Plant Physiol. (1996) 111: 1021–1029

Sucrose Phosphate Synthase Expression at the Cell and Tissue Level Is Coordinated with Sucrose Sink-to-Source Transitions in Maize Leaf[1]

Wan-Hsing Cheng[2], Kyung H. Im[2], and Prem S. Chourey*

Program in Plant Molecular and Cellular Biology (W.-H.C., P.S.C.), Departments of Plant Pathology (K.H.I., P.S.C.) and Agronomy (P.S.C.), University of Florida and United States Department of Agriculture-Agricultural Research Service (P.S.C.), Gainesville, Florida 32611–0680

Immunohistological analyses for sucrose phosphate synthase (SPS) show that the protein is localized in both bundle-sheath cells (BS) and mesophyll cells (M) in maize (*Zea mays*) leaves. In young leaves, SPS protein was predominantly in the BS, whereas mature leaves showed nearly equal levels of signal in both BS and M. A cell-type-specific response was also seen in light and dark treatments. Dark treatments led to reduced signal in M; however, little or no change was detected in BS. We suggest that SPS in BS is engaged in sucrose biosynthesis by both photoassimilatory and starch turnover reactions in maize leaves. In addition, we suggest that the enzyme in BS may play a major role in the early biosynthesis of sucrose in young leaves. These cell-specific changes in expression in situ were in agreement with the estimates of extractable enzyme activity from isolated BS and M of mature leaves (R. Ohsugi, S.C. Huber [1987] Plant Physiol 84: 1096–1101). In contrast, western blot analyses did not show any significant changes in the levels of SPS protein in either young or mature leaves subsequent to similar dark treatments. It is interesting that the northern blot analyses indicate that the steady-state levels of SPS transcripts were markedly reduced after dark treatments of >12 h. Overall, our results indicate that *Sps* gene expression in maize leaf is modulated at multiple levels of controls by both developmental and environmental factors.

Suc is the principal and the preferred form of sugar transported to various sink tissues in plants. The enzyme SPS (EC 2.4.1.14) plays a key role in the synthesis of Suc-P and, ultimately, Suc, through the removal of phosphate ester by Suc-P phosphatase. SPS is localized in the leaf cytosol and is believed to contribute to the control of the flux of carbon fixation into Suc. SPS enzyme/protein has been well analyzed in terms of its in vitro regulation and its kinetic properties (Stitt et al., 1988; see reviews by Huber and Huber, 1992; Huber et al., 1993).

At the molecular level, the SPS gene is cloned from leaves in maize (*Zea mays*) (Worrell et al., 1991), spinach (Klein et al., 1993), and sugar beet (Hesse et al., 1995). In maize, a 3509-bp cDNA encodes a 1068-amino acid polypeptide with a molecular mass of 135 kD. In spinach, the cDNA clone and the polypeptide are 3530 bp and 117 kD, respectively. In sugar beet, a 3635-bp cDNA of SPS codes for a 1045-amino acid polypeptide with a molecular mass of 118 kD and shows regions of strong homology with the maize and spinach SPS gene (Hesse et al., 1995). The SPS protein is seen predominantly in mature, Suc-source leaves in maize (Bruneau et al., 1991; Worrell et al., 1991); however, in spinach SPS enzyme activity, protein (Walker and Huber, 1989), and RNA are detected as soon as young leaves begin to expand (Klein et al., 1993). Neither plant shows any detectable change in steady-state levels of SPS protein during the day/night cycles, and alterations in enzyme activity are due to posttranslational modifications of the SPS protein (Walker and Huber, 1989; Klein et al., 1993). Although SPS is a predominant enzyme of the photosynthetic tissues, it is also detected in nonphotosynthetic tissues (Klein et al., 1993, and refs. therein; Reimholz et al., 1994). In maize SPS RNA, protein, and enzyme activity are readily detectable in developing endosperm (Chourey et al., 1993; P.S. Chourey, W.-H. Cheng, E.W. Taliercio, and K.H. Im, unpublished data). In endosperm the SPS transcript is about 300 bp shorter than that in the leaf.

SPS activity is known to be modulated by both Suc-sink-source interactions as well as by N metabolism by virtue of Suc being a ubiquitous carbon source in nearly all plant cells (Champigny and Foyer, 1992; Huber et al., 1992). Yet very little is known about its various aspects of gene expression and regulation. In C_4 plants although BS and M are metabolically coupled, the Calvin cycle reactions are unique to BS. Similarly, transitory starch, which is a major source of carbon skeletons for Suc synthesis during nonphotosynthetic periods, is predominantly localized in BS of the maize leaf (Downton and Hawker, 1973; Furbank et al., 1985; see review by Preiss, 1988). SPS is believed to play a

[1] This work was supported in part by U.S. Department of Agriculture-National Research Initiative Competitive Grants Program grant no. 93011214 and was a cooperative investigation of the U.S. Department of Agriculture-Agricultural Research Service and the Institute of Food and Agricultural Sciences. This is University of Florida Agricultural Experiment Journal Series no. R–05147.

[2] These two authors contributed equally to this work.

* Corresponding author; e-mail psch@gnv.ifas.ufl.edu; fax 1–352–392–6532.

Abbreviations: BS, bundle-sheath cell(s); M, mesophyll cell(s); nt, nucleotide(s); SPS, Suc-P synthase.

critical role in Suc biosynthesis by coupling the Calvin cycle and starch turnover reactions in chloroplasts with the cytosolic reactions in both photosynthetic and nonphotosynthetic environments, respectively.

Despite such compartmentalization of functions, there are conflicting reports of cellular level specificity of SPS enzyme/protein in maize leaf. Whereas Furbank et al. (1985) showed that the predominant site of Suc biosynthesis is in the M, Ohsugi and Huber (1987) demonstrated that SPS is localized in both M and BS. However, both of the studies were limited to mature leaves and were based only on enzyme activity assays. We demonstrate here, based on immunohistochemical analyses of leaf sections, that SPS protein is localized in both BS and M, and more importantly, that there are cell-type-specific alterations in response to sink-source transition during plant development between young (sink) and mature (source) leaves. A transition in sink-source activity during early plant development was previously demonstrated by Deleens et al. (1984). Based on relative abundance of ^{13}C and ^{12}C in 7- to 10-d-old maize seedlings grown in a microphytotron environment, Deleens et al. (1984) showed that the first two leaves are initially entirely heterotrophic and that a gradual change to autotrophy occurs in subsequent stages after the 10th d of development. In addition, we show here that cell-type-specific alterations also occur with light and dark treatments in response to sink-source alterations, as was shown previously in mature leaves (Ohsugi and Huber, 1987). Our studies also show that the *Sps* gene is regulated at the RNA level of expression by light/dark treatments.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Unless stated otherwise, all maize (*Zea mays*) lines used in this study were inbred lines of either the W22 or the Pio. 3165 stocks. The *lemon white* and *iojap* mutants were in unknown inbred backgrounds. Seeds were germinated and grown either in a greenhouse or a growth chamber; the growth temperature was maintained within 28 to 32°C in the former and at 30°C in the latter. Greenhouse plants were grown in the normal diurnal (light/dark) environments, whereas plants in the growth chamber were maintained with a 12/12-h day/night regime (irradiance 400 μmol m^{-2} s^{-1} with incandescent and fluorescent bulbs). The leaf samples referred to here as young and mature leaves were harvested from 7 to 10-d-old seedlings and 4- to 6-week-old plants, respectively. The harvested leaves were frozen immediately in liquid N_2 and stored at −80°C for no more than 1 week prior to the biochemical and molecular analyses.

Immunolocalization in Leaf Sections

Leaf sections were fixed, embedded in paraffin, sectioned, and immunostained essentially according to the method of Langdale et al. (1987), with a few exceptions that are noted below. The first or the second leaf from seedlings, or the sixth leaf from mature plants, was cut with a razor in the midregion of the leaf into 2- to 3-mm leaf slices and fixed in formalin acetic alcohol (Jensen, 1962) with vacuum for 2 min. Prior to the selection of midregion in young leaves for our detailed analyses reported here, we examined base, middle, and tip regions of the second leaf because of the well-demonstrated developmental gradient in young leaves (for review, see Langdale and Nelson, 1991). After fixation for 1 h, leaf slices were dehydrated through a tertiary butyl alcohol series, infiltrated with Paraplast plus paraffin, embedded, and cut into 10-μm-thick sections using a rotary microtome. Paraffin ribbons with sections on slides were deparaffined in xylene, rehydrated to 30% ethanol, and sequentially washed in distilled water and PBS. Immunolocalization was done by incubation in preimmune serum for control and polyclonal SPS antibodies raised against maize leaf SPS protein (Bruneau et al., 1991). Incubation time in the 1:500-fold diluted antibody was adjusted to get the maximum contrast between the specific binding of the antibody to the target site and the nonspecific binding similar to that seen in the control. The slides were then incubated in a solution of secondary antibody comprised of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Visualization of the signal was done using New Fuchsin chromogen (Dako, Carpinteria, CA), which resulted in a precipitate of fuchsia-colored end product at the site of the antigen. Each comparative pair was immunostained and analyzed on the same slide to minimize possible artifacts due to staining reactions.

Protein Extraction and Western Blotting Assay

Crude extracts were prepared from either the fresh or the frozen leaf samples by first grinding in liquid N_2, followed by homogenization using a 1:4 (young leaf) or 1:8 (mature leaf) tissue:buffer ratio in extraction buffer containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (w/v) Triton X-100 (Huber and Huber, 1992). The homogenate was centrifuged at 14,000 *g* for 1 min in a microfuge. Protein concentrations were estimated by the method of Lowry et al. (1951). The supernatant was kept on ice, and 0.25 volume of 4× SDS extraction buffer (1× SDS extraction buffer = 60 mM Tris, 2% SDS, 10% glycerol, and 5% mercaptoethanol) was added to the supernatant. After the sample was boiled for 3 min, total protein extracts were separated by SDS-PAGE and electroblotted to a nitrocellulose membrane. The SPS polypeptide was visualized using polyclonal SPS antibodies against maize leaf SPS protein (Bruneau et al., 1991) and a chemiluminescence kit, following the manufacturer's (DuPont NEN, Wilmington, DE) protocol.

RNA Isolation and Northern Blot Analyses

Total RNA was isolated according to the method of Wadsworth et al. (1988). Poly(A)$^+$ RNA was isolated by passing the sample through two cycles of adsorption and elution on oligo(dT)-cellulose columns (Maniatis et al., 1982). RNAs treated with glyoxal and DMSO were fractionated on 1.2% agarose gels as described by Brown (1993). RNA was transferred to a Nytran membrane

(Schleicher & Schuell) and prehybridized in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% (w/v) SDS solution. The blots were hybridized with the full-length insert of maize leaf SPS cDNA, isolated by *Eco*RI-*Bam*HI digestion of the plasmid pCGN 3807 (Worrell et al., 1991) for 16 h at 65°C, and then washed. The first two washes were in 5× SSC/5% SDS and the next two washes were in 0.2× SSC/5% SDS at 65°C.

RESULTS

Cell-Specific Localization of SPS Protein

Figure 1 represents data from immunohistochemical localization of SPS protein in cross-sections of leaves. The sections from young green leaves (7- to 10-d-old seedlings) showed a strong signal for SPS protein, as evidenced by the intense fuchsia-colored reaction product, predominantly in BS, and a low level signal in M (Fig. 1a). In contrast, the albino leaves from the *lemon white* (*lw2*) mutant seedlings (Fig. 1b) showed no SPS antigen in these cells. The albino seedlings did not survive beyond the three-leaf stage. The signal levels in both BS and M were approximately equal in leaf sections from 6-week-old mature plants (Fig. 1c). The interdependence between photosynthetic ability and SPS accumulation seen in young seedlings (Fig. 1, a and b) was also observed in mature leaves of a 6-week-old *iojap* mutant plant marked by pale green and albino sectors in the same leaf (Rhoades, 1947). No SPS protein was detected in cells corresponding to the albino sector at the extreme right end of the section, whereas cells in the pale-green sector showed SPS antigen in the adaxial portion of the leaf section (Fig. 1e). Cells in the green sector showed the normal pattern of localization (data not shown), similar to the normal mature leaf (Fig. 1c). The preimmune serum showed the low, uniform background stain in sections from mature leaf (Fig. 1f).

To further test the correlation between photosynthetic competence and SPS expression, we examined dark-grown etiolated leaves and greening leaves by transferring the etiolated seedlings to illuminated environments. As expected, cross-sections from etiolated leaves showed no SPS antigen. A uniform, faint stain constituted the background level signal in these samples (Fig. 1, g and j). Leaf sections from greening plants, however, showed a significant cell-specific increase in the SPS signal (Fig. 1, h and i); in particular, the most intense signal was restricted to BS, and only a low level signal was seen in M. Furthermore, the level of signal was time-dependent; leaf sections from plants illuminated for 48 h yielded a stronger SPS signal than those treated for only 24 h (Fig. 1, i and h, respectively).

We also examined the effect of dark environments on light-grown plants by transferring young seedlings and mature plants to uninterrupted dark environments for 24 and 48 h, respectively. In each case, there was a detectable reduction in the SPS signal, particularly in M, and very little or no change was seen in BS. These spatial changes were readily seen in a 24-h dark treatment to young seedlings (Fig. 1, k versus l), but a similar response in mature leaves was not detectable until 48 h after the treatment (Fig. 1, c versus d). Although the choice of longer durations of dark treatments (in particular, 48 h) is nonphysiological, it was done deliberately to maximize our chances of detecting specific changes, particularly at the cellular level. As pointed out by Stitt et al. (1988), the regulatory mechanisms under physiological conditions might interact to generate a balance; thus, contrasting effects might not be detectable.

Spatial specificity of SPS localization in young leaves was tested by examining sections from base, middle, and tip regions of the second leaf from seedlings (Fig. 1, m–o). Consistent with previous reports (for review, see Langdale and Nelson, 1991), a developmental gradient of the lowest and the highest SPS levels, particularly in the BS, was seen at the base and the tip, respectively (Fig. 1, m and o, respectively), and the intermediate levels were seen in the midregion. This is presumably reflective of the photosynthetic maturity within the leaf, as shown previously for other enzymes (Langdale et al., 1987). Finally, it should be noted that, although the SPS signal in various leaf sections appeared to be associated with plastids, we believe it is not possible to make any judgment concerning its intracellular location from these studies at the light microscope level. The observed labeling on chloroplasts may have been due to the lack of resolution of the immunocytochemical signal in thin areas of cytoplasm surrounding the plastids in an otherwise vacuolated cell.

Expression of SPS at RNA and Protein Level in Leaves

Figure 2 represents a RNA blot showing SPS transcripts in total and poly(A)$^+$ RNAs extracted from leaves of mature plants and young seedlings grown in light or dark environments. A maize SPS cDNA clone (Worrell et al., 1991) was used as a hybridization probe to detect the transcripts. A single hybridizing fragment slightly larger than the 28S rRNA was seen in leaves of both young seedlings and mature plants. The estimated size of approximately 3500 nt for this transcript in leaves from both developmental stages was in agreement with the findings of Worrell et al. (1991). Total RNA from mature leaves showed much greater abundance of SPS transcripts than from young leaves. Etiolated seedlings showed greatly reduced steady-state levels of the normal-sized transcripts, which were detectable in only the poly(A)$^+$-enriched fractions but not in total RNA (Fig. 2). There was, however, a smaller-sized transcript, approximately 2900 nt, in the total RNA but not in poly(A)$^+$ RNA from etiolated samples. Because the smaller-sized RNA was the same size as the *Sus1*-encoded Suc synthase 2 (SS2) transcript (Gupta et al., 1988) and because of the reported limited sequence homologies between the SPS and SS cDNAs (Worrell et al., 1991), a similar RNA blot was also hybridized to the Sus1 cDNA probe. No hybridization was seen with this probe (data not shown). We conclude that the 2900-nt transcript was a truncated/degraded form of the SPS transcript, but the significance of this observation remains to be elucidated.

Figure 3 is a RNA blot showing total RNA from plants subjected to light (Fig. 3A) and dark (Fig. 3B) treatments.

critical role in Suc biosynthesis by coupling the Calvin cycle and starch turnover reactions in chloroplasts with the cytosolic reactions in both photosynthetic and nonphotosynthetic environments, respectively.

Despite such compartmentalization of functions, there are conflicting reports of cellular level specificity of SPS enzyme/protein in maize leaf. Whereas Furbank et al. (1985) showed that the predominant site of Suc biosynthesis is in the M, Ohsugi and Huber (1987) demonstrated that SPS is localized in both M and BS. However, both of the studies were limited to mature leaves and were based only on enzyme activity assays. We demonstrate here, based on immunohistochemical analyses of leaf sections, that SPS protein is localized in both BS and M, and more importantly, that there are cell-type-specific alterations in response to sink-source transition during plant development between young (sink) and mature (source) leaves. A transition in sink-source activity during early plant development was previously demonstrated by Deleens et al. (1984). Based on relative abundance of ^{13}C and ^{12}C in 7- to 10-d-old maize seedlings grown in a microphytotron environment, Deleens et al. (1984) showed that the first two leaves are initially entirely heterotrophic and that a gradual change to autotrophy occurs in subsequent stages after the 10th d of development. In addition, we show here that cell-type-specific alterations also occur with light and dark treatments in response to sink-source alterations, as was shown previously in mature leaves (Ohsugi and Huber, 1987). Our studies also show that the *Sps* gene is regulated at the RNA level of expression by light/dark treatments.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Unless stated otherwise, all maize (*Zea mays*) lines used in this study were inbred lines of either the W22 or the Pio. 3165 stocks. The *lemon white* and *iojap* mutants were in unknown inbred backgrounds. Seeds were germinated and grown either in a greenhouse or a growth chamber; the growth temperature was maintained within 28 to 32°C in the former and at 30°C in the latter. Greenhouse plants were grown in the normal diurnal (light/dark) environments, whereas plants in the growth chamber were maintained with a 12/12-h day/night regime (irradiance 400 μmol m^{-2} s^{-1} with incandescent and fluorescent bulbs). The leaf samples referred to here as young and mature leaves were harvested from 7 to 10-d-old seedlings and 4- to 6-week-old plants, respectively. The harvested leaves were frozen immediately in liquid N_2 and stored at −80°C for no more than 1 week prior to the biochemical and molecular analyses.

Immunolocalization in Leaf Sections

Leaf sections were fixed, embedded in paraffin, sectioned, and immunostained essentially according to the method of Langdale et al. (1987), with a few exceptions that are noted below. The first or the second leaf from seedlings, or the sixth leaf from mature plants, was cut with a razor in the midregion of the leaf into 2- to 3-mm leaf slices and fixed in formalin acetic alcohol (Jensen, 1962) with vacuum for 2 min. Prior to the selection of midregion in young leaves for our detailed analyses reported here, we examined base, middle, and tip regions of the second leaf because of the well-demonstrated developmental gradient in young leaves (for review, see Langdale and Nelson, 1991). After fixation for 1 h, leaf slices were dehydrated through a tertiary butyl alcohol series, infiltrated with Paraplast plus paraffin, embedded, and cut into 10-μm-thick sections using a rotary microtome. Paraffin ribbons with sections on slides were deparaffined in xylene, rehydrated to 30% ethanol, and sequentially washed in distilled water and PBS. Immunolocalization was done by incubation in preimmune serum for control and polyclonal SPS antibodies raised against maize leaf SPS protein (Bruneau et al., 1991). Incubation time in the 1:500-fold diluted antibody was adjusted to get the maximum contrast between the specific binding of the antibody to the target site and the nonspecific binding similar to that seen in the control. The slides were then incubated in a solution of secondary antibody comprised of biotinylated anti-mouse anti-rabbit immunoglobulin and streptavidin alkaline phosphatase. Visualization of the signal was done using New Fuchsin chromogen (Dako, Carpinteria, CA), which resulted in a precipitate of fuchsia-colored end product at the site of the antigen. Each comparative pair was immunostained and analyzed on the same slide to minimize possible artifacts due to staining reactions.

Protein Extraction and Western Blotting Assay

Crude extracts were prepared from either the fresh or the frozen leaf samples by first grinding in liquid N_2, followed by homogenization using a 1:4 (young leaf) or 1:8 (mature leaf) tissue:buffer ratio in extraction buffer containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (w/v) Triton X-100 (Huber and Huber, 1992). The homogenate was centrifuged at 14,000 *g* for 1 min in a microfuge. Protein concentrations were estimated by the method of Lowry et al. (1951). The supernatant was kept on ice, and 0.25 volume of 4× SDS extraction buffer (1× SDS extraction buffer = 60 mM Tris, 2% SDS, 10% glycerol, and 5% mercaptoethanol) was added to the supernatant. After the sample was boiled for 3 min, total protein extracts were separated by SDS-PAGE and electroblotted to a nitrocellulose membrane. The SPS polypeptide was visualized using polyclonal SPS antibodies against maize leaf SPS protein (Bruneau et al., 1991) and a chemiluminescence kit, following the manufacturer's (DuPont NEN, Wilmington, DE) protocol.

RNA Isolation and Northern Blot Analyses

Total RNA was isolated according to the method of Wadsworth et al. (1988). Poly(A)$^+$ RNA was isolated by passing the sample through two cycles of adsorption and elution on oligo(dT)-cellulose columns (Maniatis et al., 1982). RNAs treated with glyoxal and DMSO were fractionated on 1.2% agarose gels as described by Brown (1993). RNA was transferred to a Nytran membrane

(Schleicher & Schuell) and prehybridized in 50 mM Pipes (pH 6.5), 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% (w/v) SDS solution. The blots were hybridized with the full-length insert of maize leaf SPS cDNA, isolated by *Eco*RI-*Bam*HI digestion of the plasmid pCGN 3807 (Worrell et al., 1991) for 16 h at 65°C, and then washed. The first two washes were in 5× SSC/5% SDS and the next two washes were in 0.2× SSC/5% SDS at 65°C.

RESULTS

Cell-Specific Localization of SPS Protein

Figure 1 represents data from immunohistochemical localization of SPS protein in cross-sections of leaves. The sections from young green leaves (7- to 10-d-old seedlings) showed a strong signal for SPS protein, as evidenced by the intense fuchsia-colored reaction product, predominantly in BS, and a low level signal in M (Fig. 1a). In contrast, the albino leaves from the *lemon white* (*lw2*) mutant seedlings (Fig. 1b) showed no SPS antigen in these cells. The albino seedlings did not survive beyond the three-leaf stage. The signal levels in both BS and M were approximately equal in leaf sections from 6-week-old mature plants (Fig. 1c). The interdependence between photosynthetic ability and SPS accumulation seen in young seedlings (Fig. 1, a and b) was also observed in mature leaves of a 6-week-old *iojap* mutant plant marked by pale green and albino sectors in the same leaf (Rhoades, 1947). No SPS protein was detected in cells corresponding to the albino sector at the extreme right end of the section, whereas cells in the pale-green sector showed SPS antigen in the adaxial portion of the leaf section (Fig. 1e). Cells in the green sector showed the normal pattern of localization (data not shown), similar to the normal mature leaf (Fig. 1c). The preimmune serum showed the low, uniform background stain in sections from mature leaf (Fig. 1f).

To further test the correlation between photosynthetic competence and SPS expression, we examined dark-grown etiolated leaves and greening leaves by transferring the etiolated seedlings to illuminated environments. As expected, cross-sections from etiolated leaves showed no SPS antigen. A uniform, faint stain constituted the background level signal in these samples (Fig. 1, g and j). Leaf sections from greening plants, however, showed a significant cell-specific increase in the SPS signal (Fig. 1, h and i); in particular, the most intense signal was restricted to BS, and only a low level signal was seen in M. Furthermore, the level of signal was time-dependent; leaf sections from plants illuminated for 48 h yielded a stronger SPS signal than those treated for only 24 h (Fig. 1, i and h, respectively).

We also examined the effect of dark environments on light-grown plants by transferring young seedlings and mature plants to uninterrupted dark environments for 24 and 48 h, respectively. In each case, there was a detectable reduction in the SPS signal, particularly in M, and very little or no change was seen in BS. These spatial changes were readily seen in a 24-h dark treatment to young seedlings (Fig. 1, k versus l), but a similar response in mature leaves was not detectable until 48 h after the treatment (Fig. 1, c versus d). Although the choice of longer durations of dark treatments (in particular, 48 h) is nonphysiological, it was done deliberately to maximize our chances of detecting specific changes, particularly at the cellular level. As pointed out by Stitt et al. (1988), the regulatory mechanisms under physiological conditions might interact to generate a balance; thus, contrasting effects might not be detectable.

Spatial specificity of SPS localization in young leaves was tested by examining sections from base, middle, and tip regions of the second leaf from seedlings (Fig. 1, m–o). Consistent with previous reports (for review, see Langdale and Nelson, 1991), a developmental gradient of the lowest and the highest SPS levels, particularly in the BS, was seen at the base and the tip, respectively (Fig. 1, m and o, respectively), and the intermediate levels were seen in the midregion. This is presumably reflective of the photosynthetic maturity within the leaf, as shown previously for other enzymes (Langdale et al., 1987). Finally, it should be noted that, although the SPS signal in various leaf sections appeared to be associated with plastids, we believe it is not possible to make any judgment concerning its intracellular location from these studies at the light microscope level. The observed labeling on chloroplasts may have been due to the lack of resolution of the immunocytochemical signal in thin areas of cytoplasm surrounding the plastids in an otherwise vacuolated cell.

Expression of SPS at RNA and Protein Level in Leaves

Figure 2 represents a RNA blot showing SPS transcripts in total and poly(A)$^+$ RNAs extracted from leaves of mature plants and young seedlings grown in light or dark environments. A maize SPS cDNA clone (Worrell et al., 1991) was used as a hybridization probe to detect the transcripts. A single hybridizing fragment slightly larger than the 28S rRNA was seen in leaves of both young seedlings and mature plants. The estimated size of approximately 3500 nt for this transcript in leaves from both developmental stages was in agreement with the findings of Worrell et al. (1991). Total RNA from mature leaves showed much greater abundance of SPS transcripts than from young leaves. Etiolated seedlings showed greatly reduced steady-state levels of the normal-sized transcripts, which were detectable in only the poly(A)$^+$-enriched fractions but not in total RNA (Fig. 2). There was, however, a smaller-sized transcript, approximately 2900 nt, in the total RNA but not in poly(A)$^+$ RNA from etiolated samples. Because the smaller-sized RNA was the same size as the *Sus1*-encoded Suc synthase 2 (SS2) transcript (Gupta et al., 1988) and because of the reported limited sequence homologies between the SPS and SS cDNAs (Worrell et al., 1991), a similar RNA blot was also hybridized to the Sus1 cDNA probe. No hybridization was seen with this probe (data not shown). We conclude that the 2900-nt transcript was a truncated/degraded form of the SPS transcript, but the significance of this observation remains to be elucidated.

Figure 3 is a RNA blot showing total RNA from plants subjected to light (Fig. 3A) and dark (Fig. 3B) treatments.

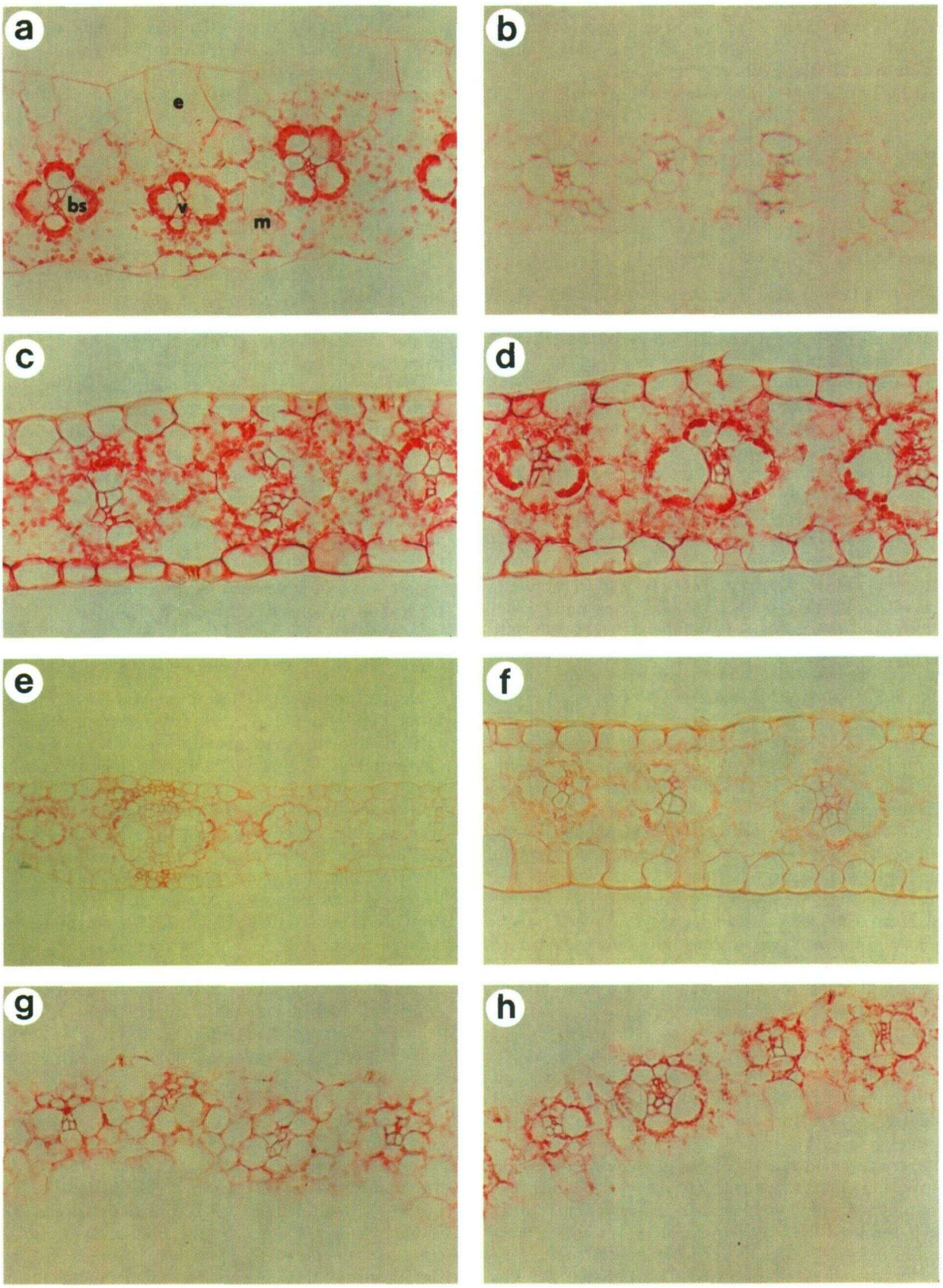

Figure 1. (Figure and legend continue on facing page.)

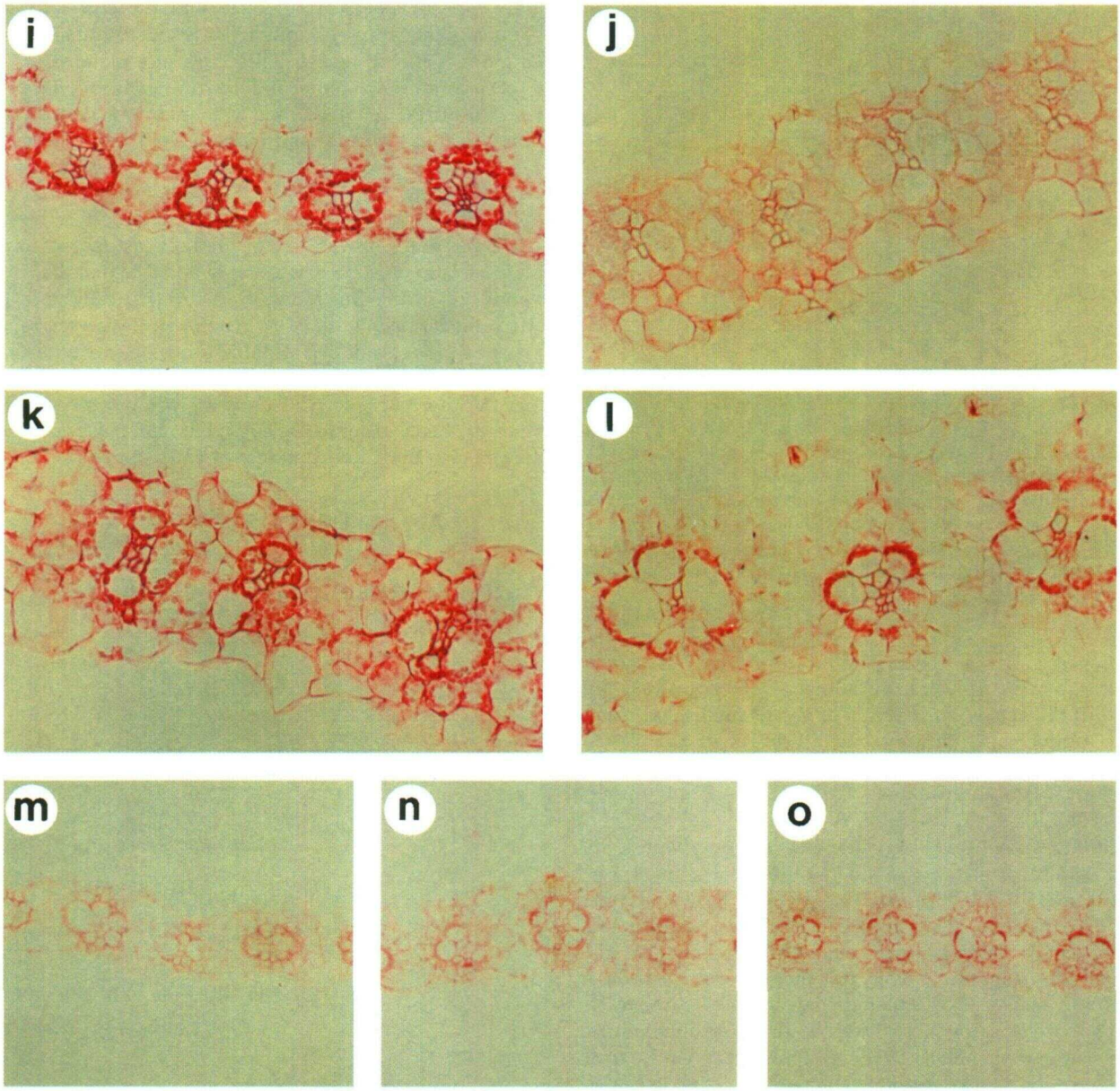

Figure 1. (Continued from facing page.) Immunolocalization of SPS in leaves from various samples. a, Young green leaf, *Lw* genotype, grown under the normal diurnal light/dark cycle. bs, Bundle-sheath cell; m, mesophyll cell; e, epidermis; v, vein. b, Young albino leaf, *lw* genotype; the same growth environment as in a. c, Mature leaf under the normal diurnal light/dark cycle. d, Mature leaf; the same growth environment as in c plus 48 h of continuous dark treatment. e, Mature leaf, *iojap* mutant; the section represents pale-green and albino sectors; cells representing the albino sector are at the right. f, Mature leaf as in c but treated with preimmune serum. g, Etiolated young leaf from dark-grown seedlings. h, Leaf from dark-grown seedlings plus a 24-h normal diurnal condition in the greenhouse. i, Leaf from dark-grown seedlings plus a 48-h normal diurnal condition in the greenhouse. j, Etiolated young leaf; the same as in g. k, Young green leaf from seedlings grown under the normal diurnal light/dark cycle. l, Young green leaf; the same as in k plus 24 h of continuous dark treatment. m to o, Young green leaf; as in k, representing sections from base (m), middle (n), and tip (o). Magnification: a to d, and f to l, ×59.4; e, m, n, and o, ×29.7.

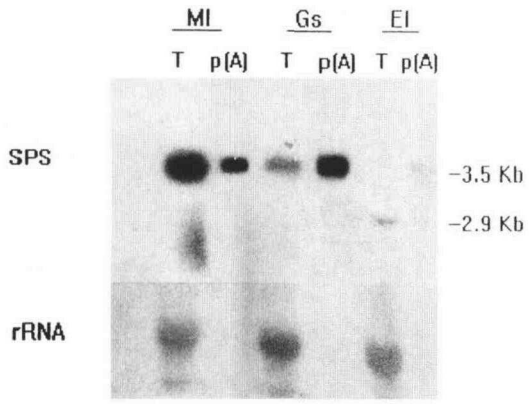

Figure 2. SPS transcripts in young and mature leaves. RNA gel blot showing SPS-specific transcripts in total RNAs (T, 20 μg) and poly(A)$^+$ RNAs (pA, 1 μg) from mature leaves (Ml) and young leaves from green (Gs) and etiolated seedlings (El). The probe was a full-length cDNA insert corresponding to the maize leaf SPS gene (Worrell et al., 1991). A single transcript of approximately 3500 nt was seen in all samples, except in the etiolated leaves, which showed a shorter transcript of approximately 2900 nt in total RNA but not in poly(A)$^+$. The same blot was hybridized with a maize rRNA probe to show rRNA levels (bottom) as an internal control.

Transfer of etiolated seedlings to uninterrupted light environments of 24- and 48-h durations led to a gradual increase in the steady-state levels of the normal-sized transcript. Conversely, the steady-state levels of SPS RNA declined when light-grown seedlings were transferred to continuous dark environments for 12-, 24-, and 48-h durations (Fig. 3B).

Figure 4 is a western blot showing SPS polypeptide in crude extracts from young and mature leaf samples. Extracts from both developmental stages have shown a single SPS polypeptide of approximately 120 kD in size. Bruneau et al. (1991) also observed an approximately 120-kD polypeptide in mature leaves, but there are no such data for young leaves. Dark-treated leaf samples, similar to those analyzed at the RNA level, were also examined by SDS-western blot analyses. Whereas dark treatment of mature plants led to no detectable change in relative levels of SPS polypeptide, extracts from young seedlings showed slightly reduced levels after 24- and 48-h treatments as compared with plants without such a treatment. As expected, etiolated leaves showed no detectable level of the SPS protein. The greening leaves, however, showed an induction of the SPS protein, the same size as in mature leaves, in a time-dependent fashion; it was in greater abundance in seedlings exposed to light for 48 h than those exposed to light for 24 h.

DISCUSSION

Altered Expression at the Cellular Level by Developmental Transition and by Photoregulation

The data in this report concerning the expression of the *Sps* gene in maize leaves are important in several respects. The most noteworthy observation concerns the cellular level specificity of SPS protein and the correlated alterations in its expression due to sink-source transitions during development and to light/dark modulations.

The immunohistological data show that the SPS protein was localized in both BS and M in mature leaves, as shown previously by Ohsugi and Huber (1987), based on enzyme activity from extractable saps from BS and M. In contrast, Furbank et al. (1985) observed an average of 10-fold excess SPS activity in M as compared with BS. There is, however, a large range of variability in these estimates, and the lowest ratio of M:BS was 2.0. It is possible that the time lag involved in the fractionation of the two cell types could account for such variability. The in situ analyses reported here, although not quantifiable, did not encounter such a delay due to the rapid fixation of leaf sections. In addition, so far there are no SPS data on young leaves that are entirely heterotrophic (Deleens et al., 1984). Here, our studies of such Suc-importing leaves have shown predominant

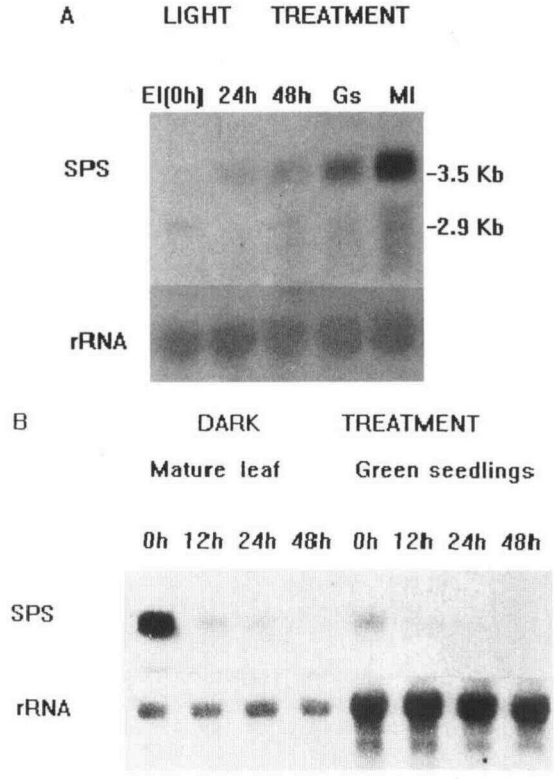

Figure 3. Photoregulation of SPS at the RNA level of expression. A, SPS transcripts in 20 μg of total RNA from samples (from left to right): El (0 h), dark-grown etiolated leaves without any light treatment; 24 h and 48 h, etiolated seedlings exposed to light for 24 and 48 h, respectively; Gs, leaves from green seedlings grown in the greenhouse under the normal diurnal environment; Ml, mature leaves from approximately 6-week-old plants grown in the greenhouse under the normal diurnal environment. B, SPS transcripts in total RNA from mature leaves (10 μg) and young leaves (20 μg) in samples (from left to right): 0 h, approximately 6-week- and 8- to 10-d-old plants grown in greenhouse under the normal diurnal environment; 12 h, 24 h, and 48 h, the same as above except plants were kept in continuous darkness for 12, 24, and 48 h, respectively. The same blots were hybridized with a maize rRNA probe to show rRNA levels (bottom) as internal controls for loading levels.

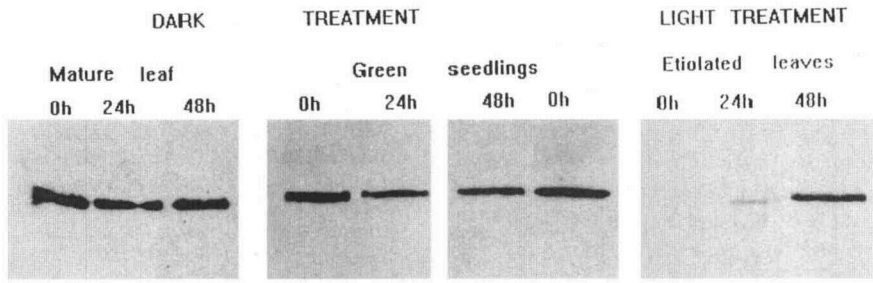

Figure 4. SDS western blot showing SPS polypeptide in crude extracts of leaves from plants exposed to light and dark environments. Mature leaves from approximately 6-week-old plants and young leaves from 8- to 10-d-old seedlings grown under the normal diurnal environment in the greenhouse were transferred to a dark growth chamber for 24 or 48 h. 0 h represents no dark treatment or no light treatment. No major change was seen in the levels of SPS protein with dark treatment. Dark-grown etiolated seedlings were transferred to an uninterrupted illuminated environment for 24 and 48 h to determine possible effects of light. A time-dependent increase in the levels of SPS protein was seen with light treatments.

localization of the SPS protein in BS. Similarly, greening leaves obtained by the transfer of dark-grown etiolated seedlings to normal light conditions also yielded a relatively strong signal in BS. No SPS protein was detected in similar leaf sections from either the albino seedlings of the *lemon white* mutant or the etiolated seedlings. We surmise on the basis of these data that the SPS protein in BS of the Suc-importing leaves was either dependent on or correlated with the photosynthetic competence of these cells.

Light is well established as a major signal in the control of BS-specific expression of the ribulose-1,5-bisphosphate carboxylase gene (Sheen and Bogorad, 1987; Langdale et al., 1988), as well as the enzymes in the Calvin cycle pathway (Hatch and Osmond, 1976). In the long chain of photoassimilatory reactions, SPS is an important cytosolic enzyme that is known to control the flux of carbon fixation into Suc. The co-localization of SPS protein with the Calvin cycle enzymes in BS, especially in greening leaves and in young leaves from light-adapted seedlings, is of specific interest. We suggest on the basis of these data that BS may play a major role in the early biosynthesis of Suc in developing leaves. It is possible that photosynthetic competence in terms of carbon fixation into Suc in BS precedes that of M; the former might export Suc to the latter for a brief time in young leaves. Photosynthetic metabolites, including Suc, are increasingly recognized as major factors in the regulation of several genes (Sheen, 1990). Wang et al. (1993) also suggested that accumulation and transport of photoassimilates may influence developmental patterns of gene expression, since the cell-type-specific changes in ribulose-1,5-bisphosphate carboxylase expression are in coordination with sink-to-source transition in amaranth leaves. Based on the SPS protein localization data shown here, we suggest that Suc synthesized in the BS may be one of the putative light-induced factors (Langdale and Nelson, 1991) that contributes to the development of photosynthetic competence of M.

The dark treatments also yielded a differential cellular response in BS and M in SPS expression. Although there was no detectable change in the signal level in BS, the levels in M were appreciably reduced; such reductions were much more rapid in young leaves than in mature leaves. Ohsugi and Huber (1987) observed a similar high percentage of whole-leaf SPS activity in BS early in dark periods. We suggest that the cell-specific response in M is related to the predominant role these cells play in the photoassimilatory biosynthesis of Suc. In dark environments, the depleted pools of Suc may down-regulate SPS expression in M, which has been shown previously in enzyme activity determinations at the whole-plant level under the diurnal control of growth environment (Furbank et al., 1985; Ohsugi and Huber, 1987; Stitt et al., 1988). In contrast, the relative insensitivity of SPS protein in BS to dark environments may indicate, a priori, that it is not light-modulated and is presumably responsible for nonphotosynthetic biosynthesis of Suc through starch turnover (Ohsugi and Huber, 1987; Farrar, 1991), since starch is strictly confined to BS in maize leaves (Downton and Hawker, 1973; Furbank et al., 1985; Preiss, 1988). Thus, based on these data, we suggest that BS may again serve as a Suc source during the nonphotosynthetic environments.

Sps Expression Is Altered at RNA Level by Light/Dark Treatments

There is substantial biochemical information concerning the regulation of SPS enzyme activity under light/dark environments in maize and spinach (summarized by Huber and Huber, 1991; Huber et al., 1993, and refs. therein). The enzyme exists in phosphorylated or dephosphorylated forms, the latter being the more active form in light-adapted plants. In vitro phosphorylation of SPS extracts from illuminated maize leaves is associated with a substantial reduction in enzyme activity to a level similar to that seen in dark-adapted plants (Huber and Huber, 1991). Thus, phosphorylation leads to much down-regulation of enzyme activity, although it does not reach a zero level. In addition, the immunohistological tests on leaf sections in these studies have clearly shown major cell-type-specific alterations in SPS protein localization with light/dark treatments, particularly in young leaves. An obvious question is how the biochemical forms of SPS protein, phosphorylated and dephosphorylated, correspond to the cellular forms described here. Although our current data do not provide an answer to this question, certain correlations between the two merit attention. For example, it is possible that the light-responsive form seen in M is the more active,

less phosphorylated form of the protein, which corresponds to the protein seen predominantly in the BS, which is also less responsive to light/dark cycles.

In addition, we have data from northern blot analyses that indicate that the *Sps* gene is also photoregulated at the RNA level of expression. In particular, there is a time-dependent increase in the steady-state levels of SPS RNA when dark-grown plants are exposed to light (Fig. 3A). Because of the relatively long time lag in the light induction of the gene, we speculate that the response is dependent on other rapid, light-responsive events prior to the accumulation of detectable levels of transcripts, as described previously for light-induction of PEP carboxylase genes in maize leaves (Schaffner and Sheen, 1992). Similarly, there are significant reductions in the steady-state levels of SPS transcripts caused by dark treatments in both young and mature plants. To the best of our knowledge, modulation of *Sps* expression at the RNA level due to the light/dark treatments in maize has not been demonstrated previously. Klein et al. (1993) observed reduced steady-state levels of SPS RNA in spinach plants adapted to a low level of irradiance. A control at the RNA level of expression may be a normal diurnal mode of regulation. Whether these alterations are due to changes in the rate of gene transcription and/or differential stabilities of RNA remains to be analyzed. We speculate that the RNA level changes in *Sps* gene expression are due to metabolic effects, contributed mainly by significant reductions in both SPS and nitrate reductase activities associated with the diurnal changes (Champigny and Foyer, 1992; Huber et al., 1992). These two enzymes control major points in the metabolic pathways relating to Suc and nitrate assimilation, respectively.

It is interesting that Suc is demonstrated to control the expression of the nitrate reductase gene at the RNA level in Arabidopsis (Cheng et al., 1992). Similarly, Hesse et al., (1995) demonstrated that exogenous feeding of Suc to leaves in sugar beets is associated with a marked reduction in the steady-state levels of SPS mRNA. Thus, SPS may belong to an increasingly large number of genes in which the expression is under metabolic control by Suc and/or hexose sugar (Mass et al., 1990; Sheen 1990; Graham et al., 1994; see review by Thomas and Rodriguez, 1994). Furthermore, our data also suggest that SPS RNA in maize leaves has a much shorter half-life than the corresponding protein. It is possible that the relatively greater stability of the protein level is attributable to the posttranslational changes associated with phosphorylation/dephosphorylation in controlling the enzyme activity (Huber and Huber, 1991).

ACKNOWLEDGMENTS

We are grateful to Drs. Toni A. Voelker (Calgene, Inc., Davis, CA) for the maize SPS cDNA clone and antibodies for maize SPS, Tim Nelson and Jan Langdale (Yale University, New Haven, CT) for sharing a laboratory protocol on immunolocalization of proteins in leaf sections, and E. Echeverria, D.R. Pring, and E.W. Taliercio for critical reading of the manuscript.

Received March 15, 1996; accepted May 7, 1996.

LITERATURE CITED

Brown T (1993) Analysis of RNA by northern and slot blot hybridization. *In* FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl, eds, Current Protocols in Molecular Biology, John Wiley & Sons, New York

Bruneau JM, Worrell AC, Cambou B, Lando D, Voelker TA (1991) Sucrose phosphate synthase, a key enzyme for sucrose biosynthesis in plants. Protein purification from corn leaves and immunological detection. Plant Physiol **96:** 473–478

Champigny ML, Foyer C (1992) Nitrate activation of cytosolic protein kinase diverts photosynthetic carbon from sucrose to amino acid biosynthesis. Plant Physiol **100:** 7–12

Cheng CL, Acedo GN, Christinsin M, Conkling MA (1992) Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc Natl Acad Sci USA **89:** 1861–1864

Chourey PS, Taliercio EW, Im KH (1993) Sucrose phosphate synthase in developing kernels of maize (abstract no. 24). Plant Physiol **102:** S-6

Deleens E, Gregory N, Bourdu R (1984) Transition between seed reserve use and photosynthetic supply during development of maize seedlings. Plant Sci Lett **37:** 35–39

Downton WJS, Hawker JS (1973) Enzymes of starch and sucrose metabolism in *Zea mays* leaves. Phytochemistry **12:** 1551–1556

Farrar JF (1991) Starch turnover: its role in source-sink relations and a comparison with the role of sucrose. *In* JL Bonnemain, S Delrot, WJ Lucas, J Dainty, eds, Recent Advances in Phloem Transport and Assimilate Compartmentation. Presses Academiques, Nantes, France, pp 213–223

Furbank RT, Stitt M, Foyer CH (1985) Intercellular compartmentation of sucrose synthesis in leaves of *Zea mays* L. Planta **164:** 172–178

Graham IA, Denby KJ, Leaver CJ (1994) Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. Plant Cell **6:** 761–772

Gupta M, Chourey PS, Burr B, Still PE (1988) cDNAs of two non-allelic sucrose synthase genes in maize: cloning, expression, characterization and molecular mapping of the *sucrose synthase-2* gene. Plant Mol Biol **10:** 215–224

Hatch MD, Osmond CB (1976) Compartmentation and transport in C4 photosynthesis. *In* CR Stocking, U Heber, eds, Encyclopedia of Plant Physiology, New Series, Vol 3: Transport in Plants III. Springer, New York, pp 144–184

Hesse H, Sonnewald U, Willmitzer L (1995) Cloning and expression analysis of sucrose-phosphate synthase from sugar beet (*Beta vulgaris* L.). Mol Gen Genet **247:** 515–520

Huber SC, Huber JL (1991) Regulation of maize leaf sucrose-phosphate synthase by protein phosphorylation. Plant Cell Physiol **32:** 319–326

Huber SC, Huber JL (1992) Role of sucrose-phosphate synthase in sucrose metabolism in leaves. Plant Physiol **99:** 1275–1278

Huber SC, Huber JL, Campbell WH, Redinbaugh MG (1992) Comparative studies of the light modulation of nitrate reductase and sucrose phosphate synthase activities on spinach leaves. Plant Physiol **100:** 706–712

Huber SC, Huber JL, Pharr M (1993) Assimilate Partitioning and Utilization in Source and Sink Tissues. International Crop Science, Madison, WI, pp 789–796

Jensen WA (1962) Botanical Histochemistry. WH Freeman, San Francisco, CA

Klein RR, Crafts-Brander SJ, Salvucci ME (1993) Cloning and developmental expression of the sucrose-phosphate-synthase gene from spinach. Planta **190:** 498–510

Langdale JA, Metzler MC, Nelson T (1987) The *argentia* mutation delays normal development of photosynthetic cell-types in *Zea mays*. Dev Biol **122:** 243–255

Langdale JA, Nelson T (1991) Spatial regulation of photosynthetic development in C4 plants. Trends Genet **7:** 191–196

Langdale JA, Zelitch, I, Miller E, Nelson T (1988) Cell position and light influence C4 versus C3 patterns of photosynthetic gene expression in maize. EMBO J **7:** 3643–3651

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the Folin phenol reagent. J Biol Chem **193:** 265–275

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Mass, C, Schaal S, Weer W (1990) A feedback control element near the transcription start site of the maize *Shrunken* gene determines promoter activity. EMBO J **9:** 3447–3452

Ohsugi R, Huber SC (1987) Light modulation and localization of sucrose phosphate synthase activity between mesophyll cells and bundle sheath cells in C_4 species. Plant Physiol **84:** 1096–1101

Preiss J (1988) Biosynthesis of starch and its regulation. *In* The Biochemistry of Plants, A Comprehensive Treatise, Vol 14. Academic Press, San Diego, CA, pp 182–249

Reimholz R, Geigenberger P, Stitt M (1994) Sucrose-phosphate synthase is regulated via metabolites and protein phosphorylation in potato tubers, in a manner analogous to the enzyme in leaves. Planta **192:** 480–488

Rhoades MM (1947) Plastid mutations. Cold Spring Harbor Symp Quant Biol **11:** 202–207

Schaffner AR, Sheen J (1992) Maize C4 photosynthesis involves different regulation of phosphoenol-pyruvate carboxylase genes. Plant J **2:** 221–232

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

Sheen J, Bogorad L (1987) Differential expression of C4 pathway gene in mesophyll and bundle sheath cells of greening maize leaves. J Biol Chem **262:** 11726–11730

Stitt M, Willce I, Feil R, Heldt HW (1988) Coarse control of sucrose-phosphate synthase in leaves: alterations of the kinetic properties in response to the rate of photosynthesis and the accumulation of sucrose. Planta **174:** 217–230

Thomas BR, Rodriguez RL (1994) Metabolite signals regulate gene expression and source/sink relations in cereal seedlings. Plant Physiol **106:** 1235–1239

Wadsworth GJ, Redinbaugh MG, Scandalios JG (1988) A procedure for the small scale isolation of plant RNA suitable for RNA blot analysis. Anal Biochem **172:** 279–283

Walker JL, Huber SC (1989) Regulation of sucrose-phosphate-synthase activity in spinach leaves by protein level and covalent modification. Planta **177:** 116–120

Wang JL, Turgeon R, Carr JP, Berry JO (1993) Carbon sink-to-source transition is coordinated with establishment of cell-specific gene expression in a C4 plant. Plant Cell **5:** 289–296

Worrell AC, Bruneau JM, Summeerfelt K, Boersig M, Voelker TA (1991) Expression of maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. Plant Cell **3:** 1121–1130