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Light regulation of sucrose-phosphate synthase activity in the freezing-tolerant grass *Deschampsia antarctica*

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Abstract

Deschampsia antarctica, a freezing-tolerant grass that has colonized the Maritime Antarctic, has an unusually high content of sucrose (Suc) in leaves, reaching up to 36% of dry weight. Suc accumulation has often been linked with increased activity of sucrose phosphate synthase (SPS; EC: 2.4.1.1.14). SPS, a key enzyme in sucrose biosynthesis, is controlled by an intricate hierarchy of regulatory mechanisms including allosteric modulators, reversible covalent modification in response to illumination, and transcriptional regulation. We hypothesized that during long day conditions in the Antarctic summer *D. antarctica* can maintain high SPS activity longer by indirect light regulation, thereby leading to a high sucrose accumulation in the leaves. The objectives of this study were to investigate a possible indirect light regulation of SPS activity and the effect of cold and day length on transcriptional and protein level of SPS in *D. antarctica*. Although SPS activity did not display an endogenous rhythm of activity in continuous light, activation of SPS at the end of the dark period was observed in *D. antarctica*. This activation of SPS is possibly controlled by covalent modification, because it was inhibited by okadaic acid while the SPS protein level did not significantly change. The highest SPS activity increase was observed after 21 days of cold-acclimation under long day conditions. This increased activity was not related to an increase in SPS gene expression or protein content. High SPS activity in cold long days leading to hyper accumulation of Suc appears to be among the features that permit *D. antarctica* to survive in the harsh Antarctic conditions.

Abbreviations: Fru-6-P – fructose-6-phosphate; Glu-6-P – glucose-6-phosphate; HPTLC – high performance thin layer chromatography; LD – long day; MD – medium day; Suc – sucrose; SPS – sucrose phosphate synthase; SPP – sucrose phosphate phosphatase; SD – short day; Suc-6-P – sucrose-6-phosphate; TSS – total soluble sugars; UDP-glu – UDP glucose; UDP – uridine 5'-diphosphate

Introduction

Plant adaptation to low (non-freezing) temperatures often involves accumulation of soluble sugars. The most common and most abundant of which is sucrose (Suc) (Steponkus and Lanphear 1968; Kaurin et al. 1981, Guy et al. 1992). Suc plays a central role in higher plants as the

dominant transport sugar, as a nutrient, and as a newly recognized signal molecule (Winter and Huber 2000). In temperate grasses, Suc is the substrate for fructan synthesis (Pollock and Cairns 1991). Increased Suc accumulation in both photosynthetic (Jeong and Housley 1990; Tognetti et al. 1990; Guy et al. 1992; Holaday et al. 1992) and non-photosynthetic tissues, for example potato tuber (Geigenberger et al. 1995), is frequently linked to increased SPS activity.

The last two steps in the biosynthesis of Suc are catalyzed by the sequential action of sucrose phosphate synthase (SPS, EC: 2.4.1.1.14) and sucrose-6-phosphate phosphatase (SPP, EC: 3.1.3.24). Rapid removal of Suc-6-P by a specific and high-activity phosphatase displaces the reversible SPS reaction from equilibrium (Stitt et al. 1987), and thus, it is thought that *in vivo* SPS activity contributes to the control of Suc biosynthesis. Some evidence suggests that SPS and SPP associate to form a multienzyme complex (Huber and Huber 1996). Recently, the gene encoding SPP was cloned and sequenced, revealing that SPP contains a sequence, at the carboxy-terminus, to which SPS might bind (Lunn and MacRae 2003).

There are species-specific differences in the *in vivo* modulation of SPS (Huber and Huber 1996). Some species exhibit a marked light activation of SPS (designated as class I and class II species), whereas others do not (class III species) (Huber and Huber 1992a). In species such as tomato, corn, barley, and spinach, SPS is indirectly regulated by light, attaining high activity during the light period but very low activity in the dark (Sicher and Kremer 1984; Stitt et al. 1988; Huber and Huber 1990; Jones and Ort 1997). Light modulation of SPS activity is thought to be one mechanism to adjust the capacity for Suc biosynthesis to match the rate of photosynthesis, which can vary in response to changes in irradiance, stomatal conductance, and a wide range of other factors (Winter and Huber 2000). SPS responds to a diverse array of regulatory mechanisms including: (1) allosteric regulation by Glu 6-P (activator) and Pi (inhibitor) (Doehlert and Huber 1983; Stitt et al. 1988); (2) reversible covalent modification in response to light (Stitt et al. 1988), osmotic stress (Toroser and Huber 1997); and (3) transcriptional regulation during sink-source transition (Harn et al. 1993; Klein et al. 1993). During darkness, spinach and maize SPS are inactivated by phosphorylation of a regulatory Ser residue (Pagnussat et al. 2000). This effect is reversed under light by a type 2A protein phosphatase resulting in activated SPS as a consequence of an increase in the affinity for substrates (Fru-6-P and UDP-glu) and for Glu-6-P, a positive modulator as well as a decrease in Pi sensitivity (Huber and Huber 1996). Changes in spinach SPS activity during light–dark transitions appear to be entirely due to covalent modification of the enzyme (Huber and Huber 1992b). This indirect mechanism of light activation has been studied using specific inhibitors of protein phosphatases (okadaic acid) and kinases (staurosporine or 5′-*p*-fluoro-sulphonylbenzoadenosine, FSBA), which affect SPS activity *in vivo* (Jones and Ort 1997; Pathre et al. 2000).

There are only two vascular plants that have colonized the Maritime Antarctic. One of them, *Deschampsia antarctica,* is a freezing-tolerant grass (Bravo et al. 2001). This plant grows during the summer, when the average air temperature is near 2 °C (Zúñiga et al. 1996), with an estimated day length that ranges from about 19 h at the end of December to 13 h at the beginning of March (Kirk 1994). In the field, *D. antarctica* has an unusually high content of Suc, reaching up to 36% of dry weight (Zúñiga et al. 1996). This high amount of Suc found in *D. antarctica* may be the result of high Suc biosynthesis. In laboratory studies comparing SPS activity under V_{max} conditions (high concentrations of substrates and activator in absence of Pi) in some wild (*Deschampsia caespitosa*, *D. beringensis* and *D. antarctica*) and cultivated (wheat, barley and oat) Poaceae species we observed a 4-fold increase in response to cold and long day treatment only in *D. antarctica* (Zúñiga-Feest et al. 2003). This increase in SPS activity was positively correlated with Suc content in the leaves (Zúñiga-Feest et al. 2003). The mechanism that leads to the enchanced capacity for SPS activity in *D. antarctica* during cold acclimation and long day treatment is unknown. The objectives of this study were to investigate a possible indirect light regulation and the effect of cold and day length on SPS at the transcriptional level and on SPS protein level. We hypothesize that during long day conditions in the Antarctic summer; *D. antarctica* maintains a high SPS activity for an extended period, leading to high sucrose accumulation in the leaves. The mechanism of prolonged high SPS activity may involve transcriptional regulation of

the SPS synthesis or posttranslational activation by dephosphorylation.

Materials and methods

Plant material and growth conditions

Deschampsia antarctica Desv. (Poaceae) was collected from the Coppermine Peninsula on Robert Island, Maritime Antarctic (62°22′ S; 59°43′ W). A description of the environmental conditions of the habitat has been published elsewhere (Alberdi et al. 2002; Zúñiga et al. 1996). Plants have been reproduced vegetatively since 1997 in plastic pots, using a soil: peat mixture (3:1) and maintained at 13–15 °C in a growth chamber (Forma Scientific. Inc., Marietta, Ohio) with a photon flux density (PFD) of 100–120 μmol photons m^{-2} s^{-1} at the top of the canopy and a 16/8 h light/dark period. This irradiance corresponds to saturating PFD in laboratory grown antarctic grass plants (Edwards and Lewis Smith 1988). Plants were fertilized with Phostrogen® (Solaris, Buckinghamshire, UK) using 0.2 g l^{-1} once every 2 weeks.

Cold acclimation treatment at different day lengths

Plants grown for at least two months in 16/8 day length, 15 °C and 100 ± 10 μmol photons m^{-2} s^{-1} were transferred to 4 °C (cold acclimated) or 15 °C (non-acclimated) for 21 days and different day lengths: SD = 8/16 h, MD = 16/8 h, and LD = 21/3 h. Samples of mature leaves were taken at 0, 7, 14 and 21 days of treatment for SPS activity determinations. Samples for protein and RNA extraction were taken during the first hours (0, 2, 4) and days (2, 7, 14 and 21) of cold acclimation treatment. All samples, with the exception of those of 2 and 4 h, were collected at the middle of the photoperiod (4 hours of light at SD, 8 h at MD and 10 h at LD).

Continuous light and okadaic acid experiments

In order to discriminate light activation from an endogenous rhythm of SPS activity in *D. antarctica,* laboratory plants maintained under LD conditions, 15 °C, and 100 ± 10 μmol photons m^{-2} s^{-1} were transferred to continuous low PFD (60 ± 5 μmol photons m^{-2} s^{-1}) for 56 h, as described for discerning circadian oscillations in other species (Jones and Ort 1997). Samples of mature leaves were taken in the corresponding light or dark periods, frozen in liquid nitrogen and stored at −80 °C until used for SPS activity determination and protein and sugar extractions.

Before okadaic acid was added, samples of mature leaves were taken during light and dark periods from plants kept for at least 2 months in LD, 15 °C and 100 ± 10 μmol photons m^{-2} s^{-1}. Leaves of these plants were abraded with 400-grit Duralum powder (Electro Minerals Corp., Niagara Falls, New York), washed with distilled water and dried with tissue paper. One hour before the dark period started, 20 μM okadaic acid in a 0.5% Tween 20 solution was applied on the surface of the leaves (Jones and Ort 1997). The samples were frozen in liquid nitrogen and stored at −80 °C until used for SPS activity determination and protein extraction.

Sugar extraction

Samples of mature leaves were homogenized and extracted with ethanol (86%) for 24 h, and then centrifuged at 12,000 × *g* for 10 min. Pigments were extracted from the supernatant in a mixture of 1:3 (v/v) with chloroform, and the aqueous fraction was freeze-dried overnight. The dry residue was resuspended in 500 μl of methanol. Total soluble sugar content was determined spectrophotometrically by the resorcinol method (Roe 1934) at 520 nm, using Suc as a standard (Merck, Darmstadt, Germany). Because xylose was not detected in *D. antarctica*, we used it as internal standard to calculate carbohydrate losses during the extraction procedure. Xylose recovery was 90 ± 2%.

Suc content was determined by high performance thin layer chromatography (HPTLC) using a Silicagel plate 60 F 254 (Merck, Darmstadt, Germany) pretreated with a 0.1 M methanolic potassium phosphate solution. HPTLC plates were run five times in acetonitrile:water (85:15 v/v). Solutions of 0.1 mg/ml of Suc, glucose and fructose (Merck, Darmstadt, Germany) were used for standard curves (Lee et al. 1979). The position of Suc was visualized by the diphenylamine/aniline reagent at 105 °C and the plate was scanned at 520 nm.

Enzyme extraction

Leaf samples for dry weight determination were weighted and then dried at 60 °C for two weeks until constant weight was obtained. Leaf samples for enzyme extraction were frozen in liquid nitrogen and stored at −80 °C until used. The frozen samples were ground to a fine powder using mortar and pestle in liquid nitrogen and then suspended in extraction buffer (0.15 g of fresh tissue per 900 µl buffer), consisting of 40 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 10 mM DTT, 2% w/v PEG, 0.1% Triton X-100, and 2 mM benzamidine. The homogenized tissue was subsequently centrifuged at 12,000 × *g* at 4 °C and the supernatant was desalted and depleted of endogenous substrates using a G-25 Sephadex column that had been previously equilibrated in the assay buffer consisting of 40 mM Hepes (pH 7.5), 15 mM $MgCl_2$ and 1 mM DTT.

SPS activity assay

SPS V_{max} activity was measured under non-limiting conditions in a reaction mixture, which contained 40 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 1 mM DTT, 40 mM Glu 6-P, 10 mM UDP-glu and 20 mM Fru-6-P. During dark–light transitions, SPS V_{max} and V_{lim} activities were measured under both non-limiting and limiting (40 mM Hepes, 15 mM $MgCl_2$, 1 mM DTT, 12 mM Glu 6-P, 10 mM UDP-glu, 3 mM Fru-6-P and 10 mM Pi) conditions. UDP formation was determined by a NADH consuming reaction containing pyruvate kinase [7.2 U ml^{-1}] and, lactate dehydrogenase [11.3 U ml^{-1}]) and phosphoenol pyruvate (0.8 mM) and NADH (0.3 mM). SPS activity was measured at 30 °C following the consumption of NADH by the decrease in absorbance at 340 nm (Hauch and Magel 1998). The values are expressed in U g^{-1} dry weight (1 U represent the oxidation of 1 µmol of NADH per min at 30 °C and pH 7.5). Values were normalized, as indicated in the respective figures (Figures 1 and 2b).

Protein extraction and quantification

After treatments, 0.2 g of *D. antarctica* leaves were macerated and homogenized with 600 µl of extraction buffer at 4 °C (0.06 M Tris-HCl, pH 6.8 containing 1% SDS, 2.5% of β-mercaptoethanol and 5% glycerol) (Laemmli 1970). The extracts were heated for 2 min at 80 °C and centrifuged for 10 min in a microcentrifuge. Proteins were quantified in the supernatants using the colorimetric method of Bradford (1976) and BSA standards (Sigma Co. Saint Louis Missouri) as a calibration curve. The extracted protein was kept at −20 °C until western analysis was completed.

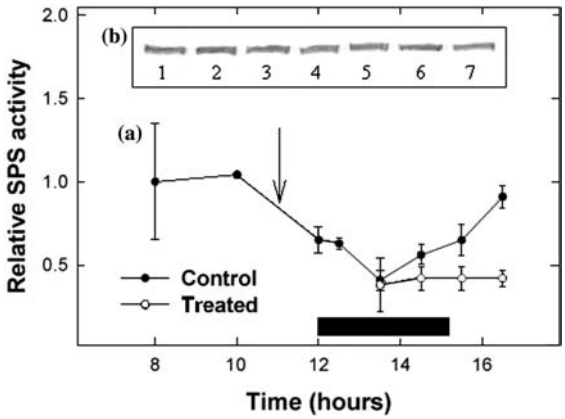

Figure 1. Light–dark variation and okadaic acid effect on SPS activity in *Deschampsia antarctica*. (a) SPS activity during light-dark–light transition in leaves of plants kept at 15 °C, LD = 21/3 h light/dark photoperiod and 100 ± 10 µmol photons m^2 s^{-1} (control) and in leaves treated with okadaic acid. Okadaic acid (20 µM) was added at abraded leaves surfaces in a 0.5% Tween-20 solution. The arrow shows the moment when okadaic acid was added. The black bar represents a dark period. The values represent the mean of four independent samples ± standard error. The initial absolute value of SPS activity at 8 h was 2.04 ± 0.7 U g^{-1} dry wt. All values shown above are relative to this control. (b) Western blot showing SPS protein during light-dark-light transition in plants kept at similar conditions described below (control). Each line was loaded with, 10 µg of total protein, lines correspond to the following samples, $1:8^{00}$, $2:10^{00}$, $3:12^{30}$, $4:13^{30}$, 5: 14^{30}, 6: 15^{30} and 7: 16^{30} h.

Western analysis

Proteins of the extracts were separated on a SDS-PAGE gel as described by Laemmli (1970). The stacking gel was 5% polyacrylamide (w/v) and the resolving gel was 10% polyacrylamide (w/v). Each well was loaded with 15 µg of total protein. Electrophoresis was run at 100 V using a Mini-Protean II (BioRad, Hercules, California). Pre-stained molecular weights standards were run on the same gel (Bio Rad). The proteins were transferred to a

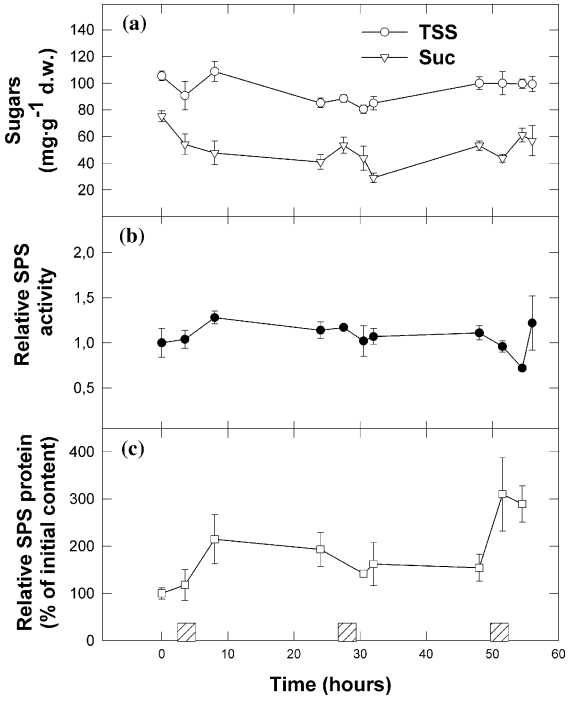

Figure 2. Effect of a continuous light treatment on sugar content, SPS activity and SPS content of *D. antarctica*. (a) Total soluble sugar (TSS) and sucrose (Suc) content. (b) Sucrose-P-synthase activity, the initial absolute value of SPS activity at 0 time was 1.35 ± 0.22 U g^{-1} dry wt. All values shown above are relative to this control and (c) relative content of SPS protein, in the leaves of *D. antarctica* plants maintained for 56 h in a low intensity continuous light (60 ± 10 μmol photons m^2 s^{-1}). The values at any time are expressed as percentage of SPS protein content with respect to the initial protein content at time zero. Values represent the mean of three independent samples ± standard error. Dashed box represent subjective night (i.e., when plants normally experience night under normal growth conditions).

polyvinilidenefluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, Massachusetts) using a Mini trans blot transfer cell (BioRad) for 1 h. The membranes were blocked with 10% non-fat powdered milk solution made in 20 mM Tris (pH 7.5) containing 500 mM NaCl (TBS buffer) after which the membrane was incubated with a rabbit polyclonal antibody raised against a consensus peptide-SPS (1:1000). The membrane was washed three times for 15 min TBS buffer containing 0.1% Tween 20. The secondary antibody was an alkaline phosphatase-goat-anti-rabbit IgG (Sigma, Saint Louis, Missouri) diluted 1:10,000 in 5% non-fat milk solution in TBS buffer. The membrane was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) in 100 mM Tris-HCl (pH 9) containing 100 mM NaCl and 5 mM $MgCl_2$. Band intensities of the westerns were densitometrically quantified with the Sigma Gel Program 1.0.5.0, Jandel Scientific.

Polyclonal antibody

A consensus peptide sequence was selected by comparing SPS amino acid sequences from different plants (Blast Program). The selected sequence corresponded to a highly conserved and antigenic 20 amino acid domain (DYLLHINHRWSHDGAKQTC) within Poaceae species (rice, wheat, maize, barley and oat). This peptide was conjugated with hemocyanine from *Concholepas concholepas* (Blue carrier, Biosonda S.A., Santiago, Chile) and injected to the rabbit following the procedures of Becker et al. (1998).

Purification of total RNA and northern blot analyses

Total RNA was extracted from leaves of control and cold acclimated plants at different times: hours (0, 2, 4) and days (2, 7, 14 and 21) using the Trizol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, California). For northern blot analysis, total RNA (20 μg) was electrophoresed on denaturing formaldehyde-1% agarose gels. After electrophoresis, RNA was transferred onto Hybond-N membranes (Amersham Pharmacia Biotech, Little Chalfont, UK), UV-cross-linked, and hybridized with the ^{32}P-labeled 790 bp cDNA Sps1 (rice) inserts, under high stringency conditions at 50 °C for 16 h in hybridization solution (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, 1% BSA). The blots were autoradiographed on Kodak Biomax MS films with intensifying screens at –80 °C. All RNA blots were hybridized with a 28S rRNA probe to confirm uniform RNA loading.

Statistics

The statistical differences in SPS activity of okadaic acid treated plants and non-treated plants during dark–light transition were

calculated using two-way ANOVA (level of significance was 0.05). The factors tested were okadaic acid addition and time of response. Statistical differences in sugar content, SPS activity and relative amount of SPS protein during continuous light treatment were calculated separately using a one-way ANOVA (level of significance was 0.05). Statistical differences in relative SPS activity and relative SPS protein content in cold acclimated and non-acclimated *D. antarctica* plants at different day lengths were calculated using three-way ANOVA (level of significance was 0.05). The factors tested were temperature, day length and time of treatment. A Tukey test was then used to identify those means with significant differences.

Results

Okadaic acid prevents the diurnal reactivation of SPS at night

Measurements of V_{max} and V_{lim} SPS activity showed similar transitions between dark and lights periods (Table 1). V_{max} SPS activity was higher during the light period than in the dark period (Figure 1). Okadaic acid pretreatment prevented the increase in V_{max} that normally occurred prior to the end of the dark period (Figure 1). Western analysis revealed that SPS protein remained relatively constant throughout the diurnal changes in SPS activity (Figure 1) indicating that the changes in SPS activity were not related to the amount of SPS enzyme present.

Light regulation of SPS activity was studied mainly in growth chambers, where there is a rapid transition from dark to light conditions. A similar pattern in SPS activity (under V_{max} conditions) was found in plants of *D. antarctica* growing under field conditions in the vicinity of Punta Arenas (55° S) during the winter. These plants also showed an increase in SPS activity at the end of the night period (data not shown). This increase occurred at a similar time of the day as in laboratory plants. During the sampling for this field experiments, the maximum noon light intensity at the top of the canopy of *D. antarctica* was close to 200 μmol photons m^{-2} s^{-1}.

SPS activity did not follow SPS relative protein content during continuous light treatment

Sugar content, SPS activity, measured as percent of initial activity, and relative protein content were determined during 56 h of continuous light at low intensity (60 ± 10 μmol photons m^{-2} s^{-1}) (Figures 2a–c). Whereas total soluble sugars (TSS) and Suc had declined significantly by the second day of continuous light (Figure 2a), SPS activity did not show significant differences (Figure 2b) even though SPS protein levels showed visible although not statistically significant (with the exception of the last two points) differences between first and second day of the treatment (Figure 2c). We did not find a correlation between SPS activity and sucrose content during continuous low light treatment ($r = -0.24$).

SPS activity is greatly enhanced by the combined effects of long days and cold without a concomitant increase in SPS protein

SPS activity and SPS relative protein content were determined during a 21 day cold acclimation

Table 1. SPS V_{max} and V_{lim} activity during dark–light transition in leaves of *Deschampsia antarctica*. Plants were kept at 15 °C, LD = 21/3 h light/dark photoperiod and 100 ± 10 μmol photons m^2 s^{-1}

Time (h)	SPS activity (U g^{-1} dry wt)	
	Limiting conditions (V_{lim})	Non limiting conditions (V_{max})
12:30	1.27 ± 0.18	1.50 ± 0.22
13:30	0.68 ± 0.09	0.85 ± 0.15
14:30	1.98 ± 0.31	2.02 ± 0.35
15:30	1.32 ± 0.20	1.55 ± 0.25

The dark period occurred between 12:00 to 15:00 h, as shown in Figure 1. Values are expressed in U g^{-1} fresh weight (1 U represents the oxidation of 1 μmol of NADH per min. at 30 °C and pH 7.5). Values represent the mean of four independent samples ± standard error.

treatment at different day lengths (SD, MD and LD). In general, cold acclimation under SD and MD conditions caused a decrease in SPS activity (Figures 3d, e) with the exceptions of SD cold acclimated plants, in which the SPS activity increased 26% after 21 days of exposure. SPS activity also increased 12% at 7 days of cold acclimation under MD conditions. LD stimulated SPS activity even in nonacclimated plants, exhibiting 49% increase after 14 days of treatment (Figure 3f). In LD cold acclimated plants, SPS activity increased 17% after 7 days and reached nearly 250% stimulation after 21 days of cold acclimation (Figure 3f). SPS relative protein content increased, at least transiently in cold acclimated plants at all day lengths (Figures 3a–c). In SD the highest increase in SPS protein content was observed after 14 days of cold acclimation (Figures 3a and 4) whereas in MD and LD the highest SPS protein contents were observed after 2 days of cold acclimation (Figures 3b, c and 4). After 21 days, the relative amount of SPS protein at MD and LD was similar between non-acclimated and cold acclimated plants at the same day length (Figures 3b, c). The absolute amount of SPS protein content was higher in SD cold acclimated plants than in MD and LD cold acclimated plants (Figure 4). Additionally, the amount of total protein extracted from leaves was three times higher in 14 day cold acclimated plants under SD compared to the amount at the beginning of the cold acclimation treatment. Under MD and LD, the amount of total protein extracted from leaves was two times higher at the end of cold acclimation treatment, compared to the content at the beginning of the treatment.

The increase in SPS activity observed after 21 days of LD treatment (Figure 3f) was not preceded by an increase in SPS protein content (Figure 3c). However, at MD, as SPS activity decreased, SPS protein content also decreased (Figures 3b, e).

The combined treatment of cold and day length showed an increase in SPS transcript level (3.4 Kb

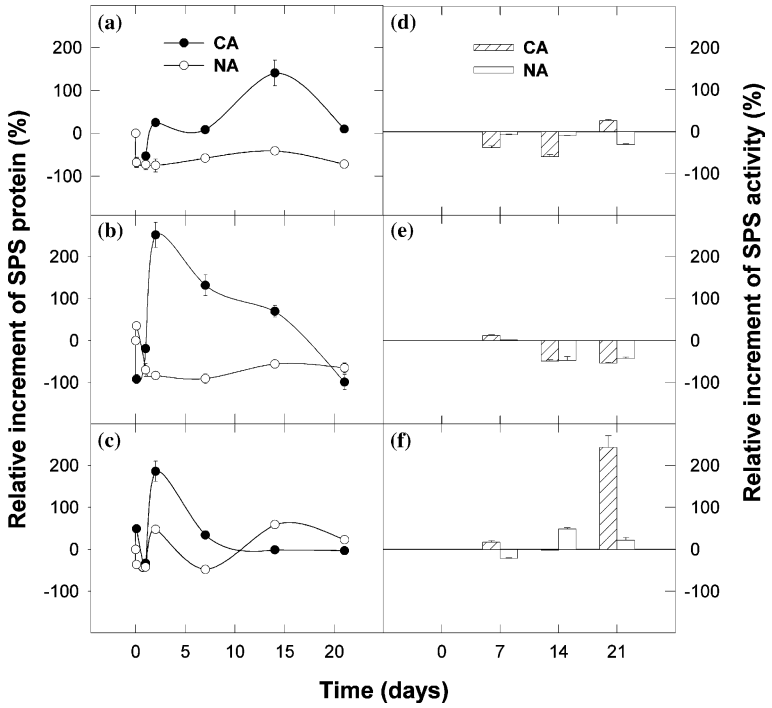

Figure 3. Effect of cold and day length on SPS activity and SPS protein content in leaves of *D. antarctica*. Left side: relative content of Sucrose-P-synthase (SPS) protein in cold acclimated plants (black circles) and in non-acclimated (white circles) at different day length: a, b and c. Right side: relative SPS activity in plants cold acclimated at 4 °C (dashed box) and in non-acclimated (white box) at different day lengths, d, e and f (SD = 8/16 h; MD = 16/8 h and LD = 21/3 h). The values are expressed in % compared with the zero time. The initial absolute value of SPS activity at 0 time was 1.40 ± 0.07 U g^{-1} dry wt. All values shown above are relative to this control. Each value represents the mean of three independent samples ± standard error.

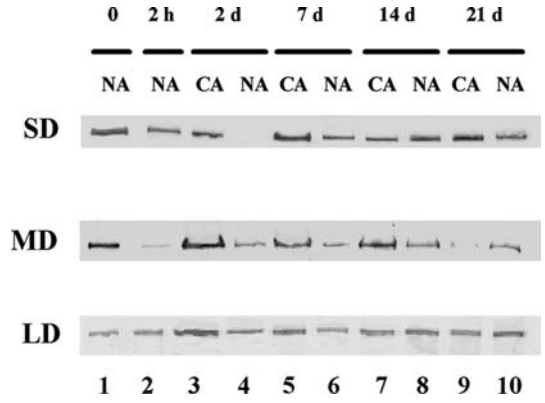

Figure 4. Effect of cold and day length on SPS protein. Western blot showing SPS in cold acclimated (4 °C) and non-acclimated (15 °C) plants for 21 days at different day lengths: SD = 8/16 h, MD = 16/8 h and LD = 21/3 h. Each line was loaded with 10 μg of total protein, lines correspond to the following samples, 1: zero time, 2: non-acclimated for 2 h, 3: cold acclimated for 2 days, 4: non-acclimated for 2 days, 5: cold acclimated for 7 days, 6: non-acclimated for 7 days, 7: cold acclimated for 14 days, 8: non-acclimated for 14 days, 9: cold acclimated for 21 days and 10: non-acclimated for 21 days.

band), only in SD plants after 14 days of cold acclimation. after which its expression decreased at 21 days (Figure 5). This increment coincides with an increase in the SPS protein content (Figure 3a).

Discussion

SPS activity was higher during light periods than early to middle of the dark period (Figure 1). This indirect light regulation of SPS in *D. antarctica* is not driven by changes in the level of SPS protein. This is similar to that shown for other species like maize and spinach in which SPS is deactivated in darkness by phosphorylation of a regulatory Ser residue. This effect is reversed in light by a type 2A protein phosphatase, resulting in an activated SPS as a consequence of an increase in the affinity for the substrates and the positive modulator (Glu 6-P), and a decrease in Pi sensitivity (Huber and Huber 1996). The light regulation observed in *D. antarctica* supports the hypothesis that during long day conditions this plant can maintain high SPS activity during the light period. *D. antarctica* grows at low temperature and long days during the Antarctic summer. In this situation, high SPS activity could be correlated with an accumulation of sucrose in leaves, which are the most exposed organs to changes in temperature. Laboratory cold acclimated plants under LD conditions show a high correspondence between leaves sucrose content and SPS V_{max} activity (Zúñiga-Feest et al. 2003). Perhaps, a high SPS activity during the Antarctic summer (long days and low temperature) may provide the necessary carbohydrate supply that allows a rapid growth of vegetative and reproductive structures.

Okadaic acid added to *D. antarctica* abraded leaves abolished the activation observed by the end of the dark period (Figure 1), similarly to what has been observed in studies with other species using phosphatase inhibitors (Jones and Ort 1997; Pathre et al. 2000). Okadaic acid is an extremely potent inhibitor of protein phosphatases-1 and -2A (Hardie 1993). Since the concentrations of protein phosphatases-1 and 2A in intact cells are usually 0.1–1 μmol/l, at least this range of inhibitor is required to completely inhibit protein phosphatases in intact cells (Hardie 1993).

In tomato plants (*Lycopersicon esculentum*), SPS is regulated by protein phosphorylation and

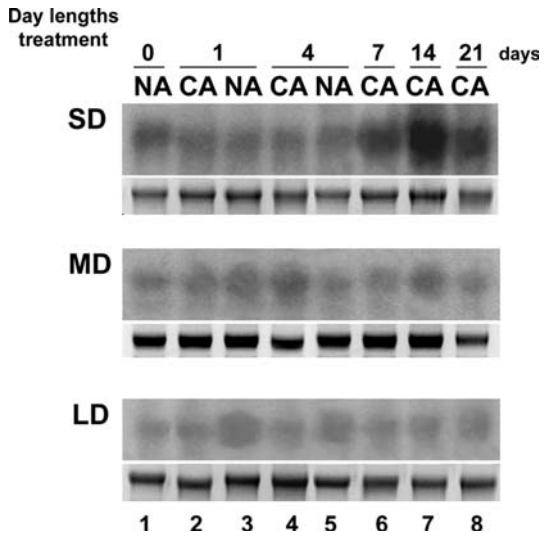

Figure 5. Effect of cold and day length on SPS expression. Northern blot showing mRNA-SPS signal in cold-acclimated plants at 4 °C for 21 days at different day lengths: SD = 8/16, MD = 16/8 h and LD = 21/3 h (above) and 28 S ribosomal RNA was used as a control (below). Each line was loaded with 10 μg of total RNA, lanes correspond at the follows samples, 1: zero time, 2: cold acclimated for 1 day, 3: non-acclimated for 1 day, 4: cold acclimated for 4 days, 5: non-acclimated for 4 days, 6: cold acclimated for 7 days, 7: cold acclimated for 14 days, 8: cold acclimated for 21 days.

shows a circadian pattern of activity. SPS is most active in its dephosphorylated state, which normally coincides with day time (Jones and Ort 1997). If phosphatase expression and activity is influenced or not by day length is something that should be studied in *D. antarctica*. An increase in SPS activity, without changes in SPS protein, could be explained by an increase in phosphatase activity in LD cold acclimated plants. Diurnal changes in SPS activity have also been observed in fully expanded leaves of cotton, and, again, were correlated positively with assimilate export rate during the photoperiod (Hendrix and Huber 1986). These results are strong support of the notion that SPS activity contributes to the control of C-flux into Suc, and thus, can be taken as an indicator of the capacity for Suc synthesis (Huber et al. 1985). The main control of SPS activity *in vivo* and sucrose biosynthesis is likely to be the cytosolic concentration of activator (Glu-6-P) and inhibitor (Pi) and the high flux of sucrose to fructans production in the vacuole. The capacity of *D. antarctica* to sustain a high SPS activity in LD cold acclimated state is most likely related to its high photosynthetic rate at low temperature, because it could maintain a high content of sugar phosphates and low Pi in the cytosol.

In contrast to the response of spinach, *D. antarctica* SPS activity presented differences between light and dark measured under both limiting (Table 1) and non-limiting conditions (Figure 1). In spinach leaves, the effect of phosphorylation on SPS activity is only evident when assayed under limiting substrate concentration (V_{lim}), near physiological conditions (Stitt et al. 1988; Lunn and Hatch 1997). In spruce needles, SPS activity was shown to be relatively insensitive to P_i, and only V_{max} (under non-limiting substrate concentration) was found to be affected by phosphorylation (Loewe et al. 1996). In *Prosopis juliflora*, both V_{max} and V_{lim} SPS activities increased in light and decreased in the dark (Sinha et al. 1997). Light modulation of SPS activity can be observed in a variety of species, but there are differences in the extent to which V_{lim} and/or V_{max} are affected (Ohsugi and Huber 1987; Winter and Huber 2000). Light modulation of SPS activity is thought to be one mechanism to adjust the capacity for Suc biosynthesis in relation to the rate of photosynthesis, which is a function of irradiance, intercellular [CO_2] and other factors (Winter and Huber 2000). A robust ability to adjust SPS activity even with high sucrose concentration could enable *D. antarctica* to sustain a high photosynthetic performance in environments like Maritime Antarctic, where irradiance changes dramatically while temperature remains low.

Endogenous rhythm of SPS activity

Deschampsia antarctica SPS activity did not show a clear endogenous rhythm of activity during continuous light treatment (Figure 2b). By the end of the period under continuous light, however, a fluctuation in activity appears to have occurred much as seen in Figure 1. Moreover, SPS activity increased prior to the state of the light period. Perhaps, there is a rhythm that was not detected because too few points were sampled. Other species, such as soybean (*Glycine max*) (Keer et al. 1985) and tomato (Jones and Ort 1997), have an endogenous rhythm of activity. The endogenous continuous light circadian rhythm of SPS activity in tomato is arrested by overnight low-temperature treatment (4 °C) (Jones et al. 1998) and impairs next-day photosynthesis by as much as 60% (Martin et al. 1981). Unlike the chilling sensitive tomato, cold tolerant plants, such as *D. antarctica*, do not have a cold sensitive endogenous rhythm of SPS activity. Indeed the capacity of *D. antarctica* to sustain high SPS activity during long cold days appears to be the foundation of its ability to grow in cold environments. There is a positive correlation between SPS V_{max} activity and sucrose content in the leaves of cold acclimated *D. antarctica* under long day conditions (Zuñiga-Feest et al. 2003). However, during continuous low light treatment we did not observe a similar correlation ($r = -0.24$). This could be due to a decrease in sucrose transport rate and activity of sugar sinks at low temperature. Therefore, in cold acclimated plants, sucrose accumulation in the leaves would follow SPS activity. At higher temperatures, sucrose could be transported or used in growth. In fact, *D. antarctica* presents the highest growth rate at long day treatment and 15 °C (Bravo et al. 2001). As expected, sucrose accumulation in leaves did not follow SPS activity under continuous light treatment at 15 °C.

Combined effect of cold and day length on SPS expression

SPS relative protein content increased in cold acclimated plants at all day lengths studied (Figures 3 and 4). In *D. antarctica*, a coincident increase in SPS transcript level and SPS relative content of protein was observed only at 14 days of cold acclimation in SD (Figures 3 and 5). Cold acclimated plants under MD and LD exhibited lower increments of SPS protein content than under SD. Perhaps, *D. antarctica* increases SPS transcript level and total protein content, as a compensatory response triggered by day length shortening, thus sustaining carbon flux to store carbohydrates reserves in crown and roots necessary for the next growing season. SD conditions used in our experiments are similar to those found in the natural habitat by the end of March (beginning of fall).

Increased levels of enzymes related with carbohydrates synthesis have been reported frequently in cold acclimated plants. Cold acclimated *Arabidopsis* has increased amounts of sucrose biosynthesis pathway enzymes, like fructose-2, 6-biphosphatase and SPS (Strand et al. 1999). In cold acclimated winter wheat, increases in sucrose synthase (SS) and SPS (Santoiani et al. 1993) expression along with higher activity levels of these enzymes (Tognetti et al. 1990; Savitch et al. 1997) were observed. In contrast with these results, the enhancement of SPS activity in *D. antarctica* was not related to more SPS transcript or SPS protein content (Figures 4 and 5). It appears that changes in protein phosphorylation control *D. antarctica* SPS activity. This mechanism is the same as observed with the regulation in plants exposed to light/dark transitions, when relative protein content did not change significantly (Figures 1). Future research on the mechanism behind the increase in SPS activity should include treatment with okadaic acid in order to abolish the high SPS activity observed in cold acclimated leaves under LD conditions. If this is the case, this experiment would demonstrate that high SPS activity observed in cold acclimated plants under long days is the result of light SPS activation by phosphatase.

Our hypothesis that during long day conditions in the Antarctic summer *D. antarctica* can maintain a high SPS activity for more time by indirect light regulation leading to high sucrose accumulation in the leaves, appears to be true. *D. antarctica* SPS showed post-transcriptional light regulation that was abolish by okadaic acid treatment. Furthermore cold acclimated plants under LD conditions induced the highest SPS activity. *D. antarctica* in contrast to other Poaceae species presents an interesting response with respect to its SPS activity when plants are exposed to cold temperatures during LD, as in the Antarctic growing season (Zúñiga-Feest et al. 2003). Several authors have raised the question of which are the special features of this plant that allow its survival in the Antarctic (Alberdi et al. 2002; Lewis Smith 2003). It appears that there are a number of features that make possible its life in the cold Maritime Antarctic. Among this features are: high freezing tolerance (Bravo et al. 2001) the presence of HSP proteins (Reyes et al. 2003), high capacity to avoid photoinhibition (Xiong et al. 1999; Pérez et al. 2004), high capacity to maintain photosynthesis rate (Edwards and Lewis Smith 1988), high SPS activity and high capacity to adjust SPS activity.

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