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Anaerobiosis Induces Transcription but Not Translation of Sucrose Synthase in Maize[1]

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ABSTRACT

This report examines the effect of anaerobic stress on the expression of sucrose synthase in maize (*Zea mays* L.). Following 24 hours of anaerobic treatment, alcohol dehydrogenase displayed the classical characteristics of induction: increased mRNA and protein levels. However, there was no detectable increase in sucrose synthase specific proteins by either native or denaturing Western blot analysis nor was there an increase in sucrose synthase activity. Anaerobic treatment did induce significantly higher steady state levels of sucrose synthase mRNA. Even though previous work has implicated sucrose synthase as an anaerobically induced protein, the data in this report suggest that sucrose synthase is not inducible at the protein level by anaerobic treatment.

Anaerobiosis in plants is characterized by a pleiotropic molecular response, *i.e.* the synthesis of most cellular proteins is generally repressed, and a set of anaerobic proteins is synthesized. In maize, this response includes at least 10 major and 10 minor polypeptides of which several have been conclusively identified (10, 12, 21). The anaerobic induction of ADH[3] in maize has been intensely studied. Both transcription and transcript stability are affected by anoxia; there is also a 110% increase in enzyme activity as a result of an increased pool size of ADH protein (8, 12, 20). However, not all of the genes that respond to anaerobic conditions do so equally, as indicated by the major and minor designations of Hake *et al.* (10). For example, ADH is induced to high cellular concentrations at both the protein and RNA levels, while aldolase responds by the induction of high levels of RNA but only a 13% increase in protein concentrations relative to control (10, 12). Furthermore, the genes that are involved in the anaerobic response are not clustered in the maize genome, indicating that while there is a coordinated response to anaerobic conditions, the degree of response is not controlled as a single chromosomal domain.

In maize, two sucrose synthase (EC 2.4.1.13) isozymes, SS1 and SS2, encoded by the *Sh* and *Ss2* loci, respectively, have been described previously (4–6). Recent evidence has indicated that the SS1 isozyme in maize is an anaerobic protein since anaerobiosis results in a significant increase in the *Sh*-encoded RNA concentrations, similar to that seen for *Adh* (17, 23). Both *Sh* and *Ss2* loci are located on chromosome 9 (9, 17) and exhibit differential expression patterns with respect to each other during the course of the development of the maize plant.

Sucrose synthase is a tetramer with a monomeric subunit size of approximately 87 kD; SS1 and SS2 are biochemically similar and share immunological cross-reactivity (4, 7). Subunits of SS1 and SS2 can copolymerize to form heterotetramers in seedlings where both genes are expressed simultaneously at approximately equal levels (6). Immunoblots of nondenaturing electrophoretic gels of seedling extracts display a five band pattern that corresponds to the two homotetramers as well as the three different heterotetramers. Conversely, protein extracts from endosperm tissue indicate no co-polymerization of the *Sh* and *Ss2* encoded monomers as seen in the root and seedling extracts (6). Previous evidence has shown that, while the expression of the *Sh* gene is affected by anaerobic conditions, the expression of the *Ss2* gene is not affected by such treatment (17, 23). This report further examines the effect of anaerobiosis on the expression of the *Sh* gene *in vivo*. Following 24 h of anaerobic treatment, a significant increase in the steady state levels of *Sh* mRNA were detectable, while there was no detectable increase in the amount of SS1 protein.

MATERIALS AND METHODS

Maize Stocks. All maize (*Zea mays* L.) lines used in this study were Pioneer inbred lines displaying a wild type or nonshrunken phenotype designated Pio 3055 and Pio 3165. Additionally, a genetic stock that displays the shrunken phenotype which contains a deletion of the entire *Sh* locus (*sh bz-m4*) was used to unequivocally identify the genetic basis of the sucrose synthase monomers and hybridizing sequences of the plants used. Seeds were germinated in flats of vermiculite and grown for 5 to 7 d in a lighted growth chamber. Seedlings to be used for anaerobic treatment were then removed from the vermiculite and submerged for 24 h in 5 mM Tris-HCl buffer (pH 7.5) through which nitrogen gas was bubbled. Following this treatment, the roots were immediately frozen in liquid nitrogen. Several roots were used for protein analysis (*i.e.* Western blots and enzymic analysis); the rest of the root tissue was used for the preparation of RNA.

RNA Analysis. Depending on the quantity of tissue available, RNA was extracted using either the method of Lizardi and Engleberg (14) or the method of Baird and Meagher (1). Messenger RNA was isolated using messenger affinity paper (mAP) from Amersham according to the manufacturer's instructions. Messenger RNA (1 μg) was separated in formaldehyde gels (16), transferred to Nytran paper (Schleicher & Schuell), and hybridized as described previously (9, 17). Probes for *Sh* and *Ss2* were near full-length cDNAs (2.5 kb) isolated and described previously (9). The *sh bz-m4* maize line was used as a control for the possible cross-hybridization of the *Sh* and *Ss2* clones. During Northern analysis, the *Sh* clone did not detect *Ss2* sequences and

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[3] Abbreviation: ADH, alcohol dehydrogenase.

the *Ss2* clone did not detect *Sh* sequences.

Protein Analysis. Western blots were done according to the method of Towbin *et al.* (25) as adapted by Chourey *et al.* (6). Protein concentrations were measured by the method of Lowry *et al.* (15). *In vitro* translations were performed using rabbit reticulocyte lysates obtained from BRL (catalog No. 8110-MET), [^{35}S]methionine (NEN)-labeled products were separated on polyacrylamide gels according to the method of Laemmeli (13) and were visualized by autoradiography. Sucrose synthase activity measurements were made as described previously (4) on partially purified protein fractions. Partial purification was done using ammonium sulfate precipitation of 0 to 30% and 30 to 50% w/v. Sucrose synthase precipitated in the 30 to 50% fraction. Following precipitation, the enzyme was resuspended in buffer (0.1 M Tris-HCl [pH 7.5], 0.1% β-mercaptoethanol), dialyzed against the resuspension buffer, then assayed immediately.

RESULTS

The induction of *Adh* RNA synthesis and protein synthesis as a result of anaerobiosis is well documented in maize (8, 10, 20, 21). Consequently, the induction of ADH was used as an internal control to determine the efficacy of the anaerobic treatment of seedlings used in this study. In Figure 1 (panel C), crude protein extracts equivalent to 10 and 30 μg of protein from 7-d-old seedlings were loaded onto denaturing (SDS-PAGE) gels and immunostained (*i.e.* Western blotted) with ADH-specific antibody. It is clear that there is significantly more ADH in the extracts from anaerobically induced seedlings than in the aerobic or untreated seedlings. Hence, the method used to induce anaerobiosis was effective and produced results similar to those reported previously (12, 23).

The accumulation of sucrose synthase was examined by immunoblot analysis using SS1 antisera. Panel A in Figure 1 is a native immunoblot of crude protein extracts equivalent to 1, 5, 10, and 20 μg of soluble protein from the same samples as those in panel C (Fig. 1), which demonstrated increases in ADH concentration as a result of anaerobic treatment. Two important observations are made. First, intensity differences between the lanes loaded with different amounts of extract were distinguishable. Second, protein extracts from nontreated control seedlings, as well as extracts from anaerobically treated seedlings, display a typical five-banded pattern of sucrose synthase tetramers. Protein extracts from anaerobically treated seedlings, however, exhibited a reproducible, slightly smeared appearance especially in the interband spaces. The significance of this observation is unclear. When the amount of protein loaded on the gel and, therefore, the concentration of sucrose synthase is low, *i.e.* lanes loaded with 1 and 5 μg of protein, it appears as if there is more sucrose synthase protein in the lanes loaded with extract from anaerobically treated plants, probably due to this smearing effect. At higher concentrations of sucrose synthase (10 and 20 μg of extract), there appears to be no staining intensity difference between the control and treated lanes. The lack of difference in the staining intensities of the individual tetrameric classes (*i.e.* S1S1S1S1 and S2S2S2S2 homopolymers, S1S1S1S2, S1S1S2S2, and S1S2S2S2 heteropolymers) of sucrose synthase molecules extracted from the control and the treated plants supports the conclusion that there is no anaerobically associated accumulation of the SS1 protein. If there was an increase in the number of SS1 monomers available for heteropolymerization as a result of anaerobic induction, then this would be manifested on the blot as a shift of the banding intensities toward the SS1 homopolymer. No such shift in the banding pattern was evident.

To clarify the results obtained with the native gel electrophoresis system, the samples described above were analyzed by denaturing gel electrophoresis. In Figure 1, panel B, varying amounts of crude protein extract equivalent to 5, 10, and 20 μg of protein were loaded onto SDS gels, transferred to nitrocellulose, and immunostained to visualize the sucrose synthase monomers. This method resolves SS1 and SS2 monomers into two distinct polypeptides. The resolution of the individual polypeptides is clearly demonstrated by using protein extracts from roots of the *sh bz-m4* genotype (labeled *sh bz-m4* in Fig. 1B), a well characterized stock that contains a deletion of the *Sh* locus (3, 4). In the *sh bz-m4* stock, the sucrose synthase activity is the result of protein coded by the *Ss2* locus (4, 7), and only a single protein band is detectable using antibody to SS1 on Western blots. This polypeptide has been designated SS2, while the slower migrating polypeptide that is not detectable in *sh bz-m4* stocks has been designated as SS1. The relevant observation from Figure 1B is that, while concentration differences are distinguishable, there is no evidence for the increased *in vivo* accumulation of SS1 or SS2 polypeptides as a result of anaerobiosis. In all cases, except in the lanes loaded with 20 μg of protein, it appears there may actually be slightly less SS1 monomer in the extracts from anaerobically treated seedlings.

The lack of detectable *in vivo* increases in SS1 or SS2 protein concentrations as a result of anaerobiosis was further confirmed by enzyme assay of both sucrose synthesis and sucrose cleavage

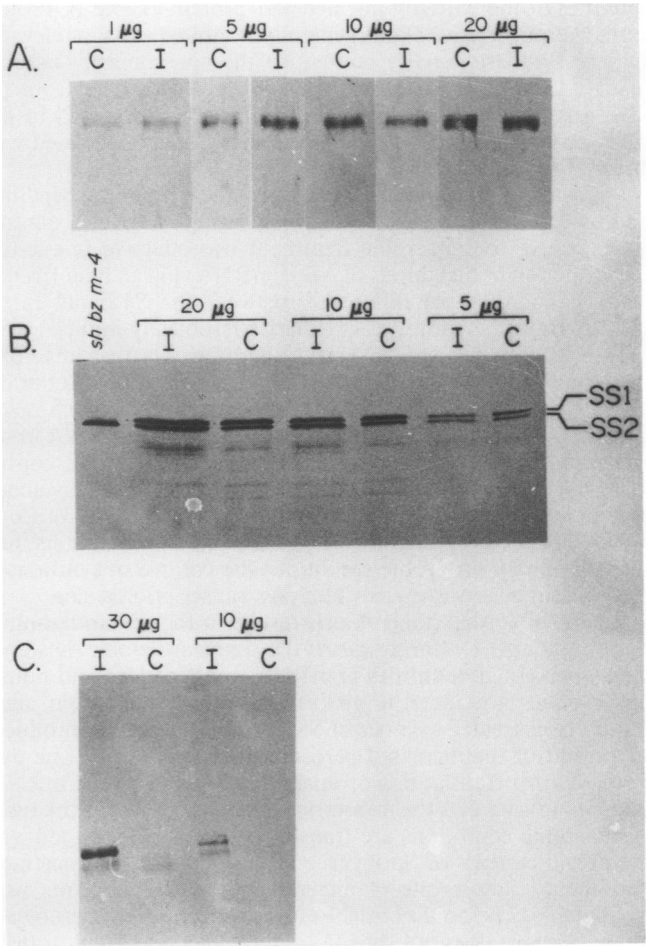

FIG. 1. Western blot of *Sh*, *Ss2*, and *Adh*-specific proteins from anaerobically induced and control seedlings. Panel A represents a native gel (lanes were rearranged for the sake of clarity but all lanes are from the same gel), while panels B and C are from denaturing gels. SS1-specific antibody was used for the blots in panels A and B; ADH-specific antibody was used in panel C. The concentrations given above the lanes indicate the amount of protein loaded; C, control seedlings; I, seedlings that were anaerobically induced. *sh bz-m4* is an *Sh* deletion line.

activities of the partially purified SS1 protein (Table I). Protein extracts from control plants generally exhibited equal or elevated sucrose synthase activity relative to the anaerobically induced plants. This is consistent with the immunoblot data from the SDS gels, which indicate that in the Pioneer lines used in this study there is no increase in SS1 or SS2 protein associated with anaerobiosis.

Messenger RNA was isolated from the remaining seedlings and used for Northern analysis, again using ADH as a control. As expected, the anaerobic treatment was effective in eliciting the induction of high levels of ADH mRNA (Fig. 2, lane 1 *versus* lane 2). The intensity differences between the *Sh* bands in the mRNA from the control *versus* the anaerobically induced seedlings (Fig. 2, lane 3 *versus* lane 4) demonstrate that anaerobic treatment also resulted in higher levels of *Sh* transcripts but did not result in higher levels of *Ss2* transcripts (Fig. 2, lane 5 *versus* lane 6). Dot blots indicate that the induction of *Sh* mRNA under anaerobic conditions was at least fivefold greater than control (data not shown). No transcriptional induction of *Ss2* by anaerobic treatment has been documented previously (17, 23). Combined with the protein abundance data, these observations indicate that under anaerobic conditions the control of synthesis of SS1 protein occurs primarily at a posttranscriptional step.

It is possible that anaerobiosis results in the accumulation of nontranslatable *Sh* transcripts. If they are translatable, however, then when equal quantities of mRNA from induced and noninduced plants are added to an *in vitro* translation system, there should be a greater number of SS1 protein molecules produced as a result of the increased percentage *Sh* transcripts. The data from *in vitro* translations of anaerobic *versus* control mRNAs (Fig. 3) indicate that the transcripts that accumulate as the result of anaerobic conditions are translatable *in vitro*. This confirms the previous work of Springer *et al.* (23), which demonstrated that the *Sh* transcripts of anaerobically induced plants were translatable. The pool of mRNA isolated from the anaerobically induced plants does not show a uniform increase for all translation products, *i.e.* some bands disappear, indicating that the increase in SS1 protein is not a fortuitous result. The position of the SS1 band was determined by immunoprecipitation (data not shown).

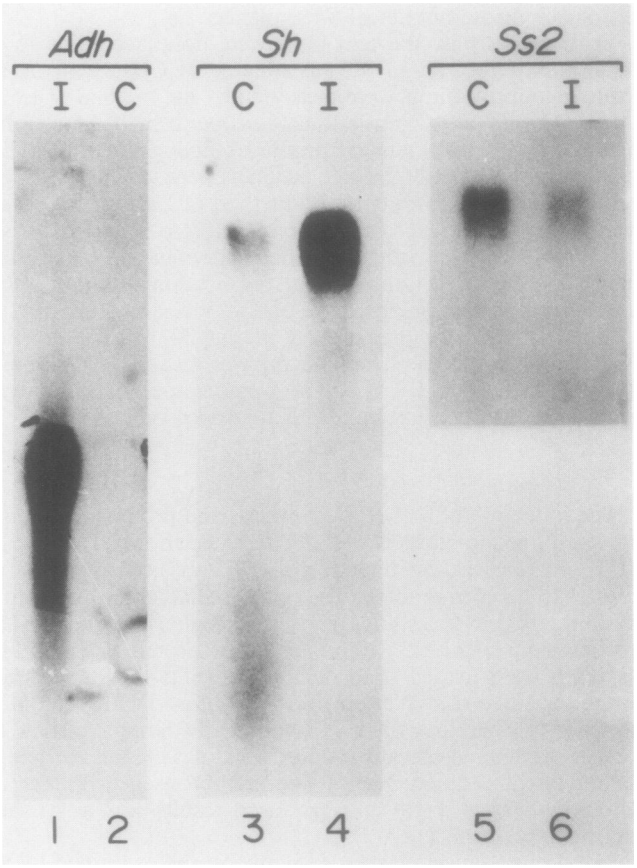

FIG. 2. Northern blots of mRNA from anaerobically treated plants *versus* control. All lanes are from the same gel and were loaded with 1 μg polyadenylated RNA. Lanes 1 and 2 were hybridized with an *Adh* probe, lanes 3 and 4 with a cDNA *Sh* probe, and lanes 5 and 6 with a cDNA probe for *Ss2*.

DISCUSSION

The data presented in this report demonstrated that, under anaerobic conditions, maize seedlings display a sharp increase in the amount of *Sh*-encoded transcript, but there is no detectable increase in the amount of sucrose synthase protein. The lack of detectable increases in sucrose synthase *in vivo* was determined by Western blot analysis of both native and denaturing gels. The same result is also obtained through the assay of sucrose synthase activity of both the synthetic and cleavage functions of the enzyme. Western blots of native gels of protein from both anaerobically treated and aerobic seedlings yielded very similar banding patterns of the sucrose synthase homotetramers and heterotetramers. Even though no increase in sucrose synthase levels was detectable, *Sh* transcripts isolated from seedlings subjected to anaerobic treatment were translatable *in vitro* and produced translation products that were recognized by antibody to sucrose synthase (23). These data suggest that even though there is an anaerobically induced increase in *Sh* transcripts, there is no detectable increase in the level of sucrose synthase protein. It is important to note, however, that there is still a significant level of sucrose synthase activity even after 48 h of anaerobiosis.

There are at least three explanations for the lack of a detectable increase in the amounts of sucrose synthase following anaerobic treatment. First, it is possible that the sucrose synthase that is being made under anaerobic conditions is very unstable or rapidly turned over and therefore undetectable in these assays. Second, the *Sh* transcripts may be translated so inefficiently that steady state levels of protein are not changed even though the number of transcripts available for translation has increased. And third, the *Sh* transcripts that are made under anaerobic conditions may not be translated. Any one of these possibilities is consistent with the data presented here.

There are two subtle, yet very important, observations that suggest there is no detectable increase in the number of active sucrose synthase molecules in the anaerobically treated plants. First, there is no shift in the intensity of the banding pattern of the tetramers as they are detectable on nondenaturing Western blots, *i.e.* the banding patterns of protein extracts from control

Table I. *Sucrose Synthase Enzyme Activities*

	Maize Lines		
	Pio 3165	Pio 3165	Pio 3055
Cleavage			
Control	0.52 ± 0.03[a]	0.85 ± 0.21	0.51 ± 0.01
24 h	0.42 ± 0.01	0.78 ± 0.09	0.30 ± 0.03
48 h	0.46 ± 0.01		
Synthesis			
Control	1.20 ± 0.03	0.37 ± 0.01	
24 h		0.34 ± 0.005	
48 h	0.36 ± 0		

[a] Values given as μg/mg protein/min ± SE. Each value represents two separate assays of protein extract from an individual experiment.

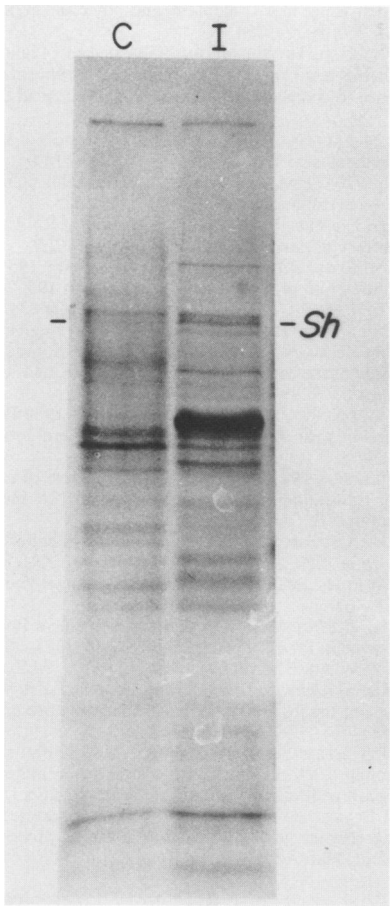

FIG. 3. *In vitro* translation of mRNAs isolated from anaerobic and control seedlings. Translations were done using the *in vitro* translation system from BRL with [^{35}S]methionine, 5 μg of the same aliquots of mRNA as those used in Figure 2 were used for the translations. The arrow indicates the position of the SS1 protein band which was determined by immunoprecipitation (data not shown).

and anaerobically induced plants are very similar. Second, the transcripts that were produced displayed no observable differences, other than concentration, on Northern blots, and these transcripts were translatable *in vitro*.

In maize seedlings, the monomers of sucrose synthase 1 (designated S1) and of sucrose synthase 2 (designated S2) can copolymerize (6). Therefore, Western blots of native gels, immunostained with antibody to sucrose synthase, display a pattern of five bands corresponding to the two homotetramers (S1S1S1S1 and S2S2S2S2) and the three heterotetramers (S1S1S1S2, S1S1S2S2, and S2S2S2S1). The staining intensity of these bands is indicative of random polymerization (predicted by binomial expansion) of the S1 and S2 monomers. There is no shift in the banding pattern of protein extracts from anaerobic *versus* aerobic seedlings—an important indicator that the sucrose synthase monomers, if they are being made, are not stable. If the number of monomers of sucrose synthase were increased, then it would be reasonable to expect the pattern of the bands detected on native Western blots to shift from the five-banded pattern, seen in extracts from control seedlings, toward the S1 homotetramer class. If the monomers of SS2 were to stabilize labile SS1 monomers, then an increased amount of staining would be seen in the S1S2 heterotetrameric classes. Finally, if the S1 monomers had a very short half-life and were not stabilized by S2 monomers, then a shift toward the S2 tetrameric class would be expected. No evidence of any shift in banding intensities is detectable on the native gels, suggesting that the amount and stability of the S1 monomers made under anaerobic conditions is similar to that made under aerobic conditions. The observation that increased amounts of SS1 protein are detectable following *in vitro* translation of the *Sh* mRNA both in this study and that of Springer *et al.* (23) suggests that the protein is at least as stable as that produced from mRNA isolated from nonstressed plants. Also, the basal level of SS1 protein (*i.e.* the same as control) is detectable in stressed seedlings indicating that some of the S1 monomers are stable.

It is possible that the translation of the induced proportion of the *Sh* transcripts is spatially or temporally separated from the *Ss2* transcripts such that heteropolymerization of S1 and S2 monomers is not possible. This is apparently the case in the immature endosperm where only the two homotetrameric proteins are detectable (6). It would therefore be necessary to invoke some process whereby the *Sh* transcripts made under anaerobic conditions are different from those made under aerobic conditions. Mechanisms such as alternative transcription initiation sites, trans-splicing, or some similar means would have to be invoked; however, none of these would necessarily be detectable by Northern analysis.

The observation that the induced transcripts are translatable *in vitro* suggests that the mRNA produced under anaerobic conditions is most likely structurally intact with respect to the 5′ and 3′ ends. In addition, the translated mRNA yields a polypeptide that is recognized by SS1 antisera; this also suggests that the message and protein are structurally similar if not identical to those made under aerobic conditions. The lack of evidence for a shift in the banding intensities on the Western blots, and the translatability of the message, suggests that the most reasonable explanation is that the transcripts made under anaerobic conditions either are not translated or are translated so inefficiently that protein levels are unaffected. The previous work (23) did not clearly establish the induction of SS1 protein above control *in vivo* levels. The *in vitro* translation of the *Sh* transcripts was the only data that indicated that SS1 might be an anaerobic protein. This conclusion was also supported by the observation that there was a basal level of SS1 protein under anaerobic conditions (23, and this report). Consequently, the induction of RNA synthesis and some amount of protein synthesis leads to the assertion that SS1 is an anaerobic protein.

Not all of the genes that respond to anaerobic induction do so equally (10, 12, 18). For example, aldolase (fructose-1,6-diphosphate aldolase, EC 4.1.2.13) is induced 13% over control, while ADH is induced 112% over control (15). However, at the RNA level, the aldolase transcripts were induced approximately to the same extent as those of the *Adh* gene (10). This suggests that translation of the transcripts made under anaerobic conditions is not an all or none phenomenon. This is consistent with our observation that the *Sh* transcripts made under anaerobic conditions may not be translated efficiently, if at all.

Utilization of previously synthesized transcripts has been demonstrated in other eukaryotic systems. Heat shock not only results in the transcription of 'heat shock' genes but also acts at the level of translation to utilize preheat shock transcripts following the removal of heat stress (22). It has also been shown that stored oocyte transcripts are translated during embryogenesis (24). In yeast, recent evidence has demonstrated the involvement of the product of the nuclear gene *PET111* in the translational activation of mitochondrially encoded *COXII* transcripts (19). Translational regulation of gene expression in plants also occurs as a result of nuclear-organellar subunit interactions as is the case with ribulose-1,5-bisphosphate carboxylase (2, 11). The data presented in this report suggest that some form of posttranscriptional control of the nuclear gene, *Sh*, may be operating under anaerobic conditions. In addition, recent data (EW Talercio, PS

Chourey, unpublished data) show that the transfer of seedlings to the normal growth conditions (*i.e.* aerobic) up to 6 h subsequent to the anaerobic treatment did not lead to any detectable increase in the *in vivo* level of SS1 protein.

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