Plant Physiol. (1991) 96, 887–891
0032-0889/91/96/0887/05/$01.00/0

Received for publication August 21, 1991
Accepted March 1, 1991

Sucrose Synthase Expression during Cold Acclimation in Wheat[1]

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ABSTRACT

When wheat (*Triticum aestivum*) seedlings are exposed to a cold temperature (2–4°C) above 0°C, sucrose accumulates and sucrose synthase activity increases. The effect of a cold period on the level of sucrose synthase (SS) was investigated. Using antibodies against wheat germ SS, Western blots studies showed that the amount of the SS peptide increased during 14 days in the cold, when plants were moved from 23°C to 4°C. The level of SS diminished when plants were moved back to 23°C. Northern blots of poly(A)$^+$ RNA, confirmed a five- to sixfold induction of SS in wheat leaves during cold acclimation. These results indicate that SS is involved in the plant response to a chilling stress.

A sudden decrease in the temperature at which plants are growing is known as a chilling stress (16). Its effect in plant metabolism may be different according to plant species, but it is generally accompanied by an immediate cessation of plant growth while photosynthesis continues at a decreased rate. The result is a net accumulation of photosynthetic products due to the growth stoppage (16). In many plants this is seen as an accumulation of sucrose and of oligosaccharides derived from it (2, 13, 28). Calderón and Pontis (2) showed that the activity of SS[3] (UDP-glucose: D-fructose-2-glucosyl transferase, EC 2.4.2.13), one of the sucrose metabolizing enzymes, starts to increase within 1 h after the beginning of the cold stress and continues rising during the acclimation period (28). The increased activity of SS may be due to an activation or to a *de novo* synthesis.

The genes encoding SS have been studied thoroughly in maize, where two, Sh1 and Ss2, have been described (4, 6, 7, 20). Echt and Chourey (10) have demonstrated that the corresponding isoenzymes have partial antigenic identity and very similar kinetic properties. The sucrose synthase corresponding to Sh1 gene is a homotetramer composed of subunits of 92 kD which is mainly expressed in the endosperm (5, 27, 30). The genes have been cloned and sequenced (11, 12, 30).

We report here, using antibodies against wheat germ SS that cross react with leaf SS (15), and an heterologous clone from maize (11), that the amount of SS increases during cold acclimation, suggesting that the enzyme may be involved in the response of plants to a chilling stress.

MATERIALS AND METHODS

Biological Material

Wheat (*Triticum aestivum* L. cv San Agustin INTA) seeds were kindly supplied by the Instituto Nacional de Tecnologia Agropecuaria (Balcarce, Argentina). A cDNA clone (pKS500) corresponding to the maize Sh1 sucrose synthase was kindly provided by Dr P. Starlinger (Köln, FRG).

Growth Conditions

Wheat plants were grown in vermiculite in a chamber at 23°C for 7 d with a day/light regime of 14/10 h. Irradiance at the plant canopy was 100 $W \cdot m^{-2}$. Seven days following emergence, seedlings were transferred to 4°C with the same day/night regime and irradiance. Chilling treatments were always started at the beginning of daytime (0700 h). Similarly, plants were always returned to 23°C at the same time of the day to minimize the effect of diurnal fluctuations in enzyme activity (28).

Protein Extraction

Wheat leaves were excised, weighed (0.5 g), and ground in liquid nitrogen with a mortar and pestle. The powder was suspended in 500 μL of 100 mM Hepes buffer (pH 7.5), 2% ethylene glycol, 10 mM $MgCl_2$, 2 mM EDTA, 20 mM mercaptoethanol, and 0.5 mM PMSF. Extracts were centrifuged at 13,000*g* for 30 min, and the clarified supernatant was immediately used for different determinations.

Proteins were quantified by the method of Bradford (1).

Preparation of SS Antibodies

Specific antibodies against SS were prepared in rabbits, using an enzyme purified from wheat germ according to Salerno and Pontis (25) with two further purification steps: gel filtration on Bio-Gel A-1.5 m followed by preparative electrophoresis on polyacrylamide gel. Fractions with SS activity from the chromatography on Bio-Gel were concentrated in an Amicon ultrafiltration cell with PM-10 membrane,

[1] Supported by Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET). M. D. C., H. G. P., and G. L. S. are Career Investigators of the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET), Argentina. E. J. Z. is a fellow of CONICET.

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[3] Abbreviation: SS, sucrose synthase.

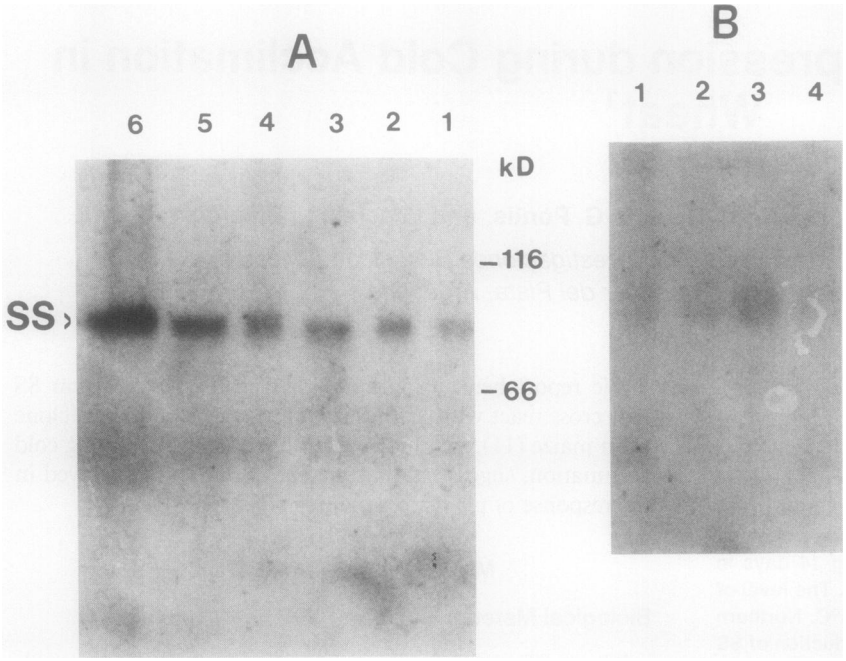

Figure 1. SS level during cold acclimation and deacclimation. A, SS accumulation in wheat leaves acclimated at 4°C; 20 μg of proteins from wheat seedlings were loaded on each lane of a 10% SDS-PAGE gel and immunoblotted (see "Materials and Methods"). Seedlings were grown at 25°C for 7 d, moved to 4°C ($T = 0$), and kept in the cold for 14 d. Lanes 1 to 6: 0, 1, 3, 7, 10, and 14 d at 4°C. B, Reversal of SS accumulation during deacclimation. SDS-PAGE and immunoblot of proteins from seedlings grown: at 25°C for 7 d (lane 1) or 14 d (lane 2); 7 d at 25°C and moved to 4°C for 7 d more (lane 3); 7 d at 25°C, moved to 4°C for 7 d and moved back to 25°C for 3 d (lane 4). Extraneous spots in lanes 3 and 4 of panel B occurred in film processing.

layered on a 7.5% native polyacrylamide gel, and electrophoresed for 14 h. Strips from the gel were analyzed for SS activity (15). The band corresponding to the enzyme was cut, homogenized in 3 mL of Freund's complete adjuvant, and injected intramuscularly into a white rabbit (15). The procedure was repeated three times with intervals of 20 d. Ten days after the final injection, 30 mL of blood was collected by cardiac puncture and immunoglobulins were partially purified by ammonium sulfate fractionation and dialysis (8).

SDS-Polyacrylamide Electrophoresis and Immunoblotting

Proteins (20 μg) were separated by SDS-PAGE, according to Laemmli (14) with a 10% separating slab gel and transferred to nitrocellulose membranes as described (17). Nonspecific protein binding sites were blocked by incubating the membranes in PBS (20 mM sodium phosphate, 150 mM sodium chloride [pH 7.2], 2% bovine albumin) containing 1% nonfat powdered milk for 1 h at room temperature. Nitrocellulose filters were then incubated overnight at 4°C in primary antibody (1:500, rabbit anti-SS). The antigens were visualized after incubating the filters in a secondary antibody (alkaline phosphatase conjugated goat anti-rabbit) followed by developing with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

DNA Isolation and Blot Analysis (Southern Blot)

Total DNA was prepared from wheat (var Trigal 800) coleoptiles grown in the dark to reduce contamination from chloroplast DNA. Twenty grams of tissue were powdered in liquid nitrogen and were extracted with 50 mM Tris buffer (pH 8.0) containing 8 M urea, 0.35 M NaCl, 0.2 M EDTA, 1% sarkosyl, and 5% phenol. The mixture was extracted as described (26). DNA (10 μg) was restricted with about 100 units of endonucleases (Pharmacia) according to the manufacturer's protocol.

The DNA digested with *Eco*RI or *Hin*dIII was fractioned by agarose gel electrophoresis (0.8% agarose) and stained with ethidium bromide. The gel was irradiated on a UV lightbox for 10 min. DNA was denatured and transferred onto nylon membranes (Hybond N, Amersham) according to the manufacturer's directions. After the blotted membranes were baked at 80°C for 2 h, they were prehybridized for 5 h at 42°C in 5 × SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, 0.033 sodium pyrophosphate, 100 μg/mL denatured herring sperm, and 20% formamide. Hybridization for 16 h was performed with the same buffer (low stringency) system containing the DNA probe (50 μCi/μg) produced by random priming (Amersham). A cDNA clone (pKS500) corresponding to the maize Sh1 sucrose synthase (12, 20) was used. The insert was purified by low melting gel electrophoresis and by Elutips (Schleicher & Schuell). Following hybridization, blots were washed twice in 5 × SSC, twice in 2 × SSC, 0.1% (w/v) SDS. Washes were of 1 h duration and at 50°C. After washing, blots were exposed to x-ray film (Kodak X-AR5) for about 4 d at −70°C with intensifying screens.

RNA Isolation and Northern Blot

Total RNA was prepared from leaves ground in liquid nitrogen by two precipitations with lithium chloride 2 M as described (17). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose (Pharmacia) chromatography.

Total RNA (15 μg) or poly(A)$^+$ RNA were electrophoresed in 1.2% agarose gels containing formaldehyde (20%) and blotted onto Nytran (Schleicher and Schuell) membranes (Hybond, Amersham) according to the protocol which accompanied the filters. Blots were prehybridized for 5 h at 42°C with a solution consisting of 5% SSC, 10% Denhardt's solu-

tion, 0.5% SDS, 0.033 M sodium pyrophosphate (pH 7), 20% formamide, 250 μg denatured salmon sperm DNA previously sonicated and heat-denaturated, and 100 μg yeast tRNA. Hybridizations were carried out overnight under the same conditions but containing 50 ng of the radioactive probe. The blots were washed with 5% for 30 min at 50°C and then washed twice for 30 min with 2% SSC at 50°C. The blots were autoradiographed at −80°C and developed after 24 h.

In Vitro Translation and Immunoprecipitation

Poly(A)$^+$ RNA (2 μg) was translated in a reticulocyte lysate system from Amersham. SS peptide was immunoprecipitated from *in vitro* translation mixture by addition of 60 μL protein A-Sepharose in 0.75 M sodium chloride and 1% Triton X-100, and 70 μL of SS antibodies at 4°C for 12 h. The pellet was washed five times with 0.75 M sodium chloride and 5% Triton X-100. Polypeptides labeled with [^{35}S]methionine were fractionated by SDS-PAGE and autoradiographed (one month).

RESULTS

SS activity has been shown to increase when wheat plants were submitted to a cold shock (2, 29). To test if the increase is due to a change in the concentration or in the activity of the enzyme, whole cell extracts from plants acclimated at 4°C for 1, 3, 7, 10, and 14 d were assayed with the specific SS antibodies. Western blots shown in Figure 1A indicate that the amount of SS protein increased during cold acclimation. The level of increase reached four- to fivefold when plants were acclimated for 14 d. A similar increase in SS activity was reported by Calderon and Pontis (2) in cold stress plants. Tognetti *et al.* (28) have shown that wheat plants acclimated at 4°C return to the metabolic levels they had prior to the cold shock when plants are moved back to room temperature (23°C). Western blots indicate that SS levels returned also to the one existing before the cold shock (Fig. 1B). The modification of the protein level may be due to an enzyme synthesis or to a different rate of protein degradation. This question may be solved by studying mRNA levels.

To study SS expression in wheat, we used a recombinant plasmid (pKS500) containing a cDNA fragment of the Sh locus of maize that we tested first for its homology with wheat DNA sequences. Total DNA from wheat seedlings was isolated, purified, and digested with *Eco*RI and *Hin*dIII as described. Under low stringency conditions, we detected a 4 kb band (Fig. 2) indicating at least a 70% homology. The effect of cold acclimation on SS mRNA level was investigated. Total RNA was prepared from control seedlings (25°C) and from cold acclimated seedlings, and used for the isolation of poly(A)$^+$ RNA through a oligo(dT)-cellulose column. In the mRNA blot analysis (Fig. 3A), a 3 kb band appeared increased upon cold acclimation to approximately five- to sixfold (lane 1) of the control (lanes 2 and 3). When mRNA from the 14 d cold-acclimated seedlings was translated *in vitro* and immunoprecipitated with the SS antibodies, a 92 kD peptide was revealed after autoradiography (Fig. 3B) that corresponds with the mol wt of the SS subunit. This result indicates the presence of SS mRNA in cold acclimated wheat plants.

DISCUSSION

Exposure of plants from temperate and cold climatic zones to chilling temperatures leads to an accumulation of fructans (9, 22, 24), which is preceded by an increase in the level of sucrose in the plant cell (3, 23). This increment may be caused by the low demand for photosynthates at low temperature (21). During growth under this condition the metabolism undergoes an adaptation process (16). When wheat seedlings were exposed to chilling temperatures the activity of SS started to increase within 1 h after the beginning of the stress (2). Calderón and Pontis suggested that the increase in SS activity was connected with the transport of sucrose to the vacuole where it is used for fructan synthesis or it accumulates as sucrose.

This report presents evidence that during cold acclimation SS protein level rises. Moreover, the presence of an increased amount of SS mRNA in plants acclimated to 14 d of cold seems to rule out the possibility of a lower rate of degradation of the enzyme during the period at 4°C. However, the protein level returns to the one found at 23°C when plants are

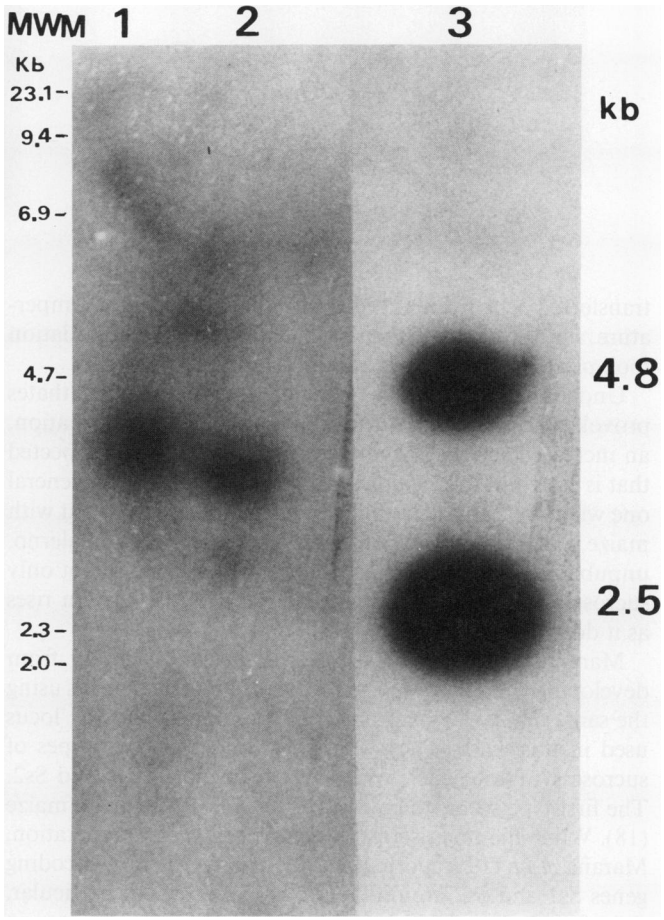

Figure 2. Southern blot analysis of wheat genomic DNA; 20 μg DNA was restricted with *Eco*RI (lane 1) and *Hin*dIII (lane 2). The SS probe used was from the pKS500 insert (see "Materials and Methods"). Control, pKS500 (lane 3); MWM, mol wt markers, lambda DNA *Hin*dIII digest.

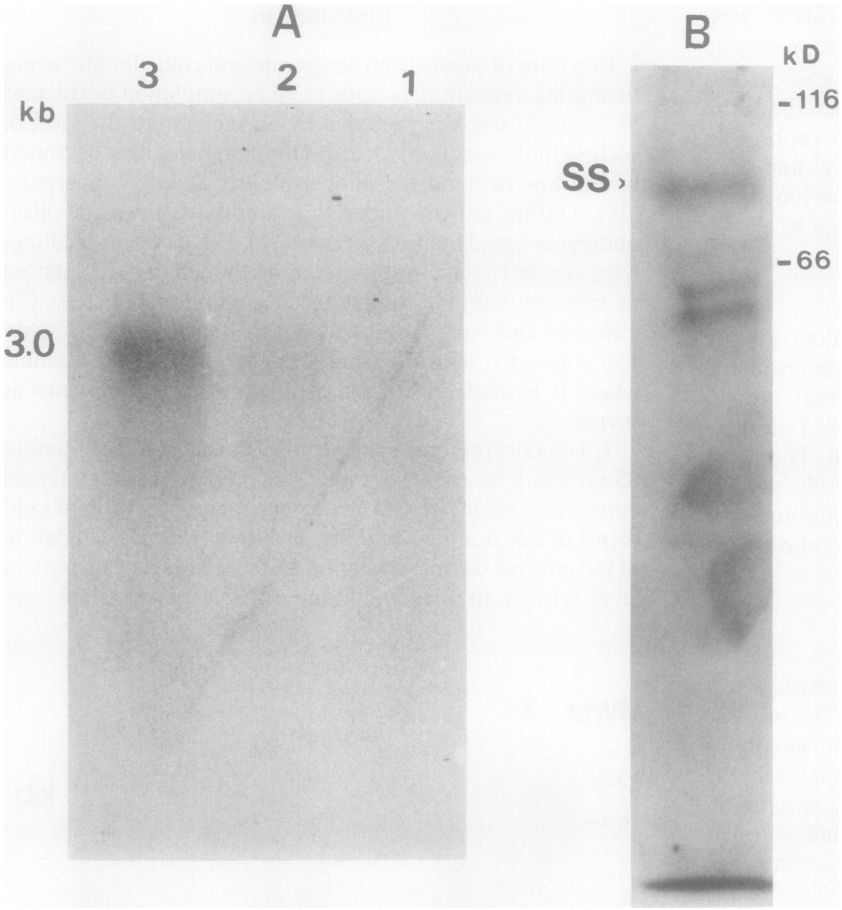

Figure 3. A, Northern blot analysis of poly(A)$^+$ RNA from wheat leaves probed with the pK500 insert. Seedlings were grown at 25°C for 7 d (lane 1) or 14 d (lane 2), or 7 d at 25°C and 7 d at 4°C (lane 3). B, Immunoprecipitation of *in vitro* translation products of poly(A)$^+$ RNA from cold acclimated leaves. ^{35}S-Labeled translation products from mRNA of seedling grown 7 d at 25°C and moved to 4°C for 7 d more were immunoprecipitated with the SS antibodies followed by SDS-PAGE and autoradiography.

transferred back from a cold environment to a warm temperature, suggesting that either an increased enzyme degradation took place or its synthesis has diminished.

Under the condition of low demand for photosynthates provoked by the chilling shock and subsequent acclimation, an increased supply of cytoplasmic sucrose may be expected that is stored in the vacuole. This condition may be a general one whenever a plant is chilled. Experiments carried out with maize, a plant that does not accumulate fructans (GL Salerno, unpublished data) bear this contention out, because not only sucrose synthase activity increases but also the protein rises as it does in wheat.

Maraña *et al.* (18) have screened a cDNA library from developing wheat endosperm for sucrose synthase clones using the same maize cDNA probe corresponding to the Sh1 locus used in this work. They were able to identify two types of sucrose synthase genes, which were designated Ss1 and Ss2. The first type seems to be equivalent to Sh1 gene from maize (18). While the manuscript of this report was in preparation, Maraña *et al.* (19) reported that the two types of SS encoding genes Ss1 and Ss2 are differentially expressed. In particular, they found that the Ss1 mRNA level sharply increases in response to anaerobiosis and cold shock, confirming our results.

In conclusion it seems that SS is one of the proteins that is preferentially expressed during cold suggesting that the enzyme may be involved in the response of plants to a chilling stress. It remains to be seen if the hypothesis suggested here regarding the role of SS is borne out by experimental data.

ACKNOWLEDGMENTS

The able technical assistance of Ing. Clara Fernández and Miss Carmen Rodriguez is gratefully acknowledged.

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