suc6P and sucrose contents in the control and selected *alc*-SPPi transgenic lines after ethanol induction.

(A) Time course of Suc6P levels. (B) Time course of sucrose levels.

Tobacco plants (42 days old) were induced with 100 ml 1% (v/v) ethanol via root drenching. Leaves were followed being ca. 5 cm in size at the time point of induction. Suc6P and sucrose contents were determined in these leaves at the indicated time. Suc6P levels were given in percentage of that in the control at each time point. Bars show the SD of six individual plants.

4 Discussion

4.1 *SPP and sucrose synthesis in autotrophic tissues of N. tabacum plants*

4.1.1 SPP is not rate limiting for sucrose synthesis in tobacco source leaves

SPP catalyses the final step of sucrose biosynthesis which is the hydrolysis of Suc6P into sucrose and inorganic phosphate. The latter is transported back into the chloroplast where it sustains ATP synthesis and regeneration of ribulose-1,5-bisphosphate. While the regulatory roles of other enzymes in sucrose biosynthesis, such as SPS or cFBPase, are well established, little is known about the contribution of SPP to control of flux through the pathway. Recent evidences showed that the activities of SPS and SPP were mutually dependent and the two enzymes might associate to form a multienzyme complex (Salerno *et al*., 1996; Echeverria *et al*., 1997), which opened the possibility that SPP could have a role in metabolite channeling between the two enzymes and could contribute to control of flux through the pathway. In order to obtain a better insight into the *in vivo* role of this enzyme, transgenic tobacco plants with a reduced level of SPP expression have been created using an RNAi approach. Removal of up to 80% of SPP activity had only minor effects on leaf sucrose content and plant growth behavior. Using transgenic plants showing only a moderate reduction in enzyme activity the control coefficient for SPP in the pathway of sucrose biosynthesis was estimated to be close to zero. These data are in agreement with earlier reports that SPP activity by far exceeds that of SPS and thus SPP is likely to exert no major control over flux through the pathway (Hawker and Smith, 1984). The fact that ca. 80% of SPP activity can be “lost” before any marked effect on sucrose synthesis can be observed suggest that the enzyme is unlikely to be tightly coupled to the SPS complex *in vivo* – certainly there does not seem to be a requirement for a 1:1 stoichiometry between SPS and SPP as has been suggested before (Lunn et al., 2000).

4.1.2 Repression of SPP alters photosynthetic carbon partitioning in favor of starch and leads to a starch excess phenotype

Reduced SPP activity in the transgenic tobacco plants led to a shift in carbon partitioning from sucrose to starch, which is reminiscent of the changes observed in the transgenic potato plants with reduced levels of TPT or cFBPase, respectively (Heineke *et al.*, 1994; Riesmeier *et al.*, 1993; Zrenner *et al.*, 1996a). However, in the latter two transgenic plants, excess starch built up in the day can be remobilized at a much higher rate during the following dark period maintaining synthesis and export of sucrose. Hence, the effects exerted during the day are compensated, which leaves growth of these transgenic plants largely unaffected as compared to that of the wild type plants. (Heineke *et al.*, 1994; Riesmeier *et al.*, 1993; Zrenner *et al.*, 1996a). Recent evidence suggests that maltose, instead of triose-phosphate, is the major metabolite exported from the chloroplast into the cytosol during starch breakdown at night (Niittylä *et al.*, 2004). Maltose is further metabolized in the cytosol by a specific glucosyltransferase eventually providing the substrates for sucrose synthesis (Chia *et al.*, 2004), thereby bypassing the reactions catalyzed by TPT and cFBPase, respectively. With respect to SPPi plants, the starch excess phenotype after a prolonged dark period, and the massive accumulation of Suc6P and the reduced sucrose level at the end of the night indicate that they lack the possibility to compensate for the block in sucrose synthesis by using an alternative pathway, which is consistent with the fact that the lesion is downstream of the point where sucrose synthesis during the day and during the night converge. Given the higher rate of starch synthesis in isolated leaf discs and the accumulation of maltose in leaves of SPPi plants at the end of the dark period, it seems likely that the starch-excess phenotype found in the transgenic lines is caused by an increased build up as well as by an impaired breakdown of starch. The accumulation of maltose might further indicate that either the maltose transporter exporting maltose from the chloroplast into the cytosol (Niittylä *et al.*, 2004) or the glucosyltransferase catalyzing the transfer of one glucosyl unit of maltose to glycogen and releasing the other (Chia *et al.*, 2004) is prone to feedback inhibition if the downstream intermediates cannot be efficiently further metabolized. Contrary to SPPi tobacco plants, *Arabidopsis* plants with antisense repression of SPS displayed no redistribution of assimilated carbon towards starch and leaf starch content was actually decreased in these plants (Strand *et al.*, 2000). Seemingly, the metabolic changes brought about by a block of sucrose synthesis at the point of the SPP reaction can trigger markedly different responses with respect to carbon partitioning than a block in the step catalyzed by SPS.

4.1.2.1 Effects on starch synthesis

It is generally assumed that the reaction catalyzed by AGPase represents the first rate-limiting step in starch synthesis in the leaves. However, the higher rate of starch synthesis in isolated leaf discs in SPPi lines occurred irrespective of a reduction in AGPase activity of approximately 50%. Previous studies with AGPase antisense potato plants revealed that a reduction of this enzyme to less than 40% residual activity was necessary before it became limiting for starch synthesis (Müller-Röber *et al*., 1992), indicating that certain factors other than the overall AGPase activity measured *in vitro* are more important in control of starch synthesis. It is well known that AGPase is subject to allosteric regulation with 3-PGA acting as an activator and P_i as an inhibitor (Sowokinos, 1981; Preiss, 1988). In SPPi plants, despite the tremendous build up of phosphorylated intermediates in form of Suc6P, there is no clear evidence for an allosteric activation of AGPase since 3-PGA steady-state levels remained low, the ATP/ADP ratio was largely unaffected in SPPi lines 31 and 18, respectively, and levels of overall free phosphate increased in all lines as compared to the control. This is something reminiscent of several previous studies, as pointed out in the introduction, in which the rate of starch synthesis was observed to be uncoupled of changes in the level of 3-PGA in leaves (Krapp *et al*., 1991; Krapp and Stitt, 1995; Westram *et al*., 2002). It is assumed that the main significance of the allosteric regulation of AGPase is more likely to be to rapidly increase the recycling of Pi in the stroma when there is transient imbalance between photosynthesis and triose phosphate export although it may in some conditions be part of a regulatory sequence that links sugar accumulation to an increase of starch synthesis (Hendriks *et al*., 2003). In this context, the recently discovered mechanism of posttranscriptional regulation of AGPase by redox is more important since it allows starch synthesis to be increased in response to rising sucrose without an increase of the 3-PGA to Pi ratio as a necessary intervening step (Hendriks *et al*., 2003). Recently, Peter Geigenberger and colleagues demonstrate that T6P but not Suc6P is involved in the redox regulation of AGPase in addition to sucrose, and increased T6P level results in a higher level of monomerization of AGPase thus leading to the activation of the enzyme (personal communication). Another recent study showed that feeding sucrose to *Arabidopsis* plants could induce the accumulation of T6P (Schluepmann *et al*., 2004). Therefore, it can be envisaged that rising sucrose in leaves might lead to the accumulation of T6P which consequently results in the monomerization of AGPase and eventually increases starch synthesis. Interestingly, T6P levels were increased by 2 to 5-fold in the SPPi plants as compared to the control despite the reduced sucrose contents. These data indicate that a signaling transduction chain with T6P as a key component might be stimulated and AGPase might be activated in SPPi plants, which probably accounts for the increased starch synthesis

observed in leaf disc experiments. To capture the *in planta* status of AGPase a sophisticated extraction and assay protocol needs to be applied in order to prevent *post extracto* dimerization and thus inactivation of the enzyme (Tiessen *et al*., 2002; Hendriks *et al*., 2003). This might explain the obvious discrepancy between carbon partitioning experiments and measurement of overall AGPase activity using a conventional enzymatic assay in SPPi plants (Müller-Röber *et al*., 1992). The data shown here also put forward an open question, what is the initial signal acounting for the ehanced starch synthesis in SPPi plants. Obviously, the levels of 3-PGA and sugars can be excluded. It might be that the increased Suc6P levels might be the trigger which leads to an increase of T6P and eventually an activation of AGPase (Fig. 4-1).

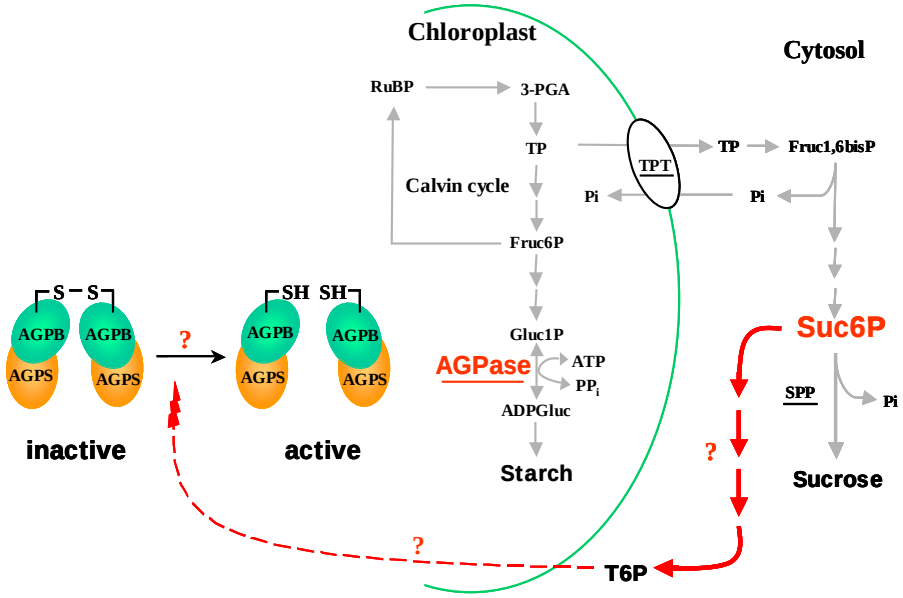


Fig. 4-1: Hypothesis of redox regulation of AGPase in starch synthesis in SPPi transgenic tobacco plants with T6P as a potential intermediatory signaling molecule.

Plant AGPase, a heterotetramer, is under redox regulation with reversible formation of an intermolecular Cys bridge between the AGPB subunits. Monomerization of AGPase leads to activation of the enzyme. In SPPi transgenic tobacco plants, it is hypothesized that the increased Suc6P levels might lead to augmented levels of T6P. The latter might act as a signaling molecule involved in redox regulation of AGPase (Peter Geigenberger, personal communication), leading to activation of AGPase and eventually an enhanced rate of starch synthesis. The unclear processes are indicated by question marks.

Although several points in this hypothetical model are just speculations at the moment, they will be experimentally addressed in the future. First of all, further experimentation would be necessary to provide direct evidence that a shift from the dimeric to the monomeric form of

AGPase causes the increase in starch synthesis observed in the SPPi plants. In order to provide the direct link between the increased level of T6P and the enhanced starch synthesis in SPPi plants, it would be necessary to coexpress an enzyme involving the degradation of T6P in SPPi plants to see whether the accelerated starch synthesis can be reversed or not. T6P phosphatase (TPP) and T6P hydrolase (TPH) from *E.coli*, which catalyse the dephosphorylation of T6P into trehalose and the hydrolysis of T6P into glucose and G6P, respectively (Schluepmann et al., 2003), are ideal to meet this purpose. Since the levels of Suc6P in SPPi plants are much higher than the physiological concentration of Suc6P in wild type plants, the importance of this model in regulating starch synthesis in wild type plants would be called in question. To address this question, transgenic plants with slight increase of Suc6P level would be favored. It might be possible to find such transgenic plants from the segregating transgenic line SPPi 36 which was used for the determination of the control coefficient for SPP in the pathway of sucrose synthesis. An alternative way is to construct SPP silenced transgenic plants using an antisense approach that is supposed to be less effective than RNAi approach and therefore provides more chances to obtain weakly silenced transgenic plants. These works should be carried out in the future.

4.1.3 Feedback regulation and Pi limitation of photosynthesis

P_i in addition to light and CO_2 represents a prime substrate for photosynthesis in higher plants. Early reports showed that accumulation of phosphorylated metabolites due to the slow sucrose synthesis rate would lead to a P_i limitation of photosynthesis (Stitt, 1997; Stitt *et al.*, 1987). The organic phosphoester-pool in SPPi plants is at least doubled as compared to the control owing to the massive accumulation of Suc6P in these plants. This could impose similar consequences on photosynthesis as the ones observed after feeding of phosphate-sequestering agents to leaf tissues. Incubation of detached leaves in solutions containing mannose or 2-deoxyglucose strongly affects photosynthesis since these compounds are readily phosphorylated *in vivo* but cannot be further metabolized. As a result cytosolic P_i is sequestered into organic compounds and becomes limiting (Harris *et al.*, 1983; Klein and Stitt, 1998). However, upon analysis of the steady-state levels of metabolites this explanation seems unlikely to account for the limitation of photosynthesis in SPPi tobacco plants. Firstly, there is no increase in 3-PGA or decrease in the ATP/ADP ratio as would be expected when photosynthesis is P_i-limited (Stitt, 1997). Secondly, although the sum of phosphoesters measured in SPPi plants is higher there is a concomitant increase of total P_i content eventually

resulting in a strong decrease of the 3-PGA/P_i ratio. In plants, the cytosolic P_i pool is relatively constant and excess P_i accumulates in the vacuole (Foyer and Spencer, 1986), from which it is gradually released if cytosolic P_i is depleted allowing for short-term adjustment of P_i available in the cytoplasm (Rebeille *et al*, 1983). Although it is difficult to estimate which proportion of the extra P_i found in SPPi plants is metabolically accessible, elevated P_i levels observed in these plants can be interpreted as a long-term adaptation preventing Pi limitation of photosynthesis despite the tremendous build-up of Suc6P as a consequence of SPP deficiency. This response is comparable to what is observed when warm grown plants are transferred to chilling-temperatures. As a short-term effect large pools of phosphorylated intermediates accumulate owing to the inhibition of sucrose synthesis under low temperatures (Hurry *et al*., 1993; Strand *et al*., 1997), eventually leading to P_i limitation of photosynthesis. In long term, however, acclimation to low temperatures involves redistribution of P_i increasing the amount available in the cytosol and thus relieving P_i limitation despite high levels of phosphorylated intermediates (Hurry *et al*., 1993). It is conceivable that a similar mechanism operates in SPPi plant to prevent long-term P_i limitation of photosynthesis. An additional mechanism which could alleviate P_i limitation of photosynthesis in SPPi tobacco plants is that the rate of Pi recycling within the chloroplast might be increased due to the elevated rate of starch synthesis because the synthesis of starch also contributes to triose-phosphate utilization and thus to P_i recycling in the chloroplast.

Another well defined mechanism exerting feedback control of photosynthesis is sugar-mediated repression of the Calvin cycle, which has frequently been observed in plants accumulating starch and soluble sugars as a consequence of impaired sucrose loading into the phloem (Bürkle *et al*., 1998; von Schaewen *et al*., 1990; Krapp and Stitt, 1995). Since the levels of soluble sugars in SPPi plants are lower than in the control it can be ruled out that carbon-catabolite repression of photosynthesis involves hexose-induced repression of photosynthetic genes via e.g. a hexokinase-mediated signaling step as it has been shown in other systems (Jang *et al*., 1997; Rolland *et al*., 2002). Another possibility involves impaired chloroplast function as a consequence of excessive starch accumulation interfering with CO_2 diffusion or rupturing chloroplasts (Nafziger and Koller, 1976; Cave *et al*., 1981; Grub and Mächler, 1990).

Taking into account the results of CO_2 gas exchange measurements it seems likely that the decrease in photosynthetic activity is largely attributable to an induced reduction of protein content and a concomitant decrease of Rubisco content. In this context, the reduction of chlorophyll content is of minor importance.

Although a substantial reduction of photosynthetic rate was observed in SPPi plants in CO_2 gas exchange measurements, an appreciable higher incorporation of $^{14}CO_2$ was found in the leaf discs from dark adapted SPPi lines under saturating light and CO_2 conditions. This discrepancy might be due to the different phase of photosynthesis measured in the two experimental systems. In the CO_2 gas exchange experiment light adapted plants were used and the steady state level of photosynthesis was measured, while in $^{14}CO_2$ labeling experiment the dark adapted leaf discs were used and the induction phase of photosynthesis was recorded. The higher incorporation of label under saturating light and CO_2 in the leaf discs from dark adpated SPPi lines might be due to different induction kinetics of photosynthesis in the control and SPPi plants. In C3 plants, the first maximal rate of photosynthesis is attained within 2 to 3 min after illumination, reflecting the activation of the enzymes of the Calvin cycle. The rate declines afterwards and then gradually recovers during the next 5-10 min following the activation of the synthesis of end products such as sucrose (Stitt and Grosse, 1988). A shortening in photosynthetic induction especially the second phase of induction period could be brought about by the higher rates of endproducts synthesis. In SPPi plants, the additional labeling was exclusively incorporated into insoluble fraction, indicating a higher starch synthesis rate which might in turn help to recycle Pi within the chloroplast back to the photosynthetic sites thus shorten the second induction phase. A shortened induction phase of photosynthesis might give rise to higher incorporation of $^{14}CO_2$ in short-term labeling experiments under saturating light and CO_2. Further experimentation must be carried out in the future to provide direct evidences for a shortened induction phase of photosynthesis.

4.1.4 Suc6P does not feedback inhibit sucrose synthesis

In SPPi plants SPS expression is induced at transcriptional level, and its activity in terms of both V_{max} and V_{sel} and activation state indicated by V_{sel}/V_{max} are distinctly increased, which probably reflect an attempt to counteract the decrease in sucrose biosynthetic capacity in these plants. Earlier reports demonstrated that transcriptional regulation of SPS expression as well as posttranslational regulation of its activity occurs in response to changing sugar levels. Provision of glucose to excised sugar beet leaves strongly increased the steady state level of SPS mRNA, whereas exogenous sucrose slightly repressed expression (Hesse *et al*., 1995). In spinach, posttranslational regulation of SPS in response to rising sucrose levels leads to partial inactivation of this enzyme (Stitt *et al*., 1988). In SPPi plants the increased SPS expression level and the augmented activation state of this enzyme occur despite the decrease in the

levels of soluble sugars, which makes it tempting to speculate that in this case it is the low sucrose content acting as the signal for the induction and activation of SPS. Alternatively, the ratio between glucose and sucrose could serve as a measure to determine the cell's demand for SPS activity, however, this is unlikely since both glucose and sucrose are decreased to a similar extent in SPPi plants.

Earlier observations suggested that Suc6P is an extremely weak inhibitor of SPS activity *in vitro* (Krause and Stitt, 1990). In SPPi tobacco plants, despite of the tremendous buildup of Suc6P, the upstream intermediates in the pathway of sucrose synthesis did not accumulate, indicating that Suc6P might also play no significant role in feedback regulation of sucrose synthesis *in vivo*.

During photosynthesis, sucrose synthesis is regulated by the coordinated action of SPS and cFBPase to keep pace with the supply of fixed carbon or to allocate fixed carbon between sucrose and starch (Stitt *et al*. 1987; Stitt, 1990b). With the onset of photosynthesis, rising 3-PGA and decreasing Pi inhibit fructose-6-phosphate,2-kinase. This results in declining Fruc-2,6-bisP levels eventually leading to a stimulation of cFBPase activity. Increasing levels of hexose-P then stimulate SPS. In turn, accumulating sucrose leads to a partial inactivation of SPS and rising hexose-P leads to an activation of fructose-2,6-bisphosphatase (Stitt *et al*., 1988; Neuhaus *et al*., 1990). The resulting increase in Fruc-2,6-bisP then inactivates cFBPase. Given the stimulation of SPS activity, unaltered Fru-2,6-bisP content as well as the decrease in cFBPase activity in the SPPi transformants is unexpected, but is fully consistent with the observation that Fru6P does not accumulate. Moreover, unlike the stimulation in SPS expression at the transcriptional level, the expression of cFBPase is unaltered in SPPi plants. Considering the existence of diurnal regulation of SPS activity and the augmented SPS activity and activation state at night in SPPi plants, these data indicate that the mechanisms for the coordinated regulation of SPS and cFBPase might still operate, however novel signaling pathways are stimulated for additional up-regulation of SPS but not cFBPase in SPPi plants. The latter situation where SPS and cFBPase are regulated independently in SPPi plants resembles the one observed during phases of starch mobilization at night when cFBPase but not SPS is dispensable for sucrose synthesis. In SPPi plants, however, these changes do not achieve a net stimulation of sucrose synthesis because the initial problem (low SPP activity) is still present.

4.1.5 Sucrose synthesis in young and mature leaves

In addition to environmental factors such as light sucrose synthesis is also developmentally regulated. Previous studies revealed that SPS activity and activation state vary with leaf age in wild type tobacco plants, being greater in the fully expanded mature leaves and lower in the expanding young leaves (Baxter, *et al*. 2003). This developmental regulation mechanism operated in the control plants as well as in SPPi transgenic plants, however, the increases in SPS activity especially V_{sel} activity and activation state during leaf maturation were more pronounced in SPPi plants. Given SPS activity and its activation state in the expanding young leaves were largely unaltered between the transgenic and control plants, the further activation of SPS in the mature leaves of SPPi plants indicated either the stimulation of an existing signalling pathway involved in developmental regulation of SPS activity or the generation of some novel regulatory signal in the mature leaves of SPPi plants. Interestingly, a more pronounced decrease in the levels of soluble sugars was found in the mature leaves from SPPi plants as compared to the young leaves, which was concomitant with the elevated activation state of SPS in the mature leaves from SPPi plants, providing an additional indication that the low sugar content might be the trigger for the induction of SPS expression and activation of SPS in the mature leaves in SPPi plants. Although SPP activity in the expanding young leaves of SPPi plants was reduced to similar extent as that in the mature leaves, it led to only a slight increase in Suc6P level in the young leaves in contrast to massive accumulation in the mature leaves. The imbalance between the synthesis rate of Suc6P catalyzed by SPS and its degradation rate catalyzed by SPP might result in the accumulation of Suc6P. Interestingly, the Suc6P levels in the leaves were found to correlate with the ratio of SPS V_{sel} activity to SPP activity but not the ratio of SPS V_{max} activity to SPP activity. In the control plants SPP activity is in far excess over SPS V_{sel} activity in both young and mature leaves, thus resulting in an extremely low level of Suc6P. In the young leaves of SPPi plants, SPS V_{sel} activity is comparable to SPP activity owing to the repression of SPP, consequently leading to a slight increase of Suc6P as compared to the control plants. In the mature leaves from SPPi plants, a further increase in SPS V_{sel} activity outmatches SPP activity, therefore the synthesis rate of Suc6P outpaces its degradation rate, eventually resulting in a tremendous build up of Suc6P. These data indicate that SPS V_{sel} activity is a more important factor in the control of sucrose synthesis than SPS V_{max} activity, which is in accordance with the previous studies (Geigenberger et al. 1997; Geigenberger et al., 1999).

Taken together, the key findings in SPPi transgenic tobacco plants can be summarized as follows: (i) in tobacco SPP is abundant and does not exert significant control of sucrose

synthesis under normal conditions, (ii) the fact that ca. 80% of SPP activity can be "lost" before any marked effect on sucrose synthesis can be observed suggest that the enzyme is unlikely to be tightly coupled to the SPS complex *in vivo* – certainly there does not seem to be a requirement for a 1:1 stoichiometry between SPS and SPP as has been suggested before (Lunn et al., 2000), (iii) the loss of SPP activity results in a change in carbon partitioning in favor of starch, something that is not observed in SPS antisense plants but does occur in cFBPase antisense *Arabidopsis* plants (Strand et al., 2000).

4.2 *SPP and sucrose synthesis in potato tubers under low temperature*

4.2.1 Massive accumulation of Suc6P might compensate for the effect of decreased SPP expression on sucrose synthesis in long-term cold storage

Repression of SPP expression led to a massive accumulation of Suc6P in cold-stored transgenic tubers, which indicated an active sucrose synthesis via SPS and SPP was induced in the cold and could be effectively impeded by knocking down the expression of SPP. However, contrary to what could be expected, sucrose content was also drastically increased in the transgenics during cold storage (33-69% increase 17days in the cold and 4 to 5-fold 34 days in the cold). Sucrose synthesis rate during cold storage was estimated based on net accumulation of sugars provided that the rate of recycling of sucrose or hexose is not high. The data from Tab. 3-11 were used for the calculation and the results revealed that sucrose synthesis rate was only slightly decreased in the transgenics within the first 17 days in the cold (6,6%, 27% and 29,8% decrease in 35SPPi line 18, 8 and 5, respectively), whereas it was slightly increased in the transgenics within 34 days in the cold (38,8%, 9,4% and 6,9% increase in 35SPPi line18, 8 and 5, respectively) (Fig. 4-2). Taken together, the rate of sucrose synthesis was largely unchanged between the transgenic and control plants during long-term cold storage. In order to fully understand this dilemma, the Suc6P concentration was estimated in the potato tubers assuming that Suc6P is restricted to the cytosol with a relative

volume equal to 12% of total volume (Farre *et al*., 2001). Suc6P was present with concentration of 7-15 µM in the control plants, whereas ca. 32-43 mM in the transgenics after 17 days in the cold. The maximum *in vitro* activity of SPP at 30°C was 11,68±0,57 µmol/g F.W./h in the wild type control after 17 days in the cold, whereas the maximum *in vitro* catalysing rates of the residual SPP in the transgenics ranged from 1,66±0,21 to 1,81±0,37 µmol/g F.W./h at 30°C. These rates would be equivalent to 1,55±0,075 µmol/g F.W./h in the controls and 0,22±0,028 to 0,24±0,049 µmol/g F.W./h in the transgenics at 4°C calculated with the temperature coefficients of SPP. Sucrose synthesis rate within the first 17 days in the cold was estimated from the production of sucrose and hexose, which was 0,122 µmol/g F.W./h in the control tubers and 0,086 to 0,114 µmol/g F.W./h in the transgenics. Therefore, in the control plants the maximum SPP activity is much higher than the rate of sucrose synthesis in the cold. Considering the potato SPP has a Km of 148µM for Suc6P, limiting Suc6P could restrict SPP from reaching its maximal catalytic capacity in wild type tubers in the cold. In the transgenics, however, the residual SPP could become saturated with the massive accumulation of Suc6P and operate *in vivo* in a rate similar as the sucrose synthesis rate, therefore the decreased SPP expression largely had no long-term effect on sucrose synthesis in the cold. This is something reminiscent of the phenomenon observed in SPS silenced potato tubers, where the rate of sucrose synthesis was decreased in the transgenic tubers in a short-term labelling experiment but largely unchanged during long-term cold storage (Krause *et al*., 1998). It is suggested that increased levels in the substrates (UDPGlc and Fru6P) and allosteric activator (Gluc6P) for SPS and cold-induced changes in the kinetic properties of SPS could compensate the effects of decreased SPS expression in the SPS silenced tubers during long-term cold storage (Krause *et al*., 1998).

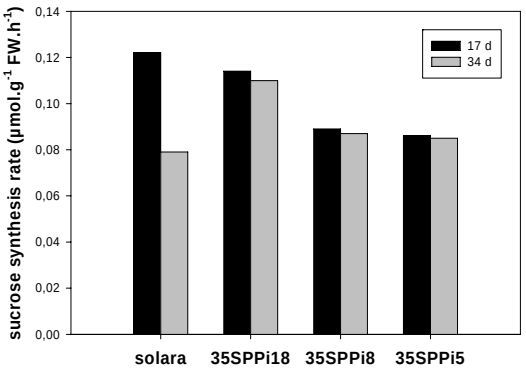


Fig. 4-2: The rate of sucrose synthesis estimated in the cold-stored tubers.

Sucrose synthesis rates during cold storage were estimated based on net accumulation of sugars (data from Tab. 3-11) within 17 days of cold storage (black columns) and 34 days of cold storage (grey columns), respectively. The calculation was carried out given that two hexose molecules are derived from a primary breakdown of sucrose.

Although sucrose synthase can catalyse either sucrose synthesis or breakdown, it is suggested that SPS rather than sucrose synthase contributes to sucrose synthesis in the cold-stored tubers (Geigenberger and Stitt, 1993; Pollock and Rees, 1975). However, it is still arguable whether sucrose synthase contributes to sucrose synthesis in the cold when the pathway through SPS and SPP become limiting in the SPPi potato tubers. This question was addressed by measuring sucrose synthase activity in the freshly harvested and cold-stored tubers. Interestingly, in both cases, sucrose synthase activity was substantially reduced in the transgenics as compared to the controls. Therefore, it seems unlikely that sucrose synthase compensates for the decreased SPP activity in sucrose synthesis during cold storage in the transgenic potato tubers.

4.2.2 Sucrose cleavage was impaired in the SPP silenced potato tubers

In principle, sucrose degradation in plants can be catalysed by sucrose synthase and invertase, respectively (ap Rees, 1984; Kruger, 1990). In cold stored potato tubers, this process mainly occurs in the vacuole and is catalysed by a vacuolar invertase (Zrenner *et al*., 1996b). The massive accumulation of sucrose without a further conversion into hexose in the transgenics in the cold indicated that sucrose catabolism via vacuolar invertase was impaired. Interestingly, cold-induced expression of vacuolar invertase was inhibited in the transgenics within the first 17days of cold storage. After 34 days in the cold, vacuolar invertase expression was induced in some transgenic tubers but largely remained unchanged in most of the transgenic tubers. Interestingly, a negative correlation between the induction of vacuolar invertase expression and the levels of Suc6P was found in the cold-stored tubers. Analysis of the expression of another cold-inducible gene, UGPase, indicated that no general inhibition of cold-induced gene expression occurred in the transgenics due to pleiotropic effects. Taken together, these data indicated that the expression of vacuolar invertase in the cold was induced by metabolic changes rather than low temperature *per se* and different signalling pathways were involved in the induction of vacuolar invertase and UGPase, respectively. The data shown here provide first evidence linking Suc6P and gene expression in plants, strongly suggesting a potential signalling function of Suc6P in regulation of carbon utilisation. In order to fully unravel the genes and pathways which might be regulated by Suc6P in the cold-stored tubers, transcriptional and metabolic profiling of cold stored SPPi tubers should be performed in the future.

In accordance with its expression on the transcriptional level, the activity of vacuolar invertase was substantially decreased in the cold-stored transgenic tubers as compared to the controls, which might account for the inability of hexose production in the presence of huge amounts of sucrose in the transgenics during cold storage. However, it is probably not the sole reason for this phenomenon. After 34 days in the cold, the activity of vacuolar invertase in the SPP silenced tubers was comparable with that observed in the VI antisense potato plants where the hexose:sucrose ratio was decreased during cold-storage while no such strong decrease of cold-induced hexose accumulation occurred (Zrenner *et al*., 1996b). It is known that specific invertase inhibitor proteins can modulate the *in vivo* VI activity (reviewed by Rausch and Greiner, 2004). Several invertase inhibitor genes have been cloned from various species (Greiner *et al*., 1998; Bate *et al*., 2004). Such an invertase inhibitor protein has been demonstrated to be present in potato plants (Matsushita and Uritani, 1976). Ectopic expression of a vacuolar invertase inhibitor from tobacco in transgenic potato tubers could drastically decrease the cold-induced hexose accumulation (Greiner *et al*., 1999). Therefore, it is possible that a vacuolar invertase inhibitor inactivates the VI *in vivo* in the transgenics and further restricts the hydrolytic cleavage of sucrose in the vacuole.

Since sucrose synthesis takes place in the cytosol while its cleavage during cold storage mainly occurs in the vacuole, sucrose has to enter the vacuole before the VI can access it. In sugar beet taproot, sucrose uptake into vacuole is an energised process and requires a sucrose/H^+ antiport (Briskin *et al*., 1985; Getz and Klein, 1994). Therefore, sucrose can be transported against the sucrose gradient across the vacuole membrane and accumulated in the vacuole in sugar beet taproot. However, no sucrose gradient is observed across the vacuole membrane in potato tubers (Farre *et al*., 2001), which indicates that sucrose loading into vacuole in potato tubers probably occurs by facilitated diffusion. At the moment, it is not clear whether this process operates normally or whether it is impaired so that sucrose cannot be loaded into the vacuole in the cold-stored transgenic tubers. Recently, a non-aqueous technique for cellular fractionation has been successfully applied to analyse metabolic compartmentation in heterotrophic tissues like potato tuber, which can help to clarify this possibility (Farre *et al*., 2001).

4.2.3 Increased inorganic phosphate might compensate the Pi-sequestrating effects exerted by massive accumulation of Suc6P in cold-stored transgenic tubers

Inorganic phosphate is an important macronutrient in plants, required for the regeneration of ATP from ADP during oxidative phosphorylation. A metabolic consequence of Pi limitation is a significant reduction in the cellular level of adenylates (Theodorou *et al*., 1991; Rychter *et al*., 1992). The tremendous build-up of Suc6P as a consequence of reduced SPP expression in the transgenic tubers in the cold would hypothetically lead to phosphate limitation because huge amounts of Pi are sequestrated into Suc6P. However, the unchanged energy charge in the transgenics as compared to the controls indicated that no phosphate limitation occurred to restrict ATP regeneration from ADP. Interestingly, a strong increase in the level of inorganic phosphate was observed in the growing tubers from the transgenic plants and even after harvest and cold storage, the Pi pool in the transgenic tubers was still substantially larger than that in the controls. Therefore, one explanation for the absence of phosphate limitation despite the massive sequestration of Pi in Suc6P is that the elevated Pi content in the transgenics probably compensates the Pi-sequestrating effects exerted by Suc6P, something similar to that in the SPPi tobacco leaves.

Taken together, the key findings in 35SPPi transgenic potato tubers can be summarized as follows: (i) the loss of SPP activity can impede the flux through the pathway of sucrose synthesis in cold-stored tubers; however, the massive accumulation of Suc6P might in turn compensate for the effect of reduced SPP expression on sucrose synthesis in long-term cold storage, leaving the long-term sucrose production largely unchanged in the cold-stored 35SPPi tubers, (ii) the levels of Suc6P in the cold-stored tubers were negatively correlated with the cold induction of vacuolar invertase, providing first evidence of a potential signaling function for S6P in regulation of gene expression in plants.

4.3 *Tuber-specific silencing of SPP in transgenic potato plants*

Constitutive silencing of SPP in 35SPPi transgenic potato plants resulted in severe phenotypical alterations in the shoots, which might impose profound influences on tuber metabolisms. In order to reduce the potential pleiotropic effects from the shoots, the tuber-

specific patatin (B33) promoter was used to control the expression of the SPP-RNAi cassette in transgenic potato plants. The expression of SPP was not influenced in the leaves of transgenic plants as revealed by western analysis and Suc6P levels, whereas SPP protein was virtually eliminated from the transgenic tuber. These data demonstrate tissue-specific silencing of endogenous genes can be achieved by using tissue-specific promoter to control the expression of dsRNA. These results are in consistence with the earlier reports that endogenous genes are resistant to systemic gene silencing (Palauqui *et al*., 1997; Palauqui and Vaucheret, 1998; Voinnet *et al*., 1998). Thus, no pleiotropic effect from the shoots imposed on BSPPi tuber, which makes it a better system to study sucrose (re)synthesis in this tissue than 35SPPi tuber.

4.3.1 Sucrose (re)synthesis in growing and stored tubers

In this study, no phenotypic alteration was observed during tuber growth, tuber yield was not affected and starch content was not influenced in BSPPi tubers as compared to the control tubers. Reduced SPP activity left the levels of sucrose largely unchanged in the growing tubers from BSPPi plants as compared to the controls, probably because sucrose (re)synthesis only accounts for a small proportion of the total sucrose contents in growing tubers. Suc6P levels were slightly increased in the growing tubers from BSPPi plants (2 to 6-fold increase as compared to the controls), indicating sucrose (re)synthesis was impaired at the point of SPP. However, under the growth conditions used in this study the impaired sucrose (re)synthesis had no influence on starch synthesis and tuber yield, which is in accordance with the observation in SPS antisense tubers (Geigenberger *et al*., 1999). After harvest in the control tubers the levels of glucose and fructose rose up and a substantial increase was observed 7 days post harvest (dph), while the level of sucrose was progressively decreased. A similar tendency in the changes of hexose contents after harvest was observed in the BSPPi tubers. With respect to sucrose levels, the tendency for a decrease was not as obvious in BSPPi tubers as observed in the control tubers. Sucrose levels were slightly higher in the tubers from BSPPi line 3 and 11 at 7 dph and were significantly higher in the tubers from all the three transgenic lines at 10 dph than those in the controls. In order to get a better understanding of sucrose synthesis in the stored tubers from BSPPi plants, a time course of Suc6P levels were followed after harvest. Surprisingly, a tremendous build-up of Suc6P with a 500 to 1200-fold increase within one week occurred in BSPPi tubers after harvest while its level was constantly low in the control tubers. The concomitant accumulation of Suc6P and sucrose in the BSPPi stored

tubers is reminiscent of the phenomenon observed in 35SPPi cold-stored tubers. Taken together, these data indicated that the pathway for sucrose synthesis was strongly stimulated after harvest in BSPPi tubers, however due to the persistence of the initial problem (low SPP activity) it was impeded at the step of SPP and led to the massive accumulation of Suc6P. At the moment it is not clear yet whether sucrose synthesis was also stimulated in the control tubers or not. Previous studies revealed that loss of water in tubers after harvest could stimulate sucrose synthesis through the actions of SPS and SPP (Geigenberger *et al*., 1997; Geigenberger *et al*., 1999). It is conceivable that the environmental changes might cause water loss in tubers after harvest in this study, which consequently resulted in the stimulation of sucrose synthesis observed in BSPPi tubers. However, at the moment the possibilities could not be ruled out that other factors might also contribute to the stimulation of sucrose synthesis in BSPPi tubers after harvest. For example, sucrose synthesis might be indispensable for the tubers to adjust themselves to the physiological and biochemical changes after harvest. Reduced SPP activity in BSPPi tubers presumably resulted in a lower end-product synthesis rate, which might feedback stimulate the entry through the pathway. In this context, the rate of sucrose synthesis through SPS and SPP might be more important for the cells to adjust to the metabolic changes after harvest than the steady state level of sucrose.

4.3.2 Respiration and sucrose (re)synthesis in BSPPi tubers

The respiration rates in the growing tubers were comparable in BSPPi and control tubers, and were decreased to a similar extent in all the lines within 6 dph. However, a strong induction in respiration was observed in BSPPi stored tubers, which started after 6 dph in BSPPi line 11 and 13 dph in the other two BSPPi lines. The higher respiration rate in BSPPi tubers during storage is reminiscent of the other transgenic tubers over-expressing a yeast-derived invertase in the cytosol, or a bacterial sucrose isomerase (suc-iso) in the apoplasm or a bacterial sucrose phosphorylase in the cytosol (Trethewey *et al*., 1998; Trethewey *et al*., 2001; Hajirezaei *et al*., 2003). In the latter three transgenic tubers, perturbation of sucrose metabolism is carried out by either converting sucrose into glucose and fructose (invertase expressors), or isomerising sucrose to palatinose (suc-iso expressors) or phosphorolysing sucrose into fructose and Glu1P (sucrose phosphorylase expressors). All three interventions result in a low level of sucrose in the cells. By comparison of these three transgenic tubers, hypotheses were put forward that either the low concentration of sucrose observed in all these three transgenic tubers or a signal related to the increased levels of metabolites downstream of the sucrose cleavage was

responsible for the increased respiration rate (Trethewey *et al*., 2001; Hajirezaei *et al*., 2003). With respect to BSPPi tubers, the respiration rate was not altered in the growing tubers compared to the control, which was contrary to the observations in cytosolic invertase and sucrose phosphorylase over-expressors where the increased respiration rates also occurred in the growing tubers (Trethewey *et al*., 1998; Trethewey *et al*., 2001; Hajirezaei *et al*., 2003). Given the unaltered sucrose concentration in BSPPi growing tubers, the unchanged respiration rate indicated that sucrose (re)synthesis via SPS and SPP plays no major role in the stimulation of respiration rate in growing tubers. After harvest, however, the pathway for sucrose synthesis was strongly induced in BSPPi tubers as indicated by a tremendous build-up of Suc6P within the first 3 days. Considering that respiration rate was comparable in BSPPi and control tubers within the first 6 days after harvest, these data indicated that Suc6P also has no apparent role in the induction of respiration. The levels of sucrose were not decreased in BSPPi tubers as compared to the control tubers at 7 dph, actually even increased by 34% and 98% in the tubers from BSPPi line 3 and 11, respectively, although sucrose synthesis was impaired at the step of SPP. Therefore sucrose contents might not be the trigger for the stimulation of respiratory pathways in BSPPi tubers as suggested in the other transgenic tubers (Trethewey *et al*., 1998; Trethewey *et al*., 2001; Hajirezaei *et al*., 2003). Possible reasons for the stimulation of respiration in BSPPi tubers during storage will be further discussed in the following part. Nevertheless, the stimulation of respiration in BSPPi tubers was concomitant with the phenotypic alterations, indicating that stimulation of respiration might be involved in burning-down the storage reserves in BSPPi tubers. Under normal conditions, the cytochrome pathway with COX as the terminal oxidase is the main respiration pathway in plant cells, which is coupled (through the generation at the three sites of a proton motive force) to the synthesis of ATP from ADP and Pi via oxidative phosphorylation. However, plant mitochondria also possess non-phosphorylative pathways including several NAD(P)H dehydrogenases and the AOX which can functionally bypass the energy conservation sites in the electron transport (Douce and Neuburger, 1989; Soole and Menz, 1995; Day and Wiskich, 1995). The AOX couples the oxidation of ubiquinol with the reduction of oxygen to water, thus bypassing the last two sites of energy conservation normally associated with the cytochrome pathway (Day and Wiskich, 1995; Vanlerberghe and McIntosh, 1997; Parsons *et al*., 1999). These non-phosphorylative pathways might help to dissipate the excess energy or reductant in form of heat (Millenaar and Lambers, 2003). The expression of alternative oxidase was strongly induced in BSPPi tubers during storage, which might contribute to the stimulated respiration. The strong induction of AOX in BSPPi tubers

during storage indicated that the energy produced during burning-down the storage reserves might be dissipated in form of heat.

4.3.3 Sucrose synthesis, phosphate limitation and phenotypic alteration in BSPPi tubers

The stimulation of sucrose synthesis in BSPPi tubers after harvest resulted in a massive accumulation of Suc6P, which consequently sequestered an equal amount of phosphate. Therefore phosphate limitation due to massive accumulation of Suc6P would be expected in BSPPi tubers during storage. Unlike 35SPPi tubers that contained more Pi when harvested, BSPPi tubers contained similar levels of Pi during growth as compared to the control tubers. Pi level in the control tubers was rather constant during storage, whereas a 32% decrease in the Pi level occurred in the tubers from the most severely affected line BSPPi 11 at 3 dph and a 52% decrease at 7 dph. However, the decreases in Pi contents was much less than the amount of phosphate sequestered in Suc6P, indicating that some other phosphate pool was mobilised to sustain a relatively constant pool of Pi in BSPPi tubers during storage. Earlier evidences suggested that under phosphate limitation certain phosphate pools like phospholipids would be mobilised to maintain a relatively constant Pi level (Andersson, *et al*. 2003; Jouhet, *et al*., 2003). On the other hand, the decrease in Pi levels might lead to phosphate limitation that could consequently restrict the oxidative phosphorylation that is coupled with the cytochrome pathway, and induce the expression of AOX that can dissipate the excess electrons without coupling to the production of ATP (Dry *et al*., 1987; Juszczuk *et al*., 2001; Parsons *et al*., 1999). The induction of AOX might help to prevent overreduction of electron transport chain components; otherwise the excess electrons overflowing the electron transport chain would lead to the over-production of reactive oxygen species that are deleterious to the cells (Parsons *et al*., 1999). These changes in BSPPi tubers might leave them facing a paradoxical situation. On one hand, a large energy demand came from the stimulation of sucrose synthesis which might consequently lead to a phosphate limitation due to the massive accumulation of Suc6P; on the other hand, the shift of the terminal oxidase from COX to AOX due to the phosphate limitation would restrict the production of ATP. Thus an imbalance between the energy demand and supply would be formed, which might in turn stimulate the respiration in BSPPi tubers. Such changes might also indicate that the function of mitochondria is probably perturbed in BSPPi tubers. The mitochondrium is not only an organelle responsible for energy production, but also a well-known organelle involved

in cell death (Ferri and Kroemer, 2001). The perturbation of the function of mitochondria might eventually lead to cell death in BSPPi tubers during storage. However, to obtain a clear understanding of the changes in BSPPi tubers, unbiased approaches must be applied in the future, e.g. metabolic profiling and transcriptome profiling. Further experiments also have to be done with respects to the energy charge states, the capacity of different respiratory pathways and the production of reactive oxygen species in BSPPi tubers during storage.

Taken together, the key findings in BSPPi transgenic potato tubers can be summarized as follows: (i) under normal growth conditions, sucrose (re)synthesis through the actions of SPS and SPP does not play an important role in the control of the net rate of starch accumulation and tuber yield, (ii) reduction of SPP activity in a tuber-specific way resulted in a massive accumulation of Suc6P, a stimulation of respiration and eventually necrotic cell death in the tubers after harvest, indicating the pathway of sucrose (re)synthesis is necessary either to cope with water loss due to the environmental changes after harvest or to adjust the tubers to the metabolic changes after harvest.

4.4 *Temporal and spatial control of post-transcriptional gene silencing in transgenic plants*

Down regulation of endogenous genes via PTGS using sense or antisense constructs is a crucial tool to assess gene function in transgenic plants. Recent findings indicate that expression of self-complementary hairpin RNAs greatly enhances the efficacy of such experiments (Chuang and Meyerowitz, 2000; Levin *et al*., 2001; Smith *et al*., 2000; Stoutjesdijk *et al*., 2002; Waterhouse *et al*., 1998). Although these approaches have been proven to be extremely useful, they are not without problems. One drawback of these studies is that constitutive gene silencing often entails pleiotropic effects on growth and development of the transgenic plants, which complicate the interpretation of the phenotype and might mask true gene function. Furthermore, if expression of the target gene is essential for early growth or regeneration during tissue culture, vital plants might not be recovered. The data shown here demonstrate that temporal and spatial control of gene silencing in transgenic plants can be achieved using ethanol inducible RNAi system based on the *alc* gene switch.

4.4.1 Ethanol inducible gene silencing

Two genes involved in chlorophyll biosynthesis, namely c*hl*I and *gsa*, were used to test down regulation by ethanol inducible expression of antisense fragments and dsRNA constructs, respectively. Suppression of either of the two target genes was assumed to result in a loss of pigmentation due to reduced chlorophyll biosynthesis (Höfgen *et al*., 1994, Papenbrock *et al*., 2000). However, only inducible RNAi constructs giving rise to dsRNA were effective in triggering gene silencing, as revealed by rapid development of the characteristic phenotype after induction with ethanol. Inducible antisense constructs designed to express antisense fragments alone failed to interfere with expression of the target gene which was further confirmed by Northern blotting. These results are consistent with the assumption that dsRNA is a much stronger trigger of PTGS than ssRNA, possibly by circumventing the initial conversion of ssRNA into dsRNA by an RNA dependent RNA polymerase (Béclin *et al*., 2002, Dalmay *et al*., 2000). First signs of phenotype development occurred at 36 hpi for *alc-chl*Ii plants to 48 hpi for *alc-gsa*i plants. Given similar patterns of the production of dsRNA in these two transgenics upon induction, the disparities in phenotype development most likely reflect differences in mRNA and protein turnover rates of the respective endogene. The time point of phenotype development was highly reproducible in several independent induction experiments and is much more rapid than the 10 days to 3 weeks which have been reported to be necessary for phenotype induction using various virus based gene silencing systems (Gosselé *et al*., 2002, Hiriart *et al*., 2002; Ratcliff *et al*., 2001; Turnage *et al*., 2002). Using a single induction, the phenotype persisted for approximately 9 days which is considerably shorter than what has been shown for VIGS (Gosselé *et al*., 2002; Ratcliff *et al*., 2001; Turnage *et al*., 2002). However, reiterated ethanol treatment permits to maintain stable silencing for extended periods which should enable to follow the full sequence of consequences of reduced gene expression whenever desirable.

To determine the degree of silencing which could be achieved by inducible expression of dsRNA constructs on the biochemical level, the decline in chl content in phenotypically affected leaves was taken as an indirect measure for chl biosynthetic capacity. A reduction in chl of approximately 80% was achieved in *alc-gsa*i plants, which is in the range of what has been reported for transgenic tobacco plants constitutively expressing *gsa* antisense RNA (Höfgen *et al*., 1994). Transgenic tobacco plants constitutively silenced for *chl*I were shown to have approximately 40% of the chl content as compared to the control (Papenbrock *et al*., 2000). Inducible silencing of c*hl*I using the *alc* system reduced the chlorophyll content in

phenotypically affected leaves to approximately 3% of that before induction indicating a much stronger effect on *chl*I expression than in constitutively silenced plants. It is reasonable to assume that in case of constitutive silencing there is a considerable selection against the reduction of Mg-chelatase activity below a certain threshold level which does not support plant growth under autotrophic conditions. Thus, in some cases inducible PTGS using the *alc* system is likely to provide a valid strategy to obtain a degree of silencing which otherwise prevents the regeneration of viable transgenics.

4.4.2 Features of gene silencing using ethanol inducible RNAi system

According to the current model of PTGS, dsRNAs expressed from transgenes are subsequently processed by an enzyme called Dicer, which has RNaseIII domains. The resulting 21 to 25-nucleotide short interfering RNAs (siRNAs) are subsequently incorporated into RISC (RNA induced silencin complex) to guide cycles of specific RNA degradation. The primary siRNAs can also be used as primers to synthesize dsRNAs via an RNA-dependent RNA polymerase with the target genes serving as templates. The Dicer enzyme would then process the newly synthesized dsRNAs into secondary siRNAs which can guide new cycles of specific RNA degradation, and thus a self-sustained cycle of siRNA generation and target mRNA degradation is formed. Therefore PTGS can be maintained without the presence of the initial triggers once it is initiated (Ruiz, *et al*., 1998; Baulcombe, 2004). In case of the ethanol inducible RNAi system, the production of dsRNAs from the transgenes only lasts till 48 hpi due to the transient expression from the *alc* promoter. However, the silencing event persists in the initially silenced leaves in *alc*-*fnr*i and *alc*-SPSi plants at least 10 dpi, which indicates gene silencing can be maintained in these leaves independent of the initial trigger. It is conceivable that a similar mechanism as mentioned above operates to maintain gene silencing in the initially silenced leaves in the ethanol inducible RNAi system. The maintenance of gene silencing in the initially silenced leaves provides another possibility besides the reiterated treatments with ethanol to follow the full sequences of the consequences of reduced gene expression in ethanol inducible RNAi system.

Another interesting characteristic of gene silencing is that it can act non-cell autonomously, meaning that it can be induced locally and subsequently spread throughout the organism, implying the existence of a mobile silencing signal (Klahre *et al*., 2002; Palauqui *et al*., 1997; Voinnet *et al*., 1998). Little is known about the nature of the signal, but the fact that the sequence specificity corresponding to the initiator dsRNA is retained in the systemic

silencing indicates the signal either is an RNA or that it has an RNA component (Boutla *et al*., 2002; Mlotshwa *et al*., 2002; Baulcombe, 2004). Evidences suggest that highly expressed transgene are much better targets for systemic silencing than are endogenous genes (Palauqui *et al*., 1997; Palauqui and Vaucheret, 1998; Voinnet *et al*., 1998), indicating that the amount of target RNA is important in establishing systemic silencing in response to the mobile signal or a yet unidentified mechanism protecting endogenous genes from systemic silencing. Nevertheless, this is consistent with the finding that using the *alc* system local silencing of *chl*I could be achieved by confined ethanol treatment of a single leaf. In this case no other parts of the plant but the treated leaf displayed any visible signs of *chl*I silencing, indicating that no systemic spread of PTGS occurred. Spatial control of gene silencing is desirable in some situations, for instance to study the function of widely expressed genes on an organ or tissue basis without affecting the entire plant. On the other hand, the system allows for the investigation of physiological perturbations on the whole plant level which were caused by local silencing of a particular gene.

4.4.3 Advantages of the ethanol inducible RNAi system

In summary, the data presented here demonstrate the utility of the *alc* gene system to achieve transient gene silencing by inducible expression of dsRNA constructs. The *alc* system offers an enormous flexibility with respect to time point of induction, expression level, spatial control, duration of expression and is applicable to a variety of plant species. In contrast to other chemically regulated systems, ethanol, or alternatively acetaldehyde, are comparatively benign inducers and exert only minimal physiological side effects in concentrations necessary for induction (Junker *et al*., 2003). These attributes greatly enhance the reproducibility of silencing experiments, which is of particular importance in metabolic studies requiring a large population of uniformly silenced individuals. The system provides a powerful tool to investigate molecular and physiological alterations associated with repression of a target gene at temporal and spatial resolution. Thus, dissection of primary and secondary effects of gene silencing should be greatly facilitated allowing more precise predictions of gene function.

Recently, an alternative system to achieve chemical-regulated inducible gene silencing has been described which takes advantage of 17-estradiol-inducible recombination to trigger the expression of an intron-containing inverted repeat RNA (CLX system; Guo *et al*., 2003). In this case induction of the system leads to permanent activation of PTGS mimicking expression from a constitutive promoter. However, due to the nature of the inducer the CLX

system might not be readily applicable to soil-grown plants and thus its use in physiological studies is limited.

4.5 *Ethanol-inducible silencing of SPP in transgenic tobacco plants*

In order to dissect the possible primary and secondary effects caused by reduction of SPP activity, SPP was down-regulated in the transgenic tobacco plants using ethanol-inducible RNAi system. Despite the low abundance of SPP transcript in the cells, it could be effectively knocked down using ethanol-inducible RNAi system. SPP activities in *alc*-SPPi line 79 and 81 were markedly decreased 3 dpi, which was concomitant with the strong phenotypic alterations and a slight increase in Suc6P levels in the young leaves. However, the levels of sucrose in these leaves were largely unaltered in the transgenics as compared to the control at 3 dpi. Therefore, either decreased SPP expression, small changes in Suc6P content or the perturbation of the flux through the pathway for sucrose synthesis might be responsible for the phenotypic alterations observed in *alc*-SPPi tobacco plants. SPP activities were further decreased in the alc-SPPi transgenic line 79 and 81 at 6 dpi, which were comparable with that obtained in moderately affected transgenic tobacco plants with constitutive reduction of SPP expression. Accordingly, Suc6P levels were further increased by 2 folds and more than 3000 folds in *alc*-SPPi line 79 and 81, respectively.

Taken together, ethanol inducible RNAi system can be applied to down-regulate SPP in transgenic tobacco plants, which might be of use in dissecting the possible primary and secondary effects caused by constitutive reduction of SPP activity. However, ethanol inducible RNAi system functions preferentially in young leaves rather than in mature leaves which might render the situation complicated in case of SPP because the pathway of sucrose synthesis is under developmental control and mainly active in mature leaves as revealed in the constitutive SPP silenced tobacco plants. Since down-regulation of target genes would persist in the initial silenced tissues using ethanol inducible system, it is still possible to study the consequences of inducible knocking-down of SPP in mature leaves by following a longer time course. However, due to the time constraint, it is not possible to perform this experiment in this project. This work should be carried out in the future.

5 Summary

Sucrose is the main transport carbohydrate in most of higher plants, forming the interface between photosynthetically active source tissues and heterotrophic sink tissues. The formation of sucrose from UDP-glucose and fructose-6-phosphate occurs via a two-step reaction catalyzed by sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP), with sucrose-6-phosphate (Suc6P) as the intermediate. SPS has been identified as a major control point of the whole pathway. The role and significance of SPP in regulating sucrose biosynthesis and carbon partitioning in plants has not been studied. However, recent evidences that SPS and SPP might form a complex *in vivo* opened the possibility that SPP could have a role in metabolite channeling between the two enzymes and could contribute to control of flux through the pathway. The pathway of sucrose synthesis in plants is comparable to that of trehalose synthesis in yeast and plants with respect to the similarities of the structure and organization of the enzymes and the structural similarities of the intermediates and products. Remarkably, trehalose-6-phosphate, the intermediate of trehalose synthesis pathway, has been shown to possess important signaling functions in yeast and plants. A similar function in signaling has also been proposed for Suc6P, although until now no experimental evidence exists for such a proposal. The aims of this work were, on one hand, to examine the *in planta* role of SPP in regulation of sucrose synthesis and carbon partitioning in photosynthetically active source tissues and heterotrophic sink tissues; on the other hand, to investigate the potential impact of Suc6P on plant metabolisms. To this end, the genes encoding SPP were cloned from tobacco and potato and down-regulation of SPP expression was achieved via an RNA interference approach in transgenic tobacco and potato in a constitutive, or tissue-specific, or inducible way. Molecular, biochemical and physiological analyses were subsequently carried out.

The key findings of this work can be summarized as follows:

1) In tobacco leaves, SPP is abundant and does not exert significant control over sucrose synthesis under normal conditions. The fact that ca. 80% of SPP activity can be “lost” before any effect on sucrose synthesis can be observed suggests that the enzyme is unlikely to be tightly coupled to the SPS complex *in vivo* – certainly there does not seem to be a requirement for a 1:1 stoichiometry between SPS and SPP as has been previously suggested. Transformants with less than 10% of wild-type SPP activity displayed a range of phenotypes including inhibition of photosynthesis, chlorosis and reduced growth rates. The loss of SPP activity in transgenic tobacco leaves resulted in a shift in carbon partitioning in favor of starch. The acceleration of starch synthesis

was concomitant with the increased levels of T6P and the accumulation of Suc6P in SPPi tobacco plants while 3-PGA levels were slighly and soluble sugars were markedly decreased. It was proposed that rising Suc6P might be the trigger leading to the increase of T6P levels which resulted in the redox activation of AGPase and the enhancement of starch synthesis in SPPi plants. The transgenic leaves were unable to mobilize transient starch during extended periods of darkness and as consequence showed a starch- and maltose-excess phenotype. This indicated that no alternative mechanism for carbon export was activated in SPPi transgenic tobacco plants. In SPPi tobacco plants SPS expression was induced at transcriptional level, and its activity in terms of both V_{max} and V_{sel} and activation state indicated by V_{sel}/V_{max} were distinctly increased, while the expression of cFBPase was unchanged and its activity was slightly decreased. These data indicated a particular signaling pathway was activated in SPPi tobacco plants to specifically stimulate SPS but not cFBPase. It was postulated that low sugar content might be the signal feedforward stimulating SPS.

2) In potato plants, SPP protein is constitutively present in growing and stored tubers. Down-regulation of SPP using different promoters resulted in distinct metabolic phenotype and eventually distinct fate of the tubers. Under normal growth conditions, sucrose (re)synthesis through the actions of SPS and SPP does not play an important role in the control of the net rate of starch accumulation and tuber yield as suggested in early studies using SPS as a target. However, this pathway becomes important after harvest either because it is required to cope with water loss due to the environmental changes after harvest or because it is indispensable to adjust the tubers to the metabolic changes after harvest. Down-regulation of SPP in a tuber-specific way resulted in a massive accumulation of Suc6P after harvest, a stimulation of respiration and eventually necrotic cell death in the tubers. The reason for this drastic metabolic changes and whether Suc6P might act as a signal to trigger this process is currently unknown. Studies with 35SPPi tubers showed that sucrose synthesis through SPS and SPP was stimulated by the low temperature (4-6°C), and the loss of SPP resulted in a tremendous build-up of Suc6P in the cold-stored tubers. The massive accumulation of Suc6P might in turn compensate the effects of decreased SPP expression on sucrose synthesis in transgenic tubers during long-term cold storage, leaving the long-term sucrose production largely unchanged in the cold-stored 35SPPi tubers. However the repression of SPP triggered profound changes that strongly decreased the cold-induced hexose accumulation in the transgenic tubers. One of the changes was that the

induction of vacuolar invertase in the cold was inhibited in 35SPPi tubers. Interestingly, the levels of Suc6P in the cold-stored tubers were negatively correlated with the cold induction of vacuolar invertase, providing first evidence of a potential signaling function for S6P in the regulation of gene expression in plants.

3) Ethanol inducible RNAi system but not inducible antisense system can trigger strong gene silencing in an inducer dependent manner. The data also demonstrate that ethanol inducible RNAi system offers enormous flexibility with respect to time point, duration and spatial control of gene silencing. In contrast to the inducers in other chemically regulated systems, ethanol is a comparatively benign inducer and exerts only minimal physiological side effects in concentration necessary for induction. These attributes render ethanol inducible RNAi system extremely useful in metabolic studies. Its utility in studying carbon metabolisms was preliminarily explored in *alc*-SPPi transgenic tobacco plants.

6 Zusammenfassung

In den meisten höheren Pflanzen dient Saccharose als Haupttransportform von Photoassimilaten und stellt damit das Verbindungsglied zwischen photosynthetisch aktiven source-Geweben und heterotrophen sink-Geweben dar. Über die Rolle im Energiestoffwechsel hinaus, ist Saccharose an der Regulation der Genexpression in verschiedensten Stoffwechselwegen beteiligt. Die Bildung von Saccharose aus UDP-Glukose und Fruktose-6-phosphat wird durch zwei Enzyme Saccharose-6-phosphat Synthase (SPS) und Saccharose-6-phosphat Phosphatase (SPP) katalysiert, wobei Saccharose-6-phosphat (Suc6P) als Zwischenprodukt entsteht. SPS wurde als ratenlimitierendes Enzym für den gesamten Stoffwechselweg beschrieben, während die Rolle der SPP für die Regulation der Saccharose-Biosynthese bisher nicht untersucht wurde. Aufgrund der Annahme, dass die SPS mit der SPP ein Enzymkomplex bildet, wurde über eine regulatorische Funktion der SPP spekuliert. Die Synthese von Saccharose weist, was die Struktur und Organisation der beteiligten Enzyme und die Ähnlichkeiten von Intermediaten und Produkten betrifft, erstaunliche Parallelen zur Synthese von Trehalose auf. Bemerkenswerterweise wurde für das Zwischenprodukt der Trehalose-Synthese, Trehalose-6-phosphat (T6P), eine Funktion als Signalmetabolit bei der Regulation des Kohlenhydratstoffwechsels nachgewiesen. Über eine ähnliche Rolle von Suc6P wurde verschiedentlich in der Literatur spekuliert. In der vorliegenden Arbeit sollte die Rolle der SPP in der Regulation von Synthese und Verteilung von Saccharose in source- und sink-Geweben von Pflanzen untersucht werden. Darüber hinaus sollte untersucht werden, ob Suc6P möglicherweise eine Funktion als Signalmetabolit zukommt. Zu diesem Zweck wurden zunächst SPP kodierende cDNAs aus Tabak und Kartoffel isoliert. Anschließend wurde die Expression der SPP in transgenen Tabak- und Kartoffelpflanzen durch RNA-Interferenz (RNAi) inhibiert, wobei neben konstitutiver RNAi auch gewebespezifische bzw. chemisch induzierbare Repression der SPP Aktivität erreicht wurde. Die erhaltenen transgenen Pflanzen wurden einer detaillierten molekularen und biochemischen Analyse unterzogen.

Die Ergebnisse der Arbeiten lassen sich wie folgt zusammenfassen:

1) In source-Blättern von Tabakpflanzen ist die SPP Aktivität nicht limitierend für die Saccharose-Synthese und erst eine Reduktion um ca. 80% wirkt sich auf Wachstum und Entwicklung der Pflanze aus. Offensichtlich ist ein stöchiometrisches Verhältnis von 1:1 zwischen SPP und SPS, wie vorher in der Literatur beschrieben, nicht für

einen effizienten Ablauf der Saccharose-Synthese notwendig. Transgene Pflanzen mit weniger als 10% SPP-Restaktivität zeigten phänotypische Veränderungen einhergehend mit einer Verringerung der Photosyntheserate, der Ausbildung von Blattchlorosen und einem verringerten Wachstum. Die Verteilung von Photoassimilaten in diesen Pflanzen war stark zu Gunsten von Stärke verschoben. Neben einem ca. 1000-fach erhöhten Suc6P Gehalt war in den transgenen Tabakpflanzen auch der Gehalt an T6P signifikant erhöht, was möglicherweise zur redox-Aktivierung der AGPase führt und dadurch zur Erhöhung der Stärkesyntheserate ohne Veränderungen im 3-PGA Gehalt in diesen Pflanzen beiträgt. Hier ergeben sich erstmals Hinweise auf eine Verbindung des Suc6P Gehaltes mit dem Signalmetabolit T6P und damit ist auch eine Signalfunktion von Suc6P möglich. Darüber hinaus waren die transgenen Pflanzen im Gegensatz zum Wildtyp nicht in der Lage transitorische Stärke während der Dunkelphase vollständig zu mobilisieren was zu einem Stärke-Überschuss-Phänotyp führte. Die Aktivität der SPS in den transgenen Pflanzen war sowohl auf V_{max} als auch auf V_{sel} Ebene erhöht, was auf einen höheren Aktivierungszustand des Enzyms hindeutet. Interessanterweise war die Aktivität des zweiten ratenlimitierenden Enzyms der Saccharose-Synthese, die cytosolische Fruktose-1,6-bisphosphat Phosphatase, nicht erhöht, so dass beide Enzyme offensichtlich unabhängig voneinander durch verschiedene Signaltransduktionswege reguliert werden können.

2) Die SPP wird sowohl in wachsenden als auch in gelagerten Kartoffelknollen konstitutiv exprimiert. Die Verwendung verschiedener Promotoren bei der Repression der SPP durch RNAi führt zu unterschiedlichen phänotypischen Veränderungen der transgenen Kartoffelknollen. Wie die Knollen-spezifische Repression der SPP durch Verwendung des B33 Promotors gezeigt hat, spielt die Saccharose-(Re)synthese in Kartoffelknollen via SPS und SPP unter normalen Wachstumsbedingungen keine wichtige Rolle für die Regulation des Kohlenstoffflusses in Stärke, wie ursprünglich in der Literatur vorgeschlagen. Erst nach der Ernte wird er Fluss durch diesen Stoffwechselweg erhöht, wahrscheinlich um die Anpassung an veränderte Umweltbedingungen zu ermöglichen, was insbesondere auf die Osmoregulation der Knolle zutrifft. Die transgenen Knollen akkumulieren nach der Ernte große Mengen an Suc6P, haben eine erhöhte Atmungsrate und zeigen Anzeichen von induziertem Zelltod. Die Ursache für diese drastischen phänotypischen Veränderungen und ob Suc6P hierbei möglicherweise eine Rolle als Signalmetabolit spielt, konnte nicht

abschließend geklärt werden. Transgene Kartoffelknollen in denen die Repression der SPP durch den CaMV 35S Promotor vermittelt wurde, waren nach der Ernte physiologisch nicht von entsprechenden Wildtyp-Knollen zu unterscheiden. Erst nach Lagerung bei 4 – 6 °C zeigte sich eine durch die Stimulation der Saccharose-Synthese verursachte Akkumulation von Suc6P. Interessanterweise, wirkte sich die Reduktion der SPP langfristig nicht auf die Saccharose-Synthese Kapazität der transgenen Knollen in der Kälte aus, was sich in einem unveränderten Saccharosegehalt in diesen Knollen gegenüber der Kontrolle äußerte. Dennoch war in den transgenen Kartoffeln die kälteinduzierte Akkumulation von Hexosen (cold-sweetening) weitestgehend unterdrückt. Der Gehalt an Suc6P in diesen Knollen korrelierte negativ mit der Expression der vacuolären Invertase, während andere Metabolite unverändert waren. Dies deutet erstmals auf eine Rolle von Suc6P als Signalmetabolit zur Regulation der Genexpression in Pflanzen hin.

3) Um primäre und sekundäre Effekte bei der Unterdrückung der Genexpression durch RNAi zu unterscheiden, wurde eine Ethanol-induzierbares RNAi System etabliert. Tabakpflanzen, die mit einem RNAi-Konstrukt unter der Kontrolle des Ethanol-induzierbaren Promotors transformiert waren, zeigten erst nach der Applikation von Ethanol phänotypische Veränderungen im Vergleich zur Kontrolle. Die Dauer der RNAi-vermittelten Repression konnte durch Wahl der Induktionsbedingungen verändert werden. Die vielseitigen Einsatzmöglichkeiten des Ethanol-induzierbaren RNAi Systems, besonders unter Gewächshausbedingungen, machen es zu einem wertvollen Werkzeug zur Untersuchung physiologischer Zusammenhänge in Pflanzen. Seinen Nutzen in der Analyse des Kohlenhydratstoffwechsels wurde durch die einstweilige Untersuchung von Ethanol-induzierbaren SPP-RNAi Pflanzen demonstriert.

7 Abbreviations

3-PGA	3-Phosphoglycerate
ADP	Adenosine 5’-diphosphate
AGPase	ADP-Glucose pyrophosphorylase
AMP	Adenosine 5’-monophosphate
AOX	Alternative oxidase
ATP	Adenosine 5’-triphosphate
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
cFBPase	Cytosolic fructose-1,6-bisphosphatase
chl	Chlorophyll
cw-Inv	cell wall invertase
cyt-Inv	cytosolic invertase
COX	Cytochrome *c* oxidase
dph	days post harvest
dpi	days post induction
dsRNA	double-stranded RNA
EST	Expressed Sequence Tag
Fruc-1,6-bisP	Fructose-1,6-bisphosphate
Fruc-2,6-bisP	Fructose-2,6-bisphosphate
Fruc6P	Fructose-6-phosphate
GFP	Green Fluorescence Protein
Gluc1P	Glucose-1-phosphate
Gluc-1,6-bisP	Glucose-1,6-bisphosphate
Gluc6P	Glucose-6-phosphate
HAD	haloacid dehalogenase
IC-MS	Ion-exchange chromatography coupled to mass-spectrometry
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEP	phosphoenolpyruvate
Pi	inorganic phosphate
PPi	inorganic pyrophosphate

RNAi	RNA interference
siRNA	short interfering RNA
SNF1	sucrose nonfermenting 1
SPP	Sucrose-6-phosphate phosphatase
SPS	Sucrose-6-phosphate synthase
ssRNA	single-stranded RNA
Suc6P	Sucrose-6-phosphate
Susy	sucrose synthase
SUT	Sucrose transporter
T6P	Trehalose-6-phosphate
TPP	trehalose-6-phosphate phosphatase
TPS	trehalose-6-phosphate synthase
TPT	Triose-phosphate translocator
UDP-Gluc	Uridine 5’-diphosphoglucose
VI	vacuolar invertase

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9 Appendix

I. Sequences

I.1 DNA sequence and derived amino acid sequence of sucrose-phosphate phosphatase 1 from *N. tabacum* (NtSPP1; Acc. No.: AY729655)

```
atggatcagctaaccagtgccgcacgtctcatgatagtctcagatcttgaccataccatg 60
 M  D  Q  L  T  S  A  A  R  L  M  I  V  S  D  L  D  H  T  M
gttgatcatcatgatcctgagaacctttctctgcttaggtttaatgctttatgggaggcc 120
 V  D  H  H  D  P  E  N  L  S  L  L  R  F  N  A  L  W  E  A
aattatcgtgaaaactccttgttagtgttctcaactgggagatcacctacccttttacaag 180
 N  Y  R  E  N  S  L  L  V  F  S  T  G  R  S  P  T  L  Y  K
gagttgagaaaagagaagcccatgctaaccccagatattaccattatgtctgtggggact 240
 E  L  R  K  E  K  P  M  L  T  P  D  I  T  I  M  S  V  G  T
gaaataacttatggtaactctatggagccagatgatggttgggaagcatttttaaatgat 300
 E  I  T  Y  G  N  S  M  E  P  D  D  G  W  E  A  F  L  N  D
aagtgggatcggaaaatagtgacagaggagacaagcaaatttcctgaactcacccttcag 360
 K  W  D  R  K  I  V  T  E  E  T  S  K  F  P  E  L  T  L  Q
tcagaaacagagcagcgaccacacaaggtcagtttctatgttcagaaagacaaggctcaa 420
 S  E  T  E  Q  R  P  H  K  V  S  F  Y  V  Q  K  D  K  A  Q
gatataacgggaactctttccaagcgcttggaagaacgtgggttggatgtcaaaataatt 480
 D  I  T  G  T  L  S  K  R  L  E  E  R  G  L  D  V  K  I  I
tatagcggagggatggatttggacattttgccacaaggtgctggcaaaggacgagcactt 540
 Y  S  G  G  M  D  L  D  I  L  P  Q  G  A  G  K  G  R  A  L
gcatatttgcttaagaaattaaagagtgagggcaagttaccaaacaacacgcttgcctgt 600
 A  Y  L  L  K  K  L  K  S  E  G  K  L  P  N  N  T  L  A  C
ggtgactctggaaatgatgctgagcttttcagtatcccagatgtttatggtgtgatggta 660
 G  D  S  G  N  D  A  E  L  F  S  I  P  D  V  Y  G  V  M  V
gcgaatgcacaggaggagttattacaatggcgtgctgcaaatgcaaaagatagtccaaaa 720
 A  N  A  Q  E  E  L  L  Q  W  R  A  A  N  A  K  D  S  P  K
gtaattcatgcaacagagagatgtgccgcgggtataatacaagcaattgggcatttcaac 780
 V  I  H  A  T  E  R  C  A  A  G  I  I  Q  A  I  G  H  F  N
ctgggaccaaatacctctcctagagatgttacagatatgtcagactgcaagatggagaat 840
 L  G  P  N  T  S  P  R  D  V  T  D  M  S  D  C  K  M  E  N
tttgttcctgcttatgaagtcgtcaaattttacttgtttttcgagaaatggaggcgtgga 900
 F  V  P  A  Y  E  V  V  K  F  Y  L  F  F  E  K  W  R  R  G
gaaattgagaattctgaccttcacttgtcaaacctgaaagcagtttgtagaccatccggt 960
 E  I  E  N  S  D  L  H  L  S  N  L  K  A  V  C  R  P  S  G
acttttgtgcacccatctggagttgagaaatatcttgaggactgcataaatacattgaga 1020
 T  F  V  H  P  S  G  V  E  K  Y  L  E  D  C  I  N  T  L  R
acttgtcacggtgacaaacagggtaaacaatttcgtatttgggttgatctagtgttacct 1080
 T  C  H  G  D  K  Q  G  K  Q  F  R  I  W  V  D  L  V  L  P
acacaggttggttcagattcatggttagtgagtttcaagaaatgggagctttgtggcgaa 1140
 T  Q  V  G  S  D  S  W  L  V  S  F  K  K  W  E  L  C  G  E
gagcgacaatgttgcataactactgtcttgttaagttcaaagaatgtgacggtcgcggat 1200
 E  R  Q  C  C  I  T  T  V  L  L  S  S  K  N  V  T  V  A  D
gggctcacttggacacatgtgcatcagacttggctgcagggagcagcagcaagtgactcc 1260
 G  L  T  W  T  H  V  H  Q  T  W  L  Q  G  A  A  A  S  D  S
gcgtcctggttcttttaa 1278
 A  S  W  F  F  -
```

I.2 DNA sequence and derived amino acid sequence of sucrose-phosphate phosphatase 2 from *N. tabacum* (NtSPP2; Acc. No.: AY729656)

```
atggatcagctaaccagtgccgcacgtctcatgatagtctcagatctagaccatacaatg 60
 M  D  Q  L  T  S  A  A  R  L  M  I  V  S  D  L  D  H  T  M
```

```
gtagatcatcatgatgccgagaacctttctctgcttagatttaatgctttatgggaggcg 120
 V  D  H  H  D  A  E  N  L  S  L  L  R  F  N  A  L  W  E  A
aattatcgtgataactctttgttagtgttctcaactgggagatcacctacactttacaag 180
 N  Y  R  D  N  S  L  L  V  F  S  T  G  R  S  P  T  L  Y  K
gagttgaggaaagaaaagcccatgctaaccccagatattactattatgtcggtgggaact 240
 E  L  R  K  E  K  P  M  L  T  P  D  I  T  I  M  S  V  G  T
gaaataacatatggtaactctgtggtgcctgatgatggttgggaagcttttctaaataac 300
 E  I  T  Y  G  N  S  V  V  P  D  D  G  W  E  A  F  L  N  N
aagtgggacagaaagatagtaacagaggagactagcaagtttcctgaactcactctacag 360
 K  W  D  R  K  I  V  T  E  E  T  S  K  F  P  E  L  T  L  Q
tcagaaacggagcagcgaccacacaaggtcagtttctatgttcagaaagacaaagcacaa 420
 S  E  T  E  Q  R  P  H  K  V  S  F  Y  V  Q  K  D  K  A  Q
gatataatgaaaactctttccaagcgcttcgaagaacgtgggctggatgtcaaaataatt 480
 D  I  M  K  T  L  S  K  R  F  E  E  R  G  L  D  V  K  I  I
tacagtggaggcatggatctagatatattaccacaaggtgctggcaaaggacaagcactt 540
 Y  S  G  G  M  D  L  D  I  L  P  Q  G  A  G  K  G  Q  A  L
gcatatttgcttaagaaattgaagagtgagggaaaattaccaaacaacacccttgcctgt 600
 A  Y  L  L  K  K  L  K  S  E  G  K  L  P  N  N  T  L  A  C
ggtgactctgggaatgatgctgagctattcagtatcccagatgtgtatggtgtaatggta 660
 G  D  S  G  N  D  A  E  L  F  S  I  P  D  V  Y  G  V  M  V
gctaatgcacaggaggaattattgcaatggcatgctgcaaatgcgaagaataatcctaaa 720
 A  N  A  Q  E  E  L  L  Q  W  H  A  A  N  A  K  N  N  P  K
gtaattcatgcaacagagaggtgtgctgccggtatcatacaagctattggtcattccaac 780
 V  I  H  A  T  E  R  C  A  A  G  I  I  Q  A  I  G  H  S  N
ctaggtccaagtacctcccctagagatgttatggatttgtcagactgcaagatggagaac 840
 L  G  P  S  T  S  P  R  D  V  M  D  L  S  D  C  K  M  E  N
tttgttcccgcctatgaagttgtcaaattttacctatttttttgagaaatggaggcgtgga 900
 F  V  P  A  Y  E  V  V  K  F  Y  L  F  F  E  K  W  R  R  G
gaaattgagcattctgagcattacctgtcaaaccttaaagcagtgtgtagaccatctggt 960
 E  I  E  H  S  E  H  Y  L  S  N  L  K  A  V  C  R  P  S  G
acttttgtccacccatctggtgttgagaaatccctccaggaatgtgtaactttattcggg 1020
 T  F  V  H  P  S  G  V  E  K  S  L  Q  E  C  V  T  L  F  G
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 T  C  H  G  D  K  Q  G  K  Q  F  R  I  W  V  D  Q  V  L  P
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 V  Q  V  G  S  D  S  W  L  V  S  F  K  K  W  E  L  S  G  E
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 D  R  R  C  C  I  T  T  V  L  L  S  S  K  N  K  T  V  A  D
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 G  L  T  W  T  H  V  H  Q  T  W  L  N  G  A  A  A  S  D  S
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 A  S  W  F  F  -
```

I.3 DNA sequence and derived amino acid sequence of sucrose-phosphate phosphatase 1 from *S. tuberosum* (StSPP1)

```
ctttacaaaagaagcccaaaggaaattgaatttttttctcaaagaagtacaaaaattaa 60
ctctctttttctaaatatatgccttctcttaagctaagaatcaatttttcaggtttccct 120
cattgctgttactgctgctccactcccaaaatcccttctttcactgcatttcagagtgaa 180
gctaatattaaatcaaccatggatcagctaaccagtgccgcacgtctcatgatagtctca 240
                  M  D  Q  L  T  S  A  A  R  L  M  I  V  S
gatcttgactatacaatggtcgatcatcatgatcccgagaacctctctctgcttaggttc 300
 D  L  D  Y  T  M  V  D  H  H  D  P  E  N  L  S  L  L  R  F
aatgctttatgggaagccaattatcgcgaaaactccttgttagtgttctcaactgggaga 360
 N  A  L  W  E  A  N  Y  R  E  N  S  L  L  V  F  S  T  G  R
tcaccaactctttacaaggagttgagaaaagagaagcccatgctaaccccagatattacc 420
 S  P  T  L  Y  K  E  L  R  K  E  K  P  M  L  T  P  D  I  T
attatgtctgtggggactgaaataacatatgggaactctatgttgccagatgatggttgg 480
 I  M  S  V  G  T  E  I  T  Y  G  N  S  M  L  P  D  D  G  W
gaagcatttttaaatgacaaatgggatcggaaaatagtgatggaggagacgaaaaaattt 540
 E  A  F  L  N  D  K  W  D  R  K  I  V  M  E  E  T  K  K  F
```

```
cctgaactaacacttcagtcagaaacggagcagcgacctcacaaggtcagtttctatgtt 600
 P  E  L  T  L  Q  S  E  T  E  Q  R  P  H  K  V  S  F  Y  V
cagaaagacaaggctcaagatataatgaaaaatctttccaaacgcttggaagaacgtggg 660
 Q  K  D  K  A  Q  D  I  M  K  N  L  S  K  R  L  E  E  R  G
ttggatgtcaaaataatttacagtggagggatggctttagatattttgcctcaaggtgct 720
 L  D  V  K  I  I  Y  S  G  G  M  A  L  D  I  L  P  Q  G  A
ggcaaaggacaagcacttgcgtatttgctgaagaaattaaaaagtgagggtaaattacca 780
 G  K  G  Q  A  L  A  Y  L  L  K  K  L  K  S  E  G  K  L  P
aacaacacgcttgcctgtggtgactctggaaatgatgctgagcttttcagcatcccggat 840
 N  N  T  L  A  C  G  D  S  G  N  D  A  E  L  F  S  I  P  D
gtttatggtgtgatggtagcgaatgcacaggaggaattattacaatggcatgctgcaaat 900
 V  Y  G  V  M  V  A  N  A  Q  E  E  L  L  Q  W  H  A  A  N
gcgaaagataatcctaaagtaattcatgcaacagagagatgtgccgcaggtataatacaa 960
 A  K  D  N  P  K  V  I  H  A  T  E  R  C  A  A  G  I  I  Q
gccattgggcgtttcaacctgggaccaagtacctctcccagagacgttaccgacatgtca 1020
 A  I  G  R  F  N  L  G  P  S  T  S  P  R  D  V  T  D  M  S
gactgcaagatggagaacgttgttcctgcttatgaaatcgtcaaattttacctgtttttc 1080
 D  C  K  M  E  N  V  V  P  A  Y  E  I  V  K  F  Y  L  F  F
gagaaatggaggcgtggagagattgagaattctgacctttacttgtcgaacctgaaagca 1140
 E  K  W  R  R  G  E  I  E  N  S  D  L  Y  L  S  N  L  K  A
gtttgtagaccatctggtacttttgtgcacccatctggagttgagaaatct 1191
 V  C  R  P  S  G  T  F  V  H  P  S  G  V  E  K  S
```

I.4 DNA sequence and derived amino acid sequence of sucrose-phosphate phosphatase 2 from *S. tuberosum* (StSPP2)

```
ccaattcccatttcaggttgaagccaatttacatcaatcatggatcggctaaccagtgct 60
                                          M  D  R  L  T  S  A
gcacgtctcatgatagtctcagatcttgaccatacaatggtagatcatcacgattccgag 120
 A  R  L  M  I  V  S  D  L  D  H  T  M  V  D  H  H  D  S  E
aacctttctctgcttaggttcaatgctttatgggaagccaattatcgtgataactctttg 180
 N  L  S  L  L  R  F  N  A  L  W  E  A  N  Y  R  D  N  S  L
ttagtgttctctactgggagatcacctacactttacaaggaattaaggaaagaaaagccc 240
 L  V  F  S  T  G  R  S  P  T  L  Y  K  E  L  R  K  E  K  P
atgctaaccccagatattacaattatgtctgtgggaactgaaataacatatggtaacgct 300
 M  L  T  P  D  I  T  I  M  S  V  G  T  E  I  T  Y  G  N  A
atggtgcctgatgatggttgggaaacatttctgaataacaagtgggatagaaagatagta 360
 M  V  P  D  D  G  W  E  T  F  L  N  N  K  W  D  R  K  I  V
acagaggagacaagcaagtttcctgaactcagtctgcagtcagaaacagagcagcgacca 420
 T  E  E  T  S  K  F  P  E  L  S  L  Q  S  E  T  E  Q  R  P
cacaaggtcagtttctatgttcagaaagagaaagctcaagatataatgaaaactctttcc 480
 H  K  V  S  F  Y  V  Q  K  E  K  A  Q  D  I  M  K  T  L  S
aagcgcttggaagaacgtgggctggatgtcaaaataatttacagtggagggatggatcta 540
 K  R  L  E  E  R  G  L  D  V  K  I  I  Y  S  G  G  M  D  L
gatatattaccacagggtgctggcaaaggacaagcacttgcatatctgcttaagaaactg 600
 D  I  L  P  Q  G  A  G  K  G  Q  A  L  A  Y  L  L  K  K  L
aagagcgagggaaaattaccaagcaacacccttgcctgcggcgactccgggaatgacgct 660
 K  S  E  G  K  L  P  S  N  T  L  A  C  G  D  S  G  N  D  A
gaattattcagtatcccagatgtgtatggtgtaatggtagctaatgcgcagaaggaatta 720
 E  L  F  S  I  P  D  V  Y  G  V  M  V  A  N  A  Q  K  E  L
ctgcagtggcatgctgcaaatgcaaaaaataatcccaaagtaattcatgcatcagagagg 780
 L  Q  W  H  A  A  N  A  K  N  N  P  K  V  I  H  A  S  E  R
tgtgccgccggtatcatacaagccattggtcatttcaaactaggtccaagtacctcccca 840
 C  A  A  G  I  I  Q  A  I  G  H  F  K  L  G  P  S  T  S  P
agagacgttacggatttgtcagattgcaagatggacaactttgttcctgcctatgaagtt 900
 R  D  V  T  D  L  S  D  C  K  M  D  N  F  V  P  A  Y  E  V
gtcaaattttacctgtttttgagaaatggaggcgtggagaaattgagcattctgagcat 960
 V  K  F  Y  L  F  F  E  K  W  R  R  G  E  I  E  H  S  E  H
tatctgtcaaacctgaaagcagtgtgtataccatctggtacttttgttcacccatctggt 1020
 Y  L  S  N  L  K  A  V  C  I  P  S  G  T  F  V  H  P  S  G
```

```
gttgagaaatcccttcaggaatgtgtaacttcattcggaacatgtcatgctgacaagcag 1080
 V  E  K  S  L  Q  E  C  V  T  S  F  G  T  C  H  A  D  K  Q
gggaaacaatatcgtgtttgggtcgatcaagttttaccttcacaggttggttcagactca 1140
 G  K  Q  Y  R  V  W  V  D  Q  V  L  P  S  Q  V  G  S  D  S
tggttagtgagtttcaagaagtgggagctctctggtgaagacatgcgatgctgcataacc 1200
 W  L  V  S  F  K  K  W  E  L  S  G  E  D  M  R  C  C  I  T
acagtcctattaagttcaaagaataagactgttgcagacgggctcacttggactcacgta 1260
 T  V  L  L  S  S  K  N  K  T  V  A  D  G  L  T  W  T  H  V
catcagacatggctgcacggtgatgcagcaagtgactccgcaacctggttcttttagatt 1320
 H  Q  T  W  L  H  G  D  A  A  S  D  S  A  T  W  F  F  -
gtcatctcagtgtattaactctgaaaattccgcacccctttaccagttcacacccagaa 1380
taaacacaac 1390
```

II. Oligonucleotides

name	sequence	application
FB223	5’-ATGGATCAGCTAACCAGTCGCCGCAC-3’	5’ primer NtSPP for cloning
FB225	5’-CTAAAAGAACCAGGACGCGGAGTCACT-3’	3’ primer NtSPP for cloning
CS51	5’-AAGGATCCAATATGGATCAGCTAACCAGTGCCGCAC-3’	5’ primer NtSPP2 for pMal-c2
CS52	5’-AAGTCGACCTAAAAGAACCAGGACGCGGAGTCACT-3’	3’ primer NtSPP2 for pMal-c2
FB228	5’-GGATCCATGGATCAGCTAACCAGTGCC-3’	5’ primer NtSPP2 for pUC-RNAi
FB229	5’-GTCGACTACCATTACACCATAACACATC-3’	3’ primer NtSPP2 for pUC-RNAi
CS9	5’-GGATCCAGGAGTTGATGCCAGGAGGTGTAAA-3’	5’ primer *gsa* for pUC-RNAi
CS10	5’-GTCGACGCCTTCCTCCGTATGCACCTACTG-3’	3’ primer *gsa* for pUC-RNAi
CS13	5’-GGATCCCGTTCAATTCAGATCCAGATGACC-3’	5’ primer *chl*I for pUC-RNAi
CS14	5’-GTCGACCCTCTCAATCCATCGACATTTAGTTC-3’	3’ primer *chl*I for pUC-RNAi
CS15	5’-AGGGATCCAGAGCCCAAGTGACCACTGAGGC-3’	5’ primer *fnr* for pUC-RNAi
CS16	5’-TGGTCGACTGCTCTCTGCTCACAGCAAAGTCC-3’	3’ primer *fnr* for pUC-RNAi
CS40	5’- ATGGCAGACGGTGAGGATATTCA-3’	5’ primer actin for RT-PCR
CS41	5’-GCCTTTGCAATCCACATCTGTTG-3’	5’ primer actin for RT-PCR
CS86	5’-GATCCTCTGGATGGATCTTCCAACA-3’	5’ primer cFBPase for RT-PCR
CS87	5’- TGTTCGATGAACATCAGCTACCAT-3’	3’ primer cFBPase for RT-PCR

Publication lists

Publications

2003 – Chen, S., Hofius, D., Sonnewald, U. and Börnke, F. Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. *Plant J*. 36, 731-740.

2005 – Chen, S., Hajirezaei, M., Peisker, M., Tschiersch, H., Sonnewald, U. and Börnke, F. Decreased sucrose-6-phosphate phosphatase level in transgenic tobacco inhibits photosynthesis, alters carbohydrate partitioning, and reduces growth. *Planta* DOI: 10.1007/s00425-004-1458-4

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2002 – Chen, S., Hofius, D., Sonnewald, U. and Börnke, F. Ethanol-inducible expression of inverted-repeat constructs efficiently triggers conditional gene silencing in plants. **–** Institutstag IPK, Gatersleben, 17. 10. 2002

2003 – Chen, S., Hofius, D., Sonnewald, U. and Börnke, F. Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. **–** Institutstag IPK, Gatersleben, 09. 10. 2003

2003 – Chen, S., Hofius, D., Sonnewald, U. and Börnke, F. Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. **–** Annual meeting of the German Genetics Society, Kassel, 26 **–** 29. 09. 2003

Patent

2004 – Ehrhardt,T., Sonnewald,U., Boernke,F. and Chen,S. Saccharose-6-phosphate phosphatase as a target for herbicides. Patent: WO 2004009808-A 19 29-JAN-2004

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Curriculum vitae

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Gatersleben, March 2005

Erklärung

Hiermit erkläre ich, dass ich die hier vorliegende wissenschaftliche Arbeit selbständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Darüber hinaus erkläre ich, dass ich den Doktorgrad nicht besitze und mich auch früher nicht um den Doktorgrad beworben habe.

Gatersleben, im März 2005

Shuai Chen