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Analysis of cDNA Clones Encoding Sucrose-Phosphate Synthase in Relation to Sugar Interconversions Associated with Dehydration in the Resurrection Plant *Craterostigma plantagineum* Hochst.[1]

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Sucrose-phosphate synthase (SPS) is a key enzyme in the regulation of sucrose metabolism, being responsible for the synthesis of sucrose 6-phosphate from fructose 6-phosphate and uridine 5′-diphosphate-glucose. We report on the isolation and characterization of cDNA clones encoding SPS from *Craterostigma plantagineum* Hochst., a resurrection plant in which the accumulation of sucrose is considered to play an important role in tolerance to severe protoplastic dehydration. Two distinct classes of cDNAs encoding SPS were isolated from *C. plantagineum*, and are represented by the clones Cpsps1 and Cpsps2. The transcripts corresponding to both cDNAs decrease to very low levels in dehydrating leaves of *C. plantagineum*. Only the Cpsps1 transcript occurs in the roots, where it is present at a higher level than in leaves and increases upon dehydration of the plant. Higher enzymatic activities have been determined in protein extracts of dehydrated tissues compared with untreated tissues, which correlates with an increase in protein levels. It is suggested that the overall regulation of SPS is strongly influenced by the changing composition of the cytoplasm in *C. plantagineum* leaves during the dehydration-rehydration cycle.

The southern-African plant *Craterostigma plantagineum* Hochst. (Scrophulariaceae) belongs to a small group of angiosperms in which the mature plants are able to tolerate severe desiccation (Gaff, 1971). The desiccated vegetative tissues rehydrate completely upon rewatering: full physiological activity is resumed within several hours, and changes caused by dehydration are gradually reversed (Bartels et al., 1990; Schneider et al., 1993; Bernacchia et al., 1996). Drought stress in plants usually causes a cessation of normal metabolism and growth, irreversible damage to membranes and proteins, and a disruption of subcellular organization (Hsaio, 1973). Therefore, the desiccation tolerance of resurrection plants suggests that any injuries produced by dehydration and subsequent rewatering are minimized by protection or repair mechanisms. Research with yeasts, animals, and fungi has shown that the disaccharide trehalose is important in surviving desiccation (Crowe et al., 1983, 1984, 1992; Colaço et al., 1992). Although trehalose is extremely rare in plants, it has been suggested that Suc and other sugars may fulfill a similar role (Crowe et al., 1992).

In the resurrection plant *C. plantagineum* the sugar composition is unusual in having an extremely high concentration of the C8 sugar 2-octulose in fully hydrated leaves (89% of the water-soluble carbohydrates; Bianchi et al., 1991). During dehydration of the plant the concentration of 2-octulose decreases, whereas the concentration of Suc in the leaves increases to 90% of the total sugar content. It is not clear by what pathway these sugar conversions occur, although structural analysis indicates that both the Glc and the Fru configurations are contained in the octulose molecule (Howarth et al., 1996). The sugar composition in roots is different from that in leaves: there is no conversion from 2-octulose to Suc during dehydration, but in the rehydrated and dehydrated state there are high constitutive levels of stachyose, another soluble sugar (Schwall et al., 1996).

Earlier studies with resurrection plants and with seeds have demonstrated that the accumulation of Suc parallels the acquisition of desiccation tolerance (Bianchi et al., 1991; Leprince et al., 1993). There are clear precedents indicating that Suc could act as an osmoprotectant in plants. A role for Suc in simple osmotic adjustment during mild drought is suggested by reports that increased Suc synthesis can be a drought response of plants that cannot withstand extreme desiccation, such as spinach (*Spinacia oleracea*; Quick et al., 1989). Furthermore, in permitting survival during severe dehydration, it appears likely that Suc can protect the cell by the formation of glass (Koster, 1991) and by direct interaction with macromolecules (Crowe et al., 1992). A potential role for glass would be in permitting a stable, quiescent state by restricting molecular diffusion (Koster, 1991).

To investigate the sugar conversions related to the desiccation tolerance of *C. plantagineum*, it was considered a priority to isolate and characterize cDNA clones encoding

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Abbreviations: RWC, relative water content; SPS, Suc-P synthase.

key enzymes such as SPS (EC 2.3.14). SPS is considered to be the major enzyme in Suc synthesis, catalyzing the production of Suc-6-P and UDP from Fru-6-P and UDP-Glc (for review, see Huber and Huber, 1996). The importance of SPS in carbohydrate metabolism has been confirmed with transgenic tomato (*Lycopersicon esculentum*) plants expressing high levels of SPS. These plants had lower levels of leaf starch and increased concentrations of Suc (Worrell et al., 1991). In spinach and maize (*Zea mays*) enzymatic activity of SPS is regulated via reversible phosphorylation (Huber and Huber, 1992), suggesting that the enzyme also has an important role in the regulation of Suc synthesis.

Here we report a detailed study of SPS cDNA clones isolated from the desiccation-tolerant plant *C. plantagineum*. Two classes of SPS transcripts were distinguished, with low levels in dehydrated leaves and distinct expression patterns in tissues in different states of hydration. The level of SPS protein, in contrast, was less variable than the mRNA level and increased during dehydration.

MATERIALS AND METHODS

Plants and callus of the resurrection plant *Craterostigma plantagineum* (Hochst.) were grown under controlled environmental conditions as described by Bartels et al. (1990). In dehydration experiments water was allowed to drain from the pots of mature plants and was then withheld for up to 9 d, by which point the leaf RWC was a small percentage of that in untreated plants. In rehydration experiments 9-d-dehydrated plants were initially fully submerged in water, and then maintained with the entire root system in water for 24 to 48 h. For ABA treatments leaves were excised 2 to 3 h into the light period and placed on water containing 1 mM ABA for 24 h. Callus was maintained on growth medium supplemented with 20 μM ABA. Tissue samples from plants 2 to 3 h into the light period for protein and nucleic acid extractions were detached and immediately frozen in liquid N_2. Leaf RWC was expressed as a percentage of the value for untreated leaves (Bernacchia et al., 1996).

SPS Extraction and Assay

Crude extracts of *C. plantagineum* tissue were prepared by grinding leaves or roots in liquid N_2 and extracting in Hepes buffer (50 mM Hepes-KOH, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mg mL^{-1} BSA, 14 mM β-mercaptoethanol, 20% [v/v] polyvinylpolypyrrolidone, 0.05% [v/v] Triton X-100, pH 7.6). After centrifuging for 15 min at 20,000*g* and 4°C, the supernatant was desalted through Sephadex G-25 columns and eluted with extraction buffer lacking polyvinylpolypyrrolidone, β-mercaptoethanol, and Triton X-100, and with 15% (v/v) glycerol. The protein concentration was measured according to the method of Bradford (1976), and an aliquot containing between 50 and 100 μg of protein was used to determine SPS activity by measuring the amount of Suc or UDP produced. For both assays the reaction mixture contained UDP-Glc (7.5 mM), Fru-6-P (7.5 mM), Glc-6-P (37.5 mM), $MgCl_2$ (5 mM), and EDTA (1 mM) in 50 mM Hepes-KOH buffer, pH 7.4, in a total volume of 0.3 mL for the Suc assay or 1 mL for the UDP assay.

Reactions were incubated at 30°C for 20 min and stopped by the addition of 0.3 mL of 30% (w/v) KOH and heating at 100°C for 15 min (Suc assay), or by heating at 100°C for 2 min (UDP assay), and were centrifuged for 2 min at 13,000*g*. Suc was assayed by adding 1 mL of anthrone reagent (150 mg of anthrone in concentrated H_2SO_4:H_2O [7.6:3, v/v]) to an aliquot of the reaction mixture, and incubating at 40°C for 40 min before measuring A_{620}. The measurement of free UDP was based on that of Stitt et al. (1988): an aliquot of the reaction mixture was added to test buffer (50 mM Tris-HCl, pH 7.6, 20 mM KCl, 1 mM $MgCl_2$, 0.4 mM PEP, 0.15 mM $NADH/H^+$) in a final volume of 1 mL, and the concentration of UDP was calculated from the decrease in A_{340} following the addition of 5 units of pyruvate kinase and 15 units of lactate dehydrogenase. For both Suc and UDP assays controls without Fru-6-P and Glc-6-P were carried through for all samples.

Nucleic Acid Preparations

Total RNA was extracted from tissues of *C. plantagineum* using the method of De Vries et al. (1986), but with the addition of two phenol extractions and one extraction with chloroform containing 1% (v/v) isoamylalcohol, before precipitation with lithium chloride. Poly(A)$^+$ mRNA was isolated by oligo(dT) chromatography (Bartels et al., 1990). Genomic DNA was isolated using the genomic tip 100/G following the instructions of the supplier (Qiagen, Hilden, Germany).

SPS cDNA Isolation

SPS cDNA sequences were amplified from the total pool of *C. plantagineum* cDNA using two degenerate primers designed with reference to the known gene sequences (Worrell et al., 1991; Klein et al., 1993; Sonnewald et al., 1993; Hesse et al., 1995). The DNA sequences of these primers, from the 5′ to the 3′ termini, were (a) GCGGAATTCGTIAA(A or G)GCITT(T or C)GGIGA(G or A)TG; and (b) GCGGGATCCTCIACIGGICCICC(A or G)TT(T or C)TT (where I represents 2′-deoxyinosine 5′-triphosphate). The predicted length of sequence amplified (lying between the primers) was just under 300 nucleotides.

cDNA for the PCR was synthesized in a standard first-strand reaction (Huynh et al., 1985) using 2 μg of poly(A)$^+$ RNA from dried leaves with 20 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Philadelphia, PA). The cDNA was subjected to 35 cycles of amplification in a 20-μL reaction mixture containing 1.4 μM of each primer, 0.63 mM each of dATP, dTTP, dGTP, and dCTP, 1.5 mM $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase, with the buffer supplied by the manufacturer (Amersham). Each amplification cycle consisted of 40 s of denaturation at 94°C, 40 s of annealing at 50°C, and 3 min of extension at 68°C. The restriction enzyme recognition sites for *Eco*RI and *Bam*HI in primers 1 and 2, respectively, facilitated cloning of the amplified products into the pBluescript SK+ vector. The amplified cDNA sequence was used as a spe-

cific probe to screen 8.0×10^4 plaque-forming units of a λ-ZAP II (Stratagene) cDNA library constructed from mRNA of ABA-treated *C. plantagineum* callus tissue (Eggestein, 1993).

Protein Analyses

For the preparation of proteins, tissue was ground in liquid N_2 and was directly dissolved in Laemmli sample buffer (Laemmli, 1970). The protein concentration was determined according to the method of Bradford (1976) using the Bio-Rad kit after removal of SDS by precipitation with potassium phosphate. Loading on gels was also checked by staining the blot with Ponceau-S before probing. Electrophoresis under denaturing conditions was performed as described by Laemmli (1970) using 7.5% (w/v) SDS-polyacrylamide gels. Western blots were probed with the SPS antiserum at a dilution of 1:1,000 prior to incubation with anti-rabbit IgG (Sigma; 1:10,000 dilution). The protein-antibody complex was detected using the chemiluminescence (ECL, Amersham) western-blotting detection system from Amersham. The blots were scanned with a laser densitometer (LKB 2202 Ultrascan, Bromma, Sweden) attached to an integrator (model 2220, LKB), and the blots were normalized for comparison of protein levels.

Preparation of Antiserum

From a Cpsps1 cDNA clone, a *Bam*HI-*Sal*I fragment corresponding to Cpsps1 from nucleotides 744 to 1852 was ligated into the expression vector pGEX-4T-2 (Smith and Johnson, 1988), yielding a translational fusion with glutathione *S*-transferase. A 67-kD protein was expressed upon exposure of plasmid-containing *Escherichia coli* to 0.4 mM isopropylthio-β-galactoside, and was partially purified by isolation of inclusion bodies (Schmidt et al., 1986). The protein was further purified by preparative SDS-PAGE, and the protein-containing band was excised from the gel and then freeze-dried for direct injection into a rabbit (Eurogentec, Seraing, Belgium).

Nucleic Acid Analyses

Northern-blot (RNA) and Southern-blot (DNA) analyses were carried out as described by Bartels et al. (1990). For Cpsps1 a *Sal*I fragment was isolated, which corresponded to nucleotides 439 to 1852; for Cpsps2, a *Kpn*I fragment was isolated from the 3′ end starting at nucleotide 2170. These fragments were labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1984). The specificity of the two probes was verified by Southern-blot analysis: the probes did not cross-hybridize with each other. For northern-blot analysis equal amounts of RNA were loaded, which was monitored by reprobing the blots with a ubiquitin-cDNA clone (Gausing and Barkardottir, 1986).

DNA Sequencing and Sequence Analysis

The cDNA clones were sequenced on both strands using the dideoxynucleotide chain termination method (Sanger et al., 1977) with a sequencing kit (Prism, Applied Biosystems). The reactions were resolved on a DNA sequencer (model 373A, Applied Biosystems). Analyses of both nucleic acid and amino acid sequences were performed using software from the University of Wisconsin Genetics Computer Group (version 8; Devereux et al., 1984).

RESULTS

SPS Enzyme Activities

During a dehydration series protein extracts were prepared from leaves and roots, and it was found that the SPS activity, as measured by the formation of Suc, increased between approximately 3-fold (in leaves) and 2-fold (in roots) during dehydration (Fig. 1). Absolute values were in the range of 28.4 (in fresh leaves) to 100.8 (in dried leaves) nmol Suc min^{-1} mg^{-1} protein and 87.2 (in untreated roots) to 165.3 (in dried roots) nmol Suc min^{-1} mg^{-1} protein. The

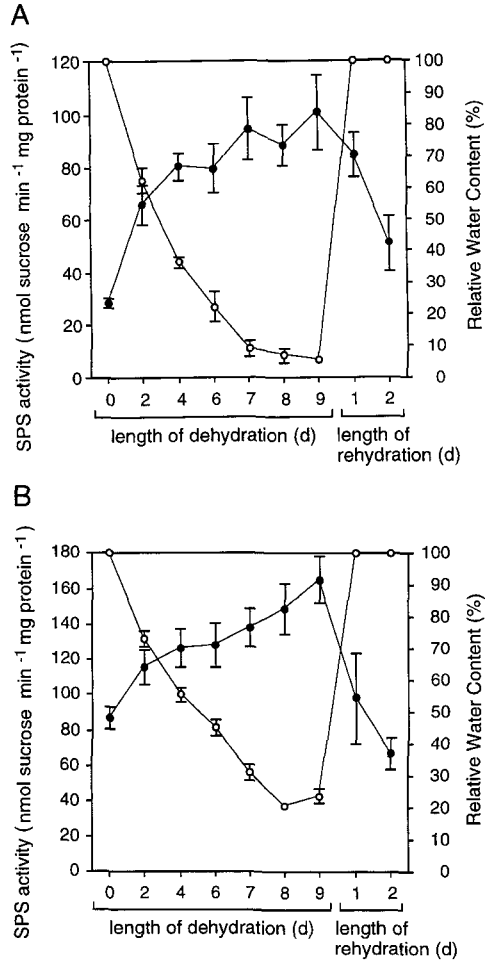

Figure 1. Measurements of SPS activity in crude protein extracts of *C. plantagineum* in leaves (A) and roots (B) during dehydration and rehydration (●). Plants were dehydrated for up to 9 d and then rehydrated for up to 2 d. Water loss is expressed as RWC (○). The untreated tissue samples were set to 100% RWC. Each data point is the mean value of three independent experiments (with duplicate samples) ± SE from a pool of three plants for each treatment.

increases in enzymatic activity were reversed during rehydration and reached the level of unstressed plants within 48 h of rehydration.

When SPS activity was estimated by measuring the formation of UDP, values were severalfold lower than those obtained by measuring Suc. This was most likely due to UDPase activity, which was measured to be between 200 and 300 nmol Pi mg^{-1} protein min^{-1} in untreated leaves, and increased during the early phases of dehydration by about a factor of two. Nevertheless, both approaches for determining SPS activity showed a similar increase during dehydration. These observations of enzymatic activity suggest a role for SPS in the sugar interconversions associated with dehydration in *C. plantagineum*.

Two Classes of cDNA Clones Encoding SPS

Nine SPS cDNA clones were obtained from screening a dehydration-related cDNA library from *C. plantagineum*, and partial sequencing showed that they have high similarity to SPS sequences from other plant species. The cDNA clones could be grouped into two distinct classes represented by the two clones Cpsps1 and Cpsps2, which have divergent sequences and appear to define two classes of SPS in *C. plantagineum* (Fig. 2A); on the nucleotide level they are 63% identical when compared over the whole sequences.

The Cpsps1 cDNA is 3436 nucleotides in length, and comprises a 44-nucleotide 5′ leader sequence, a coding region of 3162 nucleotides, and a 3′ untranslated region of 230 nucleotides. It is predicted to contain 1054 amino acid residues in the encoded polypeptide, resulting in a protein with a calculated molecular mass of 119 kD and a pI of 6.08. In contrast, the Cpsps2 cDNA is 3602 nucleotides long, consisting of a 183-nucleotide 5′ leader sequence, a coding region of 3243 nucleotides, and a 3′ untranslated region of 176 nucleotides. The deduced protein is 1081 amino acids in length, corresponding to a molecular mass of 121 kD and a pI of 6.09. The predicted polypeptide sequences for both of these clones are shown in Figure 2A and compared with SPS protein sequences from a range of plant species. Like all other SPS proteins, the sequence of CpSPS1 and CpSPS2 begins with MAGN. A Ser residue, believed to be a regulatory phosphorylation site (McMichael et al., 1993), is conserved in CpSPS1 at position 150 and in CpSPS2 at position 160.

The relationships between the different SPS proteins are illustrated in a dendrogram (Fig. 2B), which shows that the Cpsps1 cDNA clone is closely related to SPS genes from dicot plants; in contrast, the Cpsps2 sequence is more similar to monocot SPS genes. At the amino acid level, the predicted CpSPS1 polypeptide is most closely related to that from potato (*Solanum tuberosum*; DNA Data Bank of Japan accession no. X73477), with 82% identity. It also shows 79% identity with the SPS from citrus (*Citrus unshiu* Marc.; Komatsu et al., 1996), 74% with that from sugar beet (*Beta vulgaris*; Hesse et al., 1995), and 71% with that from spinach (*Spinacia oleracea*; Sonnewald et al., 1993), but only 58 and 55% identity with SPS proteins from maize (*Zea mays*; Worrell et al., 1991) and rice (*Oryza sativa*; Sakamoto et al., 1995), respectively. In contrast to CpSPS1, the CpSPS2 polypeptide sequence is more closely related to SPS proteins from the monocot plants maize and rice (70% identity to both) than to the dicot SPS of spinach (58% identity), sugar beet (59% identity), citrus (58%), and potato (57%) (Fig. 2A). When compared with each other, the predicted polypeptides of CpSPS1 and CpSPS2 are only 57% identical (73% similar), which means that they are more closely related at the sequence level to SPS from other species than to each other.

To analyze the organization of the SPS genes in *C. plantagineum*, genomic DNA fragments were hybridized with the Cpsps gene-specific probes (Fig. 3). For Cpsps1, in lane 2 of the blot where the gene is known not to have been cut in its exon regions, and for Cpsps2 in both lanes, the probes detected up to three hybridizing fragments, suggesting that both Cpsps1 and Cpsps2 are organized in small gene families with probably not more than three members.

Tissue-Specific Expression of the SPS Genes

To analyze the tissue-specific expression of the SPS transcripts, RNA extracted from leaves, roots, and untreated and ABA-treated callus was hybridized with gene-specific probes in northern blots (Fig. 4). In all experiments the Cpsps1 transcript was more abundant than the Cpsps2 transcript. Cpsps1 hybridized to a single transcript size of approximately 3500 nucleotides in all tissues tested (Fig. 4). A similar-sized transcript was also found in leaves with the Cpsps2 cDNA probe, but the gene was not expressed at all in the roots (Fig. 4). In callus tissue the Cpsps2 cDNA probe hybridized to two transcripts, one weakly expressed and about 3500 nucleotides in length, and a more predominantly expressed transcript of larger size. The transcript levels were not appreciably affected by ABA treatment (Fig. 4).

Expression of SPS in Response to Dehydration and Rehydration

Hybridization experiments performed with RNA blots from leaves and roots of the different treatment groups (Fig. 5) revealed that changes occurred in the transcript levels of the Cpsps1 and Cpsps2 genes in response to the hydration state of the tissue. From a constitutive level in the fresh leaves (100% RWC) an increase in both Cpsps1 and Cpsps2 transcript levels was observed early in dehydration (this was more pronounced for Cpsps2), with a subsequent decrease to extremely low levels in the dried plants for both transcripts. During rehydration Cpsps transcripts started to accumulate again in leaves within 24 h. In roots the Cpsps1 transcript increased early in dehydration to a slightly higher level, which was then maintained until full desiccation. The Cpsps2 transcript was not detected under any condition in roots.

SPS Protein Expression

To analyze the expression of SPS at the protein level, polyclonal antibodies were generated (for details, see "Ma-

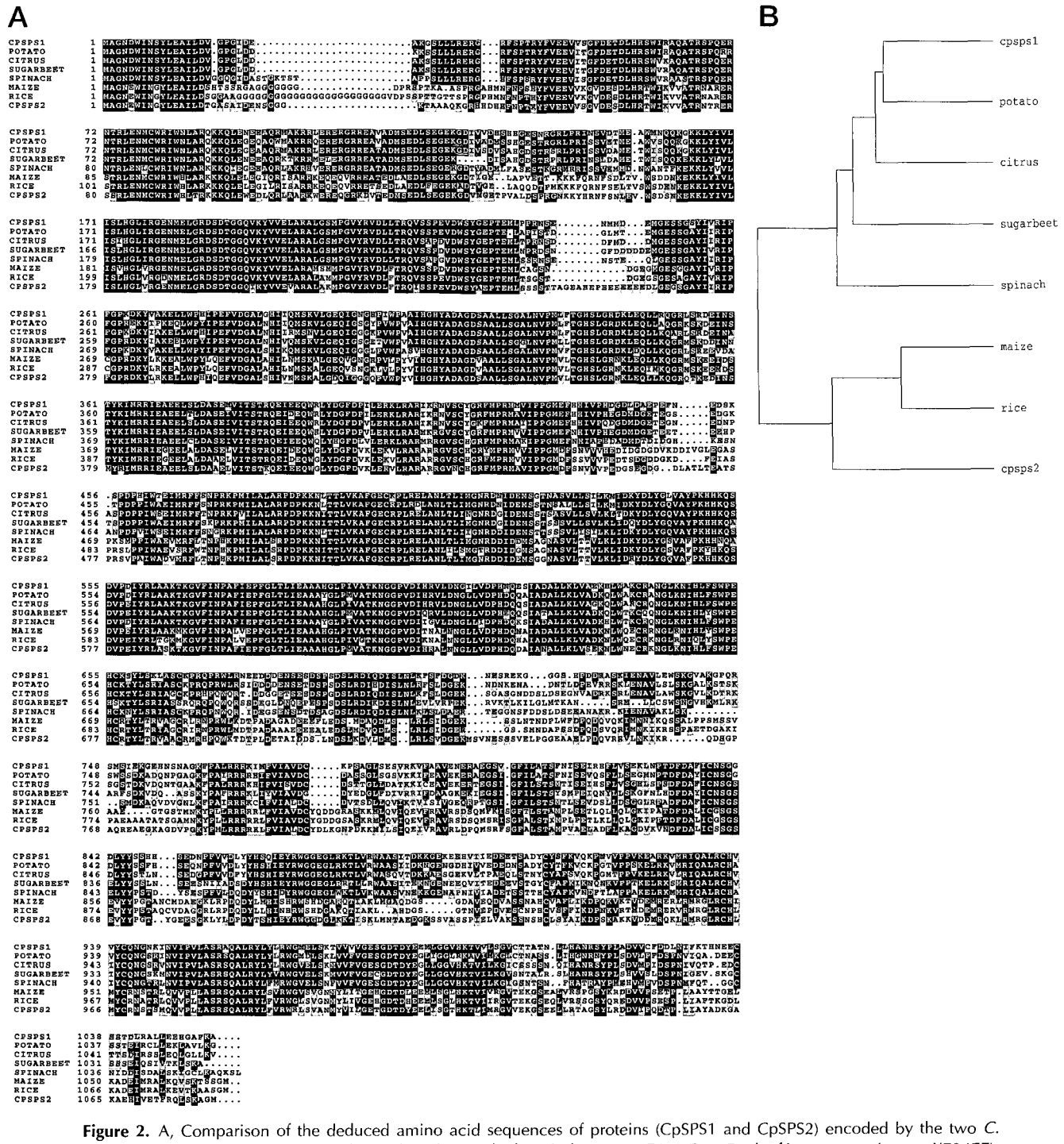

Figure 2. A, Comparison of the deduced amino acid sequences of proteins (CpSPS1 and CpSPS2) encoded by the two *C. plantagineum* SPS cDNAs with SPSs from other plants, which include: potato (DNA Data Bank of Japan accession no. X73477), citrus (Komatsu et al., 1996), sugar beet (Hesse et al., 1995), spinach (Sonnewald et al., 1993), maize (Worrell et al., 1995), and rice (Sakamoto et al., 1995). The comparison was produced using the PILEUP program of the University of Wisconsin Genetics Computer Group. Gaps indicated by dots were introduced to optimize the alignment. Identical amino acids are shaded in black; similar amino acids are in gray. B, Dendrogram established with the PILEUP program to demonstrate the relationships between the different SPS sequences. The references for the sequences are the same as in A.

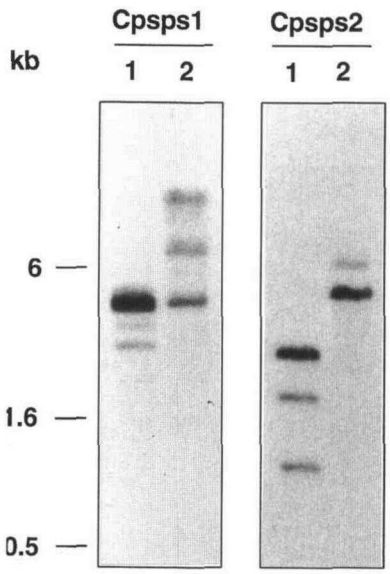

Figure 3. Southern-blot analysis showing the representation of the genes corresponding to cDNAs Cpsps1 and Cpsps2 in the *C. plantagineum* genome. Two blots were produced using genomic DNA that had been digested with either *Hin*dIII (lanes 1) or *Bgl*II (lanes 2), and were probed with Cpsps1 or Cpsps2 as shown. The region of the Cpsps1 cDNA used to produce the gene-specific probe contains one *Hin*dIII site.

terials and Methods"). The antibodies potentially recognize proteins encoded by Cpsps1 and Cpsps2. Western-blot analysis performed using protein extracted from leaves and roots of the different treatment groups showed that the SPS protein level changed only slightly, in contrast to large changes in the mRNA level (Fig. 6). The amount of SPS protein in both leaves and roots showed a slight increase during dehydration but was not appreciably altered by rehydration (Fig. 6A). ABA did not alter the amount of protein in leaves or callus tissues (Fig. 6B). The molecular mass of the protein band was estimated to be over 116 kD. Under the chosen experimental conditions no smaller protein bands were detected.

DISCUSSION

This report describes the molecular analysis of SPS cDNA clones from *C. plantagineum* and the expression characteristics of their corresponding transcripts. SPS was targeted because the encoded enzyme has a key role in Suc synthesis, which may be an important component of dehydration tolerance. This assumption was strengthened by the observation that the enzymatic activity of SPS increased in leaves and roots during dehydration of *C. plantagineum*.

Two Classes of SPS Transcripts

Detailed analysis of the SPS genes in *C. plantagineum* revealed that two different classes of transcripts (Cpsps1 and Cpsps2) can be distinguished based on primary sequence and on distinct expression patterns. In all other plant species (with the exception of citrus [Komatsu et al.,1996] and sugar cane [database accession nos. AB 001337 and AB 001338]) only one type of expressed SPS gene has been reported. It is possible that the two distinct classes of SPS transcripts in *C. plantagineum* are related to the unique sugar conversions associated with desiccation tolerance.

Comparisons between the sequences reported here for *C. plantagineum* and all published SPS DNA and protein sequences show a high degree of homology. Based on sequence similarity, a closer evolutionary relationship is suggested between CpSPS1 and the SPS from dicot than from monocot plants. Conversely, CpSPS2 was more closely related to the monocot sequences than to those of the dicot species. A Gly-rich region in the N-terminal region is one of the distinctive features of the other two monocot polypeptides, and in fact the CpSPS2 protein contains three Gly residues in the relevant position (amino acids 27–29 in Fig. 2A), supporting a close affiliation with the monocot genes. It is notable that the relationships of CpSPS1 and CpSPS2 with the dicot and monocot species, respectively, are closer than their relationship with each other, suggesting that the divergent SPS sequences from dicot and monocot species may be a reflection of functionally important features.

The close relationship between the *C. plantagineum* Cpsps cDNA clone sequences and other SPS sequences that were demonstrated to encode active proteins suggests that the *C. plantagineum* transcripts encode active SPS enzymes. However, the contribution of both SPS classes to the activities cannot easily be distinguished without protein purification and sequencing. Nevertheless, because the Cpsps2 transcript was not detected in roots, the enzyme activity in these organs must be derived from the Cpsps1 gene family.

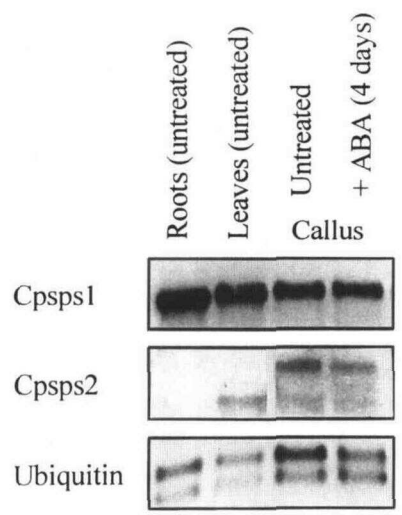

Figure 4. Northern-blot analysis from *C. plantagineum* showing the basic expression of the two classes of SPS transcripts Cpsps1 and Cpsps2. The northern blot was produced using poly(A)$^+$ RNA (2 μg in each lane of the gel) from untreated roots, leaves, and callus, and from callus that had been grown in a medium with 20 μM ABA for 4 d. The blot was first probed with Cpsps2, and then exposed to radiographic film for 5 d; hybridization was then repeated with the Cpsps1 probe, and the blot was again exposed to film for 5 d. Hybridization was later repeated with the ubiquitin probe (Gausing and Barkardottir, 1986) to indicate the RNA loading.

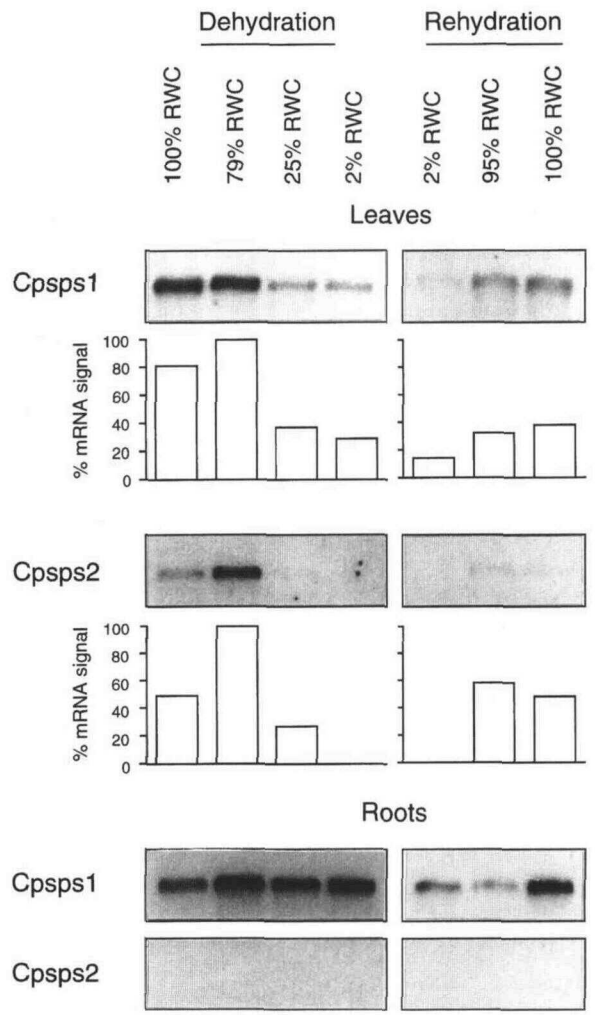

Figure 5. Northern-blot analysis showing the expression of mRNA transcripts corresponding to the two Cpsps cDNA clones in the leaves and roots of plants of *C. plantagineum* at stages during dehydration and rehydration. The untreated tissue samples were set to 100% RWC. Stages during dehydration included: after 1 d of drought (79% RWC in the leaves), after 4 d of drought (25% RWC), and after 9 d of drought (2% RWC). Stages during rehydration included: after 24 and 48 h of supplying water (95 and 100% RWC in the leaves, respectively). The blots were produced using 2 μg of poly(A)$^+$ RNA in each lane of the gel. The blots were either probed with the Cpsps1 or Cpsps2 probe and were exposed to radiographic film for 5 d. Hybridization was repeated with a ubiquitin probe (Gausing and Barkardottir, 1986) on all blots to indicate the RNA loading. The bar diagrams represent densitometric analyses of the relative mRNA levels in leaves during dehydration and rehydration.

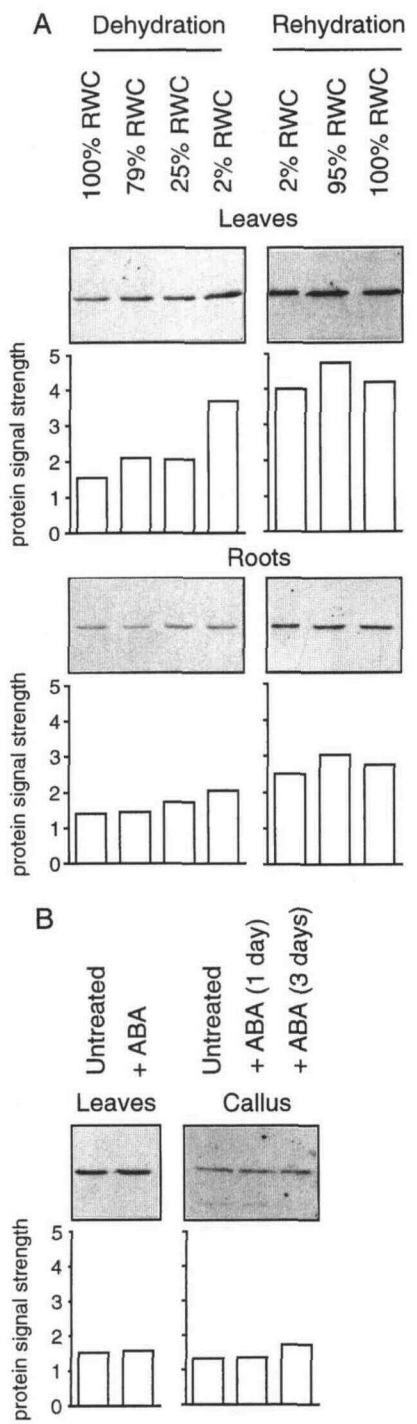

Figure 6. Western-blot analysis showing the level of SPS in protein extracts from the leaves and roots of plants of *C. plantagineum*, at stages during dehydration and rehydration (A), and from ABA-treated leaves and callus (B). The dehydration and rehydration treatments were as described in Figure 5, and the ABA treatments for callus were as described in Figure 4. The ABA treatment of detached leaves involved incubation for 24 h with 1 mM ABA. The different blots were produced using a constant amount of protein (between 30 and 60 μg) in each lane of the gel. Loading was also checked by staining the blots with Ponceau-S before probing. The SPS antiserum was used as a probe. The bar diagrams show densitometric values that have been normalized to represent the amount of SPS protein in 10 μg of total protein extracts.

The different expression patterns of both *C. plantagineum* SPS transcripts suggest that they may be involved in functionally different pathways. The modulation of Cpsps2 expression by dehydration suggests that Cpsps2 may be necessary for stress-related sugar metabolism in leaves, whereas Cpsps1 may encode mainly a constitutively expressed SPS enzyme. This interpretation agrees with a recent report on SPS isoforms in potato, in which different forms of the SPS protein were associated with tissue-

specific expression during development and with acclimation to low temperature (Reimholz et al., 1997).

The fact that Cpsps2 hybridizes to two transcripts of differing sizes among RNAs extracted from callus can be interpreted in two ways: either a third type of SPS transcript exists or the larger transcript represents an incompletely processed form of Cpsps2. It is interesting in this context that studies of kinetic properties of SPS enzymes suggested three classes of SPS, differing in their allosteric regulation and their potential for activation/inactivation in vivo. These different forms of SPS, however, were associated with different plant species (Huber and Huber, 1992).

Regulation of SPS Gene Expression

The results of the present study indicate that SPS gene expression is in part regulated at the mRNA level by transcriptional activation or mRNA stability. The regulation includes organ-specific expression (Cpsps2 is not present in roots) and modulation by the water status of the tissue. Organ-specific expression of SPS transcripts has also been reported for other plant species: the SPS transcripts were detected mainly in leaves of spinach or rice (Klein et al., 1993; Sakamoto et al., 1995) or in roots of sugar beet (Hesse et al., 1995). The transcript distribution in *C. plantagineum* is different: distinct SPS transcripts are present in leaves, roots, and callus.

SPS enzyme activity in the leaves of *C. plantagineum* appears to increase, together with Suc accumulation, during dehydration. The experiments we have presented indicate that the increase in enzyme activity may be accounted for by increasing levels of SPS protein. However, during the periods when the highest extractable SPS activity was found, the transcript levels were at their lowest, suggesting additional regulatory mechanisms. The increasing enzymatic activity of SPS may reflect the activation state of the enzyme rather than just the amount of protein. It has been shown for other plant species (mainly spinach) that phosphorylation of SPS is a critical factor in determining SPS activity (Huber and Huber, 1992; McMichael et al., 1993).

Based on immunological and kinetic studies, it has been suggested that SPS exists in interconvertible forms that differ in their phosphorylation status, and thus result in SPS proteins with different substrate and effector affinities (Weiner, 1995; Huber and Huber, 1996). The Ser in position 158 of spinach SPS (see Fig. 2A) has been identified as critical for phosphorylation (McMichael et al., 1993), and the Ser residues in CpSPS1 (position 150) and in CpSPS2 (position 160) are directly comparable. The sequence conservation extends to the residues preceding the regulatory Ser residue, and includes the seven-amino acid SPS kinase target sequence as defined by McMichael et al. (1995). It thus seems likely that, like the enzyme from spinach, the SPS from *C. plantagineum* can undergo reversible phosphorylation to regulate its activity.

CONCLUSIONS

The increase in activity of SPS in the leaves *of C. plantagineum* coincides with the periods when major Suc synthesis occurs. It is therefore assumed that SPS contributes to the net synthesis of Suc. The expression pattern of SPS in leaves is different from that in roots. The need for such differences may be to support different patterns of Suc synthesis. This correlates with the biochemical analyses that showed only traces of octulose but a high concentration of stachyose in the roots (Schwall et al., 1996). The situation in roots highlights the difficulty of understanding the reasons for the expression patterns of an enzyme that is a critical component of normal Suc metabolism and yet is associated with a phenomenon as dramatic as dehydration tolerance. The specific cDNAs isolated in the present research should allow more specific questions concerning the role of Suc metabolism in dehydration tolerance to be addressed.

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