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Regulation of Sucrose Phosphate Synthase by Gibberellins in Soybean and Spinach Plants[1]

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ABSTRACT

Exogenous applications of gibberellins (GAs) increased the extractable activity of leaf sucrose phosphate synthase (SPS) in soybean (*Glycine max* [L.]) and spinach (*Spinacia oleracea* [L.]). The response to GA applications was detectable within 2 h postapplication and was still observed 6 h, 24 h, and 7 d after treatment. When paclobutrazol, a GA biosynthesis inhibitor, was applied to intact soybean and spinach plants, decreased extractable SPS activity resulted within 24 h following the treatment. Different methods of GA application (spray, injection, capillary wick, and excised leaf systems) produced similar effects on SPS activity of soybean leaves. Protein synthesis in soybean leaves appeared to be necessary for GA-promoted SPS activity because gibberellic acid only partially reversed the inhibitory effect of pretreatment with cycloheximide. Levels of SPS protein from crude extracts of spinach plants were measured by a dot blot technique using monoclonal antibodies against SPS. Application of gibberellic acid to spinach leaves increased levels of SPS protein 2 h, 24 h, and 7 d after treatment. The results suggest that, in both soybean and spinach, GA is one of the endogenous hormonal factors that regulate the steady-state level of SPS protein and, hence, its activity.

SPS[3] plays an essential role in the regulation of photosynthetic sucrose formation. Consequently, several studies have been directed at explaining the mechanisms that directly or indirectly regulate SPS activity in leaves of higher plants. Prior research has indicated that SPS activity appears to be regulated at two distinct levels (1, 17). The first is a metabolic "fine" control of enzyme activity, which is exerted by metabolic effectors that modify kinetic properties. The second is a "coarse" control, which refers to slower modifications in extractable V_{max} and may be caused by protein modification or turnover.

In many species, the activity of SPS changes diurnally. There appear to be several different mechanisms behind these diurnal fluctuations (17). In soybean leaves, there have been indications of an endogenous rhythm in SPS enzymic activity (3, 10). In certain other species, such as barley (15), maize (16), and spinach (18), SPS activity has been reported to change rapidly according to light/dark transitions and involves a mechanism of light activation of the enzyme. More recently, Huber et al. (6) characterized several species according to the extent of light modulation of the corresponding SPS enzyme. Three main groups were identified: one in which light activation involves an increase in V_{max} of the enzyme (barley, maize). A second group responds to light activation by alterations in certain kinetic properties of the SPS enzyme, such that activation is only apparent when SPS is assayed with the "limiting" substrate condition and in which light activation has no effect on V_{max} (spinach). Finally, there are species that are essentially unaffected by light/dark transitions (soybean) (6). This implies that not only may SPS be controlled by a number of factors, but also that its regulation is species specific, with possible variations in protein structure (17).

In the present study, the specific objective was to investigate the role of GA in the natural regulation of SPS activity in two different species (soybean [*Glycine max* L. Merr. cv Evans] and spinach *Spinacia oleracea* L. cv Bloomsdale]) in which the mechanisms of regulation of SPS activity are believed to differ greatly (6). In one set of experiments, the aim was to determine the effect of GAs on the activity of SPS by comparing the effect of several concentrations of the plant hormone, and to examine the kinetics of the GA effect. In addition, preliminary experiments were conducted to determine whether the GA effect involves activation of the existing enzyme or increased steady-state levels of the SPS protein.

MATERIALS AND METHODS

Plant Material and Chemicals

Soybean (*Glycine max* [L.] Merr. cv Evans) and spinach (*Spinacia oleracea* [L.] cv Bloomsdale) plants were planted in 10-cm plastic pots filled with soil consisting of a 1:1:2 (v/v) mixture of sand:soil:peat. Unless otherwise specified, soybean plants were grown in growth chambers that were maintained at 26/19°C (day/night) temperatures with a 14-h photoperiod. The PPFD at the top of the plant canopy was 400 μmol m^{-2} s^{-1} provided by a combination of cool-white fluorescent

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[3] Abbreviations: SPS, sucrose phosphate synthase; GA, gibberellin; F-6-P, fructose-6-phosphate; UDP-G, UDP-glucose.

Table I. *Response of Soybean Leaf SPS Activity to Applications of 10^{-6} M GA_3 and 1.7×10^{-4} M Paclobutrazol*

Plants were sprayed by hand with GA_3, paclobutrazol, or water (control) until run-off. Leaf sampling occurred at the same time. Values are the means ± SE of three replicates.

	SPS Activity	
	1.7×10^{-4} M paclobutrazol[a]	10^{-6} M GA_3[b]
	μmol of product g^{-1} fresh wt h^{-1}	
Control	39.8 ± 2.9	52.1 ± 4.5
2 h	ND[c]	70.2 ± 3.1
6 h	25.6 ± 2.0	61.3 ± 6.2
24 h	21.4 ± 3.0	78.2 ± 10.5

[a] Leaf tissue (fourth node) of 28-d-old plants was harvested 3 h into the photoperiod. [b] Leaf tissue of the fourth fully expanded leaf of 34-d-old plants was harvested 3 h into the photoperiod. [c] ND, Not determined.

lamps (F48T12/CW/WHO Philips bulbs) and incandescent lamps (60-W Philips bulbs).

Spinach plants were grown under similar conditions as described for the soybeans except for a 9/15-h (day/night) photoperiod and temperatures were maintained at 22/16°C (day/night). All plants were watered daily and were fertilized with a 20:20:20 (N:P:K) Peters' mixture and Peters' micronutrients twice a week, starting at the appearance of the first true leaf.

Fully expanded leaves of 4-week-old soybean and 6- or 9-week-old spinach plants were utilized in these studies. In all instances, when leaf tissue was harvested, it was immediately frozen either in liquid nitrogen or on dry ice. Samples were then stored at −80°C before extraction and assay of SPS.

Paclobutrazol was obtained from ICI Americas Inc. Unless otherwise indicated, all other chemicals were obtained from Sigma Chemical Co.

Enzyme Extraction

Unless otherwise indicated, the extraction of frozen leaves was accomplished by grinding the tissue with a precooled mortar and pestle using a 1:5 (w/v) tissue:buffer mixture with polyvinylpolypyrrolidone. The extraction buffer contained 50 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 2 mM GSH, 2% (w/v) PEG-20, and 0.5% (w/v) BSA. The homogenate was then squeezed through eight layers of cheesecloth. The filtrate was centrifuged at 27,000*g* for 15 min at 0 to 4°C. The pellet was discarded, and the resulting supernatant was immediately desalted on Sephadex G-25 (Sigma) columns equilibrated with the extraction buffer and then utilized for SPS assay.

Enzyme Assay

SPS was assayed as described by Kerr et al. (9) by measuring F-6-P-dependent sucrose (+sucrose-P) formation from UDP-G. The assay mixture contained 28 mM UDP-G, 10 mM F-6-P, 15 mM $MgCl_2$, 10 mM NaF, and 50 mM Hepes-NaOH (pH 7.5). An aliquot of the leaf extract was added to the assay mixture to initiate the reaction. The mixtures were incubated at 30°C for 10 min, and the reaction was terminated by adding 70 μL of 1 N NaOH. Unreacted fructose and F-6-P were then destroyed by placing the tubes in a boiling water bath for 10 min. After the assay mixtures were cooled, volumes of 250 μL of 0.1% (w/v) resorcinol and 750 μL of 30% (v/v) HCl were added, and the mixtures were incubated at 80°C for 10 min. The tubes were allowed to cool, and the absorbance at 520 nm was determined.

Dot Blot Analysis of SPS Protein Levels

The levels of SPS protein in crude extracts of spinach leaf tissue were determined using an ELISA as described by Walker and Huber (21). The monoclonal antibodies specific for spinach SPS were obtained using the same procedures and material described by Walker and Huber (20, 21).

RESULTS

Effect of GA on SPS Activity in Soybean Leaves

The role of GAs in the regulation of SPS activity was investigated by applying GA_3 exogenously. The effect of GA_3 at 10^{-6} M on enzyme activity occurred as early as 2 h following the GA treatment (Table I). The increase in SPS activity in response to GA_3 was also observed 6 and 24 h after hormonal treatment (Table I). We further examined the effect of four different modes of exogenous applications of GA on the response of SPS activity. Spraying intact leaves (10^{-6} M GA_3), injection through the petiole of 10 μL GA_3 (10^{-4} M), application of 10^{-6} M GA_3 via a capillary wick system (2), and dipping petioles of excised leaves in a GA_3 solution (10^{-6} and 10^{-4} M) for 4 h all elicited an increase in the extractable activity of SPS in the leaves (Table II).

In an attempt to deplete leaves of their endogenous GAs, paclobutrazol (PP333), a potent GA biosynthesis inhibitor

Table II. *Effect of Different Modes of GA_3 Application to Soybean Plants on the Extractable Activity of SPS*

	SPS Activity			
	Application via capillary wick system[a]	Spray intact leaves[b]	Injection through the petiole[c]	Excised leaf[d]
	μmol of product dm^{-2} h^{-1}			
Control (water)	131 ± 16	143 ± 38	162 ± 15	123 ± 2
10^{-6} M GA_3	253 ± 9	227 ± 37	ND[e]	333 ± 57
10^{-4} M GA_3	ND	ND	218 ± 33	158 ± 33

[a] GA_3 was applied using a capillary wick system as described by Brun et al. (2), and samples were collected 24 h after the start of application. [b] Plants were hand-sprayed with GA solution until run-off, and samples were harvested 24 h posttreatment. [c] Injection of 10 μL of 10^{-4} M GA_3 solution; sampling occurred 6 h later. [d] Petioles of excised leaves were placed in the GA_3 solutions for 4 h. [e] ND, Not determined.

(13), was applied to intact soybean plants. After 1.7×10^{-4} M paclobutrazol was applied, a reduction in extractable SPS activity was observed 6 and 24 h later (Table I).

The question of whether GAs caused synthesis of SPS protein or merely activated the preexisting SPS enzyme in soybean leaves was first tested using the protein synthesis inhibitor cycloheximide. Treating excised leaves and leaf discs with 3.6×10^{-4} M cycloheximide for 2 h significantly inhibited the extractable activity of SPS (Table III). To determine whether GA was capable of reversing the inhibition of SPS activity caused by cycloheximide, GA_3 was next added to the cycloheximide-pretreated leaf systems for 2 h. The results indicated that GA_3 does partially restore SPS activity, but the levels of response were still lower than those of the GA-treated controls (Table III).

Effect of GA_3 on SPS Activity and Enzyme Protein in Spinach Leaves

Spinach responded to GA_3 treatment similarly to soybean leaves by an increase in SPS activity. The response to GA application appears to shift according to the time of the treatment and GA concentration. GA_3 at 10^{-6} and 10^{-5} M were equally effective in increasing SPS activity when leaf samples were taken 2 h after treatment. However, at 24 h postapplication, 10^{-6} M GA_3 elicited the highest increase in enzyme activity (Table IV). It is noteworthy that the GA effect on SPS activity persisted for at least the 7-d experimental period (Table IV).

An effect of paclobutrazol on SPS activity similar to that detected in soybean leaves was observed in spinach. SPS activity was decreased by about 60% 24 h after paclobutrazol application; however, paclobutrazol increased enzyme activity when leaves were sampled 7 d following the treatment (Table IV). This increase in activity could be due to the long-term effects of paclobutrazol on other components of photosynthetic sucrose biosynthesis. In fact, reports have indicated that some of the long-term effects of paclobutrazol are delay of senescence, increase in Chl content, and eventual increase in photosynthetic activity (14, 17).

Immunochemical quantification of SPS protein in spinach leaves was our means of investigating the nature of the GA effect on leaf SPS. Recent research by Walker and Huber (20) led to purification and characterization of spinach SPS using monoclonal antibodies. These spinach-specific antibodies were used in the present study to quantitate SPS protein in spinach. However, when these antibodies were tested for cross-reactivity with SPS isolated from soybean, they did not bind to the active enzyme (20). This obstacle prevented our investigating the effect of GAs on the levels of SPS protein in soybean leaves.

We investigated the GA effect on SPS protein levels to examine the relationship between the regulation of the amount of SPS protein and its activity. The activity of SPS in leaf extracts was assayed under two different conditions, as described by Stitt et al. (18) and Walker and Huber (21). The enzyme activity was first tested under low-substrate ("limiting") conditions, in which the assay included lower substrate concentrations (10 mM UDP-G and 3 mM F-6-P) and the addition of 10 mM Pi, an inhibitor. In the limiting assay, changes in enzyme activity would reflect alterations in enzyme protein level and/or phosphorylation status of the enzyme (5). The second assay ("V_{max}" conditions) contained high substrate concentrations (10 mM UDP-G and 10 mM F-6-P) in the absence of Pi. Under the latter condition, alterations in activity would reflect some form of protein modification that affected maximum catalytic activity or amount of SPS protein present in the leaf.

When leaves from 9-week-old spinach plants were used in the experiment, the results provided evidence that GA_3 increased levels of SPS protein. This augmentation (32%) was apparent 2 h after 10^{-6} M GA_3 was applied and was still present 7 d following the treatment (61%, Table V). This change in the amount of SPS protein was also accompanied by some alteration in the kinetic properties of the enzyme. This is supported by the observation that the activation state increased only slightly 24 h and 7 d after the treatment (Table V). A similar experiment conducted on 6-week-old spinach plants confirmed the GA effect on SPS protein level (data not shown). Moreover, GAs were added to the purified SPS enzyme in vitro. When SPS was assayed under these conditions, there was no direct effect of the hormone on enzyme activity (data not shown).

Table III. *SPS Activity in Soybean Leaf Extracts Pretreated with Cycloheximide and Incubated in GA_3*

All treatments were sampled and assayed at the same time as the control.

	SPS Activity	
	Excised leaves	Leaf discs
	μmol of product g^{-1} fresh wt h^{-1}	
Control (water)	20.0 ± 2.8	13.1 ± 0.2
Cycloheximide (3.6×10^{-4} M for 2 h)	10.7 ± 1.4	10.1 ± 0.6
GA_3 control (10^{-6} M for 2 h)	24.1 ± 1.9	15.6 ± 0.5
3.6×10^{-4} M cycloheximide[a] + 10^{-6} M GA_3	15.8 ± 2.1	12.7 ± 0.7

[a] Excised leaves or leaf discs were pretreated with cycloheximide for 2 h and then transferred to GA_3 solution for another 2-h incubation before tissue was analyzed for SPS activity.

Table IV. *Time-Course Responses of SPS Activity from Spinach Leaf Extracts following Treatment with GA_3 and Paclobutrazol*

Spinach plants were 9 weeks old at the start of the experiment. Five replicates were used per treatment. Plants were sprayed with GA_3, paclobutrazol, or water (control), and sampling occurred 3 h into the photoperiod.

	SPS Activity		
	2 h	24 h	7 d
	μmol of product g^{-1} fresh wt h^{-1}		
Control	20.0 ± 0.8	26.8 ± 0.9	15.2 ± 0.8
10^{-6} M GA_3	24.4 ± 2.3	41.8 ± 5.3	21.7 ± 2.4
10^{-5} M GA_3	24.8 ± 2.4	21.0 ± 1.6	27.3 ± 1.1
1.7×10^{-4} M paclobutrazol	18.6 ± 1.1	10.8 ± 3.8	33.9 ± 8.0

Table V. *Variations in Enzyme Activity Protein Level and Activation State of SPS in Response to GA Treatment*

Spinach plants were 9 weeks old at the start of the experiment. Plants were sprayed with GA_3 or water (control), and sampling occurred 3 h into the photoperiod with four replications.

	SPS Activity[a]		Activation State[b]	SPS Protein
	Limiting	V_{max}		
	μmol of protein g^{-1} *fresh wt* h^{-1}		%	*μg* g^{-1} *fresh wt*
2 h				
Control	21.2 ± 0.6	70.7 ± 0.5	30	8.7 ± 1.2
10^{-6} M GA_3	23.0 ± 1.9	79.0 ± 2.4	29	11.5 ± 1.6
10^{-5} M GA_3	26.8 ± 1.8	88.1 ± 5.7	30	8.8 ± 0.4
24 h				
Control	23.3 ± 2.0	81.0 ± 5.7	29	9.4 ± 1.0
10^{-6} M GA_3	26.1 ± 1.1	78.4 ± 3.3	33	10.3 ± 1.4
10^{-5} M GA_3	32.1 ± 4.1	88.7 ± 4.3	36	9.0 ± 1.7
7 d				
Control	17.6 ± 2.4	54.6 ± 4.0	32	7.2 ± 1.0
10^{-6} M GA_3	25.5 ± 1.8	70.8 ± 3.4	36	11.6 ± 0.5
10^{-5} M GA_3	31.0 ± 2.3	79.8 ± 2.6	39	16.1 ± 1.3

[a] Enzyme extraction and assay in this experiment were performed as described by Huber et al. (5). [b] The activation state of the enzyme is defined as the ratio of activities under limiting versus saturating substrate conditions (5).

DISCUSSION

SPS has been the subject of several studies in the past decade. However, no conclusive information has been reported characterizing the regulation of the activity of this enzyme in soybean. Nevertheless, there have been reports indicating that SPS activity in soybeans is subject to distinct diurnal fluctuations that are due to an endogenous rhythm (3, 10). Close examination of the effect of GAs on leaf SPS in this study indicates that this plant hormone may serve as a natural modulator of SPS activity in leaves. Results of previous investigations indicate that the GA effect on SPS activity is a typical hormonal response (3). The results obtained following the application of paclobutrazol (a GA biosynthesis inhibitor) gave a clear indication that some level of GA in the leaf is necessary to maintain a base level of SPS activity. Moreover, the increase in enzyme activity was detected within 2 h of adding the plant hormone and was maintained through a 24-h treatment period. This demonstrates that a fast response is elicited by the GA on SPS activity. Earlier reports have indicated such fast responses (2–4 h) in other GA-promoted enzyme systems, such as α-amylase and protease (7). However, the characteristics of this response could be of a different nature, occurring either by alteration of the activity of SPS or via modulation in protein turnover (synthesis versus degradation). The response obtained following the application of a protein synthesis inhibitor, cycloheximide, may be an indication that protein synthesis is necessary for a GA-promoted SPS activity. Similar results have been shown in other enzyme systems (8, 19). However, we cannot exclude the possibility that the effect of cycloheximide on SPS activity could be of an indirect nature. This inhibitor has been reported to modify several parameters of photosynthetic carbohydrate synthesis, such as photosynthesis and control of stomatal function, and to alter levels of key and abundant photosynthetic enzymes (4, 11, 12).

Studies of spinach leaf SPS established that GA exerts an effect on the activity of the enzyme similar to that observed in soybean leaves. We are the first to report (this paper) the GA-promoted synthesis of SPS protein. Furthermore, the comparisons of GA effects on kinetic properties and levels of SPS protein in spinach leaves indicate that the influence of GAs in the present system involves mainly the coarse control of spinach leaf SPS activity, because the results indicated that changes in enzyme activity, in response to GA treatment, were largely the result of increased levels of SPS protein as opposed to activation of preexisting enzyme. It is important to note that GA treatment showed no significant effect on leaf total protein content (data not shown).

CONCLUSION

In the present study, GAs were clearly identified as one of the possible signals in the regulation of SPS activity in soybean and spinach leaves. Although previous studies have established the existence of kinetic and regulatory differences in leaf extract SPS from these two species, our investigations demonstrated that the response of this enzyme to increased levels of GA was similar in both species.

It is very significant that most of the increase in the extractable leaf spinach SPS activity appears to be attributable mainly to increased levels of enzyme protein. Any minor changes in the interconversions of the kinetic forms of the enzyme that we have observed in this study could be an indirect GA effect by modifications of levels and sensitivity to allosteric regulators, such as glucose-6-P and Pi.

Considerable progress has been made in recent years toward understanding the processes involved in hormonal control of protein synthesis. Much of the information available concerning GAs and regulation of enzyme levels has been obtained from aleurone layers of certain cereals (7). However, the accumulation of SPS in leaf cytosol in response

to GA presents an excellent opportunity for further studies of GA action on enzyme production.

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