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Circadian Regulation of Sucrose Phosphate Synthase Activity in Tomato by Protein Phosphatase Activity[1]

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Sucrose phosphate synthase (SPS), a key enzyme in sucrose biosynthesis, is regulated by protein phosphorylation and shows a circadian pattern of activity in tomato. SPS is most active in its dephosphorylated state, which normally coincides with daytime. Applying okadaic acid, a potent protein phosphatase inhibitor, prevents SPS activation. More interesting is that a brief treatment with cycloheximide, a cytoplasmic translation inhibitor, also prevents the light activation of SPS without any effect on the amount of SPS protein. Cordycepin, an inhibitor of transcript synthesis and processing, has the same effect. Both of these inhibitors also prevent the activation phase of the circadian rhythm in SPS activity. Conversely, cycloheximide and cordycepin do not prevent the decline in circadian SPS activity that normally occurs at night. These observations indicate that SPS phosphatase activity but not SPS kinase activity is controlled, directly or indirectly, at the level of gene expression. Taken together, these data imply that there is a circadian rhythm controlling the transcription of a protein phosphatase that subsequently dictates the circadian rhythm in SPS activity via effects on this enzyme's phosphorylation state.

The ubiquitous nature of clock-controlled processes in virtually all living organisms suggests a fundamental biological relevance. Indeed, circadian rhythms are involved in diverse processes including daily sleep/wake cycles in humans (Edmunds, 1988), gene transcription in prokaryotes (Kondo et al., 1993), as well as leaf movements (Darwin, 1880; Satter and Galston, 1981), stomatal conductance (Hennessey and Field, 1992), and photosynthesis (Britz et al., 1987; Hennessey and Field, 1991) in higher plants. Attempts to understand both the mechanism and purposes of circadian oscillations in higher plants have revealed numerous genes that exhibit circadian regulation of transcription (e.g. Otto et al., 1988; Kondo et al., 1993; Zhong et al., 1994; Anderson and Kay, 1995).

Surprisingly, by comparison, there are relatively few examples of circadian rhythms in enzyme activity. One reason for this disparity is that the protein products are often abundant and quite stable, so that a circadian oscillation in gene transcription and subsequent translation often does not result in any detectable oscillation at the protein level. This, for example, is the case for cab and Rubisco activase (Martino-Catt and Ort, 1992). Another probable reason that few plant enzymes have been reported to have circadian oscillations in activity is that circadian activity is easily obscured by robust light-regulated activity. However, because circadian rhythms are observed in numerous whole plant processes, as mentioned above, it is apparent that many enzymes must have circadian rhythms in activity. Circadian oscillations in enzyme activity could be conferred in various ways, including cycling protein levels, cycling substrate concentrations, changing levels of allosteric effectors, or changing protein phosphorylation or redox state. For example, circadian oscillations in nitrate reductase activity in *Gonyaulax polyedra* result from circadian fluctuations in the nitrate reductase protein level (Ramalho et al., 1995). On the other hand, the circadian rhythm in PEPc activity in CAM plants is generated by circadian changes in the PEPc protein phosphorylation state, which are in turn the result of circadian oscillations in PEPc protein kinase activity (Carter et al., 1991).

Our interest in circadian rhythms in enzyme activity has focused on reactions that might underlie the chilling sensitivity of photosynthesis in chilling-sensitive plant species. An overnight low-temperature treatment (4°C) impairs next-day photosynthesis by as much as 60% in chilling-sensitive plant species such as tomato (Martin et al., 1981). The mechanism for this inhibition of photosynthesis is unknown; however, one striking effect of the chilling treatment is to delay the progress of the circadian clock regulating the transcription of certain nuclear-encoded genes (Martino-Catt and Ort, 1992). Our interest in the mechanism of the chilling sensitivity of photosynthesis led us to investigate ways that uncoordinated gene expression or enzyme activity, as a result of low temperature-induced mistiming of transcriptional circadian rhythms, might lead to feedback inhibition in the metabolic reactions of photosynthesis. Our results led us to focus on SPS, a key enzyme in Suc biosynthesis and an enzyme that has been shown to have an endogenous rhythm in activity in at least one plant species (Kerr et al., 1985).

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Abbreviations: cab, chlorophyll *a/b* binding protein; LSU and SSU, large and small subunits of Rubisco; PEPc, PEP carboxylase; PVPP, polyvinylpolypyrrolidone; SPS, Suc phosphate synthase.

SPS catalyzes the penultimate step in the Suc biosynthetic pathway, forming Suc-6-P from UDP-Glc and Fru-6-P. SPS has been purified from spinach and has a subunit molecular weight of approximately 120 kD (Walker and Huber, 1989a; Salvucci et al., 1990; Sonnewald et al., 1993). Conflicting reports suggest that the active form of SPS is a homodimer (Salvucci et al., 1990), a homotetramer (Walker and Huber, 1989a), or both (Sonnewald et al., 1993). Metabolic flux analysis indicates that control of the Suc biosynthetic pathway is shared between SPS and cytosolic Fru-1,6-bisphosphatase (Stitt, 1989). The subsequent conversion of Suc-6-P to Suc is thought to exert little if any control on Suc biosynthesis (Krause and Stitt, 1992). The important role that SPS plays in regulating this pathway was confirmed by the transgenic overexpression of maize SPS in tomato. The resulting high levels of SPS activity altered carbon partitioning between Suc and starch as well as root:shoot ratios of the transformed plants (Worrell et al., 1991; Galtier et al., 1993, 1995).

Considering the important role that SPS plays in carbon metabolism, the regulation of SPS activity has appropriately received a considerable amount of attention. SPS activity is modulated at several levels; however, the protein's phosphorylation state is the largest determinant of catalytic activity in species that exhibit this form of regulation (Huber et al., 1989a, 1989b; Weiner et al., 1992). In these species SPS activity is negatively regulated by protein phosphorylation. Studies on the regulation of SPS kinase and SPS phosphatase are under way in several laboratories, and it appears that the modulation of SPS phosphatase activity controls the diurnal modulation of the SPS phosphorylation state (Weiner et al., 1992, 1993). In addition to protein phosphorylation, SPS activity is further regulated allosterically by Glc-6-P and Pi (Doehlert and Huber, 1984) and, in the case of developing leaves, SPS protein levels also change (Walker and Huber, 1989b). Many plant species show diurnal regulation of SPS, i.e. light regulation (Huber et al., 1985, 1989b), and at least one plant species has been demonstrated to exhibit endogenous rhythms in SPS activity (Kerr et al., 1985). However, in the best-studied plant species such as spinach, sugar beet, and maize, regulation of SPS activity does not appear to include a circadian component (e.g. Li et al., 1992; Weiner et al., 1992).

We have studied the regulation of SPS in tomato because of our interest in the effects of low temperature on SPS activity in chilling-sensitive plant species. Our results on the effect of low temperature on SPS activity patterns will be presented elsewhere. We demonstrate here that SPS activity exhibits diurnal and circadian rhythms in tomato, which are not the results of corresponding changes in the SPS protein level. Using inhibitors directed against protein kinases and phosphoprotein phosphatases, we have shown that the rhythm in SPS activity in tomato is almost certainly the result of changes in the protein phosphorylation state. Additional experiments using inhibitors of transcription and translation revealed that de novo transcription and subsequent translation is required for the activation phase of the circadian rhythm. Conversely, de novo transcription and translation is not required for the deactivation of SPS, although protein kinase activity is required. Together, these experiments indicate that there may be a circadian rhythm controlling the transcription of SPS phosphatase, which is in turn responsible for the circadian rhythm in SPS activity.

MATERIALS AND METHODS

Plant Growth Conditions

Tomato plants (*Lycopersicon esculentum* Mill. cv Floramerica) were raised in growth chambers from surface-sterilized seeds in Promix brand (Premier, Quebec, Canada) sterile soil. The fungicides Banrot (Grace-Sierra Horticultural Products, Milpiitas, CA) and Subdue (Ciba-Geigy, Greensboro, NC) were applied at planting and again 14 d after planting. The plants were watered daily and fertilized three times per week with Marvel 12–31-14 (Plant Marvel Lab, Chicago, IL) fertilizer supplemented with 10 mM KNO_3 and 1 mM $MgSO_4$. Tomato plants were maintained under a 14-h light (26°C), 10-h dark (21°C) diurnal regime with illumination (350–450 μmol quanta m^{-2} s^{-1} PPFD) supplied by fluorescent bulbs (General Electric, F72T12-CW-1500) supplemented with incandescent lighting. Samples were taken from fully expanded leaves 21 to 28 d after planting.

Assay of SPS Activity

SPS activity was assayed in vitro using crude tissue extracts following a protocol that was modified from the method of Huber et al. (1992). Samples were frozen in liquid nitrogen in preweighed foil envelopes and stored at −80°C until use. The samples were ground under liquid nitrogen and then suspended in extraction buffer (1 g tissue 4 mL^{-1} buffer) consisting of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% Triton X-100. The homogenate was subsequently centrifuged at 15,000*g* (4°C), and the supernatant was desalted on G-25 Sephadex spin columns that had been preequilibrated in extraction buffer minus Triton X-100.

SPS activity was determined in vitro by measuring the formation of Suc under "limiting" or V_{max} substrate conditions (Huber et al., 1989a, 1989b). The desalted tissue extract (45 μL) from above was added to 25 μL of reaction buffer, resulting in the following final concentrations for the "limiting" reaction: 10 mM UDP-Glc, 3 mM Fru-6-P, 12 mM Glc-6-P, 10 mM Pi, 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$, and 2.5 mM DTT. Phosphate was removed for the V_{max} assay and the concentration of Fru-6-P was increased to 10 mM and that of Glc-6-P to 40 mM. Samples were incubated at 30°C for 20 min and the reaction was stopped by the addition of 70 μL of 30% KOH. To remove any residual Fru-6-P from the samples that would otherwise react with the Suc detection reagent, samples were boiled for 10 min and then cooled on ice prior to the addition of 1 mL of 0.14% anthrone in sulfuric acid. For full colorimetric development, samples were incubated at 40°C for 30 min and the A_{620} was recorded. The blanks were identical to the samples but were quenched with the KOH prior to the addition of the leaf extract.

SPS activity was calculated on a chlorophyll basis. The chlorophyll concentration was assayed from the crude homogenates following an 80% acetone extraction and calculated as in the method of Graan and Ort (1984).

Production of SPS Antibodies

A truncated SPS protein was isolated from *Escherichia coli* transformed with a His-tagged potato SPS clone generously provided by Professor Mark Stitt (Heidelberg, Germany). The potato SPS clone contained bp 296 to 1488 (amino acids 60–456) of the potato SPS-4 clone (EMBL accession number X73477, cut with *Bam*HI). The clone was in the vector pQE11 and was transformed into the *E. coli* strain M15 (pREP4) (Qiagen, Chatsworth, CA).

SPS protein was isolated from a 500-mL *E. coli* culture that was induced with 2 mM isopropyl-β-D-thiogalactopyranoside. Nickel-affinity-column chromatography was used according to the Qiagen denaturing isolation protocol. The protein was eluted from the column using a descending pH gradient (pH 5.9–2.0) and then dialyzed overnight with 25 mM sodium phosphate buffer (pH 7.0). The SPS-containing fractions were identified by SDS-PAGE, and although much of the protein had precipitated during dialysis, these fractions were used by the University of Illinois Biotechnology Center to produce polyclonal antibodies from mice.

The antibodies produced by five different mice were screened by western analysis for efficacy in tomato. Three of the five mice produced antibodies that specifically recognized tomato SPS. These mice produced 7 mL of antibody containing solution that was effective for western blotting using a 1:500 dilution.

Western-Blot Analysis

Leaf punches were taken from tomato leaves using a size 4 cork borer (63 mm^2), frozen in liquid nitrogen, and stored at −80°C until use. Frozen leaf samples were ground in a modified 2× Laemmli buffer (Laemmli, 1970) with a mortar and pestle; the protein extracts were briefly vortexed with 8 mg PVPP, and then centrifuged for 2 min at 15,000*g*. The supernatant was subsequently heated at 80°C for 2 min. An equal volume of each sample was loaded on 7.5% SDS-PAGE gels and thereafter electrophoretically transferred to PVDF membrane (Millipore). After electroblotting, the PVDF membrane was blocked for 2 h with 3% gelatin, incubated with anti-SPS polyclonal antibody (1:500) for 4 h, and then probed with secondary antibody that was either ^{35}S-labeled (Amersham) or conjugated to a chemiluminescent system (ECL, Amersham). Subsequently, the blots were exposed to film (Kodak X-OMAT) and quantitated by laser densitometry (LKB Ultroscan XL, Bromma, Sweden). The quantitative relationship between the integrated absorbance of spots on the autoradiographs and the actual radioactivity or chemiluminescent emission of the sample was determined empirically for our conditions as described previously (Martino-Catt et al., 1993) to ensure that the assay was performed within its pseudolinear range.

Inhibitor Treatments

Attached leaves were lightly abraded with 400-grit Duralum powder (Electro Minerals Corp., Niagara Falls, NY), rinsed with water, blotted dry, and the inhibitors were applied directly to the leaf in a 0.5% Tween 20 solution (Cooper and Ort, 1988). Okadaic acid (10 μM) and staurosporine (100 μM) were applied to leaves in a 10-μL droplet, which was spread over the abraded leaf surface. Cordycepin (200 μg mL^{-1}) and cycloheximide (100 μg mL^{-1}) were applied following leaf abrasion by briefly submerging the leaf in the inhibitor solution. The incubation periods were as described in the figure legends. Following the experiment, treated leaves were rinsed, blotted dry, frozen in liquid nitrogen, and stored at −80°C until assayed.

The effectiveness of the cycloheximide treatment was determined by immediately following the inhibitor treatment with ^{35}S-Met in vivo labeling (Martino-Catt et al., 1993). ^{35}S-Met trans-label (250 μCi) was applied in a 25-μL droplet to the leaf after the cycloheximide treatment. After both a 30-min and a 4-h incubation period, protein samples were taken by grinding 63 mm^2 of leaf tissue in a mortar in a modified 2× Laemmli buffer (Laemmli, 1970; Martino-Catt et al., 1993). An equal volume of each sample was loaded on a 12.5% SDS-PAGE gel, transferred to PVDF by electroblotting, and visualized by autoradiography.

The effectiveness of the cordycepin treatment was demonstrated by showing the inhibition of transcription of the *cab*. Control samples were taken in the dark 3 h prior to the light cycle when *cab* transcription is at a minimum (Martino-Catt and Ort, 1992). At the same time, abraded leaves were treated with cordycepin (100 and 200 μg mL^{-1} in 0.5% Tween 20), incubated for 3 h in the dark, and then illuminated for 1 h to maximally induce *cab* gene transcription. Total RNA was isolated from frozen leaf samples (−80°C) by a small-scale hot phenol extraction (Verwoerd et al., 1989) and probed by northern-blot analysis using a pea pAB96 cDNA probe (Broglie et al., 1981). The blots were hybridized overnight with denatured probe at 63°C and washed 3 times (5 min per wash) with a solution containing 0.15 M NaCl, 0.015 M sodium citrate, and 0.1% SDS at 63°C. The northern blots were then washed 3 times (20 min per wash) in a solution of 0.03 M NaCl, 0.003 M sodium citrate, and 0.1% SDS at 63°C. After drying, the blots were exposed to Kodak X-OMAT film for 4 d.

RESULTS

SPS Activity Displays Diurnal and Circadian Rhythms in Tomato Leaves

SPS diurnal activity was monitored under "limiting" substrate conditions throughout the normal 14-h day/10-h night light cycle. Figure 1 shows a pronounced diurnal oscillation in SPS activity, exhibiting a doubling between day and night activities, which is similar to work reported by Galtier et al. (1993) for tomato. Whereas there is a slight progressive decline in activity throughout the day, onset of darkness predicates a sharp drop in SPS activity to low (nighttime) levels. The presence of an underlying circadian

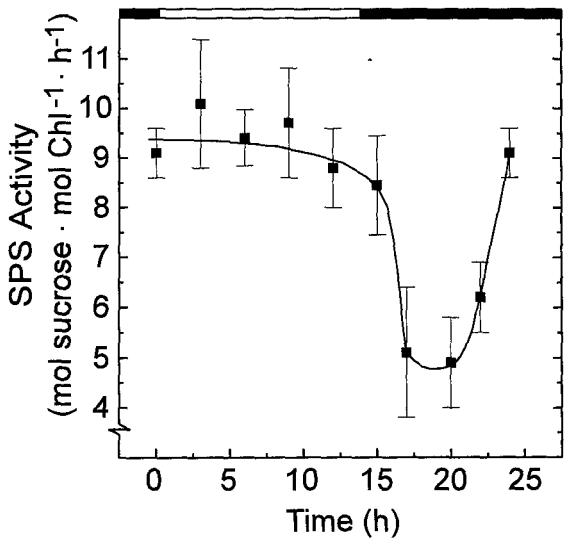

Figure 1. Diurnal oscillation of SPS activity in tomato leaves. Samples were taken throughout the normal day-night cycle as denoted by the light and dark boxes above the figure. Time = 0, Onset of the light cycle; the transition to darkness occurs at 14 h. Each point represents the mean ± SD of at least three independent samples. Chl, Chlorophyll.

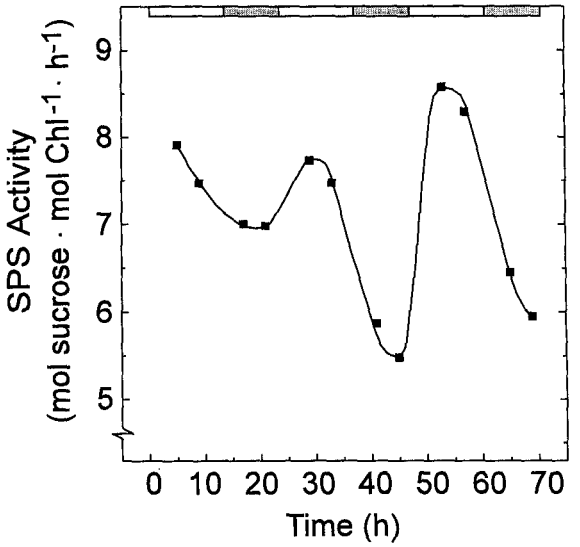

Figure 2. Circadian oscillation of SPS activity in tomato leaves. Plants were transferred to constant low light (50 μmol quanta m^{-2} s^{-1}) at the beginning of the normal photoperiod (0 h), and samples were taken over the following 72 h. The white boxes above the figure represent the normal day interval and the gray boxes represent subjective night. Each point is the average of at least two independent samples. Chl, Chlorophyll.

rhythm is indicated by the rise in dark SPS activity immediately prior to the light transition, as seen in the final two data points in Figure 1. This "anticipation" of the dark-to-light transition is a hallmark of circadian rhythms.

To further distinguish between diurnal (light-regulated) and endogenous circadian regulation of SPS activity, plants were maintained under constant conditions of light and temperature, and SPS activity was measured over the course of 3 d. Figure 2 shows a clear circadian pattern of "limiting" SPS activity in tomato. When monitored under V_{max} conditions, SPS displayed neither diurnal nor circadian regulation of activity (data not shown). The V_{max} rate was 15.7 ± 3.5 mol Suc mol^{-1} chlorophyll h^{-1}. Thus, the estimated activation state of SPS (i.e. limiting/V_{max}) was modulated between about 35 and 65% by circadian regulation. This range is nearly identical to that reported by Galtier et al. (1995) for the dark/light modulation of SPS in tomato.

The Circadian Oscillation in SPS Activity Is Not Due to Oscillations in SPS Protein Level

To determine the basis for the circadian oscillations in SPS activity, we first investigated the possibility that oscillations in SPS protein level were responsible for the changing SPS activity. Protein samples were taken throughout the 3-d constant light circadian time course at the same time points that activity was assessed in Figure 2. Leaf protein samples were separated by SDS-PAGE gel electrophoresis, transferred to PVDF membrane, and SPS was identified using mouse polyclonal anti-SPS antibody. By using secondary antibodies that were conjugated to a chemiluminescent system (ECL, Amersham) or that were ^{35}S-Met labeled, we were able to accurately monitor SPS

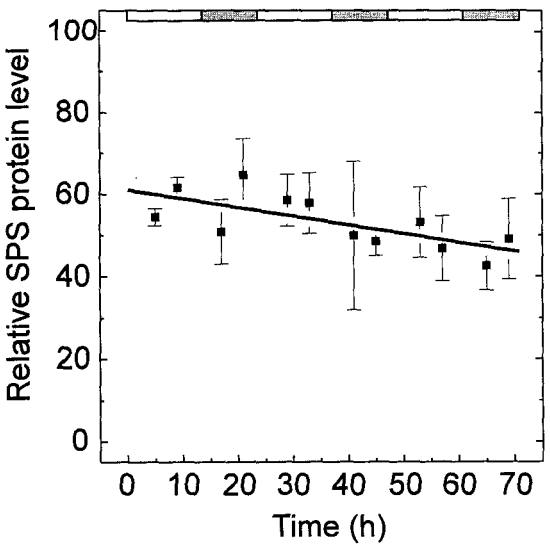

Figure 3. SPS protein level does not oscillate during a 72-h continuous light circadian time course in tomato leaves. Leaf protein samples were taken from the same leaves used to monitor SPS activity in Figure 2. Homogenates of crude leaf protein from each time point were loaded on SDS-PAGE gels and transferred to PVDF membranes. Western blots were performed using a polyclonal anti-SPS antibody coupled to either a chemiluminescent or radioisotopic secondary antibody. The exposed films were quantitated by laser densitometry. Each point represents the mean ± SD of three independent samples.

protein levels over the 3-d time course. Laser densitometry analysis demonstrated that there were no systematic oscillations in SPS protein level that could account for the circadian rhythm in SPS activity (Fig. 3). This result is consistent with our observation mentioned above that the V_{max} rate did not oscillate. Over the 3-d constant light (50 μmol quanta m^{-2} s^{-1}) time course the amount of SPS protein gradually declined by approximately 20%.

Inhibitor Studies Indicate that the Diurnal Regulation of SPS Activity Is the Result of Diurnal Cycling of the SPS Phosphorylation State

We used specific protein kinase and phosphatase inhibitors to investigate the role of protein phosphorylation in the regulation of SPS activity in tomato and to determine if the diurnal rhythm in SPS activity results from diurnal oscillations in the SPS phosphorylation state. Okadaic acid (10 μM), a specific inhibitor of primarily type 2A protein phosphatases (Mumbly and Walter, 1993), was used to inhibit SPS phosphatase activity, and staurosporine (100 μM), an inhibitor of Ser/Thr protein kinases (Tamaoki et al., 1986), was used to inhibit SPS kinase activity. These relatively high concentrations of the inhibitors were necessary because the compounds were applied to attached leaves via a mild leaf abrasion technique (Martino-Catt et al., 1993), in which only a portion of the inhibitor applied penetrates the leaf and enters the cellular cytoplasm. To investigate the specificity of okadaic acid treatment of intact tomato leaves, 1 μM of inhibitor was introduced into excised tomato leaflets via the transpirational stream. Okadaic acid inhibited tomato SPS in a concentration-dependent yet saturable fashion, consistent with specific inhibition of a phosphoprotein phosphatase (Fig. 4). The concentration of okadaic acid in the leaflet was calculated based on the mass of the leaflet and the volume of the okadaic acid solution transpired. Approximately 16 nmol g^{-1} fresh weight okadaic acid (about 1.6 μM) reduced SPS activity by 60%, which is consistent with the results obtained with spinach leaves (Siegl et al., 1990) and potato tubers (Reimholz et al., 1994).

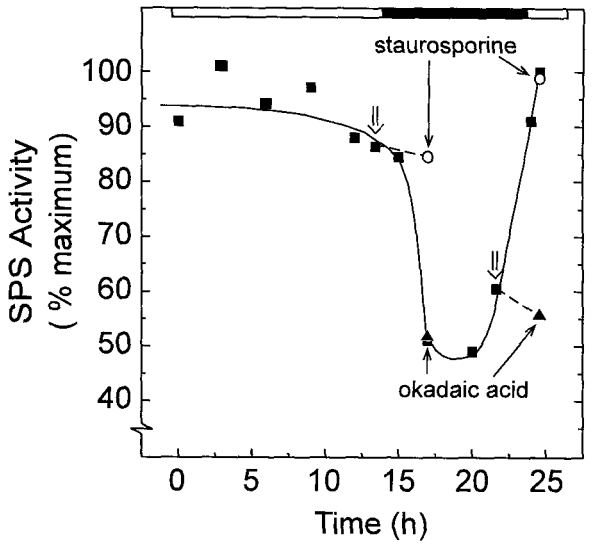

Figure 5. Effect of protein kinase and phosphatase inhibitors on SPS activity at the diurnal light/dark transitions in tomato leaves. Prior to the light-to-dark transition (13 h), 100 μL of either okadaic acid (10 μM, ▲) or staurosporine (100 μM, ○) was applied to lightly abraded, attached tomato leaves. The time of inhibitor addition is indicated by the double-lined arrow (⇓). Three hours after the transition to darkness (17 h), samples were harvested and SPS activity was assayed. To examine the dark-to-light diurnal transitions, 100 μL of okadaic acid (10 μM, ▲) or staurosporine (100 μM, ○) was applied before the transition at 22 h, and samples were taken 1 h after the light transition (25 h) for SPS activity determination. Each point represents the mean of at least four independent samples, and the standard deviations were less than 15% of the mean value.

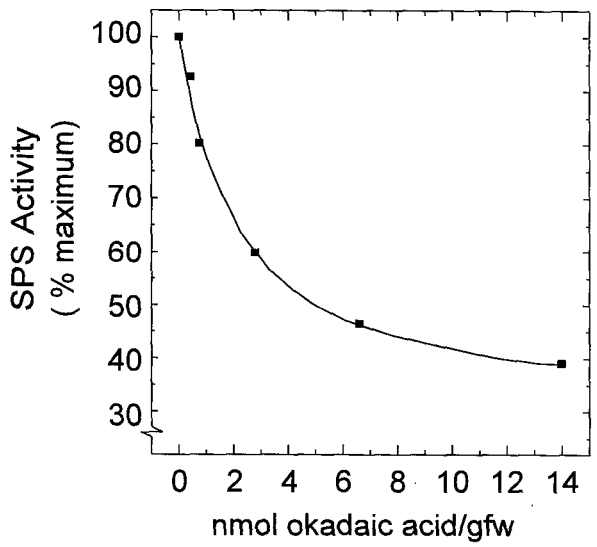

Figure 4. Okadaic acid inhibits light-dependent SPS activation in tomato. Okadaic acid (1 μM) was supplied to excised, illuminated (350 μmol quanta m^{-2} s^{-1}) tomato leaflets through the transpiration stream; control leaflets were given water. At the time the leaflets were harvested, the volume of okadaic acid solution transpired was determined and SPS activity was assayed. The amount of okadaic acid in the leaflet was estimated from the volume of okadaic acid solution transpired and the fresh weight of the leaflet. gfw, Grams fresh weight.

To investigate the role of protein phosphorylation at the light-to-dark diurnal transition, okadaic acid (10 μM, ▲) and staurosporine (100 μM, ○) were applied at 13 h, 1 h prior to the transition to darkness (Fig. 5). At 17 h samples were taken and assayed for SPS activity. Staurosporine (○) treatment prevented the conversion from the high daytime activity to the lower nighttime value, indicating that SPS kinase activity is required for the deactivation of SPS. Conversely, okadaic acid (▲) had no effect and SPS deactivated normally, showing that SPS phosphatase activity is not necessary for the deactivation of SPS.

To investigate SPS phosphorylation regulation at the dark-to-light transition, inhibitors were applied 2 h prior to the onset of light (22 h) and samples were harvested 1 h after the lights turned on (25 h). Okadaic acid (▲) strongly inhibited SPS light activation, whereas the staurosporine (○) treatment had no effect and SPS activated normally

(Fig. 5). Additionally, it should be noted that this lack of effect of staurosporine at the dark-to-light transition and of okadaic acid on SPS activity at the light-to-dark transition (see above) are important indicators that these compounds are acting specifically at the level of protein phosphorylation and not inducing broadly disruptive effects within the plants.

Taken together, these experiments support the model that the dephosphorylation of tomato SPS is required for light activation and that phosphorylation is required for dark deactivation. These results are typical for the diurnal regulation of SPS by protein phosphorylation, as demonstrated in certain other plant species (Huber et al., 1989b; Reimholz et al., 1994).

De Novo Transcription and Translation Is Required for Dark-to-Light, but Not Light-to-Dark, Transition in SPS Diurnal Activity in Tomato Leaves

We wanted to investigate if a rhythm in the expression of either SPS kinase or SPS phosphatase might underlie the diurnal oscillation that we observed in SPS activity (Fig. 1). Since neither SPS phosphatase nor SPS kinase has been cloned, it was not possible to monitor gene expression directly. Instead, we used inhibitors of transcription and translation to explore these questions.

The effectiveness of cycloheximide, which blocks translation on cytoplasmic ribosomes, was determined by labeling leaves with ^{35}S-Met immediately after applying cycloheximide (200 μg mL^{-1}). The synthesis of the chloroplast-encoded LSU of Rubisco was largely unaffected by cycloheximide treatment, whereas the synthesis of the nuclear-encoded SSU was abolished (Fig. 6). This was true for both the 15-min and 4-h incubations, demonstrating the effectiveness and selectivity of this inhibitor treatment.

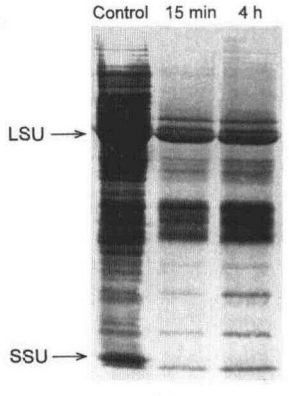

Figure 6. Cycloheximide is an effective inhibitor of cytoplasmic protein synthesis when applied to tomato leaves via leaf abrasion. Tomato leaves were lightly abraded, rinsed, and briefly submerged in 200 μg mL^{-1} cycloheximide in 0.5% Tween 20. Control leaves were abraded and submerged briefly in 0.5% Tween 20. At 15 min and 4 h following cycloheximide treatment, crude leaf protein was extracted from cycloheximide-treated and control leaves following a 15-min pulse label with ^{35}S Met (250 μCi). Thereafter, leaf proteins were separated by SDS-PAGE gel electrophoresis, dried, and autoradiographed. Gels were loaded with a constant amount of protein per lane.

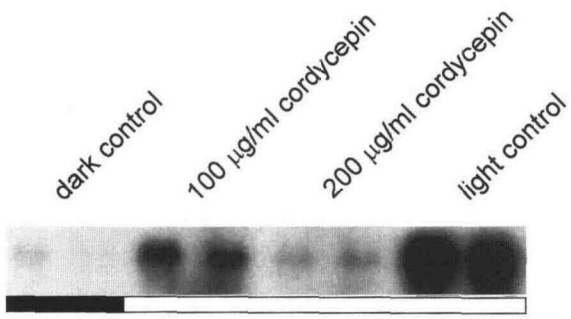

Figure 7. Cordycepin inhibits *cab* gene transcription when applied via a leaf abrasion technique. Cordycepin was applied at a time in the diurnal rhythm when cab transcription is at a minimum, at 21 h. At this time a dark control sample was harvested and leaves were abraded, rinsed, and briefly submerged in either 0.5% Tween 20, 100 μg mL^{-1} cordycepin in 0.5% Tween 20, or 200 μg mL^{-1} cordycepin in 0.5% Tween 20. The attached leaves remained in the dark for 3 h and were then illuminated for 1 h to maximize *cab* gene expression. Samples were taken for RNA isolation at 25 h. Gels were loaded with constant total RNA per lane and northern blots were probed with labeled *cab* cDNA. Each lane represents an individual treatment, and each experiment was repeated three times, with consistent results.

In many eukaryotic systems cordycepin acts most potently to inhibit cytoplasmic polyadenylation, perhaps acting directly on poly(A) polymerase (Duncan, 1995). However, this activity is superseded at higher concentrations, where cordycepin inhibits transcription directly, most likely as a chain-terminating adenosine analog (Cline and Rhem, 1974; Seeley et al., 1992). The effectiveness of cordycepin in intact tomato leaves was investigated by monitoring *cab* gene transcription, which has a well-defined circadian rhythm. Tomato *cab* gene transcription is very low at night 2 to 4 h prior to the dark-to-light transition (Tavladoraki et al., 1989; Martino-Catt and Ort, 1992; Piechulla, 1993). In the dark prior to the lights-on transition, *cab* transcription increases and, after the lights-on transition, *cab* is massively transcribed. Figure 7 is a northern blot showing that the dark control (21 h) sample had virtually no *cab* RNA, and that the Tween 20 light control had a very large amount of *cab* RNA. Treatment with 100 μg mL^{-1} cordycepin substantially reduced the *cab* mRNA level, whereas the 200 μg mL^{-1} treatment essentially abolished light-induced *cab* accumulation, demonstrating that cordycepin was present in the leaf at sufficient concentrations to inhibit at the level of gene transcription.

Cycloheximide (200 μg mL^{-1}, Δ) and cordycepin (200 μg mL^{-1}, ●) were applied at the light/dark transitions identically to the application of the phosphorylation inhibitors described in Figure 6. For the light-to-dark transition, neither cycloheximide (Δ) nor cordycepin (●) treatment had any effect on the normal diurnal deactivation of SPS (Fig. 8). This shows that the de novo transcription and translation of SPS kinase is not necessary for SPS deactivation, although we have shown that SPS kinase activity is required (Fig. 6). At the dark-to-light transition, both cycloheximide (Δ) and cordycepin (●) completely abolished the light activation of SPS, implicating a requirement for both

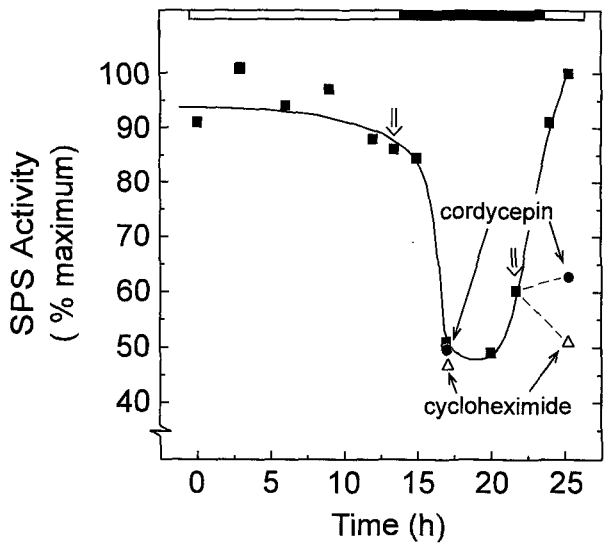

Figure 8. Effect of transcription and translation inhibitors on SPS activity at the diurnal light/dark transitions in tomato leaves. Prior to the light-to-dark transition, at 13 h, control samples were taken and cycloheximide (200 μg mL^{-1}, Δ) or cordycepin (200 μg mL^{-1}, ●) was applied to lightly abraded, attached tomato leaves. The time of inhibitor addition is shown by the double-lined arrows (⇓). After the transition to darkness, at 17 h, samples were taken and SPS activity was assayed. To examine the dark-to-light transition, cycloheximide (200 μg mL^{-1}, Δ) and cordycepin (200 μg mL^{-1}, ●) were applied in the dark at 22 h and samples were taken 1 h after the dark-to-light transition, at 25 h. Each point represents the mean of at least four samples and the standard deviation in all cases was less than 15% of the mean.

the transcription and subsequent translation of SPS phosphatase for SPS light activation (Fig. 8). It seems clear that while SPS kinase activity is required for dark-deactivation, the kinase is already present and need not be newly synthesized. On the other hand, our data support the idea that SPS phosphatase must be transcribed and translated in the morning when phosphatase activity is required to activate SPS. If so, it follows that the diurnal rhythm in SPS activity results from the transcriptional regulation of the SPS phosphatase gene(s).

Does Circadian Regulation of SPS Activity Result from Circadian Transcriptional Control of a Protein Phosphatase Gene?

The above results on diurnal regulation indicate that the circadian rhythm in SPS activity may be the result of a circadian rhythm in the transcription of SPS phosphatase. To determine if the circadian rhythm in SPS activity is driven by protein phosphorylation in a similar manner to the diurnal rhythm, we examined the effect of these inhibitors during the 3-d constant light time course (Fig. 9). Okadaic acid (10 μM, ▲) and cordycepin (200 μg mL^{-1}, ●) were applied to attached leaves at a low point in the circadian rhythm (45 h). Six hours later, at 51 h, samples were harvested for SPS activity assays. Both okadaic acid (▲) and cordycepin (●) inhibited the rise in SPS circadian activity (Fig. 9). In the companion experiment, where staurosporine (100 μg mL^{-1}, ○) and cordycepin (200 μg mL^{-1}, ●) were applied at a high point in SPS circadian activity (57 h) and samples were harvested 6 h later (63 h), staurosporine (○) inhibited the decline in SPS activity, whereas cordycepin (●) had no effect. These data are consistent with the premise that changes in protein phosphorylation state are required for the circadian oscillation in SPS activity and that an endogenous rhythm in the transcription of a protein phosphatase gene drives the circadian oscillation of the SPS phosphorylation state in tomato.

DISCUSSION

We have shown that SPS activity, assayed under "limiting" conditions, exhibits a diurnal and circadian rhythm in tomato (Figs. 1 and 2). To our knowledge, this is the first report of a circadian rhythm in SPS activity, although an endogenous ultradian rhythm with a period of about 12 h has been reported in *Glycine max* (L.) Merr. cv Ransom (Kerr et al., 1985). We think it is likely that circadian regulation of SPS activity will be observed in other plant species; however, it should be recognized that circadian rhythms in enzyme activity can be difficult to detect. In tomato it was not possible to detect a rhythm in constant darkness because SPS rapidly deactivated. Although we

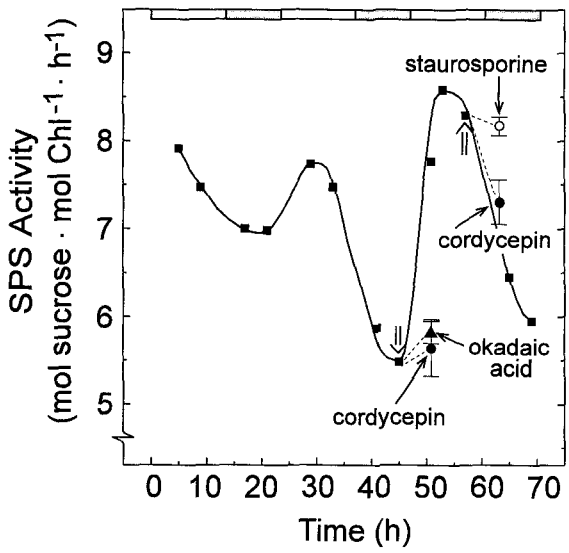

Figure 9. Effect of inhibitors on the circadian rhythm controlling SPS activity in tomato leaves. At a low point in the SPS activity rhythm (45 h), control samples were taken and leaves were treated with okadaic acid (10 μM, ▲) or cordycepin (200 μg mL^{-1}, ●). The time of the application of the inhibitors is indicated by the double-lined arrows (⇓). After 6 h (51 h), the inhibitor-treated leaves and 0.5% Tween 20-treated controls were harvested and SPS activity was determined. These data were normalized to the circadian rhythm profile at 45 h. At a high point in the SPS activity rhythm (57 h), control points were taken and the leaves were treated with staurosporine (100 μM, ○) or cordycepin (200 μg mL^{-1}, ●). Six hours later (63 h) the treated and control leaves were harvested and SPS activity determined. These values were normalized to the circadian rhythm profile at 57 h. Each point represents the mean and standard deviation of four samples. Chl, Chlorophyll.

were able to clearly discern a circadian oscillation in SPS activity under constant low-light (50 μmol quanta m^{-2} s^{-1}) conditions, increasing the illumination level to the growth light intensity (450 μmol quanta m^{-2} s^{-1}) obscured the rhythm and caused SPS activity to slowly ramp upward. Western analysis of SPS protein levels under this high-light condition demonstrated a gradual increase in SPS protein level, doubling in 56 h, which is coincident with the increase in enzyme activity (Jones and Ort, 1995). This is in marked contrast to the 20 to 25% drop in SPS protein levels over the 3-d low-light time course (Fig. 3), where there is a robust circadian oscillation in SPS activity (Fig. 2). It is not unusual to lose circadian rhythmicity under constant high-light conditions; for example, the bioluminescent rhythm of *Gonyaulax* desynchronizes under constant high-light conditions (Sweeney and Hastings, 1957). Additionally, whereas it may be possible that SPS protein level indirectly affects the oscillation in SPS activity, it is clear that the circadian oscillation in SPS activity is not the result of a circadian oscillation in SPS protein level (Fig. 3). Although the amount of SPS protein declines over the 3-d time course, it does not exhibit day-night oscillations.

Tomato SPS activity is specifically inhibited by increasing concentrations of okadaic acid (Fig. 4), confirming that tomato is among those species that employ phosphorylation regulation of SPS, which is consistent with the suggestion of Galtier and coworkers (1993). It is interesting to note in Figure 4 that the maximum inhibition of SPS activity by okadaic acid appears to be approximately 50% that of control activity, which is effectively the SPS activity seen in the dark. These results suggest that there is a persistent activity, equivalent to about one-third of the V_{max} rate, that arises either from a basal activity level for the phosphorylated form of SPS, or a separate population of SPS that is not regulated by phosphorylation-dephosphorylation. Although the need for some SPS activity in the dark is clear (e.g. for the mobilization of starch at night for translocation), and it is reasonable that dark activity would be independent of phosphorylation control, there is no assurance that these commonly employed in vitro assay conditions accurately reproduce the actual in situ rates.

Inhibitor studies with okadaic acid and staurosporine indicate that the circadian rhythm in SPS activity is driven by a circadian oscillation in the protein phosphorylation state of SPS in tomato (Figs. 5 and 9). To our knowledge this is the first evidence for a circadian oscillation in protein phosphorylation state in C3 plants. The only other example of circadian oscillations in protein phosphorylation state in plants is that of PEPc in CAM plants, which is driven by circadian oscillations in the activity of PEPc protein kinase (Carter et al., 1991).

The diurnal and circadian activation of SPS requires transcription in tomato, as evidenced by the effects of specific inhibitors of transcription and translation (Figs. 8 and 9). Our results in tomato, demonstrating a requirement for transcription and translation for diurnal and circadian activation of SPS, support and extend the work by Huber et al. (1992), which shows that transcription and translation are required for the light activation of spinach SPS. Of particular interest is the indication that circadian regulation of SPS phosphatase gene transcription drives the circadian rhythm in SPS activity. We suggest that SPS phosphatase message and protein pools turn over rapidly so that the circadian regulation of SPS phosphatase gene transcription controls the timing of SPS phosphatase activity, which is in turn the primary determinant of the activation state of SPS in tomato. In experiments similar to ours, Comolli et al. (1996) recently reported that protein phosphatase inhibitors alter the circadian pattern of bioluminescent emission in *Gonyaulax* and concluded that protein Ser/Thr phosphatases are required for the operation of the circadian system in this dinoflagellate. In fact, we cannot currently eliminate the possibility that the effects we see with okadaic acid are on some feature of the circadian system of tomato rather than directly on SPS dephosphorylation. However, our interpretation of these results in tomato is consistent with results in spinach (Weiner et al., 1992), where substantial light-induced changes in extractable SPS phosphatase activity were detected but no diurnal changes in the extractable activity of SPS kinase were seen. We have recently initiated experiments to directly investigate our notion of a circadian rhythm in SPS phosphoprotein phosphatase activity in tomato.

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