

Investigating the role of the thiol-regulated enzyme sedoheptulose-1,7-bisphosphatase in the control of photosynthesis

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Sedoheptulose-1,7-bisphosphatase (SBPase; EC 3.1.3.37) catalyses the dephosphorylation of sedoheptulose-1,7-bisphosphate in the regenerative phase of the Calvin cycle. Antisense plants with reduced levels of SBPase have decreased photosynthetic capacity and altered carbohydrate status, leading to modifications in growth and development. The catalytic activity of SBPase is regulated by light via the ferredoxin/thioredoxin system. Recently, the amino acids within the SBPase

protein involved in this regulatory mechanism have been identified and a deregulated, permanently active form of the enzyme has been produced using site-directed mutagenesis. This paper explores how transgenic *Nicotiana tabacum* cv. Samsun plants, containing the deregulated form of the SBPase enzyme, may lead to a better understanding of the in vivo role of light activation of this important Calvin cycle enzyme.

Introduction

Sedoheptulose-1,7-bisphosphatase (SBPase; EC 3.1.3.37) is unique to the C₃ photosynthetic carbon reduction cycle (Calvin cycle), where it catalyses the dephosphorylation of sedoheptulose-1,7-bisphosphate. This reaction takes place in the regenerative phase of the C₃ cycle, where the CO₂ acceptor molecule, ribulose-1,5-bisphosphate, is regenerated from triose phosphates through a series of sugar condensation and carbon rearrangement reactions (Woodrow and Berry 1988, Geiger and Servaites 1995).

The SBPase gene has been cloned from a number of different plant species, where it is located in the nuclear genome (Raines et al. 1992, Willingham et al. 1994, Hahn et al. 1998). The *Arabidopsis* SBPase gene is present as a single copy sequence as is the case in *Chlamydomonas* (Willingham et al. 1994, Hahn et al. 1998). However, in hexaploid wheat the organisation is more complex, perhaps as a result of gene duplication (Devos et al. 1992). SBPase gene expression is regulated by light, development and levels of hexose sugars (Willingham et al. 1994, Jones et al. 1996). In dark grown wheat and *Arabidopsis* seedlings the level of SBPase mRNA is very low but on transfer to light increases by at least 20-fold (Willingham et al. 1994). In studies using the

primary wheat leaf, Calvin cycle enzyme mRNA levels, including SBPase, were very low in cells containing immature plastids, but increased significantly (5–20-fold) in the mid-section where the cells are fully expanded and the chloroplasts mature (Raines et al. 1991, Willingham et al. 1994). The regulation of SBPase gene expression is likely to be largely at the level of transcription, as is the case for the nuclear encoded Rubisco small subunit genes (Dean et al. 1989). The wheat and *Arabidopsis* SBPase genes both contain a number of DNA sequence motifs which have been identified as having a role in the transcriptional regulation of other photosynthetic genes (Miles et al. 1993, Willingham et al. 1994). The upstream sequence of the *Chlamydomonas* SBPase gene also contains elements important for directing light-regulated expression (Hahn et al. 1998). However, as yet only one putative transcription factor, WF-1, present in wheat leaf nuclei, has been identified as interacting with an SBPase gene upstream sequence (Miles et al. 1993).

The activity of the SBPase enzyme is regulated by light. On transfer from darkness to light, the catalytic activity of SBPase increases as a result of light-modulated activation by thioredoxin *f* (Breazeale et al. 1978, Wirtz et al. 1982).

Further regulation of SBPase activity results from changes in stromal Mg^{2+} levels and pH which also occur as a result of illumination; indeed, the substrate for SBPase is sed-1,7-bP- Mg^{2+} (Portis et al. 1977, Purczeld et al. 1978, Nishizawa and Buchanan 1981, Woodrow and Walker 1982, Woodrow et al. 1984, Cadet and Meunier 1988). An additional level of regulation may result from the association of SBPase into complexes with other Calvin cycle enzymes within the stroma, possibly improving the efficiency of the cycle by facilitating the channelling of intermediates between enzymes (Suss et al. 1993). The highly regulated catalytic activity of SBPase, together with data from modelling studies (Pettersson and Ryde-Pettersson 1989), has suggested that this enzyme may play an important role in the control of carbon flux through the Calvin cycle.

This review focuses on two main areas of SBPase research in which significant advances have recently been made. Firstly, analysis of the structure of this enzyme leading to the identification of the cysteine residues involved in redox regulation and secondly, the use of transgenic technology to manipulate the levels of SBPase activity, revealing the importance of this enzyme in the control of photosynthetic carbon fixation.

Location of the regulatory cysteines in SBPase

In common with several Calvin cycle enzymes SBPase is virtually inactive in the dark but activity increases by more than 10-fold within minutes of illumination (Laing et al. 1981, Wirtz et al. 1982). This light activation of SBPase is mediated through reducing power produced by the photosynthetic light reactions. Reducing power is transferred from ferredoxin to thioredoxin *f* in a reaction catalysed by the enzyme ferredoxin/thioredoxin reductase. Thioredoxin then binds to the inactive SBPase enzyme in a stable complex and reduces the regulatory disulphide bond (Geck et al. 1996,

Jaramillo et al. 1997). This activation mechanism involves the formation of protein-protein mixed disulphide bonds followed by the release of the reduced SBPase protein and the formation of oxidised thioredoxin (Brandes et al. 1996). Reduction of the disulphide bond in the SBPase protein changes the conformation of the active site, resulting in activation of the enzyme.

As a first step towards the identification of the cysteine residues responsible for light activation of SBPase, a comparison was made between the derived amino sequences for SBPases from wheat, spinach, *Arabidopsis* and *Chlamydomonas*. The initial hypothesis was that the position of the cysteines involved in thiol regulation would be conserved in all species and from the alignment (Fig. 1) it can be seen that four cysteine (Cys) residues, at positions 52, 57, 86 and 90, are conserved in all SBPases. In addition, Cys10 and Cys35 are conserved in all the higher plant sequences. In order to identify the specific cysteines involved in thiol regulation, site-directed mutagenesis was used to individually change each of these 6 cysteine codons to serine codons in a wheat SBPase cDNA clone (Dunford et al. 1998a,b). When the resulting mutant proteins were expressed in *E. coli* and their activity assayed in the presence or absence of reductant, only the mutant enzymes with Cys52 and Cys57 replaced by serine displayed redox insensitive activity (Table 1). These data suggested that amino acids, Cys52 and Cys57, were the regulatory cysteines in SBPase. Although amino acid sequence comparisons show that SBPase is closely related to FBPase (Raines et al. 1992, Martin et al. 1996), information from mutagenesis studies has revealed that the regulatory cysteine residues in these two proteins are located in different positions (Jacquot et al. 1995, 1997b). The feature that they have in common is that the redox active cysteines are distant from the catalytic site. This is in contrast to PRKase, where the cysteines involved in thiol regulation are some 39 amino acids apart and are located

	15	30	45	60	75	90
AtSBP	SNGASTVTKCEIGQS	LEEFQAQATPDKGLR	TLLMCMGEALRTIAF	KVRTASCGGTACVNS	FGDEQLAVDMLADKL	LFEALQYSHVCKYAC
So1SBP	--NSSLVTKCELGDS	LEEFQAKATTDKGLI	RLLMCMGEALRTIGF	KVRTASCGGTQCVENT	FGDEQLAIDVLADKL	LFEALNYSHFCKYAC
TaSBP	ASRAALTTRCAIGDS	LEEFQKATPDKNLI	RLICMGEAMRTIAF	KVRTASCGGTACVNS	FGDEQLAVDMLADKL	LFEALEYSHVCKYAC
Ch1SBP	SRRTAVLTQAKIGDS	LAEFQVATPDKPLR	HVMMSMAEATRTIAH	KVRTASCGGTACVNS	FGDEQLAVDMVADKL	LFEALKYSHVCKLAC
	*		*	*	*	*
	105	120	135	150	165	180
AtSBP	SEEVPELQDMGGPVE	GGFSVAFDPLDGSSI	VDNFTVGTIFGVWP	GDKLTGITGGDQVAA	AMGIYGPRTTYVLAV	KGFPGTHEFLLLDG
So1SBP	SEELPELQDMGGPVD	GGFSVAFDPLDGSSI	VDNFTVGTIFGVWP	GDKLTGVTGRDQVAA	AMGIYGPRTTYVLAL	KDYPGTHEFLLLDG
TaSBP	SEEVPELQDMGGPVE	GGFSVAFDPLDGSSI	VDNFTVGTIFGVWP	GDKLTGVTGGDQVAA	AMGIYGPRTTFVVAL	KDCPGTHEFLLLDG
Ch1SBP	SEEVPEPVDMMGG---	EGFCVAFDPLDGSSS	SDTNFAVGTIFGVWP	GDKLTNITGREQVAA	GMGIYGPRTVFCIAL	KDAPGCHEFLLMDG
	195	210	225	240	255	270
AtSBP	KWQHVKTTEIAEGK	MFSPGNLRATFDNSE	YSKLIDYYVKEKYTL	RYTGGMVPDVNQIIV	KEKGIFTNVTSPATAK	AKLRLLEFVAPLGLL
So1SBP	KWQHVKTTEINEGK	LFCPGNLRATSDNAD	YAKLIQYYIKEKYTL	RYTGGMVPDVNQIIV	KEKGIFTNVTSPATAK	AKLRLLEFVAPLGLL
TaSBP	KWQHVKTTSIGEGK	MFSPGNLRATFDNPD	YDKLVNYYVKEKYTL	RYTGGMVPDVNQIIV	KEKGIFTNVTSPATAK	AKLRLLEFVAPLGLL
Ch1SBP	KWMHVKTTHIGEGK	MFAPGNLRATFDNPA	YERLINFYLGKEYTL	RYTGGIVPDLFQIIV	KEKGVFTNLTSPPTK	AKLRILFEVAPLALL
	285	300	315	330		
AtSBP	IENAGGFSS-DGHK-	SVLDKTIINLDDRTQ	VAYGSKNEIIRFEET	LYGTSRLKN-VPIGV	TA	
So1SBP	IEKAGGHSS-EGTK-	SVLDIEVKNLDDRTQ	VAYGSLNEIIRFEKT	LYGSSRLEEPVAVGA	AA	
TaSBP	IEKAGGHSS-DGKQ-	SVLDKVISVLDERTQ	VAYGSKNEIIRFEET	LYGSSRLEASATVGA	TA	
Ch1SBP	IEKAGGASSCDGKAV	SALDIPILVCDQRTQ	ICYGSGIEVRRFEFY	MYGTSRPFSEKVA		

Fig. 1. Alignment of SBPase amino acid sequences from *Arabidopsis* (*Arabidopsis thaliana*, AtSBP; Willingham et al. 1994), spinach, (*Spinacia oleracea*, So1SBP; Martin et al. 1996) wheat (*Triticum aestivum*, TaSBP; Raines et al. 1992) and *Chlamydomonas reinhardtii* (Ch1SBP, Hahn et al. 1998). Amino acids are numbered according to Dunford et al. (1998a,b). Cysteines conserved in all of the higher plant sequences are indicated by asterisks.

Table 1. Identification of the redox active cysteines in wheat SBPase. The 6 conserved cysteine residues identified in Fig. 1 were individually changed to serine residues and the activities of the oxidised and reduced forms of the enzymes determined by spectrophotometric detection of Pi released from SBP (Dunford et al. 1998b)

	Wild-type	C10	C35	C52	C57	C86	C90
Ratio oxid/red SBPase activity	5.18	8.33	5.00	1.07	1.00	2.33	9.87

within the active site region of this protein. This work suggests that in each case thiol regulation has evolved independently in response to the appearance of oxygenic photosynthesis (Buchanan 1991; reviewed in Jacquot et al. 1997a).

The similarity between the primary amino acid sequences of SBPase and FBPase has enabled modelling studies to be carried out, based on the extensive crystallographic data available on the pig FBPase structure (Ke et al. 1991). This has indicated that the regulatory cysteines, Cys52 and Cys57, may be located in a flexible loop, near to the junction between the two subunits of the homodimer. The next challenge in this area of SBPase research will be to use crystallographic techniques to resolve the 3D structure of SBPase and to reveal the structural details of the allosteric changes occurring during redox regulation, as has been done for chloroplastic FBPase (Chiadmi et al. 1999). An understanding of the molecular interactions between SBPase and thioredoxin during the light activation process should develop from such studies.

Transgenic plants with altered SBPase activity

In recent years, studies in plant metabolic pathways, including the C3 cycle, have made extensive use of transgenic plants to investigate the importance of individual enzymes directly (Stitt and Sonnewald 1995). Genetic manipulation was used to alter SBPase levels in transgenic plants and the effect on photosynthetic carbon fixation was measured. Metabolic control analysis was then applied to quantify the contribution that SBPase makes to the control of photosynthetic carbon fixation. For linear metabolic pathways the flux control coefficient can vary from 0 for an enzyme that makes no contribution to control to 1 for an enzyme that exerts total control (Kacser and Porteous 1987). Using an antisense construct, SBPase expression was lowered in transgenic tobacco plants, producing recombinants with a range of SBPase protein and activity levels (Harrison et al. 1998). To determine the relationship between SBPase activity and photosynthetic carbon fixation, the rate of photosynthesis was measured under light-saturating conditions, and either ambient (A_{sat}) or saturating CO_2 (A_{max}), in antisense plants with a range of SBPase levels (Fig. 2). The amount of control exerted by SBPase on photosynthetic carbon fixation, the flux control coefficient, was calculated from the slopes of logarithmic plots of A_{sat} and A_{max} against enzyme activity and values of 0.31 and 0.54 obtained, respectively (Fell 1997). These data show that SBPase exerts greater control over carbon flux when photosynthesis is measured in saturating CO_2 . These results are in keeping with data from the analysis of transgenic plants with reduced levels of Rubisco which show that the control of flux does not reside

solely with Rubisco but is shared with other enzymes in the cycle, particularly when photosynthesis is measured in ambient conditions. In contrast, Rubisco has almost total control over the rate of carbon fixation in conditions of high light and high temperature, when the oxygenation reaction is favoured and the rate of photorespiration is high (Stitt and Schulze 1994). The data available from the analysis of transgenic plants with reduced levels of individual Calvin cycle enzymes have revealed that the control of carbon fixation is shared between Rubisco, SBPase and aldolase (Hudson et al. 1992, Stitt and Schulze 1994, Harrison et al. 1998, Haake et al. 1998, 1999). In addition, these results have shown very clearly that the distribution of control between these enzymes is not constant and can vary, depending on environmental conditions. Interestingly, several highly regulated enzymes in the cycle, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, phosphoribulokinase, were shown to make little contribution to the control of photosynthetic carbon flux, under the

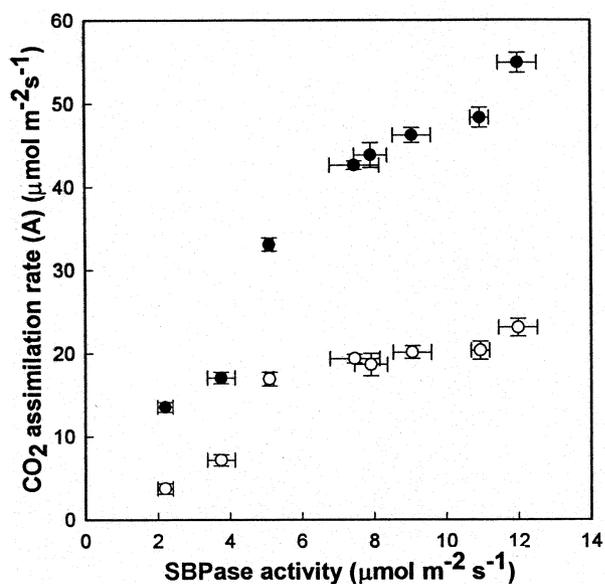


Fig. 2. The response of photosynthetic carbon assimilation to reductions in SBPase activity. Photosynthesis was measured under light saturating ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) conditions in ambient CO_2 (350 ppm; open circles) and saturating CO_2 (closed circles) in two consecutive leaves (leaves 7 and 8) on reaching full expansion, using a portable open gas exchange system (CIRAS-1, PP-Systems, Hitchin, UK). Plants were grown in the greenhouse, with light levels in excess of $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperatures of between 25 and 30°C . Data points are the mean \pm SE ($n = 5$ for WT plants, for the transgenic antisense plants two duplicate measurements were made from consecutive leaves). SBPase activity was determined in samples from the same leaves used for photosynthesis measurements, immediately frozen in liquid nitrogen and assayed according to Harrison et al. (1998).

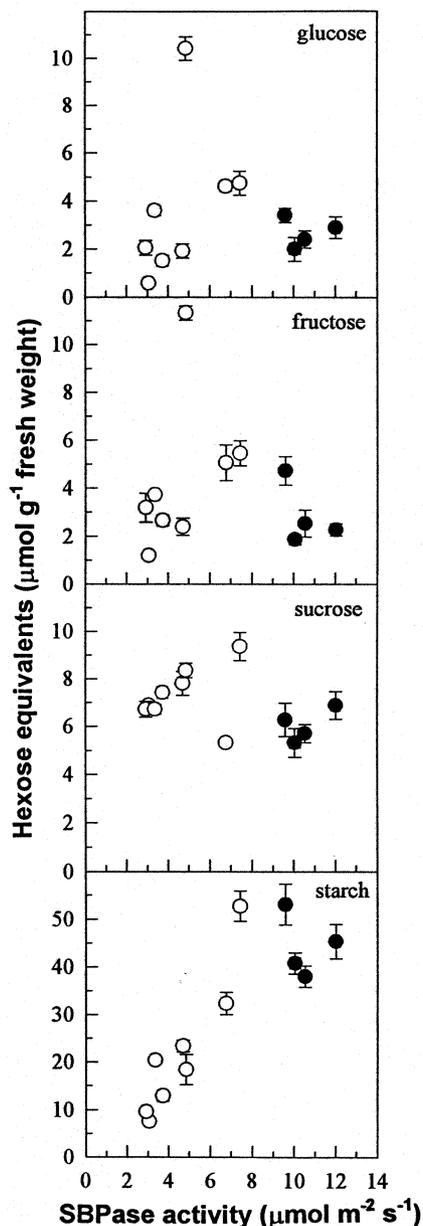


Fig. 3. Carbohydrate levels in tobacco plants with reduced SBPase activity. Samples for carbohydrate analysis were harvested at the end of the light period from fully expanded leaves (leaf 8) from a second set of plants grown as for photosynthesis analysis (Fig. 2) and frozen immediately in liquid nitrogen. Glucose, fructose and sucrose levels were determined using an enzyme-based protocol (Stitt et al. 1989); starch was measured in the ethanol-insoluble pellet (Stitt et al. 1978). Data points for the WT plants (filled symbols) are the mean \pm SE ($n = 4$), and for the transgenic antisense plants (open symbols) are the mean of triplicate measurements of single extracts from individual leaves on each plant.

conditions in which they were analysed (Kobmann et al. 1994, Paul et al. 1995, Price et al. 1995). However, the predictions for the control exerted by chloroplastic FBPase on carbon fixation may have been underestimated because the antisense plants were grown in low light conditions.

SBPase is positioned at the branch point in the cycle where intermediates are either channelled towards regenera-

tion of the CO_2 acceptor or are exported from the cycle for carbohydrate biosynthesis. To address the question of whether reducing the level of SBPase perturbs the balance of carbon flux to the starch and sucrose biosynthetic pathways, carbohydrate levels were measured in the fully expanded source leaves of SBPase antisense plants. Plants with moderate reductions in SBPase activity had no significant reduction in glucose, fructose or sucrose levels (Fig. 3). In the plants with more severe reductions in SBPase activity, sucrose levels were maintained close to that of wild-type (WT) plants. In contrast, the reductions in starch content were significant in these plants. These results show that plants with reduced SBPase activity maintain the flux of carbon to sucrose at the expense of starch biosynthesis. This change in carbon partitioning has been observed in all of the Calvin cycle antisense transgenic plants, suggesting that it is a general response to reduced photosynthetic carbon flux (Kobmann et al. 1994, Stitt and Schulze 1994, Paul et al. 1995, Price et al. 1995, Haake et al. 1998, 1999, Harrison et al. 1998). What is interesting about the SBPase antisense plants is that starch levels decreased linearly, in response to reductions in SBPase activity (Fig. 3), to the extent that in some of the antisense SBPase plants, starch was barely detectable by iodine staining, regardless of developmental stage (Fig. 4).

Investigating light activation of SBPase in vivo

An important question remaining is the role of light activation of individual enzymes, such as SBPase, in controlling the flux of carbon through the Calvin cycle. Thiol-mediated light/dark regulation of Calvin cycle enzyme activity may act, in part, as a simple on/off switch to prevent futile cycling of Calvin cycle intermediates in the dark, using ATP in the process and reducing the availability of erythrose-4-

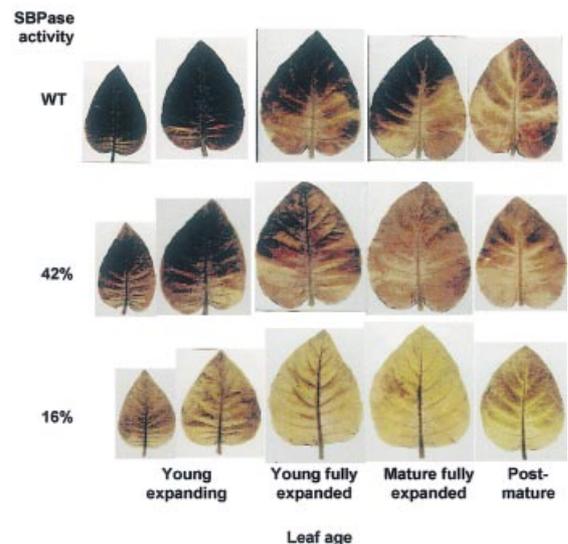


Fig. 4. Starch content of leaves at different developmental stages harvested from greenhouse-grown (as for photosynthesis; Fig. 2) WT and two SBPase antisense plants at the end of the light period. The SBPase activity (% WT) of a newly fully expanded leaf from the plant is indicated. Starch was visualized by iodine staining.

phosphate for the shikimate pathway. In addition, thiol modulation of the Calvin cycle may regulate the photosynthetic flux of carbon in response to rapid short-term alterations in the light environment, such as shading and sunflecks (Buchanan 1980, Scheibe 1991, Jacquot et al. 1997a). The availability of fully active deregulated mutants of SBPase (Dunford et al. 1998b), produced using site directed mutagenesis, will enable this question to be addressed in vivo. Transgenic plants, expressing this fully active mutant form of the enzyme, are being produced and will be used to investigate the physiological consequences of deregulation of SBPase activity. This use of transgenic technology is one approach likely to further our understanding of the role of SBPase in Calvin cycle metabolism.

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