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A CDPK type protein kinase is involved in rice SPS light modulation

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A protein kinase activity that can phosphorylate and inactivate rice (*Oryza sativa*) sucrose-phosphate synthase (SPS; UDP-glucose: D-fructose-6-phosphate-2-glucosyl transferase, EC 2.4.1.14) was measured in extracts prepared from leaves exposed to light-dark transitions. Enzyme activity present in extracts from dark leaves was about 5-fold higher than the activity in extracts from leaves that had been collected in the light. The protein kinase (named R-SPSK) was purified about 100-fold from dark leaves and its biochemical properties were studied. The micromolar dependence of Ca^{2+} exhibited by R-SPSK, and its response to calmodulin antagonists was similar to the properties associated with members of the plant Calcium-Dependent Protein Kinase (CDPK) family. Two modulators of SPS activity, Pi and Glc-6-P, were examined for an effect on R-SPSK. While Glc-6-P did not affect R-SPSK activity, Pi drastically increased the kinase activity. Taken together, these data provide evidence that SPS may be regulated by a CDPK type protein-kinase whose activity is modulated by light-dark transitions and stimulated by Pi, the negative effector of SPS activity.

Introduction

Sucrose-phosphate synthase (SPS; UDP-glucose: D-fructose-6-phosphate-2-glucosyl transferase, EC 2.4.1.14) has a key role in sucrose biosynthesis. SPS regulation involves allosteric modulation by Glc-6-P (activator) and Pi (inhibitor) (Doehlert and Huber 1983, Stitt et al. 1988), and reversible covalent modification in response to light (Stitt et al. 1988, Huber et al. 1989, Huber and Huber 1991, 1992a, 1992b), sucrose accumulation (Stitt et al. 1988) and osmotic stress (Toroser and Huber 1997).

Phosphorylation of specific seryl residues affects the activation state of SPS in spinach leaves in response to light/dark conditions (Huber and Huber 1992a,1992b, 1996). Three phosphorylation sites have been suggested to be of regulatory significance. Serine^{158} (Ser^{158}) is the residue proposed for light/dark modulation of SPS activity in spinach and most other higher plant SPSs contain a seryl residue at an analogous position in their sequences (Huber and Huber 1996). For example, in both rice and maize leaf SPS, the regulatory phosphorylation site has been identified as Ser^{162} (Huber and Huber 1996). However, an exception is the constitutive form of SPS cloned from sugar cane, which lacks a Ser residue at this position (Sugiharto et al. 1997). Phosphorylation of Ser^{158} reduces the activation state of spinach leaf SPS, by altering affinities for substrates and effectors, without affecting maximum catalytic activity. The significance of Ser^{158} phosphorylation for dark inactivation of SPS in vivo was demonstrated by site-directed mutagenesis experiments (Toroser et al. 1999).

The major protein kinase that phosphorylates Ser^{158} in spinach SPS has been designated PKIII (protein kinase III), which is a 150-kDa Ca^{2+}-independent kinase that tends to co-purify with SPS (McMichael et al. 1995). The catalytic subunit of PKIII is around 60 kDa and is recognized by antibodies against SNF1-like proteins (Douglas et al. 1997). Another protein kinase, designated as PKI, appeared to be a monomeric protein and was able to phosphorylate and inactivate spinach SPS in vitro in a Ca^{2+}-dependent manner (McMichael et al. 1995). PKI, is thought to phosphorylate SPS-Ser^{229}, a site suggested for interaction with 14-3-3 proteins (McMichael et al. 1995, Toroser and Huber 1998, Moorhead et al. 1999). PKI activity was dependent on

Abbreviations – CDPK, Calcium-Dependent Protein Kinase; R-SPSK, rice SPS kinase; SPS, sucrose-phosphate synthase; TFP, trifluoperazine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphtalensulphonamide.

micromolar concentrations of Ca^{2+} for activity and has been shown to be a CDPK (Douglas et al. 1997). Studies with the maize leaves have identified a protein kinase that phosphorylates and inactivates SPS in a strictly Ca^{2+}-dependent manner (Huber et al. 1995). Similarly, the osmotic-stress activation of SPS is also thought to involve Ca^{2+}-dependent phosphorylation. This activity is catalysed in spinach leaf by PKIV, which phosphorylates SPS-Ser^{424} (Toroser and Huber 1997).

Metabolite regulation of SPS kinase activity has been proposed in spinach leaf (Huber and Huber 1991). Particularly, Glc-6-P was reported as an inhibitor of the PKIII-catalysed phosphorylation of SPS, although high IC50 values were reported (McMichael et al. 1995). In a more recent study with the SNF1-related protein kinase, Glc-6-P inhibited the catalytic activity of PKIII (Toroser et al. 2000). However, studies with highly purified SNF1-related protein kinases phosphorylating SPS protein (one of them, HRK-C, corresponds to PKIII) did not show metabolite regulation (Sugden et al. 1999). Thus, inhibition of SPS kinase activity by Glc-6-P is still somewhat controversial.

Since two kinds of protein kinase activities were suggested for SPS light modulation (Ca^{2+}-dependent and Ca^{2+}-independent), the regulation of the phosphorylation state of SPS is quite complex. Consequently, a study of factors that regulate kinase activity in vitro may help to identify mechanisms that control the phosphorylation status of the enzyme in vivo. In this paper, the regulatory properties of a rice protein kinase that can phosphorylate and inactivate SPS (named R-SPSK) are reported. Biochemical analysis and response to calmodulin antagonists indicate that R-SPSK may be a member of the plant Calcium-Dependent Protein Kinase (CDPK) family. Light modulation and effect of metabolites suggest that this R-SPSK could be involved in the regulation of sucrose biosynthesis in rice.

Materials and methods

Plant material

Rice (*Oryza sativa* L. cv. Blue Bell) seeds were provided by Cámara Argentina de Arroz, Paraná, Entre Ríos, Argentina. Seeds were germinated and grown in a growth chamber. The temperature was maintained within 28 ± 2°C with a 14/10 day/night^{-1} regime (irradiance 200 µmol m^{-2} s^{-1}). Plants were watered with Hoagland and Arnon solution as described (Salerno et al. 1991).

Protein extraction and protein kinase partial purification

All steps were carried out at 0–4°C. Plant material was weighed and powdered under liquid nitrogen with mortar and pestle. Homogenates were prepared by stirring the powder in a buffer (2 ml g^{-1} tissue) containing 100 m*M* HEPES-NaOH pH 7.5, 5 m*M* *β*-mercaptoethanol, 10 m*M* $MgCl_2$, 2 m*M* EDTA, 0.5 m*M* PMSF, 2% (v/v) ethyleneglycol and 20% (v/v) glycerol (buffer A). Extracts were filtered through sintered glass units and centrifuged twice at 12 000 *g* for 15 min (crude extract). The supernatant was adjusted to 5% (w/v) PEG, stirred for 15 min and centrifuged at 12 000 *g* for 15 min. The supernatant solution obtained was then adjusted to 30% (w/v) PEG following a similar procedure. The protein pellet was re-solubilized in buffer A. The solution was clarified by filtration through a 0.22-µm membrane filter and applied to a Mono-Q column (Pharmacia Biotech, Piscataway, NJ, USA). The column was washed with a buffer containing 50 m*M* MOPS-NaOH pH 7.5, 10 m*M* $MgCl_2$, 2.5 m*M* DTT, 1 m*M* EDTA, 0.5 m*M* PMSF, 3 µg ml $^{-1}$ leupeptin and 3 µg ml $^{-1}$ aprotinin (buffer B) until the A_{280} decreased to baseline. The bound proteins were eluted with a 100-ml linear gradient from 0 to 0.5 *M* NaCl in buffer B at a flow rate of 0.5 ml min^{-1}. Fractions were collected, assayed for peptide kinase activity and those containing high enzyme activity were pooled, concentrated in an ultrafiltration membrane cone (Centriflo Amicon, MA, USA) and further purified by Superose-12 chromatography using as elution buffer 50 m*M* Tris-HCl pH 7.5 containing 10 m*M* $MgCl_2$, 2.5 m*M* DTT, 1 m*M* EDTA, 0.5 m*M* PMSF, 3 µg ml^{-1} leupeptin, 3 µg ml^{-1} aprotinin and 10% glycerol (buffer C). Protein was eluted at a flow rate of 0.5 ml min^{-1} in buffer C. Fractions with high kinase activity were pooled, concentrated in an ultrafiltration membrane cone (Centriflo Amicon, MA, USA) and used for biochemical characterization.

Protein determination

Quantification of proteins was performed according to Bradford (1976) using bovine serum albumin as standard.

Enzyme assays

SPS activity was assayed as reported (Salerno et al. 1998) by quantifying sucrose-phosphate (as sucrose) formed by the thiobarbituric acid method (Salerno et al. 1991). The effect of a phosphorylation treatment on SPS activity was detected by using two different assay conditions as described (Pagnussat et al. 2000): (1) selective assay (v_{sel}), which incubation mixture contains 1 m*M* Fru-6-P, 3 m*M* Glc-6-P, 5 m*M* Pi, 10 m*M* UDP-Glc, 20 m*M* $MgCl_{2\ and}$ 100 m*M* HEPES-NaOH pH 7.5; (2) non-selective assay (V_{nsel}) that contains 10 m*M* Fru-6-P, 30 m*M* Glc-6-P, 10 m*M* UDP-Glc, 20 m*M* $MgCl_{2\ and}$ 100 m*M* HEPES-NaOH pH 7.5. The activation state (defined as: v_{sel} Vnsel − 1 100) for rice SPS was determined as described (Siegl et al. 1990).

To determine R-SPSK activity, a synthetic peptide (KFQRNFSELTV) was designed from the regulatory phosphorylation site (Ser^{162}) of rice leaf SPS (Huber and Huber 1996). Typically, a 50-µl reaction mixture contained 0.1 mg ml^{-1} of synthetic peptide, 10 µl of partially purified protein kinase (or crude extract) and 50 m*M* MOPS-NaOH pH 7.5 buffer containing 10 m*M*

$MgCl_2$, 2.5 m*M* DTT, 10 m*M* orthovanadate, 10 m*M* glycerophosphate, 10 m*M* NaF and $CaCl_2$ or EGTA as was indicated in each experiment. Reactions were started by the addition of 100 m*M* ?γ^{32}P]ATP (500 cpm pmol^{-1}). Following a 10-min incubation at room temperature, a 20-µl aliquot of the reaction mixture was spotted onto a 2 ×2 cm square of phosphocellulose paper (P81, Whatman, Clifton, NJ, USA). The papers were then washed in 250 ml of 75 m*M* H_3PO_4 three times at 10 min per wash to remove unincorporated ATP. ^{32}P incorporated to the synthetic peptide was determined by scintillation counting on a liquid scintillation counter (Wallacay 1214, Pharmacia Turku, Finland). One enzyme unit (U) is defined as the activity that catalyses the incorporation of 1 pmol of [^{32}P] Pi min^{-1}.

In vitro phosphorylation and autoradiography

A 50-µl reaction mixture containing 10 µl of purified R-SPSK and 50 m*M* MOPS-NaOH pH 7.5 buffer containing 10 µl of purified SPS (according to Salerno et al. 1998), 10 m*M* $MgCl_2$, 2.5 m*M* DTT, 10 m*M* orthovanadate, 10 m*M* glycerophosphate, 10 m*M* NaF and $CaCl_2$ or EGTA as was indicated in each experiment. Reactions were started by the addition of 100 m*M* ?γ^{32}P]ATP (500 cpm pmol^{-1}). At different times of incubation at room temperature, reactions were stopped by addition of sample buffer (Laemmli 1970). The proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane, which was autoradiographed. The position of the phosphorylated polypeptides corresponding to SPS were identified using anti-SPS polyclonal antibodies (Salerno et al. 1998) in a Western blot experiment.

Results

Rice sucrose-phosphate synthase kinase partial purification

In a preliminary experiment, to study the behaviour of the SPS-kinase activity with respect to light-dark transitions, protein kinase activity was determined after exposing rice plants to a photoperiod of 14 h. A sucrose-phosphate synthase kinase (R-SPSK) activity was measured in total extracts of mature leaves harvested at different times of day as indicated, using a synthetic peptide as substrate (see Material and methods) in the presence of Ca^{2+} or EGTA (Fig. 1). Enzyme activity was about 5-fold higher in the dark extracts than in the light ones, but only in the presence of Ca^{2+}. Similarly, when light and dark extracts were chromatographed through an anion exchange column (DEAE-Sephacel), R-SPSK activity was 3- to 8-fold higher in the fractions eluted from the dark extracts than in those from the light extracts (not shown). Therefore, R-SPSK purification was performed from leaves harvested during the dark period. On account of the instability of the enzyme activity, a sequence of fast steps was used to optimize the purification yield (Table 1). Elution profiles of the two chromatographic steps used for the purification of R-SPSK are shown in Fig. 2. The PEG fraction was chromatographed through a Mono-Q column, and R-SPSK activity eluted as a single peak at about 0.20–0.24 *M* NaCl (Fig. 2A). The concentrated Mono-Q eluant was subjected to a gel filtration through a Superose-12 column, from where a single peak of R-SPSKI Ca^{2+}-dependent activity was obtained. The protein fraction corresponding to the Superose-12 elution step resulted in an approximate 100-fold increase in R-SPSK specific activity and it was used for the kinase characterization. The relative molecular mass of R-SPSK was estimated as 60 kDa in this step.

R-SPSK phosphorylates and inactivates SPS

The ability of R-SPSK to phosphorylate the SPS protein was investigated. A preparation of purified rice SPS (Salerno et al. 1998), free of endogenous protein kinase activity, was incubated with [γ^{32}P]ATP in the presence of Ca^{2+} or EGTA. The resulting protein products were electrophoresed and transferred onto a nitrocellulose membrane, which was autoradiographed (Fig. 3A). The position of the phosphorylated polypeptides corresponding to SPS was identified in the membrane by immunodetection (Fig. 3B). Phosphorylation of SPS polypeptides was only detectable in the presence of Ca^{2+} (Fig. 3A,B, lanes 2). Additionally, the effect of phosphorylation on the activation state of SPS was studied. An aliquot of purified rice SPS was incubated in the presence of R-SPSK in a phosphorylating medium containing [γ^{32}P]ATP. A control experiment was performed incubating SPS and R-SPSK plus the synthetic peptide used for the kinase purification. After different incubation times, SPS activity was measured, its activation state was calculated and ^{32}P incorporation to SPS poly-

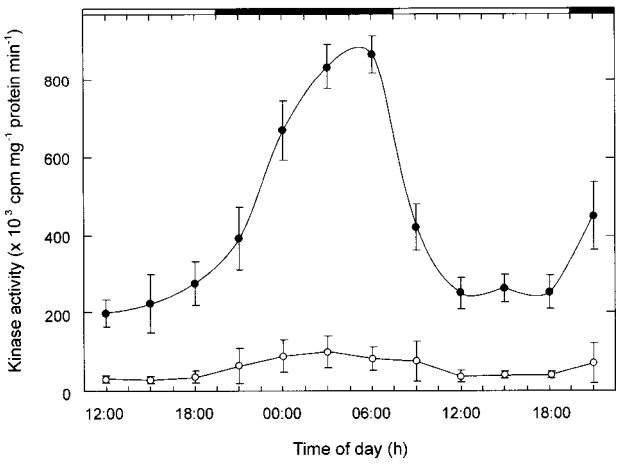

Fig. 1. SPS-kinase activity in rice leaf extracts. Activity was measured in crude extracts of leaves harvested at the times indicated during the light or the dark period in the presence of 1 m*M* EGTA (?) or Ca^{2+} (?). Each point corresponds to the mean value of three independent experiments (each done in duplicate); bars indicate standard deviation.

Table 1. Summary of the purification procedure of SPS-kinase from rice leaf.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg $^{-1}$)	Yield (%)	Purification factor
Crude extract	96.9	11 540	119	100	1
PEG 6000	31.4	8450	269	73	2.3
Mono-Q	7.60	5070	667	44	5.6
Superose-12	0.14	1860	12 962	16	108

peptides was quantified (Fig. 3C,D). The results show that SPS inactivation follows the increase of the enzyme phosphorylation by R-SPSK. Furthermore, when the synthetic peptide was added to the incubation medium, both SPS inactivation and phosphorylation of the enzyme were affected (Fig. 3C,D).

The effect of Glc-6-P and of Pi, SPS modulators, on R-SPSK activity were studied (Fig. 4). While Glc-6-P did not modify R-SPSK activity, physiological concentrations of Pi dramatically stimulated the kinase activity (approximate 6-fold increase).

R-SPSK: its identification as a CDPK-type protein kinase

The effect of Ca^{2+} concentration on R-SPSK activity was studied in a standard protein kinase assay mixture at different concentrations of free cation using Ca^{2+}/EGTA buffers (Bartfai 1979). The kinase activity was stimulated by micromolar Ca^{2+} concentrations, to a maximum of about 7-fold in the presence of 5 μ*M* Ca^{2+}.

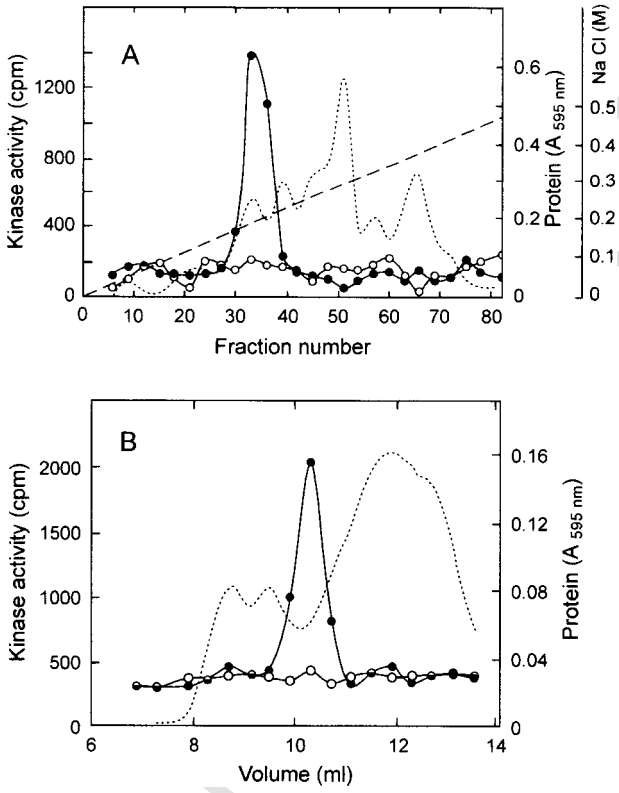

Fig. 2. R-SPSK activity elution profiles for chromatography on Mono-Q (A) and Superose 12 (B) columns. R-SPSK activity was measured in the presence of 1 mM EGTA (?) or Ca^{2+} (?). Details of the procedures are described in Materials and methods. The dotted line indicates the progress of the salt gradient used in R-SPSK elution.

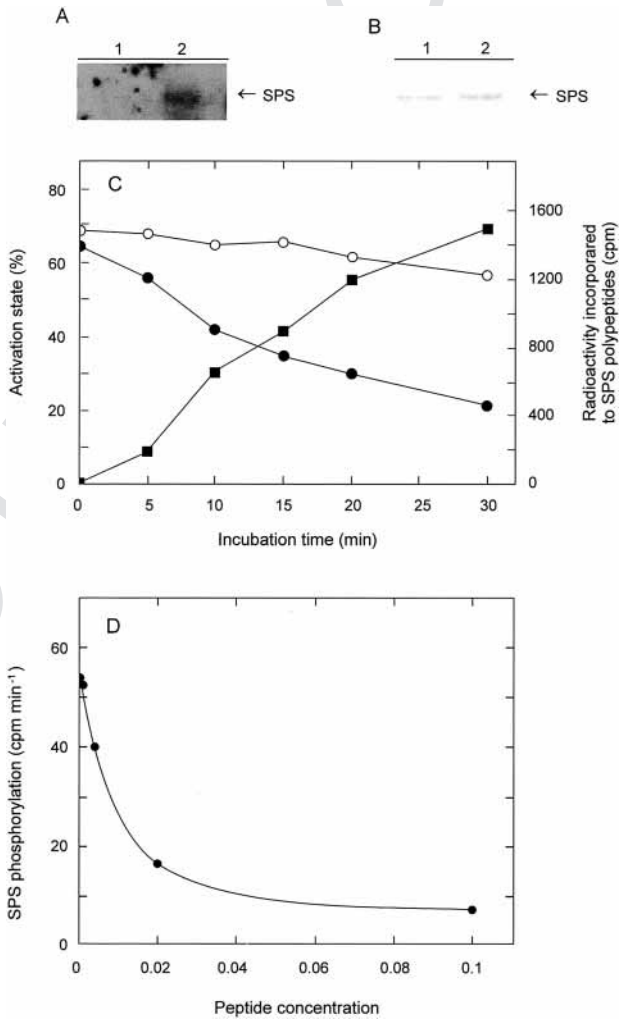

Fig. 3. SPS phosphorylation by purified R-SPSK and its effect on SPS activation state. A and B: rice SPS was incubated with ?γ^{32}P]ATP and partially purified R-SPSPK in the presence of EGTA (lane 1) or Ca^{2+} (lane 2). Products were separated by SDS-PAGE, transferred to a membrane and autorradiographed (A) followed by Western blot (B). C: SPS activation state and radioactivity incorporated to SPS polypeptides were determined after different times of incubation of purified SPS with R-SPSK. Activation state (?); cpm incorporated to SPS (?). As a control, parallel determinations of activation state were performed by adding to the incubation medium the competitor peptide that mimics SPS (?). D: SPS phosphorylation by R-SPSK was measured including the synthetic peptide to the incubation medium.

At higher Ca^{2+} concentrations, a decrease in the kinase activity was observed (Fig. 5). Since the micromolar dependence of Ca^{2+} for activity exhibited by R-SPSK are indicative of a CDPK-type protein kinases (Roberts 1993, Stone and Walker 1995), the effect of trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphtalensulphonamide (W7), two calmodulin antagonists, were tested (Fig. 6). Both compounds inhibited R-SPSK activity. However, addition of TFP caused a higher degree of inhibition (IC50 = 55 μ*M*) than W7 (IC50 = 145 μ*M*).

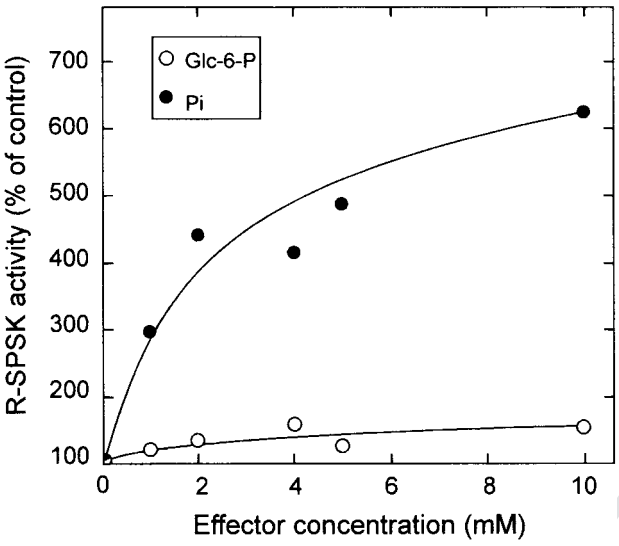

Fig. 4. Effect of the SPS modulators (Glc-6-P and Pi) on R-SPSK activity. 100% activity was 5 pmol P ml^{-1} min^{-1}.

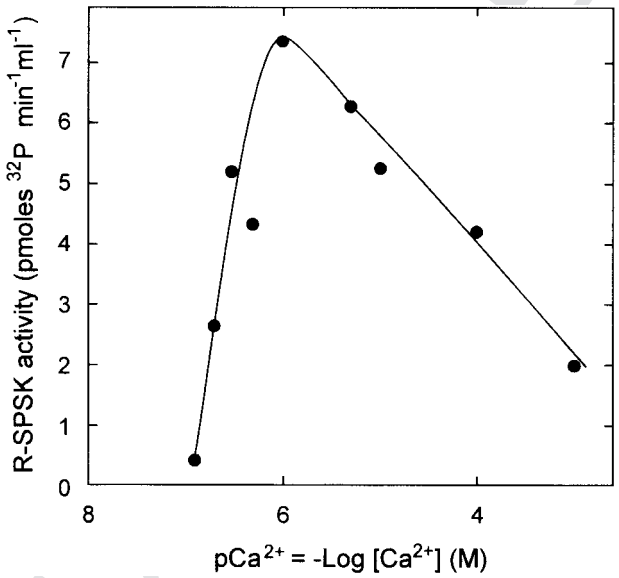

Fig. 5. Effect of calcium ion concentration on R-SPSK activity.

Discussion

We have identified a novel protein kinase activity related to light modulation of SPS. This sucrose-phosphate synthase kinase activity (R-SPSK) was able to phosphorylate rice SPS on a regulatory site, as the phosphorylation treatment affected the activation state of the enzyme (Fig. 3C). Phosphorylation of SPS protein and of the synthetic peptide used as substrate was Ca^{2+}-dependent, and the maximum activity obtained using the synthetic peptide was at 5 μ*M*. This value was reported as the cytoplasmic level of free Ca^{2+} in plant cells (Williamson and Ashley 1982, Brownlee and Wood 1986) and also this concentration typically activates plant CDPKs (Roberts 1993). The calmodulin antagonists W7 and TFP, caused inhibition of R-SPSK activity at concentrations that are similar to those recorded for CDPKs from apple

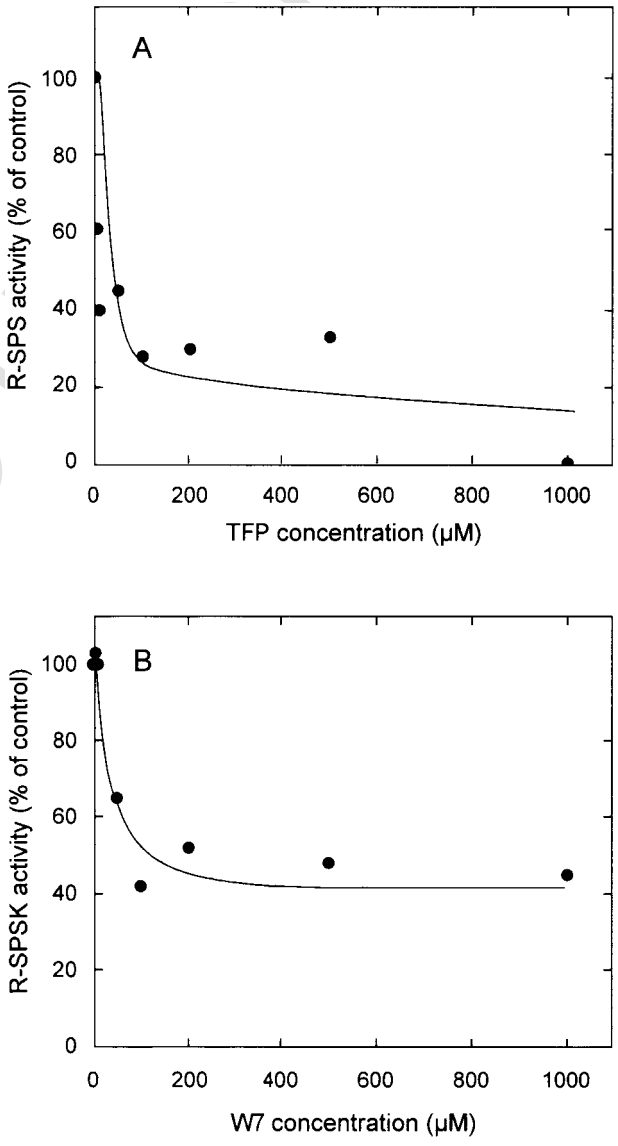

Fig. 6. Effect of the calmodulin antagonists TFP (A) and W7 (B) on R-SPSK activity. 100% activity was 5 pmol P ml^{-1} min^{-1}.

(Battey and Venis 1988), peanut (Das Gupta 1994) and *Vicia faba* (Li et al. 1998). These data suggest that SPS phosphorylation—and consequently sucrose biosynthesis—may be calcium regulated processes and indicate that R-SPSK could be a member of the plant CDPK family. These results are in line with those previously published for maize SPS-kinase. In contrast, spinach SPS phosphorylation appears to be a Ca^{2+}-independent event. It is known that the amino acids surrounding a phosphorylation site may be determinants of protein kinase specificity. As maize and rice SPSs have identical conserved regulatory domains, it may be expected that the same type of protein kinase is acting at this site. On the contrary, spinach and other dicot species differ in the residues that are surrounding the Ser^{158} phosphorylation site (Huber and Huber 1996). These differences could explain in part the results obtained herein.

Previous studies on SPS regulation were based on the light modulation of the protein phosphatase activity (a member of the PP2A family) responsible for SPS activation (Huber and Huber 1996). In this work, we show that a rice protein kinase activity that is able to phosphorylate SPS (R-SPSK) could be modulated by light in accordance with SPS activity regulation. The differences in R-SPSK activity detected in total extracts from leaf harvested during the dark and the light period (Fig. 1) suggest that rice R-SPSK activity is associated with light-dark transitions. This result could not be attributed to differences in phosphatase activity, since phosphatase inhibitors were used in all kinase activity assays, and the same behaviour was seen when R-SPSK activity was measured in crude extracts from dark and light that were separated by anion-exchange chromatography (data not shown). The mechanism of the regulation of R-SPSK by light-dark transition is still unknown, but, if analogous to other protein kinases, it could involve protein phosphorylation (Ranjeva and Boudet 1987).

R-SPSK activity was not inhibited by Glc-6-P (SPS activator), as was described for spinach SPS-kinase (Huber and Huber 1991); however, it exhibited a strong activation by Pi (SPS inhibitor) (Fig. 4). This effect on SPS-kinase has not been previously reported. In spite of the differences observed between rice and spinach SPS-kinase activity, the results presented here are in general agreement with the scheme for SPS regulation proposed by Huber and Huber (1996). During the dark period, the rate of triose-P export from the chloroplast to the cytoplasm falls, leading to an increase of cytosolic Pi concentration. On the contrary, the Glc-6-P concentration is very low (Heldt et al. 1977). Thus, rice SPS-kinase would be activated due to the high Pi concentration present in cytoplasm during the dark period and phosphorylation of SPS would take place. On the other hand, the phosphorylated SPS present in the dark period is very sensitive to Pi inhibition (Toroser et al. 1999), and consequently its activity is drastically reduced. In this way, Pi has a double role in SPS regulation: as allosteric effector of SPS (inhibitor) and as activator of SPS-kinase.

Collectively, our results suggest a key role for the SPS-kinase in the regulation of sucrose biosynthesis. Its activity could be modulated in response to light-dark transitions and to changes in Pi and Ca^{2+} levels. Consequently, Ca^{2+} may be included as a regulatory factor in the sucrose biosynthesis pathway while Pi would fulfil a double role regulating both SPS activity and the phosphorylation state of SPS.

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