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Metabolic Repression of Transcription in Higher Plants

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Using freshly isolated maize mesophyll protoplasts and a transient expression method, I showed that the transcriptional activity of seven maize photosynthetic gene promoters is specifically and coordinately repressed by the photosynthetic end products sucrose and glucose and by the exogenous carbon source acetate. Analysis of deleted, mutated, and hybrid promoters showed that sugars and acetate inhibit the activity of distinct positive upstream regulatory elements without a common consensus. The metabolic repression of photosynthetic genes overrides other forms of regulation, e.g., light, tissue type, and developmental stage. Repression by sugars and repression by acetate are mediated by different mechanisms. The identification of conditions that avoid sugar repression overcomes a major obstacle to the study of photosynthetic gene regulation in higher plants.

INTRODUCTION

Photosynthesis involves the most important metabolic pathways in the vegetative phase of higher plants. Among the essential substrates for photosynthesis, CO_2, water, and light, light is well documented to be an important regulator of photosynthetic gene expression (Simpson et al., 1986; Kuhlemeier et al., 1987; Schell, 1987; Ha and An, 1988; Benfey and Chua, 1989; Ueda et al., 1989). However, little is known about the contribution of other metabolites to the regulation of photosynthetic gene expression, even though metabolic regulation is well known at the level of enzyme activity in photosynthesis (Woodrow and Berry, 1988). Because a large number of genes are involved in photosynthetic pathways, it is reasonable to speculate that some type of metabolic regulation might exist to maintain economical use of the pathways.

To date, the study of photosynthetic gene regulation has relied solely on transgenic plants (Kuhlemeier et al., 1987; Schell, 1987; Benfey and Chua, 1989). Although informative, the study of transgenic plants is cumbersome and limited for routine analysis to a few easily transformed dicot species. The multicellular nature of transgenic plants also creates difficulties for the study of metabolic regulation. In contrast, transient expression in protoplasts is convenient and powerful, and has been used successfully to study gene regulation by plant hormones (Marcotte et al., 1988), UV light (Lipphardt et al., 1988), heat shock (Callis et al., 1988), and anaerobic stress (Walker et al., 1987). However, the study of photosynthetic gene regulation in protoplasts has not been vigorously pursued because it is widely believed that photosynthetic genes are suppressed in mesophyll protoplasts by osmotic stress (Fleck et al., 1982; Vernet et al., 1982). In the course of developing a protoplast transient expression method to study photosynthetic gene regulation, I discovered that the inhibition of photosynthetic gene expression in protoplasts is likely the result of photosynthetic end product repression by sucrose and glucose. Sucrose and glucose are two commonly used osmotica in tobacco protoplast culture (Nagy and Maliga, 1976) in which repression of photosynthetic genes was shown previously (Fleck et al., 1982; Vernet et al., 1982). Here, I present evidence that the transcriptional activity of seven photosynthetic gene promoters is repressed specifically and coordinately by sugars. Although glucose repression in *Escherichia coli* and yeast is a type of catabolite repression, the glucose repression in photosynthetic higher plants should probably be called a type of anabolite repression because excess sugars inhibit the photosynthetic anabolic pathways. To determine whether photosynthetic genes are subject to other forms of metabolic regulation, further studies with various reagents were carried out. These experiments demonstrated that acetate, a common carbon source for green algae, also mediated anabolite repression in higher plants. Analysis of deleted, mutated, and hybrid promoters showed that sugars and acetate inhibited the activity of distinct positive upstream regulatory elements.

RESULTS

Transient Expression in Maize Mesophyll Protoplasts

To determine whether maize mesophyll protoplasts can be used for transient expression assays, two constructs

that contain the cauliflower mosaic virus 35S (35S) promoter and two reporter genes that encode chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) were electroporated into freshly isolated maize mesophyll protoplasts. The experiment was repeated six times for each construct to evaluate the sensitivity and the reproducibility of electroporation and CAT and GUS assays. As shown in Table 1, the 35S promoter directed a high level of CAT and GUS expression in maize mesophyll protoplasts. Both CAT and GUS activities could be detected in cell extracts prepared from less than 100 protoplasts electroporated with 35SCAT or 35SGUS. Although both CAT and GUS assays are very sensitive, the fluorogenic assay for GUS activity (Jefferson, 1987) has a 10-fold higher background and more errors among repeated samples than the isotopic and phase extraction assay for CAT activity (Seed and Sheen, 1988).

Sugar Repression

To determine whether photosynthetic gene promoters are active in mesophyll protoplasts, a chimeric gene was constructed with the maize C_4 pyruvate phosphodikinase (PPDK) gene (*C_4ppdkZm1*) promoter fused to the CAT coding sequence and the 3′ sequence of the nopaline synthase gene (*nos*). The *C_4ppdkZm1* encodes the chloroplast isoform of PPDK, which is the key enzyme of the C_4 pathway in C_4 photosynthesis. As shown in Figure 1, a high level of CAT activity was detected after plasmid DNA containing this chimeric gene was electroporated into freshly explanted maize mesophyll protoplasts cultured in 0.6 M mannitol solution. A similar plasmid without the *C_4ppdkZm1* promoter did not direct CAT activity. To determine whether sucrose or glucose, two photosynthetic end products commonly used as stabilizers for protoplast culture, would inhibit the activity of a light-regulated photosynthetic gene promoter, electroporated protoplasts were cultured in solutions containing 0.3 M sucrose or glucose in addition to 0.3 M mannitol. The promoter activity of *C_4ppdkZm1* was decreased more than 15-fold in the presence of either sucrose or glucose (Figure 1). In the same experiment, two control plasmids bearing the 35S and the *nos* promoters directed similar levels of CAT activity (less than twofold difference) in mesophyll protoplasts cultured with or without sucrose or glucose (Figure 1). Therefore, the sugar inhibition of the *C_4ppdkZm1* promoter was not the result of a nonspecific inhibition of transcriptional activity in protoplasts.

Acetate Repression

With a convenient transient expression tool in hand, it is feasible to find out rapidly whether another type of metabolic repression exists in higher plants. Four more sugars, various metabolic intermediates, several common carbon sources, nitrogen sources, salts, and plant hormones were also tested for their ability to mediate repression. As shown in Table 2, acetate was found to be a potent inhibitor (more than 50-fold repression) and glycerol and fructose were found to be mild inhibitors (about 10-fold repression). Acetate is a commonly used carbon source for the heterotrophic growth of *Chlamydomonas* and has been shown to inhibit the expression of chlorophyll *a*/*b*-binding protein, an essential polypeptide for light capture in photosynthesis (Kindle, 1987). The inhibition of photosynthetic gene promoter activity by acetate in maize seems to imply the existence of similar repression in higher plants.

Metabolic Repression Is Coordinated among Photosynthetic Gene Promoters

To learn how widespread the repression effect is among other photosynthetic genes, the promoters of the C_4 phosphoenolpyruvate carboxylase gene (*C_4pepcZm1*), C_4 malic enzyme gene (*C_4meZm1*), chlorophyll *a*/*b*-binding protein

Table 1. Expression of 35SCAT and 35SGUS in Maize Mesophyll Protoplasts

Relative CAT Activity				Relative GUS Activity			
Samples	35SCAT	Samples	No Plasmid	Samples	35SGUS	Samples	No Plasmid
1	22.7	1	0.13	1	40.4	1	2.1
2	22.6	2	0.14	2	42.2	2	2.0
3	22.6	3	0.14	3	50.7	3	2.3
4	20.3	4	0.12	4	39.6	4	2.3
5	20.6	5	0.13	5	49.7	5	3.0
6	21.8	6	0.14	6	51.2	6	2.9
SD	0.98	SD	0.006	SD	4.98	SD	0.382

Results of six replicas are shown. Both CAT and GUS assays were performed with cell extracts from 2×10^3 protoplasts for 90 min. The relative CAT activity was expressed in counts per minute divided by 1000. The relative GUS activity was the direct fluorescence reading divided by 10. The experiment was repeated twice with similar results. SD: standard deviation.

Promoter and CAT fusions	Relative CAT activity (Relative GUS activity)		
	mannitol	sucrose	glucose
C4ppdkZm1 | CAT | nos (-507, +72)	10.0 (22.7)	0.7 (18.5)	0.2 (18.9)
	9.9 (26.3)	0.5 (15.2)	0.1 (19.0)
	10.4 (21.3)	0.6 (17.1)	0.2 (18.0)
35S | CAT | nos (-430, +1)	23.8	15.1	15.7
	23.7	13.3	13.6
nos | CAT | nos (-155)	11.9	8.1	8.6
	10.8	7.1	7.3
CAT | nos	0.0	0.0	0.0
	0.0	0.0	0.0

Figure 1. Transient Expression of C_4ppdkZm1CAT, 35SCAT, and nosCAT in Maize Mesophyll Protoplasts and the Effect of Sugars.

The plasmid DNA of C_4ppdkZm1CAT (55 μg), 35SCAT (50 μg), and nosCAT (50 μg) was electroporated into 3 $\times$ 10^5 maize mesophyll protoplasts. 35SGUS (30 μg) was cotransfected with C_4ppdkZm1CAT during electroporation as internal control. Each electroporated sample was divided into three parts (1 $\times$ 10^5 protoplasts each) and cultured in 1 mL of 0.6 M mannitol, 1 mL of 0.3 M sucrose plus 0.3 M mannitol, and 1 mL of 0.3 M glucose plus 0.3 M mannitol. CAT assay was performed with cell extracts from 5 $\times$ 10^4 protoplasts for C_4ppdkZm1CAT and nosCAT, and from 2 $\times$ 10^3 protoplasts for 35SCAT. Relative CAT activity was expressed in counts per minute divided by 1000. GUS assay was performed with cell extracts from 2 $\times$ 10^3 protoplasts. Relative GUS activity was the direct fluorescence reading divided by 10. Three replicas were performed with C_4ppdkZm1CAT and two replicas were performed with 35SCAT, nosCAT, and blueCAT (without a promoter). The experiment was repeated once with similar results.

genes (*cabZm1, cabZm5*) and ribulosebisphosphate carboxylase small subunit genes (*rbcSZm1* and *rbcSZm3*) were fused to the CAT coding sequence and the 3′ sequence of *nos*. Promoters of three other nonphotosynthetic genes, 35S, *nos*, and maize alcohol dehydrogenase gene (*Adh1*), were similarly fused to the CAT and *nos* 3′ sequences, as well as the promoter of a cytosolic PPDK gene (*cyppdkZm1*), which encodes the cytosolic isoform of PPDK that might be involved in carbon metabolism. As shown in Table 3 and Table 4, the activity of all photosynthetic gene promoters was repressed about threefold to 20-fold by sugars and about 20-fold to 200-fold by acetate. The repression by glucose was generally stronger than the repression by sucrose. Glycerol only repressed the activity of two photosynthetic gene promoters, *C_4ppdkZm1* (10-fold) and *cabZm1* (fivefold) (data not shown). In contrast, less than twofold repression was found for most nonphotosynthetic gene promoters by sugars and at least one nonphotosynthetic gene promoter by acetate in the same experiment (Tables 3 and 4). However, sugars but not acetate repressed the activity of the *cyppdkZm1* promoter (Tables 3 and 4). Although the precise role of the *cyppdkZm1* gene product is not known at present, these data suggest that it might be involved in

Table 2. Effects of Various Reagents on C_4ppdkZm1CAT Expression

Reagents	Relative CAT Activity	-Fold Repression
Mannitol	10.19	1.0
Sorbitol	6.11	1.7
Sucrose	0.69	14.8
Glucose	0.59	17.3
Fructose	0.90	11.3
Galactose	3.24	3.1
Lactose	11.34	0.9
Xylose	7.61	1.3
Ammonium sulfate	16.36	0.6
Potassium nitrate	11.17	0.9
Sodium pyrophosphate	10.55	1.0
Sodium phosphate	9.19	1.1
2,4-Dichlorophenoxyacetate	8.73	1.2
Benzylaminopurine	10.22	1.0
K3 salts	3.48	2.9
Pyruvate	7.22	1.4
Phosphoenolpyruvate	5.45	1.9
Oxalacetate	9.59	1.1
Malate	17.97	0.6
Glyceraldehyde 3-phosphate	7.33	1.4
Dihydroxyacetone phosphate	6.59	1.5
Aspartate	9.88	1.0
Glutamate	8.11	1.3
Isocitrate	7.21	1.4
Citrate	5.98	1.7
Succinate	4.88	2.1
Glyoxylate	3.06	3.3
Acetate	0.16	63.7
Ethanol	12.17	0.8
Glycerol	0.81	12.6

Protoplasts from 10 electroporated samples (3 $\times$ 10^5 protoplasts transfected with 50 μg of C_4ppdkZm1CAT) were pooled together and distributed (10^5 each) into 1 mL of 0.6 M mannitol solution containing various reagents. The osmolarity was maintained roughly equal to 0.6 M mannitol solution. The concentration of various sugars is 0.3 M. The concentration of other reagents is 5 mM for salts, nitrogen sources, and metabolic intermediates, 3 mM for acetate, 2 mg/L for plant hormones, and 5% (v/v) for ethanol and glycerol. K3 salt is the mix of salts in K3 medium (Nagy and Maliga, 1976). The experiment was repeated once with similar results. Similar results were obtained when the concentration of some salts and metabolic intermediates was increased up to 50 mM (data not shown). Fructose 6-phosphate (10 mM) and fructose 1,6-phosphate (10 mM) were tested without significant effect (data not shown). -Fold repression was calculated relative to the expression in mannitol. CAT activity was assayed with cell extract from 5 $\times$ 10^4 protoplasts.

Table 3. Effects of Sugars on Photosynthetic and Nonphotosynthetic Gene Promoter Activities

Promoter and CAT Fusions (Cotransfection GUS Fusion)	Relative CAT (GUS) Activity		
	Mannitol	Sucrose	Glucose
C_4ppdkZm1CAT (35SGUS)	9.6 (37.1)	0.7 (21.1)	0.4 (18.9)
C_4pepcZm1CAT (35SGUS)	7.2 (35.7)	2.3 (18.3)	0.9 (17.6)
C_4meZm1CAT (35SGUS)	5.4 (26.7)	1.3 (13.2)	0.6 (12.4)
cabZm1CAT (35SGUS)	18.2 (30.4)	4.9 (15.5)	1.8 (16.2)
cabZm5CAT (35SGUS)	21.8 (27.6)	2.0 (15.2)	1.8 (17.4)
rbcSZm1CAT (35SGUS)	22.0 (30.9)	1.7 (18.6)	0.6 (19.1)
rbcSZm3CAT (35SGUS)	7.5 (33.2)	1.6 (15.8)	0.3 (15.1)
35SCAT	34.2	20.1	19.7
nosCAT	20.7	12.7	11.9
Adh1CAT	5.6	4.7	4.3
cyppdkZm1CAT (35SGUS)	19.3 (27.6)	3.4 (17.7)	0.6 (19.6)

Cell extract from 5×10^4 protoplasts was used for CAT assay except the 35SCAT sample (2×10^3 protoplasts). Cell extract from 2×10^3 protoplasts was used for GUS assay. Relative GUS activity of cotransfection is shown in parentheses. Similar results were obtained when the experiment was repeated three times without cotransfection and once with cotransfection. The construction of photosynthetic gene promoter and CAT fusions will be described elsewhere (J. Sheen, A. Schäffner, and H. Huang, unpublished data).

both photosynthetic and nonphotosynthetic carbon metabolism. The insensitivity of *cyppdkZm1* promoter activity to acetate shows that sugar repression and acetate repression are mediated by distinct mechanisms.

The detection of the *rbcSZm1*, *rbcSZm3*, and *C_4meZm1* promoter activities in the mesophyll protoplasts was unexpected initially because the *rbcS* and *C_4me* mRNAs only accumulate in bundle sheath cells but not mesophyll cells of greening maize leaves (Sheen and Bogorad, 1986b, 1987). However, the results of in vitro labeled RNA from cell type-specific nuclei indicate that all bundle sheath-specific genes are regulated at the post-transcriptional level (J. Sheen, unpublished results). The *Adh1* and *nos* promoters are shown here to be active in maize mesophyll protoplasts. The results are not unexpected because two isoforms of alcohol dehydrogenase are found in maize leaves (Scandalios, 1974) and the *nos* promoter is active in tobacco mesophyll cells (Simpson et al., 1986).

The cotransfections of 35SGUS and cyppdkZm1GUS were used as internal controls for sugar and acetate repression, respectively (Tables 3 and 4). The 35SGUS was not included in the samples of 35SCAT, nosCAT, and Adh1CAT because of promoter interferences (data not shown). The expression of cyppdkZm1GUS in cotransfection experiments was slightly affected by other promoters (Table 4).

Sugar Repression and Acetate Repression Are Concentration Dependent and Physiologically Significant

Sugar repression allowed a low level of basal expression that was not inhibited even with 0.6 M of sucrose, as shown in Table 5. When the concentration of sucrose was decreased from 300 mM to the lower level found in normal photosynthetically active cells (30 mM) (Gerhard et al., 1987), sugar repression was no longer significant (Table 5). Therefore, it is possible that sugar inhibition is a type of feedback regulation and only occurs when the accumulation of sugars in cells is above certain physiological limits.

The effective concentration of acetate for repression has a very narrow range (1 mM to 3 mM) and is only slightly higher than the endogenous concentration of acetate (0.25 mM to 1 mM) found in leaf cells probably derived from a number of sources (Kuhn et al., 1981; Murphy and Stumpf, 1981). As shown in Table 6, when the concentration of acetate applied to protoplasts was decreased to 0.3 mM, repression was no longer detectable (less than twofold repression). These experiments suggest that acetate repression is sensitive, stringent, and physiologically significant. However, it is not clear where the sensor for acetate is located and how the signal is transmitted in the leaf cell.

Table 4. Effect of Acetate on Photosynthetic and Nonphotosynthetic Gene Promoter Activity

Promoter and CAT Fusions (Cotransfection GUS Fusion)	Relative CAT (GUS) Activity	
	No Acetate	3 mM Acetate
C_4ppdkZm1CAT (cyppdk-Zm1GUS)	25.3 (23.0)	1.5 (11.7)
C_4pepcZm1CAT (cyppdk-Zm1GUS)	8.5 (25.5)	0.1 (8.8)
C_4meZm1CAT (cyppdk-Zm1GUS)	7.5 (24.8)	0.2 (6.2)
cabZm1CAT (cyppdkZm1GUS)	24.8 (24.6)	0.3 (8.6)
cabZm5CAT (cyppdkZm1GUS)	30.1 (19.9)	0.2 (6.6)
rbcSZm1CAT (cyppdk-Zm1GUS)	13.3 (15.1)	0.5 (7.1)
rbcSZm3CAT (cyppdk-Zm1GUS)	8.9 (17.1)	0.04 (6.7)
35SCAT (cyppdkZm1GUS)	54.9 (33.3)	6.3 (19.4)
nosCAT (cyppdkZm1GUS)	28.6 (38.5)	4.8 (10.9)
Adh1CAT (cyppdkZm1GUS)	8.3 (28.1)	1.0 (8.3)
cyppdkZm1CAT	13.2	8.8

CAT assay was the same as described in Table 3. Cell extract from 10^4 protoplasts was used for GUS assay. Relative GUS activity of cotransfection is shown in parentheses. Similar results were obtained when the experiment was repeated three times without cotransfection and once with cotransfection.

Table 5. Concentration Dependence of Sugar Repression

Promoter and CAT Fusions	Sucrose Concentration and Relative CAT Activity 0 mM	30 mM	100 mM	300 mM	600 mM
C_4ppdkZm1CAT	53.85	16.20	8.65	2.10	2.05
C_4pepcZm1CAT	25.89	13.19	7.03	2.97	2.97
rbcSZm1CAT	56.66	28.73	14.70	7.70	7.42
35SCAT	181.73	150.37	115.36	100.02	-
nosCAT	23.49	12.70	9.92	9.14	-

Cell extract from 10^5 protoplasts was used for CAT assay except the 35SCAT sample (3×10^4 protoplasts). The experiment was repeated once.

Repression Is Mediated by Distinct Positive Upstream Regulatory Elements

To identify the DNA sequences responsible for repression, a set of mutated *C_4ppdkZm1* and *cabZm5* promoters was created by deletion, mutagenesis, and promoter sequence interchange and analyzed for repression by sugars and acetate. The deletions spanned multiple positive and negative upstream regulatory elements of the promoters (J. Sheen and H. Huang, unpublished results). Similar repression was observed for all promoters deleted to within 30 bp to 50 bp upstream of the TATA box. Further deletion gave weak promoter activity that did not appear to be repressed, as shown in Figure 2. These results suggested that all of the positive upstream regulatory elements were susceptible to repression. To exclude the possibility that repression might be mediated by the basal promoter elements of photosynthetic gene promoters, three hybrid promoters derived from 35S and *C_4ppdkZm1* promoters were analyzed (Figure 2). When positive upstream regulatory elements of the *C_4ppdkZm1* promoter were placed upstream of the 35S basal promoter element, expression was repressed. Conversely, when the positive regulatory elements of the 35S promoter were placed upstream of the *C_4ppdkZm1* basal promoter elements, little repression was observed (Figure 2). Moreover, when the 16-bp enhancer of the octopine synthase promoter (*ocs*) (Ellis et al., 1987) was fused to the basal promoter of *cabZm5*, or substituted two of the positive regulatory elements in the *C_4ppdkZm1* promoter (J. Sheen, unpublished results), the *ocscab5* promoter and the mutated photosynthetic promoters (C_4ppdkmut1 and C_4ppdkmut2) became insensitive to repression, as shown in Figures 3 and 4.

Table 6. Concentration Dependence of Acetate Repression

Promoter and CAT Fusions (Cotransfection)	Acetate Concentration and Relative CAT (GUS) Activity 0 mM	0.1 mM	0.3 mM	1.0 mM	3.0 mM
C_4ppdkZm1CAT	8.1	5.9	4.4	1.3	0.7
(cyppdkZm1GUS)	(15.6)	(13.4)	(13.7)	(15.4)	(16.7)
CabZm5CAT	21.7	9.3	9.6	1.2	0.2
(cyppdkZm1GUS)	(23.6)	(16.7)	(16.1)	(12.1)	(14.3)
cyppdkZm1CAT	10.8	7.8	6.2	6.7	8.5

Cell extract from 5×10^4 protoplasts was used for CAT assay. Cell extract from 10^4 protoplasts was used for GUS assay. Relative GUS activity of cotransfection is shown in parentheses. The experiment was repeated twice without cotransfection and once with cotransfection with similar results.

Thus, the positive upstream regulatory elements appear to mediate repression by sugars and acetate. The involvement of the upstream negative element as proposed in yeast glucose repression (Beier and Young, 1982; Struhl, 1985) is unlikely because the upstream deletions did not eliminate repression, and a 16-bp nonresponsive enhancer converts a photosynthetic gene promoter from responsive (cabZm5, C_4ppdkZm1) to nonresponsive (ocscab5, C_4ppdkmut1, and C_4ppdkmut2) (Figures 3 and 4). The possibility of post-transcriptional regulation can also be excluded. As shown in Figure 2, identical CAT mRNA should be synthesized from various deleted promoters, and repression depends on the presence of upstream positive elements (Figure 2). Moreover, in the case of hybrid and mutated promoters, the CAT mRNAs synthesized contain either 35S (C_4ppdk35Shyb1), *cabZm5* (ocscab5), or *C_4ppdkZm1* (35SC_4ppdkhyb1, hyb2, C_4ppdkmut1, mut2) untranslated leader sequences, but repression depends exclusively on the type of upstream positive elements (Figures 2, 3, and 4).

DISCUSSION

The experiments reported here demonstrate that, as in unicellular bacteria and yeast, genes involved in metabolic

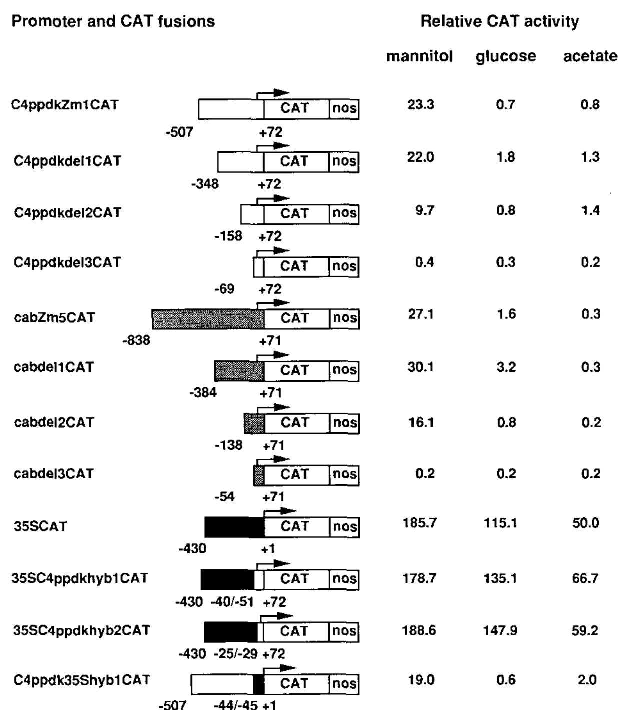

Figure 2. Effects of Glucose and Acetate on the Expression of Deleted and Hybrid Promoter/CAT Fusions.

The plasmid DNA of various promoter and CAT fusions (in equal molar ratio) were electroporated into 3×10^5 maize mesophyll protoplasts. Each electroporated sample was divided into three parts (1×10^5 each) and cultured in 1 mL of 0.6 M mannitol, 1 mL of 0.3 M glucose plus 0.3 M mannitol, and 1 mL of 0.6 M mannitol plus 3 mM sodium acetate. CAT assay was performed with cell extracts from 10^5 protoplasts for C_4ppdkCAT, cabZm5CAT, and promoter deletions. For 35SCAT and constructs containing the 35S enhancer, cell extracts from 3×10^4 protoplasts were used for CAT assay. Relative CAT activity was expressed in counts per minute divided by 1000. The experiment was repeated twice with similar results.

pathways are also subject to regulation by the fluctuation of internal and external metabolites in multicellular higher plants. The metabolic regulation of gene expression should play a role of fundamental importance in maintaining an economical balance of the supply and demand of biomolecules in different organs of higher plants. The metabolic repression of photosynthetic genes apparently overrides other forms of regulation, e.g., light, tissue type, and developmental stage (Simpson et al., 1986; Kuhlemeier et al., 1987; Schell, 1987; Ha and An, 1988; Benfey and Chua, 1989; Ueda et al., 1989) because it is executed in young leaf cells under light. In this study, the young leaf cells (greening mesophyll protoplasts) were isolated from illuminated dark-grown seedlings, which is a well-established system for the study of the photosynthetic gene expression at an early developmental stage (Nelson et al., 1984; Sheen and Bogorad, 1986a, 1986b, 1987; Simpson et al., 1986; Mosinger et al., 1988; Lam et al., 1989). The greening mesophyll protoplasts of maize provide an abundant and homogeneous cell population, synchronized in photosynthetic gene induction and expression, for the study of photosynthetic gene regulation.

The results described here also demonstrate that transient expression in protoplasts is a viable and attractive alternative to transgenic plant analysis for the study of photosynthetic gene regulation. The previously observed inhibition of photosynthetic gene expression in mesophyll protoplasts is likely the result of end product repression by the sugars chosen to stabilize protoplasts. Substitution of sucrose or glucose with mannitol, sorbitol, or other carbohydrates releases the repression.

In transient expression, the strength of the 35S promoter is 10 times stronger than the *nos* promoter and photosynthetic gene promoters in maize mesophyll protoplasts, as also shown in tobacco mesophyll protoplasts (Harkins et al., 1990). It is curious that the expression of the 35S promoter is much higher in transient expression than in transgenic plants (Harkins et al., 1990). This might indicate that the free promoter can get easier access to *trans*-acting factors than the integrated promoter. As recently shown, the addition of the as-1 (−82 to −62 of the 35S

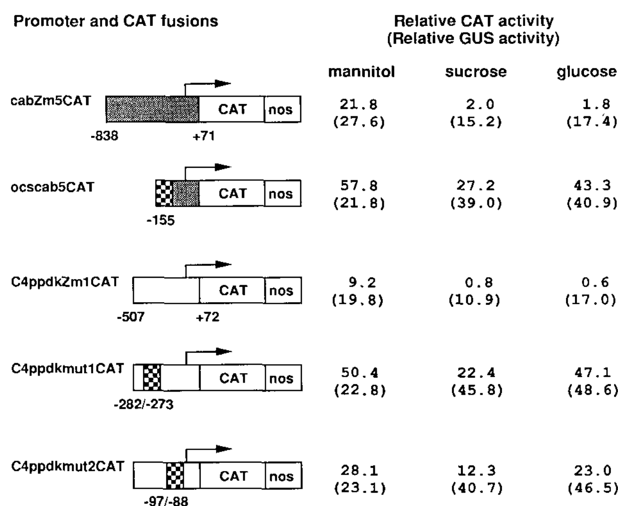

Figure 3. Effect of Sugars on the Expression of Mutated Promoter/CAT Fusions.

The position of mutated sequences is shown. CabZm5CAT and C_4ppdkZm1CAT were cotransfected with 35SGUS. ocscab5CAT, C_4ppdkmut1CAT, and C_4ppdkmut2CAT were cotransfected with nosGUS. CAT assay was performed with cell extract from 5×10^4 protoplasts. GUS assay was performed with 2×10^3 protoplasts for 35SGUS, and 10^4 protoplasts for nosGUS. The experiment was repeated once with similar results.

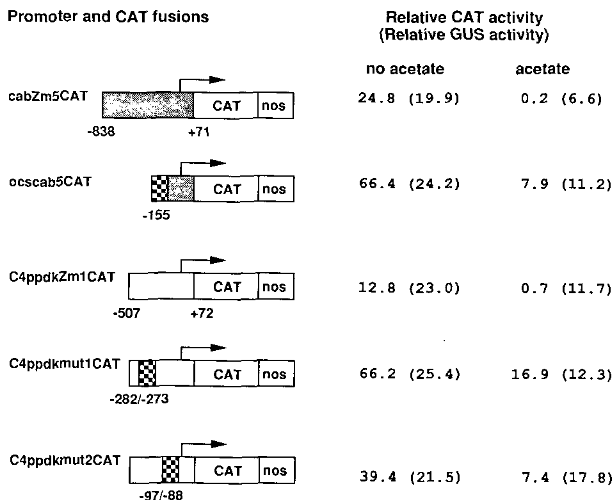

Figure 4. Effect of Acetate on the Expression of Mutated Promoter/CAT Fusions.

The cotransfection plasmid was cyppdkZm1GUS. CAT assay was performed with cell extract from 5×10^4 protoplasts. GUS assay was performed with cell extract from 10^4 protoplasts. The experiment was repeated once with similar results.

promoter) tetramer enhances leaf expression considerably in transgenic tobacco plants (Lam and Chua, 1990).

The most striking advantages of protoplast transient expression are rapidity, convenience, and versatility. Starting from the isolation of protoplasts to the collection of quantitative data, the whole process takes less than 2 days, whereas it takes months to obtain equivalent results with transgenic plants. In addition, the electroporation and CAT assay used here yield highly reproducible results when samples from the same batch of protoplasts are tested. It is especially convenient for testing a large number of constructs without relying on internal controls and the large number of duplicated samples needed for transgenic plant analysis. The method requires little tissue culture and plant care and can be applied to both dicot and monocot plants.

Glucose Repression in Maize Is a Novel Type of Feedback Regulation on Gene Expression

In *E. coli* and yeast, glucose is a preferred exogenous carbon source and can inhibit the expression of genes involved in other catabolic pathways. This inhibition is called catabolite repression (Miller and Reznikoff, 1978; Beier and Young, 1982; Struhl, 1985). In higher plants, glucose is synthesized endogenously through photosynthesis and used for starch synthesis in chloroplasts, or is converted to sucrose in the cytosol for export. The export of sucrose from leaf cells is a principal factor linking carbon assimilation at the source (photosynthetic organs) to carbon utilization at the sink (nonphotosynthetic organs) (Bonner and Varner, 1976; Stitt, 1986; Foyer, 1988). The removal of sinks causes an increase in the sucrose concentration of leaves and a severe inhibition of photosynthesis (Foyer, 1988). The feeding of the leaves with sucrose or glucose also causes a substantial increase in leaf sugar content and a significant decrease in photosynthetic capacity (Foyer, 1988; Huber, 1989). Based on these observations, it has been proposed that sugars play a crucial role in the biochemical feedback regulation of carbon assimilation by depriving the chloroplasts of orthophosphate, a metabolite essential for generating ATP by photophosphorylation during photosynthesis (Stitt, 1986; Walker and Sivak, 1986; Foyer, 1988; Huber, 1989). Therefore, sugars can act as the modulators of source and sink interaction when the production of photosynthate significantly exceeds the capacity of photosynthate utilization.

In this work, I present evidence that sugars can play another role in the feedback regulation of photosynthesis,

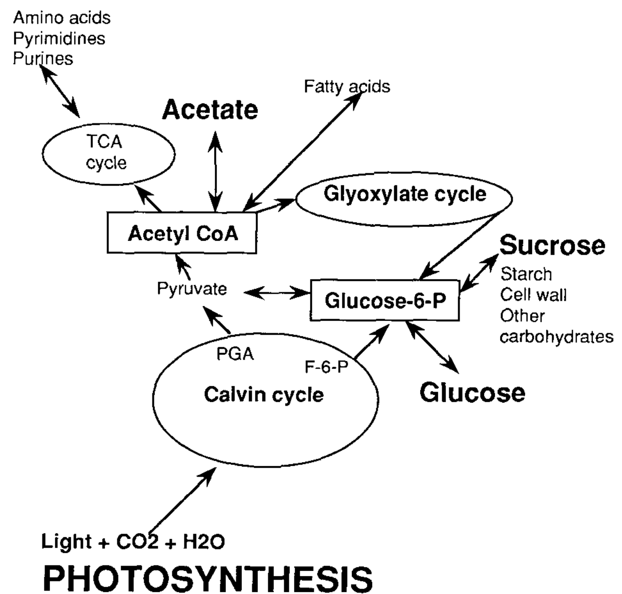

Figure 5. Acetyl-CoA Is a Central Metabolite in Plant Cells.

A simplified scheme of global cellular metabolism of plant cells is shown. Acetyl-CoA connects the carbon assimilation from photosynthesis in chloroplasts to the metabolism of amino acids, fatty acids, and nucleotides. The glyoxylate cycle in glyoxysome is most active when cells are fed with acetate or are metabolizing lipids. The gluconeogenesis through the glyoxylate cycle is unique to plant cells. One-end arrows indicate that reverse reactions are unlikely to occur. PGA is 3-phosphoglycerate. F-6-P is fructose 6-phosphate.

namely switching off the expression of photosynthetic genes. It is also possible that sugar repression is used, in part, as a regulatory mechanism for the developmental and tissue-specific expression of photosynthetic genes in higher plants. For instance, photosynthetic genes are transcriptionally more active in young developing leaves than in mature leaves, probably because mature leaves produce and accumulate more sugars than do younger leaves (Sheen and Bogorad, 1986, 1987; Nakamura and Hashimoto, 1988; J. Sheen, unpublished results). Another example is that photosynthetic genes are usually not expressed in nonphotosynthetic cells, probably because the sugar content of these cells is often higher (over 200 mM) than that found in photosynthetic cells (10 mM to 50 mM) (Gerhard et al., 1987; Griffith et al., 1987).

Sugar repression allows a basal level of expression and is reversible when the concentration of sugars decreases. The concentration dependence and leaky features of sugar repression in higher plants resemble that found in end product repression of the tryptophan operon in *E. coli* (Miller and Reznikoff, 1978; Yanofsky, 1981). Because sugars are the major end product of photosynthesis, it is more appropriate to classify the glucose repression of photosynthetic genes as a type of end product repression in higher plants. Moreover, because sugars inhibit the expression of genes involved in an anabolic pathway—photosynthesis—glucose repression in higher plants is a type of anabolite repression in contrast to catabolite repression in bacteria and yeast.

Acetate Repression Is Evolutionarily Conserved in the Plant Kingdom

Besides sugar repression, transcriptional repression of photosynthetic genes was found with acetate. The effective concentration of acetate is 100 times lower than that of sugars for transcriptional repression. This difference reflects the intrinsic concentration ranges of sugars and acetate in leaf cells (10 mM to 50 mM for sucrose and 0.25 mM to 1 mM for acetate) (Kuhn et al., 1981; Gerhard et al., 1987). Repression mediated by acetate and repression mediated by sugars are quite different. Usually, acetate repression is 10 times stronger than sugar repression for photosynthetic gene promoters, with the exception of the cytosolic *ppdkZm1* promoter that is inhibited by sugars but not acetate (Tables 3 and 4). Thus, there must be at least two pathways for metabolic repression at the transcriptional level in maize.

The reasons for acetate repression can probably be best explained by the unique ability of plant cells to use acetate for gluconeogenesis and by the central role played by acetyl-CoA (the direct cellular derivative of acetate) in the global cellular metabolism of plant cells, as shown in Figure 5 (Beevers, 1969; Fletcher and Beevers, 1970; Bassham, 1971; Lehninger, 1975; Bonner and Varner, 1976; Murphy and Stumpf, 1981). It has been shown that acetate can be used as the sole exogenous carbon source by several green algae. In algal cells fed with acetate, the glyoxysome, a single membrane-bound organelle, is involved in using acetyl-CoA for gluconeogenesis through the glyoxylate cycle (Beevers, 1969; Monroy and Schwartzbach, 1984; Gibbs et al., 1986; Steinbiss and Zetsche, 1986; Rikin and Schwartzbach, 1989). The presence of acetate inhibits the expression of photosynthetic genes under light but activates the expression of glyoxysome enzymes for the metabolism of acetate (Monroy and Schwartzbach, 1984; Gibbs et al., 1986; Steinbiss and Zetsche, 1986; Kindle, 1987; Rikin and Schwartzbach, 1989). These observations suggest that in algae acetate is a more favorable exogenous carbon source than CO_2, probably because the utilization of CO_2 involves the expression of more complex photosynthetic pathways. The repression of genes involved in other metabolic pathways by preferred exogenous carbon sources in green algae is a type of metabolic repression similar to that found in *E. coli* and yeast. However, an anabolic pathway, photosynthesis, is inhibited in green algae instead of the catabolic pathways inhibited in bacteria and yeast.

In higher plants, glyoxysomes are particularly abundant in germinating seedlings that are metabolizing fat. In these cells, acetyl-CoA plays an important role for the generation of energy through the TCA cycle and the generation of carbohydrates through the glyoxylate cycle (Figure 4) (Beevers, 1969; Huang and Beevers, 1971; Lehninger, 1975; Bonner and Varner, 1976; Kuhn et al., 1981; Murphy and Stumpf, 1981). The presence of acetate repression in the mesophyll cells of maize seedlings might suggest the adaptation of an economical strategy by higher plants to ensure the use of stored fat first before switching over to the operation of more complex photosynthetic pathways. Alternatively, plants can also absorb nutrients from organic acid-enriched soil, and the presence of excess acetate can act as a signal to convert plants to the utilization of easily available fuel from the environment. Because glyoxysomes and acetate repression are found in the plant kingdom from green algae to higher plants, the evolutionary conservation strongly suggests that they play a role of fundamental importance in cellular metabolism.

Mechanisms Mediating Metabolic Regulation in Higher Plants Differ from Those Found in Bacteria, Yeast, and Vertebrates

Mechanisms of metabolic regulation are quite different in bacteria and yeast. In bacteria, genes encoding enzymes with related metabolic functions are frequently arranged contiguously in the chromosome and are coregulated by short sequences upstream of the gene cluster. The lactose

operon and tryptophan operon are two well-known examples (Miller and Reznikoff, 1978). In yeast, genes encoding related enzymes of metabolic pathways are not arranged in gene clusters and are frequently scattered among several chromosomes (Hinnebusch and Fink, 1983; Giniger et al., 1985). However, the genes encoding amino acid biosynthesis enzymes from different pathways are regulated through a consensus sequence and its binding protein GCN4 (Hinnebusch and Fink, 1983; Hope and Struhl, 1985). Although it is not yet clear whether the glucose repression in yeast is controlled by a consensus sequence, it has been proposed to be regulated by negative regulatory elements (Beier and Young, 1982, Struhl, 1985). As in yeast, photosynthetic genes in higher plants are located on different chromosomes (Shiaoman et al., 1989; Weber and Helentjaris, 1989). Thus far, the analysis of seven different photosynthetic gene promoters of maize has not demonstrated any overt consensus sequences among the upstream regulatory elements (J. Sheen, A. Schäffner, and H. Huang, unpublished results). In this work, analysis of deleted, mutated, and hybrid promoters showed that distinct positive upstream regulatory elements but not basal promoter structures or negative elements were responsible for mediating repression.

Recently, it has been proposed that CCAAT/enhancer-binding protein (C/EBP) may play a role in the regulation of genes involved in energy metabolism (gluconeogenesis and lipogenesis) in vertebrates (McKnight et al., 1989). One piece of evidence is that the promoters of at least five genes involved in energy metabolism are *trans*-activated by C/EBP (McKnight et al., 1989). Because the consensus sequence for the binding of C/EBP is present in only one (*CabZm5*) out of seven photosynthetic gene promoters, the metabolic repression of photosynthetic genes in higher plants apparently uses mechanisms unlike that of vertebrates.

Although metabolic repression in higher plants might be regulated by protein-protein interactions instead of protein-DNA interactions, the involvement of degenerate binding sites cannot be ruled out. The isolation and analysis of the genes encoding photosynthetic gene activators and the use of a protoplast transient expression method will allow rapid elucidation of these interesting mechanisms without recourse to transgenic plants.

METHODS

Plant Growth and Protoplast Isolation

Protoplasts were isolated from 20-hr illuminated leaves of 10-day-old maize seedlings that had been kept in the dark at 25°C. The middle part of the second leaves (about 6 cm in length) was cut to 0.5-mm strips with new razor blades and digested in an enzyme solution containing 1% (w/v) cellulase RS, 0.1% (w/v) macerozyme R10 (both from Yakult Honsha, Nishinomiya, Japan), 0.6 M mannitol, 10 mM Mes (pH 5.7), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.1% BSA (w/v) for no more than 3 hr at 23°C. Protoplasts were released by shaking on a rotary shaker at 80 rpm for 10 min and were filtered through a 70-μm nylon filter. Protoplasts were collected by centrifugation at 100 *g* for 2 min, washed in cold 0.6 M mannitol solution once, centrifuged, and resuspended at 2×10^6/mL in cold 0.6 M mannitol. The yield was about 5×10^6/g. The viability was about 99% estimated by staining with fluorescein diacetate (Sigma) and examined by fluorescence microscopy. Mesophyll protoplasts with chloroplasts contributed about 95% of the total population.

CAT and GUS Chimeric Constructs with Various Promoters

C_4ppdkZm1CAT was constructed by fusing the 5′-flanking region (−507 to +72) of the *C_4ppdkZm1* gene to the CAT coding sequence and the *nos* 3′ sequence as described (Fromm et al., 1985, 1986; Callis et al., 1988) except that the BamHI site of pUC8 was converted to a SacI site for promoter fusion. The *nos* promoter was originated from Dr. V. Walbot's laboratory but was deleted down to the Sac II site (−155) to obtain better plasmid DNA yield and expression. 35SGUS and nosGUS were generated by transferring the 35S and *nos* promoter fragments from 35SCAT and nosCAT (excised with HindIII and BamHI digestion) to the pUC19GUS (Jefferson, 1987). CyppdkZm1GUS was generated by cloning the *cyppdkZm1* promoter (−1100 to +85) to the pUC19NcoGUS (J. Dewdney, unpublished data) after the ligation of Nco linker to the 3′ end.

Electroporation and Protoplast Culture

Electroporation conditions were 400 V/cm, 200 μF, 10 msec (set pulse time), and one pulse with a Promega X-cell 450 apparatus. Each sample contained 3×10^5 protoplasts and about 50 μg of DNA in 0.3 mL of 0.6 M mannitol and 20 mM KCl. After electroporation, 10^5 protoplasts were cultured in 1 mL of 0.6 M mannitol, 4 mM Mes (pH 5.7), and brome mosaic virus (BMV) salts (Okuno et al., 1977; Loesch-Fries and Hall, 1980) at 25°C under 20 μE/m^2 of light for 20 hr (200 μE/m^2 light gave similar results). The BMV salts were omitted when the effect of various salts was tested.

CAT and GUS Assays

CAT assays were performed with cell extracts from 2×10^3 to 10^5 protoplasts in 100 μL for 90 min as described (Seed and Sheen, 1988). The phase-extraction method is quantitative, fast, inexpensive, and more sensitive compared with the conventional method by TLC plates. Because of its broad linear range (from 0.05% to 50% conversion) and low background (about 100 cpm with 0.2 μCi of ^{3}H-chloramphenicol per sample), the new method is excellent for the study of weak promoters. The relative CAT activity is expressed in counts per minute divided by 1000.

The fluorogenic assay for GUS activity was performed with cell extracts from 2×10^3 to 10^4 protoplasts in 200 μL for 90 min as

described (Jefferson, 1987). The relative GUS activity is the direct fluorescence reading divided by 10.

Because protoplast activity and substrates for CAT and GUS assays were not identical in each experiment, the data from different experiments were not combined.

Deletion, Promoter Interchange, and Mutagenesis

The deleted promoters of *C_4ppdkZm1* were generated by Bal31 digestion from the 5′ end as described (Maniatis et al., 1982). The promoter sequences of *C_4ppdkZm1*, from −507 to +72, from −348 to +72, from −158 to +72, and from −69 to +72, were inserted into the 35SCAT plasmid (Fromm et al., 1985, 1986; Callis et al., 1988) digested with HindIII and SacI to generate C_4ppdkZm1CAT, C_4ppdkdel1CAT, C_4ppdkdel2CAT, and C_4ppdkdel3CAT, respectively. The deleted promoters of cabZm5CAT were generated by SpeI, BssHII, and DraI digestions (Sullivan et al., 1989). The promoter sequences of *cabZm5*, from −838 to +71, from −384 to +71, from −138 to +71, and from −54 to +71, were inserted into the blueCAT plasmid (J. Sheen, unpublished data) digested with EcoRI and StyI, SpeI and StyI, SmaI and StyI, and SmaI and StyI to generate cabZm5CAT, cabdel1CAT, cabdel2CAT, and cabdel3CAT, respectively. The hybrid promoters were generated by Bal31 digestions from both the 5′ and 3′ ends of the 35S and *C_4ppdkZm1* promoters and fusions with HindIII linkers. The 35SC_4ppdkhyb1CAT and 35SC_4ppdkhyb2CAT were created by inserting the 35S promoter sequences, from −430 to −40 and from −430 to −25, into the C_4ppdkZm1CAT deletions at −51 and −29 positions with HindIII linkers, respectively. The C_4ppdk35Shyb1CAT was created by inserting the *C_4ppdkZm1* promoter sequences from −507 to −44 into the 35SCAT deletion at −45 position with HindIII linkers. The ocscab5 was generated by ligating the 16-bp *ocs* enhancer (ACGTAAGCGCTTACGT) (Ellis et al., 1987) to a truncated *cabZm5* promoter at −100. The mutated *C_4ppdkZm1* promoters were first generated by site-specific mutagenesis with primers containing 10-bp mismatches flanked by 15-bp homologous sequences on both ends as described (Kunkel, 1985). Then the 16-bp *ocs* enhancer was inserted into the mutated sites digested with EcoRV and XbaI to create C_4ppdkmut1 and C_4ppdkmut2.

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