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Regulation of sucrose phosphate synthase of the sweet potato callus is related to illumination and osmotic stress

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Abstract. Sucrose phosphate synthase (SPS) was purified from sweet potato callus grown under different conditions, including illumination, darkness, and osmotic stress. The properties of the enzymes purified from the different conditions were compared. Since sucrose synthase (SS) could also be purified using the purification process, the expression patterns and properties of the two enzymes were also compared. The SPS purified from the calli tissue medium under illumination had an optimum pH of 7.5. The enzyme had an optimum reaction temperature of 37°C, and maintained stable activity at pH 6 to 8. It was allosterically regulated using either Glc 6-P or Pi. However, the enzyme purified from the tissues grown under osmotic stress conditions was not allosterically regulated using Glc 6-P or Pi. The maximal activity of SPS and maximal protein content of the illumination grown calli appeared on the 14th day after the culture was transferred into a new medium. This was compared with the peak value on the 7th day for the samples under osmotic stress. The maximum activity for SS was observed on the 14th day. The dark grown cells had very low SPS, but normal SS activities. A histological study revealed an abundance of starch granules in the osmotic stressed cells. The in situ hybridization technique showed that the illumination and osmotic stress conditions induced the accumulation of SPS mRNA. The PAGE and enzyme activity assay data showed that, in addition to enhancing SPS gene expression, the activation of SS gene expression isozymes may be achieved under the illumination and osmotic stress conditions.

Keywords: Dark; Illumination; Osmotic stress; Sucrose phosphate synthase; Sucrose synthase; Sweet potato callus.

Introduction

Higher plants synthesize sucrose through two processes. The first and the major method employs sucrose phosphate synthase (EC 2.4.1.14; SPS), a soluble enzyme located in the cytoplasm that catalyzes the reaction:

UDPGlc + Fru6-phosphate $\rightleftharpoons$ UDP + Suc6'-phosphate

Suc 6'-phosphate is then dephosphorylated by Suc-P phosphatase (EC 2.3.1.14; SPP) to produce sucrose as the final product (Huber and Huber, 1996). The second is sucrose synthase (EC 2.4.1.13; SS) which catalyzes a reversible reaction:

UDP – glucose + Fructose $\rightleftharpoons$ Sucrose + UDP

Sucrose is the main carbohydrate used by most plant species to translocate photo assimilates from the leaves to non-photosynthetic tissues. In addition, it is the most common carbon source added to plant tissue cultures. Sucrose is transported by a combination of symplasmic and apoplasmic pathways to the phloem, where it is loaded using a proton-driven symporter (Riesmeier et al., 1994). In the phloem, sucrose is translocated by a mass flow to sink tissues, where it is cleaved by SS to produce UDPG and fructose or it is hydrolyzed by invertase (EC 3.2.1.26) to yield glucose and fructose (Giaquinta, 1983). They are then used for other biosynthetic pathways as energy and sources of structural components. SPS has also been proposed to be an important limiting step in the source-sink relationship, in grain filling in cereals (Prioul and Schwebel-Dugue, 1990), in sugar accumulation in fruits (Hubbard et al., 1990; Dali et al., 1992) and in cold stress acclimation (Guy et al., 1992).

SPS undergoes many regulatory mechanisms. First, SPS is subjected to allosteric regulation by Glc 6-P (activator) and Pi (inhibitor) (Douglas and Huber, 1983; Stitt et al., 1988). Secondly, it can be reversible and covalently modified in response to light (Stitt et al., 1988; Huber et al., 1989; Huber and Huber, 1991, 1992), accumulation of sucrose (Stitt et al., 1988) or osmotic stress (Toroser and Huber, 1997). Finally, it is involved with transcriptional regulation during sink-source transition (Harn et al., 1993; Klein et al., 1993).

The aim of this report was to explore the SPS activity of non-photosynthetic tissues under illumination. The callus of sweet potato was induced from tubers for simplicity in manipulating the conditions. After treatment with illumination and high osmotic stress, the callus cells acquired a high starch content. Illumination and high osmotic stress were then used as two controlled factors for SPS activity. The condition of highest enzyme activity was used for the biochemical characterization of SPS.

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Materials and Methods

Plant Materials

Calli of sweet potato were induced from sweet potato tuber root (*Ipomoea batatas* cv. Tainong 57) and cultured as previously described (Wang et al., 2000). Briefly, they were subcultured by monthly transference into a fresh Murashige-Skoog medium containing 2 $\mu g \cdot mL^{-1}$ 2,4-D, 0.2 $\mu g \cdot mL^{-1}$ kinetin, 30 gL^{-1} sucrose and 8 gL^{-1} agar. Cells for experiments were harvested 35 days after routine transfer into the same medium. Calli cells were collected at 7-day intervals until 35 days of cultivation, and then they were used for further analysis.

Assay of SPS and SS

SPS activity was monitored using the anthrone test during the entire purification procedure (Huber et al., 1989). In this test, 70 µL of the reaction mixture including the extract was adjusted to a final concentration of 4 mM Fru 6-P, 20 mM Glc 6-P, 3 mM UDPG, 50 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, and 1 mM EDTA. The mixture was incubated at 37°C for 15 min before adding 70 µl 30% (w/v) KOH and heating for 10 min at 95°C. To this 1 mL 0.14% (w/v) anthrone in 95% H_2SO_4 was then added. The mixture was incubated 20 min at 37°C, and A_{650} was measured. An assay of the SS activity method was described using Nelson's modification of Somogi's method (Nelson, 1944). The method was used when various nucleotides were tested as the substrate.

Western Blotting

After electrophoresis, native-PAGE or SDS-PAGE gel plates were soaked in blotting buffer containing 25 mM Tris and 192 mM glycine at pH 8.3 for 5 to 10 min. Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Amersham Pharmacia Biotech). Protein gel blots were blocked using 5% fat-free powdered milk solution in 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl. Blots were incubated with rabbit antiserum raised against RBE1, glutathione-*S*-transferase (GST)–SPK fusion protein, GST-Suc synthase fusion protein (Urao et al., 1994) for the detection of each protein. Immunodecorated proteins were visualized with horseradish peroxidase-conjugated anti-goat secondary antibody using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and DAB substrates (Funakoshi, Tokyo, Japan). The amount of total protein was estimated using a protein assay kit (Bio-Rad).

In Situ Hybridization Technology

RNA *in situ* analysis was performed as previously described (Gavis and Lehmann, 1992). Sections of immature tissues were prepared from the callus of a sweet potato.

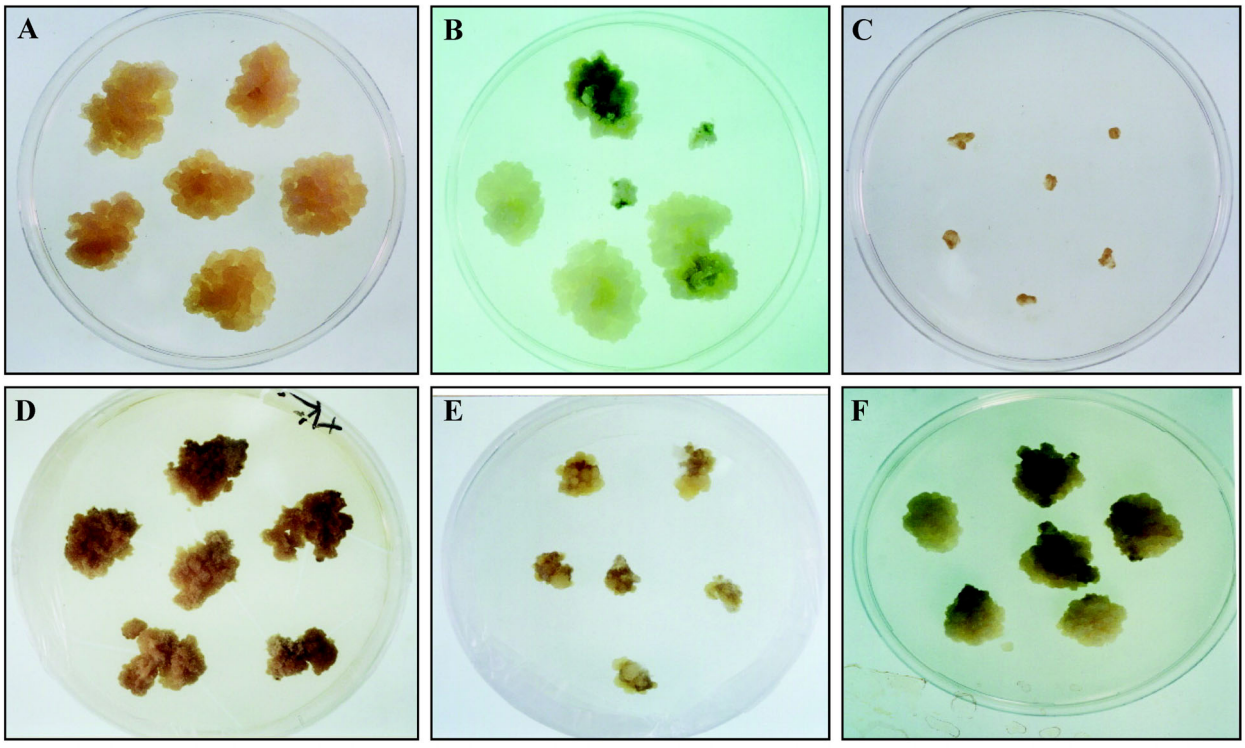

Figure 1. The changes of sweet potato tuberous calli cells with different treatment. Calli of sweet potato were induced from sweet potato tuber root and cultured on MS medium under illumination, dark and osmotic stress treatment. Cells for experiments were harvested at 35 days after routine transfer into the same medium. A, cells grown under dark; B, cells grown under illumination; C, cells growth were inhibited under osmotic stress; D, cells grew at negligible rate under osmotic stress, and as time went on, they turned brown; E and F, the new cells grown from browning cell upon illumination.

PAS Staining

Tissue slides were stained for carbohydrates with periodic acid-Schiff (PAS) stain (Segrest and Jackson, 1972) using a Gelcode glycoprotein staining kit (Sigma) according to the manufacturer's protocols.

Results

Qualitative Protein Analysis

The callus was cultured on a medium under the conditions described in the previous section (Figure 1). The Bradford method was used to estimate the total protein content. It was found that the protein expression increased during the second week (data not shown). As the osmotic stress increased, the protein content increased as well. It should be noted that the expression of protein was different when comparing the following conditions: illumination versus *sans*-illumination; high osmotic stress versus the normal; and illumination versus high osmotic stress. Different protein profiles were observed from the cultures with illumination or osmotic stress (Figure 2). This stimulation may have also induced large quantities of SPS and SS expressions.

Enzyme Properties

After osmotic stress induction or illumination, the SPS partially purified from sweet potato callus was determined to have an optimal reaction temperature of 37°C (Figure 3A). It was also discovered that at low temperatures, the solution seemed to become stable (Figure 3B). Approximately 80% of the activity was maintained at a temperature of 37°C. However, when the temperature rose above 37°C, maintenance of the activity was difficult. No activity was detected at 70°C. Therefore, the purification and the storage of SPS were carried out at low temperatures throughout the experiment.

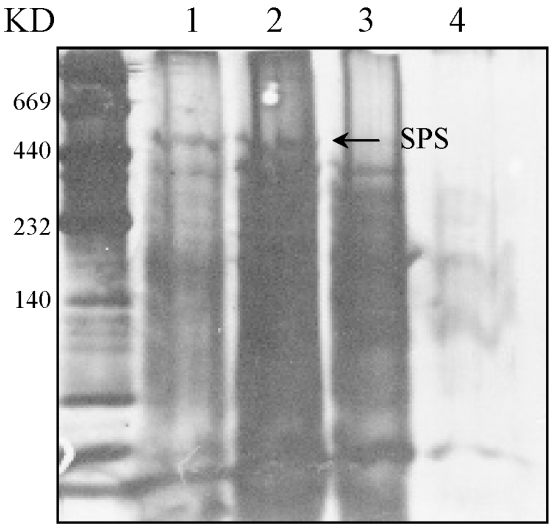

Figure 2. Silver-staining patterns of SPS on 7.5% Native-PAGE. Lane 1, illumination; lane 2, illumination and osmotic stress; lane 3, osmotic stress; lane 4, dark. It should be noted that the expression of protein varied with different treatment.

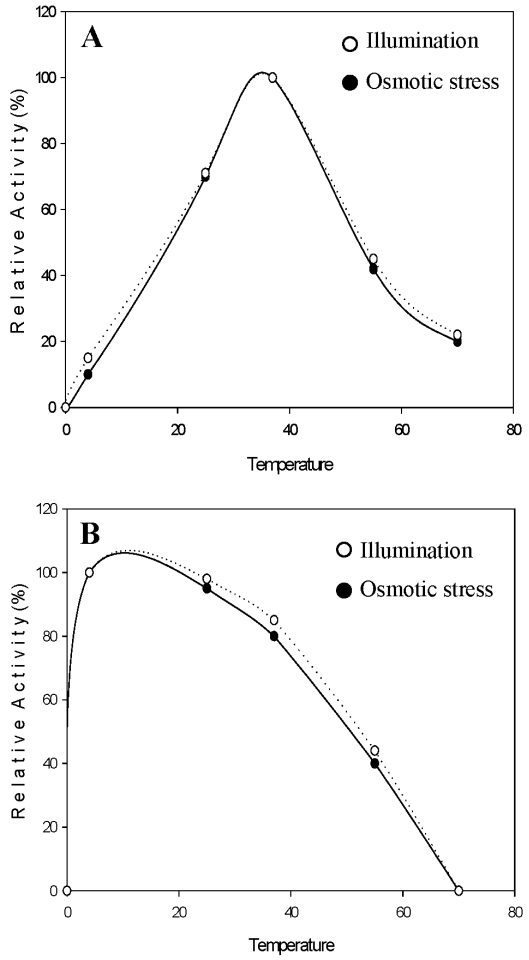

Figure 3. Biochemical characterization of SPS from sweet potato calli under osmotic stress and illumination. A, temperature dependence of the activity of SPS from sweet potato tuberous calli. After osmotic stress induction or illumination, the SPS partially purified from sweet potato calli was determined to have an optimal reaction temperature of 37°C; B, thermal stability of SPS from sweet potato tuberous calli. It is also discovered that at the low temperatures, SPS is more stable.

The optimal pH value for the enzyme reactions was determined to be pH 7.0 (Figure 4A). The enzyme exhibited high reaction activity from pH 5.5 to 9.0. However, SPS was the most stable at pH 7.0 (Figure 4B). Approximately 80% of the activity could be maintained in the range of pH 5.5 to 9.0 after 15 min of incubation (data not shown). Maintaining the activity outside that range was difficult.

SPS from callus with osmotic stress treatment was not regulated by Glc 6-P or Pi (Figure 5A-B). From the results of previous studies, SPS was obtained from photosynthetic tissues subjected to the allosteric regulation of Glc 6-P and Pi (Figure 5A-B). This suggested the existence of isoenzymes to meet the different physiological roles in the different tissues.

Comparative Analysis of Enzyme Activity

The activities of SPS and SS from calli under different conditions were recorded every 7 days (Figure 8). This was to identify the activity changes of the non-illuminated area during its growth and development. The activities of SPS and SS reached their peak during the second week (Figure 8B). However, the changes were not significant. In comparing the CBR-stained electrophoresis slides, no bands were apparent from the Native-PAGE 440 to 550 kDa (Figure 6A) or from the SDS-PAGE 116 to 140 kDa (Figure 6B), where the SPS was expected. This may suggest that the gene expression of the SPS was minimal without illumination.

The illuminated cells were placed under constant illumination of 5000 Lux (approximate wavelength of 700 nm) from 06:00 to 21:00 (with no illumination from 21:00 to 05:00) for 45 days. The activity recorded every 7 days revealed that SPS and SS had the highest activities during the second week as well (Figure 8B). Using electrophoresis analysis, several visible bands appeared on the electrophoresis gels. SPS bands were also present around 130 kDa in SDS-PAGE (Figure 7A-B).The important

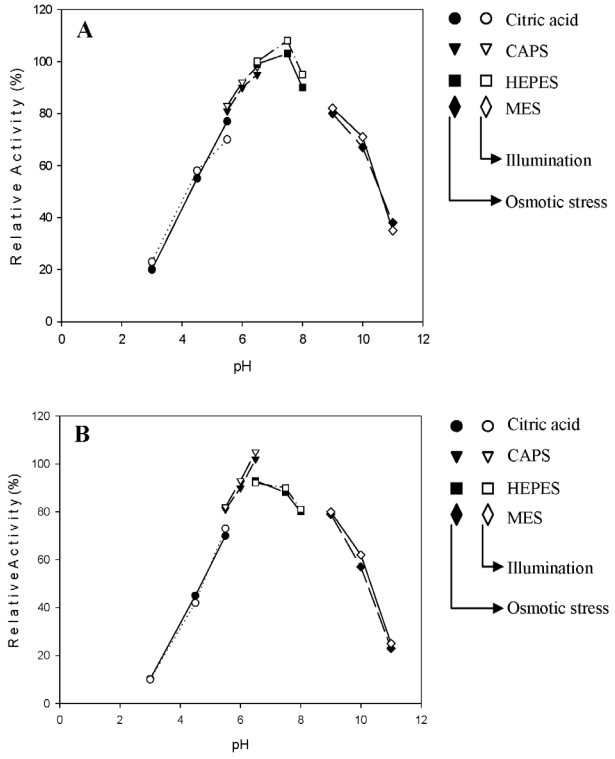

Figure 4. Biochemical characterization of SPS from sweet potato calli under osmotic stress and illumination. A, pH dependence of the activity of SPS from sweet potato tuberous calli. The optimal pH value for the enzyme reactions was determined to be pH 7.0; B, pH stability of the activity of SPS from sweet potato tuberous calli. SPS was the most stable at pH 7.0 under CAPS buffer. CAPS: 3-[cyclohexylamino]-1-propanesulfonicacid. HEPES: N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid. MES: 2-(N-morpholino)ethanesulfonic acid.

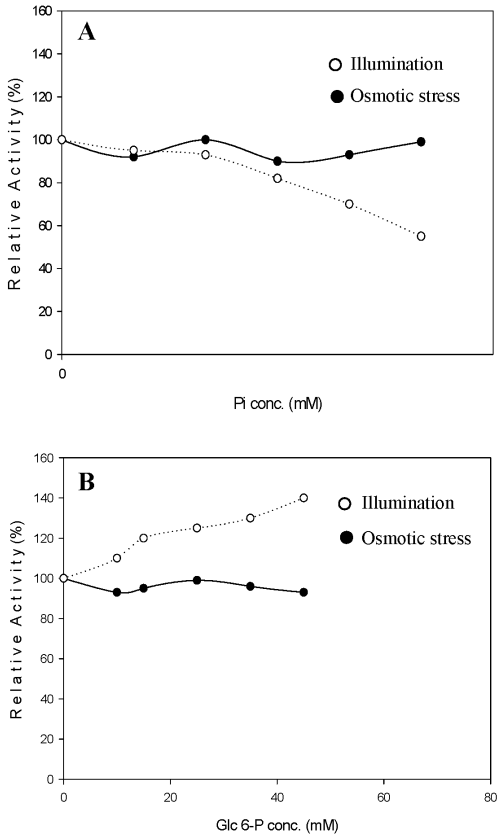

Figure 5. Biochemical characterization of SPS from sweet potato calli under osmotic stress and illumination. A, Effect of inorganic phosphate on SPS from sweet potato tuberous calli; B, Effect of Glc 6-P on SPS from sweet potato tuberous calli. SPS from calli with osmotic stress treatment was not regulated by Glc 6-P or Pi. From the results of previous studies, SPS was obtained from photosynthetic tissues subjected to the allosteric regulation of Glc 6-P and Pi.

difference was that illumination may direct the calli to transform their amyloplast into chloroplast (Figure 1B). The presence of chloroplasts suggests the occurrence of photosynthesis; hence, the detection of SPS agreed with this physiological change. The SS activities were highest during the second week (Figure 8B). In spite of the fact that sucrose encourages SS gene expression (Figure 7C-D), cells must decompose sucrose to provide monosaccharides for growth. The growth rates during the second and the third weeks were the fastest. Since most sucrose would have been decomposed after two days, the effects were minimal. The comparison between illuminated subjects and non-illuminated subjects was alone sufficient to show that illumination does indeed induce the gene expression of SPS and SS. High osmotic stress analysis for SPS and SS activity (Figure 8C) also revealed that activity was higher in batches under high osmotic stress than in those without illumination. The activity peaked during the first week for SPS and during the second week for SS (Figure 8C). When comparing the growth rate with protein content, the high osmotic stress induced SPS gene expression at the shortest lapse of time to synthesize

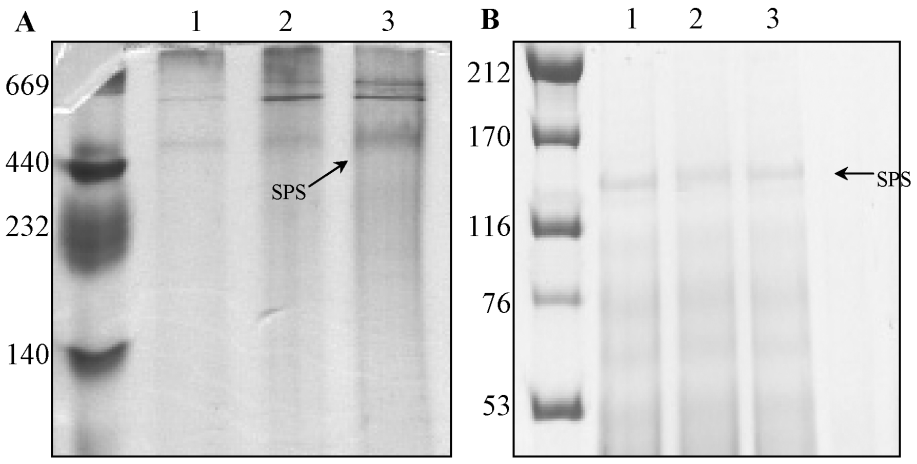

Figure 6. CBR-staining patterns of SPS on 7.5% Native-PAGE and 9.5% SDS-PAGE. Lane 1, illumination; lane 2, illumination and osmotic stress; lane 3, osmotic stress. Comparing the CBR-stained electrophoresis slides, no bands were apparent from the Native-PAGE 440 to 550 kDa (A), or from the SDS-PAGE 116 to 140 kDa (B) where the SPS was expected. The extract was fractionated with the polyethylene glycol precipitation followed by the successive chromatographic steps of DEAE-Sepharose ion exchange, Hitrap heparin affinity adsorption, and FPLC/Mono-Q columns.

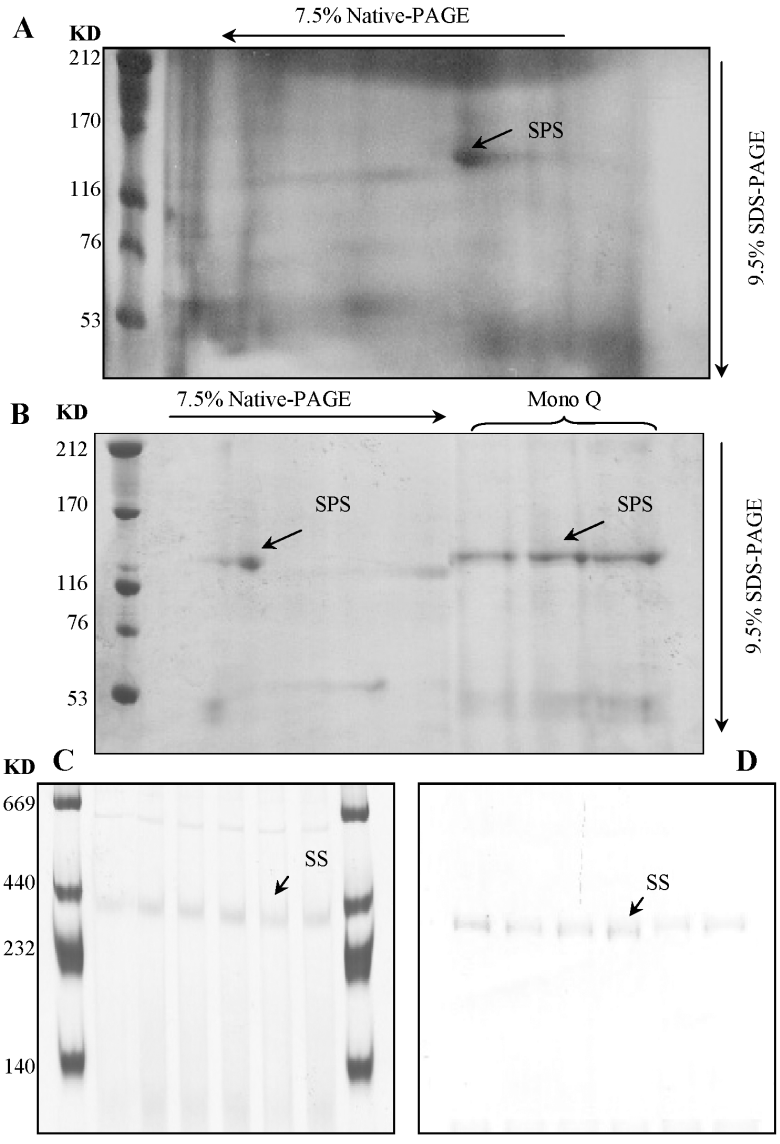

Figure 7. Silver, CBR reagent-staining patterns of SPS and immuno-staining patterns of SS on 7.5% Native-PAGE and 9.5% SDS-PAGE. Lane 1, Illumination; lane 2, illumination and osmotic stress; lane 3, osmotic stress. A, Two-dimensional PAGE of SPS staining with silver; B, Two-dimensional PAGE of SPS staining with CBR; C and D, CBR and immuno-staining patterns of SS on 7.5% Native-PAGE SPS bands were also present around 130 kDa in SDS-PAGE.

sucrose. The sucrose produced by SPS was then used as a substrate for SS or sucrose transformation as well as to synthesize starch to counter osmotic stress. When osmotic stress was present, sucrose was synthesized as a temporal solution until starch synthesis began to occupy the intracellular space. When the space was reduced, a lesser amount of solute was required to deter osmotic stress. Furthermore, cells grew at a negligible rate under osmotic stress, and as time went on, they turned brown (Figure 1D). The browning cells were further tested, and we found that the illuminated batch began to grow new cells while the non-illuminated batch showed no signs of growth (Figure 1E-F). The illuminated batch also showed a rise in SPS and SS activities (Figure 8B) whilst the activities of the batch without illumination remained negligible. This suggests that illumination may have induced the SPS gene expression for the cells that turned brown and hence lead to sucrose synthesis. The induction of the SS gene expression then decomposed the sucrose and provided the substrates needed for further growth. Thus, the cells began to grow. This showed that the cells had gone brown but had not died.

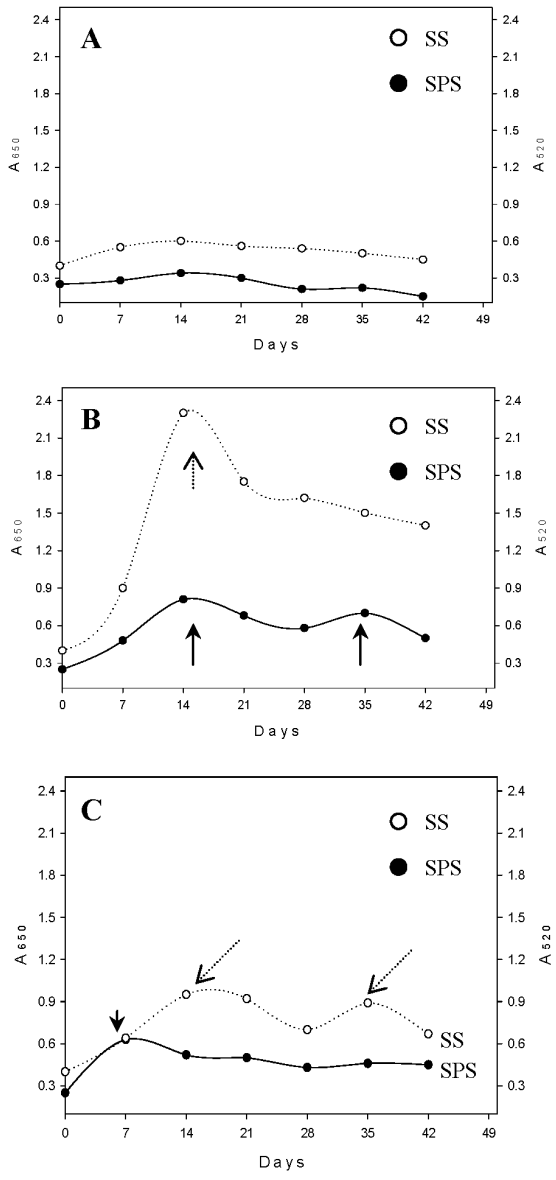

Figure 8. The changes of SPS and SS enzyme activities of sweet potato tuberous calli under different treatments. The activity recorded every 7 days revealed that SPS and SS had reached the maximum activities during the second week as well. High osmotic stress analysis for SPS and SS activity (C) also revealed that activity was higher in batches under high osmotic stress than in those without illumination. A, Dark; B, Illumination; C, Osmotic stress.

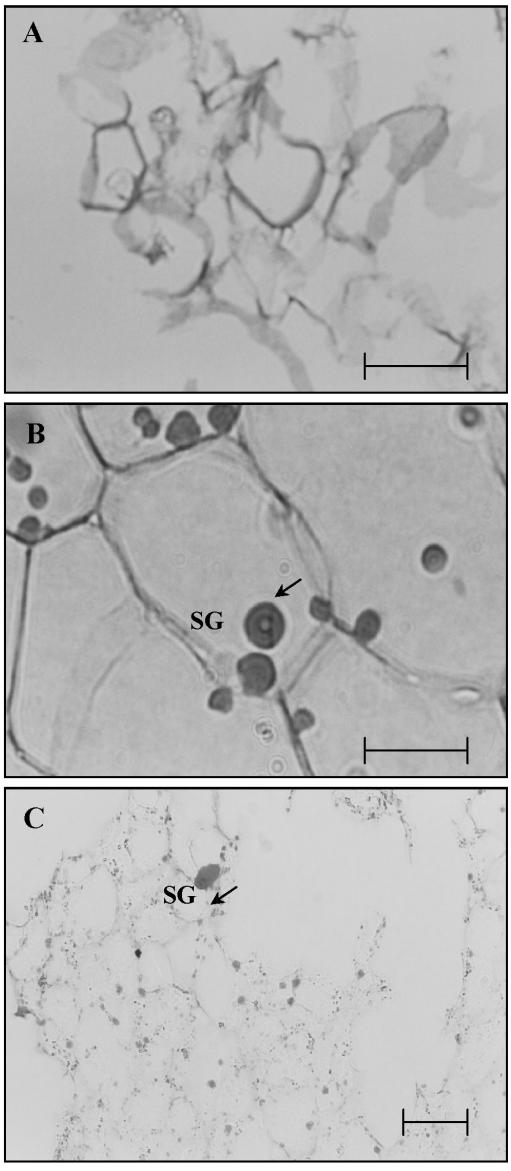

Figure 9. The PAS staining patterns of SPS from sweet potato tuberous calli under different treatment (Bar=50 mm). Fewer starch granules were observed in cells without illumination. After high osmotic stress treatment, more starch granules were produced. A, The PAS staining patterns (Dark); B, The PAS staining patterns (Illumination); C, The PAS staining patterns (Osmotic stress). SG: starch granule.

PAS Staining

The presence of carbohydrates in cells was demonstrated using PAS staining (Figure 9). Fewer starch grains were observed in cells without illumination. Furthermore, the cell walls were stronger (Figure 9A). After high osmotic stress treatment, more starch grains were produced (Figure 9C). The higher the stress level, the more starch grains were produced, and plasmolysis was observed. The cells may try to reduce the intra-extracellular pressure difference by allowing water to dissipate outwards, causing the protoplast to contract, thus separating the cell membrane from the cell wall. When the turgor pressure was reduced, and the strength of the cell wall was also reduced. In other words, the cells tried to maintain the balance in osmotic pressure by raising the intracellular concentration. To achieve this, the cells must synthesize large amounts of

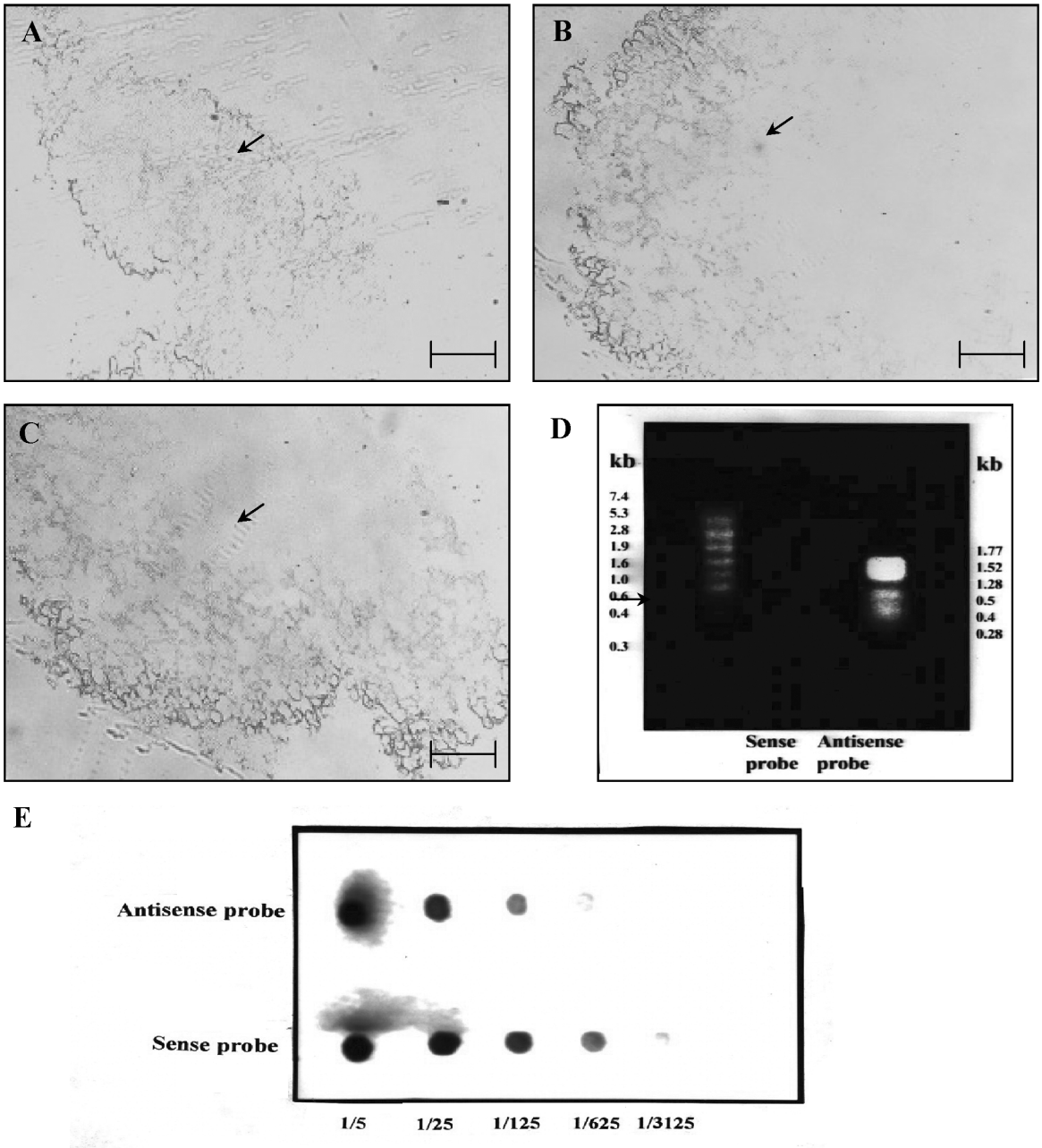

Figure 10. In situ hybridization of SPS by antisense probe in sweet potato tuberous calli under different treatment (Bar=50 mm). A, Dark; B, Illumination; C, Osmotic stress. Light and osmotic stress treatments induced the accumulation of SPS mRNA; D, Using in vitro transcription to synthesis RNA probe of SPS. The plasmid DNA containing sense and antisense cDNA would be cut to line by restriction enzyme. After addition, RNA and NTP reacted an h under 37°C. Running 1% agarose gel. E, The optimal concentration of RNA probe in *in situ* hybridization. Different concentrations of in vitro transcription RNA were dropped on the nitrocellulose membrane and stained with NBT/BCIP.

starch, activating the genes related to sucrose-starch transformation. SS activity increases immensely under high osmotic stress, but only for the first week. One possible hypothesis is that, when cells are placed under high osmotic stress, they store non-structural substances, such as sucrose.

The sucrose produced by SPS was then catalyzed by SS to get into the sugar nucleotides (UDPG and ADPG), which were used to synthesize starch, directly or indirectly. A large amount of starch occupied the intracellular space, thus, allowing the cells to raise the intracellular concentration with less solute. Most of the fructose and glucose, from sucrose decomposition, was used to synthesize starch, indirectly. This limited the cellular growth and metabolic rate and led to the synthesis of starch grains as in our results.

In Situ Hybridization Technology

We used the SPS sense and antisense cDNA as the probe (Figure 10D-E) for *in situ* hybridization (Figure 10). A great amount of the antisense hybridized with the sense mRNA under the conditions of illumination and high osmotic stress, and this accounts for the dark blue shades in the cytoplasm on Figures 10B and 10C. This means that the SPS gene became highly expressive under illumination and high osmotic stress. In contrast, subjects without illumination appeared as a much lighter color, which suggests that the light induced SPS gene expression.

Discussion

Through the research on sweet potato callus, it was noted that illumination and high osmotic stress enhanced SPS gene expression. This was mainly known through *in situ* hybridization using probes specialized for the SPS nucleic acid from tuberous root. The trend showed that the amount of SPS protein was proportional to the level of activity. This also showed that gene expression layers mainly regulated SPS from the callus. Then again, the genetic expression is a result of the induction of illumination and high osmotic stress. If purified proteins were used to determine biochemical properties, we would have noticed that stressed-induce SPS was not enhanced by Glc 6-P or inhibited by Pi. Many of the biochemical properties are similar to what was noted in previously conducted studies (Chen et al., 2001) in this laboratory. The main function of a tuberous root is to store starch. The callus from the tuberous root should at least partially maintain its original properties. Nevertheless, illuminated SPS was enhanced by Glc 6-P and inhibited by Pi. This may suggest the existence of an isoenzyme. From the tissue slides, it was noted that large quantities of starch grains were synthesized under illumination and high osmotic stress, and SPS activity increased. All evidence seems to suggest that the SPS from the callus was closely related to the cumulated starch. The finding that intracellular metabolism favored starch synthesis rather than starch decomposition remains a worthy basis for future exploration.

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甘藷癒創組織中蔗糖磷酯合成酶受光及滲透逆境之調控

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將甘藷塊根癒創組織 （callus） 經光照、黑暗與滲透逆境處理後，並分別純化出蔗糖磷酯合成酶（SPS）與蔗糖合成酶 （SS）做比較。在 SPS 之生化性質方面：光照處理下所得之 SPS，其最適反應 pH 值為 7.5，最適反應溫度為 37°C，在 pH 6~8 之間酵素活性穩定，酵素本身亦會受到 Glc 6-P 的促進及 Pi 的抑制調控。然而經由滲透逆境處理後所得 SPS，不受 Glc 6-P 及 Pi 的異位調控。另外，光照處理所得的 SPS 與 SS 最大活性及最多蛋白質含量均出現在培養第 14 天，但在高滲透逆境下 SPS 則以第 7 天的活性為最高；而 SS 仍維持第 14 天有最大活性。黑暗生長下之癒創組織 SPS 活性均很低，但SS 仍有相當的活性。以組織切片及原位組織雜交技術來分析，發現高滲透逆境下均有大量的澱粉粒累積，且光照與滲透逆境處理，可促進甘藷癒創組織內 SPS mRNA 生成量。配合電泳分析及酵素純化結果，推測在光照與高滲透逆境有助於 SPS 基因的表現及酵素活性的增加，或有可能誘導產生不同的 SPS 及 SS 異構酶，以便在甘藷塊根癒創組織生長中表現不同的生理活性。

關鍵詞：黑暗；光照；滲透逆境；蔗糖磷酯合成酶；蔗糖合成酶；甘藷癒創組織。