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# Sugar Sensing in Higher Plants

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**Sugar repression of photosynthetic genes is likely a central control mechanism mediating energy homeostasis in a wide range of algae and higher plants. It overrides light activation and is coupled to developmental and environmental regulations. How sugar signals are sensed and transduced to the nucleus remains unclear. To elucidate sugar-sensing mechanisms, we monitored the effects of a variety of sugars, glucose analogs, and metabolic intermediates on photosynthetic fusion genes in a sensitive and versatile maize protoplast transient expression system. The results show that sugars that are the substrates of hexokinase (HK) cause repression at a low concentration (1 to 10 mM), indicating a low degree of specificity and the irrelevance of osmotic change. Studies with various glucose analogs suggest that glucose transport across the plasma membrane is necessary but not sufficient to trigger repression, whereas subsequent phosphorylation by HK may be required. The effectiveness of 2-deoxyglucose, a nonmetabolizable glucose analog, and the ineffectiveness of various metabolic intermediates in eliciting repression eliminate the involvement of glycolysis and other metabolic pathways. Replenishing intracellular phosphate and ATP diminished by hexoses does not overcome repression. Because mannoheptulose, a specific HK inhibitor, blocks the severe repression triggered by 2-deoxyglucose and yet the phosphorylated products per se do not act as repression signals, we propose that HK may have dual functions and may act as a key sensor and signal transmitter of sugar repression in higher plants.**

## INTRODUCTION

Numerous physiological and biochemical studies have suggested that photosynthesis is feedback regulated by the accumulation of carbohydrates in source leaves (Neales and Incoll, 1968; Nafziger and Koller, 1976; Herold, 1980; Azcon-Bieto, 1983; Plaut et al., 1987; Foyer, 1988; Blechschmidt-Schneider et al., 1989; Sawada et al., 1989; Stitt, 1991; Goldschmidt and Huber, 1992). However, the molecular mechanism of this feedback control remains illusive. It has been proposed that reduced photosynthesis is the result of increased hexose production and cytosolic phosphate (Pi) depletion (Foyer, 1988; Huber, 1989; Goldschmidt and Huber, 1992), or the feedback inhibition of sucrose phosphate synthase that results in the accumulation of phosphorylation intermediates, the depletion of stromal Pi, and the decrease of ATP synthesis (Gerhard et al., 1987; Stitt et al., 1987; Foyer, 1988; Loughman et al., 1989; Stitt and Quick, 1989). Recently, using transgenic plants overexpressing a yeast invertase, two groups have shown independently that the accumulation of carbohydrates inhibits photosynthesis and causes stunted growth and necrotic leaves (von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991). By analyzing transgenic plants, one research team showed that photosynthesis inhibition is attributed to a decreased level of Calvin cycle enzymes and an increased level of glycolytic enzymes (Stitt et al., 1991); another group emphasized that the decrease of photosynthesis is accompanied by an increase in the osmotic pressure in leaf cells (Heineke et al., 1992). Although each model has its merit, none can satisfactorily reconcile many features of physiological studies on feedback regulation of photosynthesis (Stitt et al., 1991; Krapp et al., 1993).

An alternative mechanism underlying feedback regulation of photosynthesis could be a global repression of photosynthetic gene transcription by carbohydrates. Evidence supporting this model has emerged recently by using different experimental systems. For example, the activities of seven maize photosynthetic gene promoters are severely repressed by 100 to 300 mM glucose or sucrose in a protoplast transient expression assay (Sheen, 1990). The steady state transcript levels of ribulose bisphosphate carboxylase small subunit (*rbcS*), chlorophyll *a/b* binding protein (*cab*), and the δ subunit of the thylakoid ATPase (*atp*-δ) genes are significantly reduced within 5 hr after the addition of 50 mM glucose in an autotrophic cell suspension culture of *Chenopodium rubrum* (Krapp et al., 1993). In Arabidopsis, the light-inducible accumulation of *rbcS* transcript is dramatically repressed within 2 hr in the presence of 2% sucrose or glucose (Cheng et al., 1992). The light-dependent *cab* mRNA accumulation is also significantly reduced in rapeseed cell culture with 2% sucrose (Harter et al., 1993). These observations strongly support the concept that the control of gene expression plays a fundamental role in carbohydrate-mediated feedback or sink-regulated inhibition of photosynthesis.

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Although glucose-regulated gene expression has been studied in both prokaryotes and eukaryotes, the glucose-sensing and glucose-signaling pathways are still largely unknown. In *Escherichia coli*, where the mechanism is best understood, cAMP and its receptor protein are the major regulators of genes repressed by glucose (Saier, 1991). In budding yeast, extensive molecular genetic studies have defined both positive and negative regulators (Carlson, 1987; Entian and Barnett, 1992; Gancedo, 1992). Hexokinase (HK) PII is considered to be the major sensing molecule of catabolite repression triggered by glucose (Entian, 1980; Entian and Frölich, 1984; Ma and Bostein, 1986; Ma et al., 1989; Rose et al., 1991). Although glucose repression of the alcohol dehydrogenase II gene can be mimicked by protein kinase A phosphorylation (Cherry et al., 1989), the extent of cAMP involvement remains controversial (Mbonyi et al., 1990; Thevelein, 1991; Gancedo, 1992). In fission yeast, however, glucose repression is mediated by a cAMP-signaling pathway shown by the study of the fructose-1,6-bisphosphatase gene (Hoffman and Winston, 1991).

In mammalian cells, glucose has been shown to control gene expression both positively and negatively. For example, glucose-regulated proteins (Lee, 1987) and CCAAT/enhancer binding protein-related gene *gadd153* (Carlson et al., 1993) are repressed, whereas insulin (Nielsen et al., 1985; Welsh et al., 1985; Hammonds et al., 1987a, 1987b; German et al., 1990; Efrat et al., 1991), pyruvate kinase (Marie et al., 1993), and acetyl coenzyme A (CoA) carboxylase (Brun et al., 1993) genes are induced by glucose. Glucose transporter 2 and glucokinase are proposed to be glucose sensors in insulin secretion and gene activation (Johnson et al., 1990; Thorens et al., 1990; Epstein et al., 1992; Newgard, 1992; Tal et al., 1992; German, 1993). In some cases, glycolysis is required in glucose signaling, and cAMP (Nielsen et al., 1985; Welsh et al., 1985; Hammonds et al., 1987a, 1987b) and calcium (German et al., 1990; Efrat et al., 1991) are implicated as second messengers.

Sugar repression of photosynthetic genes is a unique phenomenon in higher plants where sugars are generated endogenously. However, evolutionarily conserved molecules and pathways may still be used for sugar sensing and signaling. To explore the sugar-sensing mechanism in higher plants, we performed a series of experiments using maize photosynthetic fusion genes and a sensitive protoplast transient expression system in which biochemical and molecular genetic manipulation is feasible (Sheen, 1990, 1991, 1993). We show that glucose and other hexoses are the direct signals in triggering repression. Studies with various glucose analogs suggest that glucose transport across the plasma membrane is necessary but not sufficient to cause repression. The sugar sensor is intracellular because glucose delivered only by electroporation triggered the same levels of repression as by uptake through the transport system. Phosphorylation of glucose, but not Pi and ATP depletion, is likely involved in signal transmission. However, sugar phosphates do not act as direct signals. By directly delivering various metabolic intermediates into protoplasts through electroporation, we further demonstrate that the signal transduction pathway of sugar repression does not overlap with downstream glucose metabolic pathways. We propose a novel role of HK as a putative sugar sensor and signal transmitter in higher plants.

## RESULTS

### Glucose Elicits Photosynthetic Gene Repression at Physiological Concentrations

Previously, it was shown that the transcriptional activity of seven maize photosynthetic gene promoters is repressed by a relatively high level (100 to 300 mM) of glucose or sucrose (Sheen, 1990). Although the sugar concentration used in those experiments is standard in plant tissue culture medium, it is not a physiological concentration in leaves. To establish that glucose represents a physiological regulator, we tested the effect of lower concentrations of glucose. Three photosynthetic fusion genes, *cabZm5-cat*, *rbcSZm1-cat*, and *C$_4$ppdkZm1-cat* (Sheen, 1990), were used as reporters to monitor repression by measuring the chloramphenicol acetyltransferase (CAT) activity that is not affected by various sugar treatments (Sheen, 1990). In C4 plants, such as maize, the *rbcS* promoter has been shown to be active in mesophyll cells (Schäffner and Sheen, 1991).

Three other fusion genes that are relatively insensitive to glucose were used as internal controls in the coelectroporation experiments (Sheen, 1990, 1993). One was a fusion between the nopaline synthase (NOS) promoter and the β-glucuronidase (GUS) gene (*nos-gus*). The second was the cauliflower mosaic virus (CaMV) 35S RNA promoter and *gus* fusion (*35S-gus*). The third was constructed by using a hybrid promoter consisting of the 5′ enhancer element of the *CaMV35S* promoter and the maize *C$_4$ppdkZm1* basal promoter and the *gus* reporter gene (*35S-C4ppdkhyb-gus*) (Sheen, 1993).

Green or greening protoplasts freshly isolated from light-grown or illuminated etiolated seedlings, respectively, were coelectroporated with CAT and GUS fusion genes. The transfected protoplasts were incubated with or without sugars for either 3 to 4 or up to 16 hr before CAT and GUS assays were performed (Sheen, 1990). To eliminate potential complications, a defined simple medium consisting of 0.6 M mannitol, 10 mM KCl, and 5 mM Mes, pH 5.7, was used. This condition not only allowed high levels of expression of transfected fusion genes in protoplasts but also made it possible to test various reagents in the incubation medium.

To show specific results, CAT expression of the reporter fusion genes was normalized by the GUS expression of internal controls. As shown in Figure 1, glucose at 1 to 10 mM was enough to cause fourfold repression of the *cabZm5* promoter activity. Little repression could be triggered by the glucose analog 3-O-methylglucose (3-OMG) at the same concentration, indicating that repression was not the result of osmotic change. Sucrose at 10 mM had much less effect, suggesting that glucose was likely the direct signal. The glucose repression of

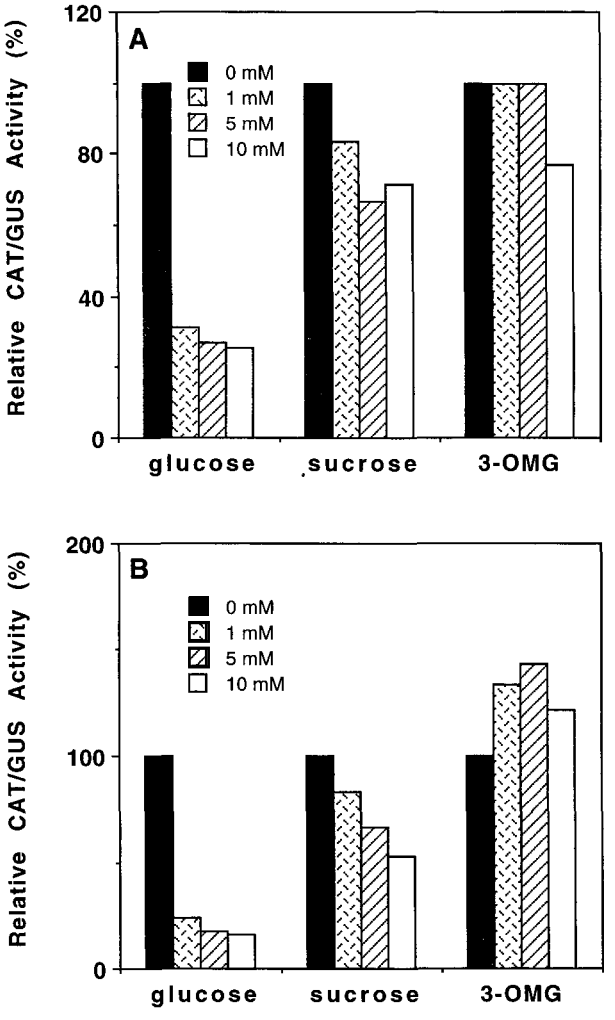


**Figure 1.** Effect of Sugars on *cabZm5-cat* Expression.

**(A)** Transfected greening maize protoplasts.
**(B)** Transfected green maize protoplasts.
Protoplasts were incubated for 16 hr before CAT and GUS assays were performed. The data are shown as relative CAT activity that is normalized by the expression of internal control *35S-C$_4$ppdkhyb-gus*. The experiment was repeated twice with consistent results. 3-OMG, 3-O-methylglucose.

*cabZm5-cat* was similar in green and greening protoplasts, but green protoplasts were more sensitive to glucose. Figure 2 shows that expression of two other photosynthetic fusion genes, *rbcSZm1-cat* and *C$_4$ppdkZm1-cat*, was also suppressed by glucose in green or greening protoplasts.

To demonstrate that glucose is taken up by protoplasts readily and is responsible for repression, intracellular concentrations of glucose and glucose-6-phosphate (G-6-P) were measured within 0.5, 1, 3, and 16 hr after transfection. As shown in Figure 3A, within 3 hr of incubation with either 1 or 10 mM glucose, five- to 10-fold repression of *cabZm5-cat* could be detected. However, the expression of the internal control *35S-gus* remained essentially constant. Figure 3B shows that there was ~5 and 15 mM increase in cellular glucose concentration with 1 and 10 mM glucose in the incubation medium, respectively. These data suggest that glucose uptake by maize protoplasts is active and rapid. On the other hand, cellular G-6-P concentration remained at a similar level within 3 hr of the incubation period (Figure 3C), suggesting that repression of *cabZm5-cat* (Figure 3A) was largely due to glucose but not to G-6-P. In fact,

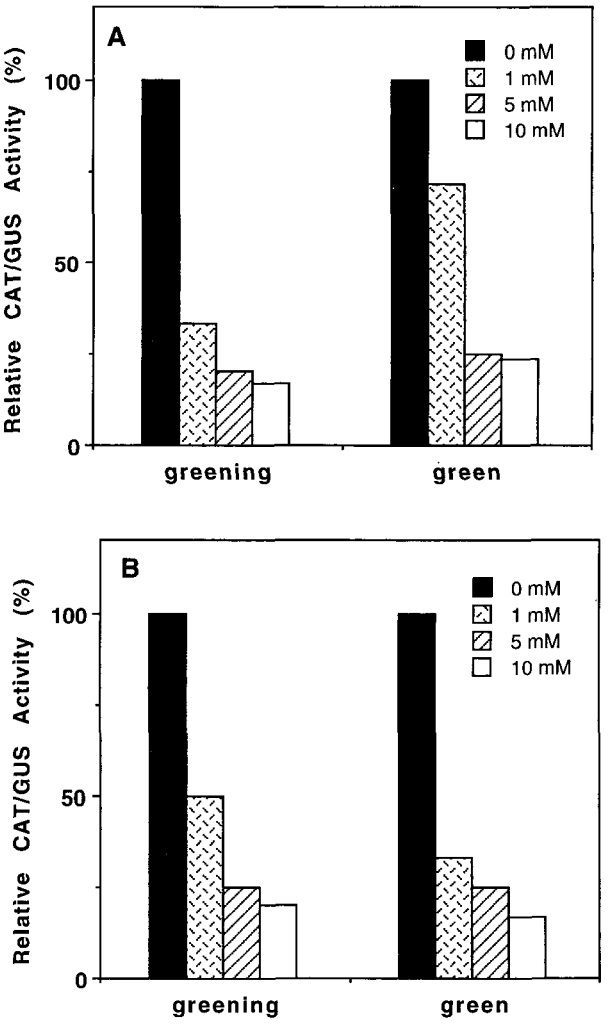


**Figure 2.** Effect of Glucose on the Expression of *rbcSZm1-cat* and *C$_4$ppdkZm1-cat* in Greening and Green Maize Protoplasts.

**(A)** Maize protoplasts transfected with *rbcSZm1-cat*.
**(B)** Maize protoplasts transfected with *C$_4$ppdkZm1-cat*.
Protoplasts were incubated with various concentrations of glucose for 16 hr before CAT and GUS assays were performed. The data are shown as relative CAT activity that is normalized by the expression of internal control *35S-C$_4$ppdkhyb-gus*. The experiment was repeated twice with consistent results.

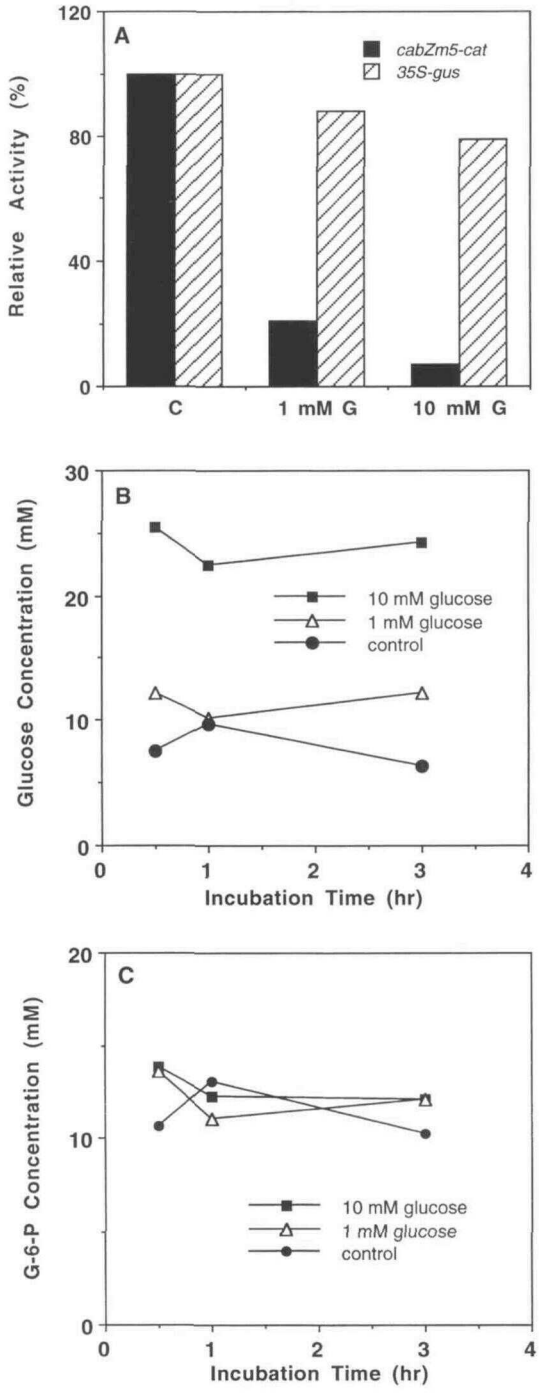


**Figure 3.** Glucose Repression Correlates with the Increase of Intracellular Glucose but Not G-6-P Levels.

**(A)** Glucose inhibits *cabZm5-cat* but not *35S-gus* expression. Incubation was for 3 hr. C, control; G, glucose.

**(B)** Intracellular glucose concentration. Samples of transfected greening maize protoplasts were taken after 0.5, 1, and 3 hr of incubation in the medium containing 0 (control), 1, or 10 mM glucose.

**(C)** Intracellular G-6-P concentration. Samples were derived from the same experiment as described above.

the G-6-P concentration decreased slightly within 16 hr of incubation (3 to 5 mM; data not shown).

### Glucose Reduces the Accumulation of the *cabZm5-cat* Transcript

Using truncated and hybrid promoters, it was shown previously by Sheen (1990) that glucose represses the transcription of *cabZm5-cat* and *$C_4$ppdkZm1-cat*. To show directly that glucose affects transcript accumulation, a sensitive reverse transcriptase–polymerase chain reaction (RT–PCR) assay was used to determine the steady state mRNA levels in electroporated protoplasts. Figure 4 shows that the CAT mRNA level controlled by the *cabZm5* promoter was significantly reduced by 10 mM glucose but not by sucrose. The reduction of CAT mRNA was specific because the GUS mRNA level regulated by the *nos* promoter remained constant with 10 mM glucose.

### Can Repression Be Triggered by Other Sugars?

In *E. coli*, yeast, and mammals, glucose is usually the predominant signal in gene regulation. To investigate the signal specificity of sugar repression in higher plants, we tested the effect of other sugars. Greening protoplasts coelectroporated with *cabZm5-cat* and *nos-gus* were incubated with mono-, di-, and trisaccharides at 10 mM. Figure 5 shows that hexoses, such as galactose and fructose, caused repression similar to that of glucose. However, mannose was very potent and specific, triggering more than 50-fold repression. Mannose has a similar chemical structure to glucose except for the orientation of hydroxy group at C-2. In general, mannose is transported into the cell and phosphorylated by HK with an efficiency comparable to glucose. However, the metabolism of mannose-6-phosphate by either hexose phosphate isomerase or hexose-

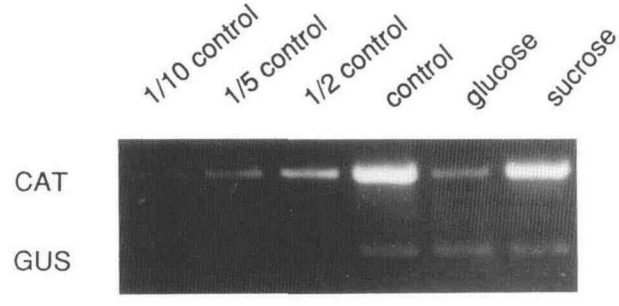


**Figure 4.** Glucose Reduces the Accumulation of *cabZm5-cat* Transcript.

Quantitative analysis of *cabZm5-cat* and *nos-gus* mRNA from transfected protoplasts incubated with or without 10 mM sugars as indicated in each lane. RNA samples were purified from transfected greening maize protoplasts incubated for 3 hr. The first three lanes from the left of the figure are serial dilutions of the control lane 4.

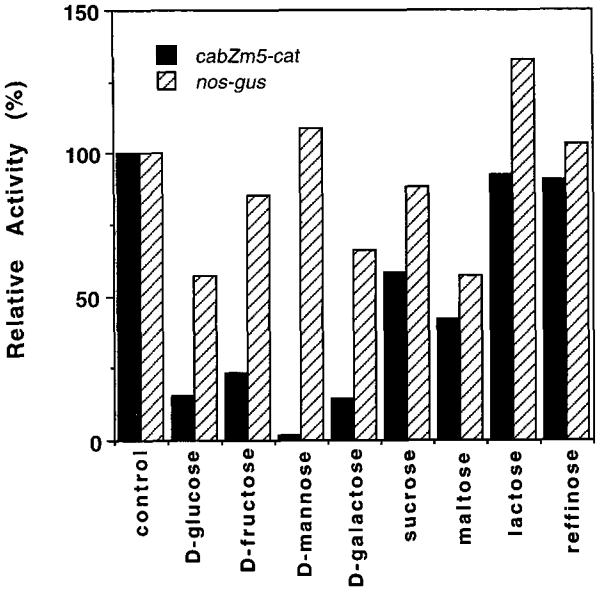


**Figure 5.** Effect of Various Sugars on the Expression of *cabZm5-cat* and *nos-gus*.

Transfected greening maize protoplasts were incubated with various sugars at 10 mM for 16 hr before CAT and GUS assays were performed. The experiment was repeated twice with consistent results.

6-phosphate dehydrogenase is inefficient (Salas et al., 1965; Sheu-Hwa et al., 1975; Loughman et al., 1989). There was approximately a twofold reduction of *cabZm5-cat* expression in the presence of sucrose and lactose but not in the presence of the trisaccharide raffinose (Figure 5). The ability of sucrose to cause repression is presumably dependent on its hydrolysis to glucose and fructose by invertase, as was suggested by Goldschmidt and Huber (1992). The repression caused by 10 mM sucrose was not as severe as that caused by glucose, suggesting that the concentration of invertase is low in maize mesophyll cells.

## Glucose Repression of Gene Transcription Is Not Caused by Phosphate or ATP Depletion

It has been proposed that the depletion of phosphate or ATP causes sugar repression of photosynthesis (Walker and Sivak, 1986; Loughman et al., 1989; Brauer and Stitt, 1990). To determine whether sugar repression of photosynthetic gene expression is also controlled by this mechanism, we examined the ability of phosphate and ATP to block glucose repression. We first tested the effect of phosphate that can be taken up readily by plant protoplasts (Lin, 1979) and is able to block the sequestration of leaf cell phosphate by mannose in excised spinach leaf (Weiner et al., 1992). As shown in Figure 6A, providing up to 50 mM phosphate in the incubation medium did not block repression caused by 10 mM glucose,

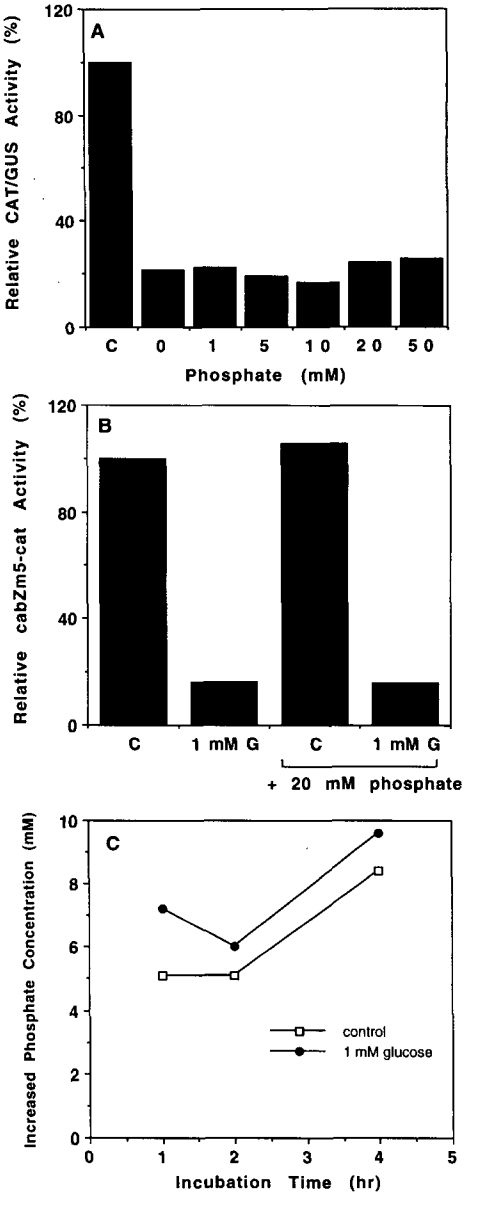


**Figure 6.** Phosphate Does Not Block the Repression of *cabZm5-cat* Caused by Glucose.

**(A)** The effect of various concentrations of phosphate on glucose repression was determined. The data are shown as relative CAT activity that is normalized by the GUS activity derived from the expression of the internal control *nos-gus*. Transfected maize greening protoplasts were incubated for 16 hr in the presence of 10 mM glucose and 0, 1, 5, 10, 20, or 50 mM phosphate. Control (C) sample was incubated in a medium without glucose and phosphate.

**(B)** Increased phosphate/glucose ratio does not block repression. Transfected maize greening protoplasts were incubated in the presence of 1 mM glucose (G) and with or without 20 mM phosphate for 4 hr before CAT assay was performed. Control (C) is the same as in **(A)**.

**(C)** Time course measurement of phosphate uptake. Transfected maize greening protoplasts were incubated in the medium containing 20 mM phosphate and without (control) or with 1 mM glucose.

suggesting that the specific repression of *cabZm5-cat* by sugar may not be due to the depletion of phosphate. Similarly, phosphate was not able to relieve the strong repression caused by either 10 mM mannose or 1 mM 2-deoxyglucose (2-dG), a potent glucose analog (data not shown).

To confirm that phosphate enters maize protoplasts efficiently, an uptake assay using $^{32}P$-labeled $KH_2PO_4$ was conducted. As shown in Figure 6B, repression of *cabZm5-cat* could be induced by 1 mM glucose within 4 hr of incubation. The presence of 20 mM phosphate in the incubation medium was not able to reverse repression. An aliquot of the same batch of protoplasts was used for phosphate uptake assay. Figure 6C shows that with 20 mM phosphate in the incubation medium, the cellular phosphate concentration estimated by $^{32}P$ uptake increased rapidly and reached a high level after 4 hr of incubation. This level remained constant throughout the 16-hr incubation period (data not shown). The presence of 1 mM glucose in the incubation medium enhanced phosphate uptake slightly. These data suggest that depletion of phosphate in cells caused by 1 mM glucose in the incubation medium is not responsible for the repression of *cabZm5-cat* transcription. Furthermore, as shown in Figure 3B, after 3 hr of incubation with 1 mM glucose and 20 mM phosphate, cellular glucose concentration rose to a maximum of 5 mM, whereas cellular phosphate concentration (estimated by $^{32}P$ uptake) increased ~8 mM (Figure 6C).

To determine whether ATP depletion is the cause of sugar repression, membrane-impermeable ATP together with both *cabZm5-cat* and the internal control *nos-gus* were delivered into maize protoplasts by electroporation (Zachrisson and Bornman, 1986; Zimmerman, 1986). Results showed that repression triggered by glucose cannot be relieved by ATP treatment (Figure 7). ATP alone enhanced the expression of *cabZm5-cat* significantly, indicating successful ATP delivery by electroporation. As shown in Figure 7, addition of ATP did not reduce the strong repression mediated by mannose, which is frequently used to deplete ATP and phosphate in vivo.

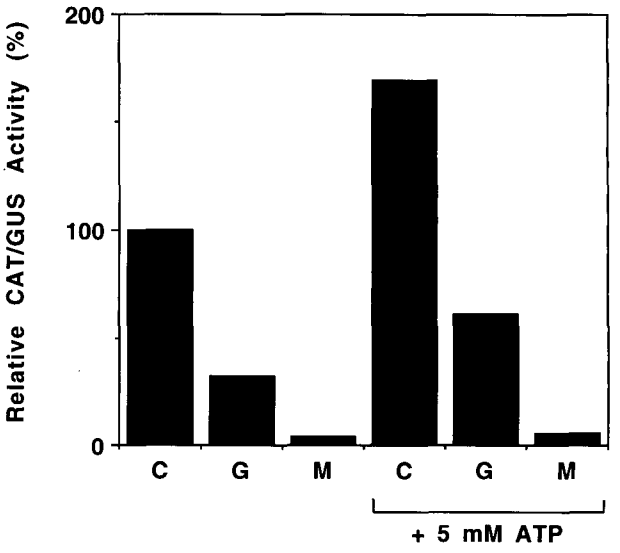


**Figure 7.** Effect of ATP on Sugar Repression.

Maize greening protoplasts were transfected with *cabZm5-cat* and *nos-gus*. ATP (5 mM) was delivered into cells by electroporation. Transfected protoplasts were incubated in the medium containing 10 mM glucose (G) or 10 mM mannose (M) for 16 hr before CAT and GUS assays were performed. The experiment was repeated three times with consistent results. The data are shown as normalized values. C, control sample without sugar incubation.

## Glucose Transport Is Required but Is Not Sufficient to Trigger Repression

We demonstrated that hexoses act as repression signals in plant leaf cells. However, it remains unclear whether sugar signals are perceived outside or inside the cell. To understand how the sugar signal is sensed and transduced, a set of glucose analogs was used to probe the sensing mechanism. We first tested the involvement of glucose transport. As shown in Figure 8, L-glucose, which cannot be efficiently taken up by the plant cell (Lin et al., 1984a, 1984b; Komor et al., 1985; Gogarten and Bentrup, 1989; Tubbe and Buckhout, 1992), did not trigger repression in protoplasts transfected with *cabZm5-cat*. However, glucose transport per se is not sufficient to induce repression because 6-deoxyglucose (6-dG) (Figure 8) and 3-OMG (Figure 1) did not cause repression. Both 6-dG and 3-OMG are taken up by cells with a comparable efficiency to glucose (Lin et al., 1984a, 1984b; Komor et al., 1985; Gogarten and Bentrup, 1989; Tubbe and Buckhout, 1992) but are not substrates for hexokinase. To further support the hypothesis that the sugar sensor is intracellular and specific, glucose or 6-dG was delivered into protoplasts by coelectroporation with reporter genes. Transfected protoplasts were incubated on ice for 10 min to allow the plasma membrane to reseal. Two washes were followed immediately by using ice-cold 0.6 M mannitol to remove extracellular sugar. Such a condition allows sugar to enter cells without much interaction with any possible surface molecules. The results showed that glucose alone but not 6-dG causes repression (Figure 9), suggesting that the sugar sensor is specific and intracellular. A similar level of repression of *cabZm5-cat* was triggered by 100 mM glucose, supplied either in the incubation medium (data not shown) or delivered by electroporation (Figure 9). Therefore, it is obvious that glucose transport is not a limiting factor and that the intracellular glucose sensor or receptor is saturable for transmitting the repression signal.

## Extensive Glucose Metabolism Is Not Required for Repression

When sugars enter into leaf cells, repression signals can be generated either directly by sugars or indirectly through further

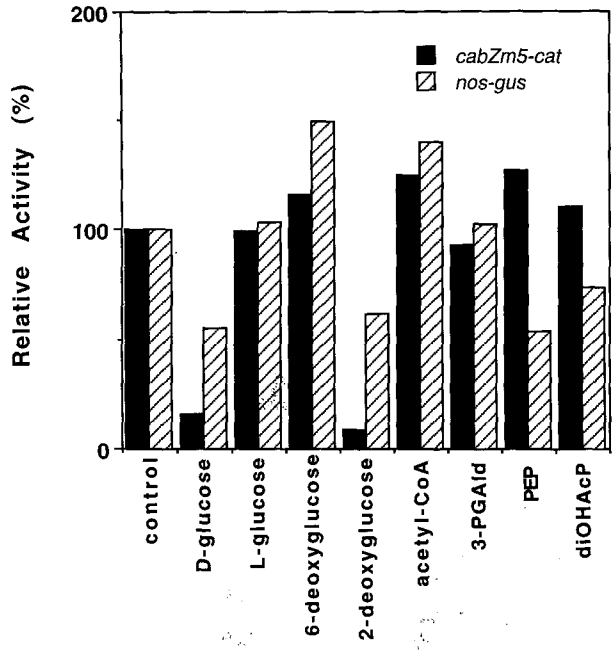


**Figure 8.** Glucose Repression Does Not Require Extensive Metabolism.

D-Glucose (10 mM), L-glucose (10 mM), 6-deoxyglucose (6-dG; 10 mM), and 2-dG (0.5 mM) were added to the protoplast incubation medium after the transfection of *cabZm5-cat* and *nos-gus*. Acetyl-CoA, glyceraldehyde 3-phosphate (3-PGAld), phosphoenolpyruvate (PEP), and dihydroxyacetone phosphate (diOHAcP) at 10 mM in the electroporation medium were delivered into cells by electroporation. Transfected maize greening protoplasts were incubated for 16 hr before CAT and GUS assays were performed. The experiment was repeated twice with consistent results.

metabolism of the sugars. To distinguish between the two possibilities, we tested the effect of 2-dG. It can be transported into leaf cells and phosphorylated by HK, but the phosphorylated product 2-deoxyglucose 6-phosphate (2-dG-6-p) cannot be metabolized further (Bessell and Thomas, 1973a, 1973b). As shown in Figure 8, 2-dG triggered severe and specific repression at a concentration as low as 0.5 mM. The repression level was similar to that caused by 10 mM mannose (Figure 5). These results suggest that extensive metabolism of sugars is not required for triggering repression. To support this conclusion further, we also investigated the effectiveness of various metabolic intermediates of the glycolytic pathway via electroporation. As shown in Figure 8, no repression was triggered by acetyl CoA, glyceraldehyde 3-phosphate, phosphoenolpyruvate, and dihydroxyacetone phosphate. It has also been shown previously that pyruvate, malate, and oxalacetate do not trigger repression (Sheen, 1990). Taken together, these data support the notion that the signal transduction pathway leading to photosynthetic gene suppression does not overlap with metabolic pathways downstream of sugar phosphates.

## Sugar Phosphates Do Not Trigger Repression

The ineffectiveness of 6-dG and 3-OMG in triggering repression indicates the potential importance of sugar phosphorylation in signal transduction because they both cannot be phosphorylated by HK (Dixon and Webb, 1979). This hypothesis is further supported by the fact that sugars and the sugar analog 2-dG, which are substrates of HK, cause repression. The immediate question then is whether phosphorylated sugars are the signals. Because sugar phosphates cannot be taken up by plant cells readily, we delivered these compounds into protoplasts by electroporation. As shown in Table 1, no repression was triggered by G-6-P, fructose 6-phosphate, fructose 1,6-diphosphate, or glucose 1-phosphate. Remarkably, we found that 2-dG-6-P, the phosphorylated product of the most potent sugar 2-dG, did not cause repression. These results show that the phosphorylated sugars are ineffective in triggering repression. It is interesting to note that the expression of both *cabZm5-cat* and *nos-gus* was enhanced by sugar phosphate treatments. This was due in part to the salt (potassium or sodium) derived from sugar phosphate compounds in the electroporation medium (Table 1).

To demonstrate that G-6-P can be electroporated into protoplasts efficiently and remain at a constant cellular concentration within a short period of incubation, an enzymatic assay was conducted to monitor its concentration over time. Figure 10A shows that the expression of *cabZm5-cat* was severely repressed by 20 mM glucose but not by G-6-P delivered by electroporation within 3 hr of incubation. An aliquot of the same

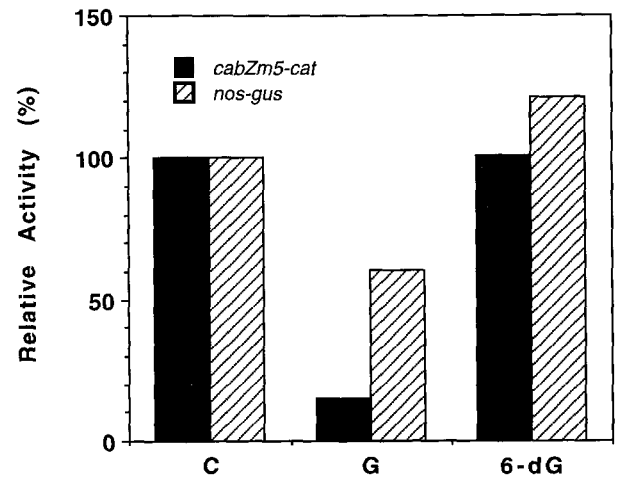


**Figure 9.** Glucose Sensing Is Likely Intracellular.

Greening maize protoplasts were coelectroporated with reporter genes (*cabZm5-cat* and *nos-gus*) and 100 mM glucose (G) or 100 mM 6 dG. Transfected cells were incubated on ice for 10 min and washed with ice-cold 0.6 M mannitol solution twice to remove extracellular glucose. Washed protoplasts were then incubated in the basal medium without sugar for 16 hr before CAT and GUS assays were performed. C, control sample without coelectroporation of sugar.

**Table 1.** Effect of Sugar Phosphate on *cabZm5-cat* Expression

| Treatment | Activity | |
|---|---|---|
| | *cabZm5-cat* | *nos-gus* |
| Glucose-6-phosphate (20 mM) | 31,850 | 1,100 |
| Control | 13,220 | 315 |
| 2-Deoxyglucose-6-phosphate (10 mM) | 34,660 | 3,630 |
| Control | 31,550 | 1,350 |
| Fructose-6-phosphate (10 mM) | 30,475 | 1,600 |
| Control | 13,810 | 565 |
| Fructose-1,6-diphosphate (10 mM) | 42,850 | 1,360 |
| Control | 12,855 | 310 |
| Glucose-1-phosphate (10 mM) | 34,815 | 1,740 |
| Control | 27,385 | 930 |

The results are shown as CAT and GUS activity. Because varying amounts of potassium or sodium salt are associated with sugar phosphate compound, controls were performed with respective salt concentration without sugar phosphates. Sugar phosphates were delivered into protoplasts by two pulses of electroporation at standard condition (see Methods).

batch of protoplasts was used for the enzymatic assay. As shown in Figure 10B, the cellular G-6-P concentration was 3 to 4 mM higher in cells coelectroporated with G-6-P than in control cells. This concentration difference remained essentially constant within the 3-hr incubation period. G-6-P concentration did not increase in cells coelectroporated with 20 mM glucose (Figure 10B), although the intracellular glucose concentration increased significantly (data not shown). These data strongly suggest that G-6-P does not cause repression and that glucose is the direct signal.

### Hexokinase as a Sensor in Glucose Signaling

Although the involvement of other unknown sugar receptors has not been eliminated, HK is considered to be a potential sugar sensor that can interact with various hexoses and 2-dG, can distinguish similar molecules such as glucose and 3-OMG or 6-dG, and is evolutionarily conserved (J.-C. Jang and J. Sheen, unpublished data).

In higher plants, depending on the species and tissues, biochemical and genetic studies have shown that there are multiple HKs (Miernyk and Dennis, 1983; Wendel et al., 1986; Doehlert, 1989; Schnarrenberger, 1990; Renz et al., 1993) that might hinder genetic analysis of the role of HK in sugar repression. To support further the involvement of HK in sugar sensing, we sought to determine the effect of specific HK inhibitors, such as glucosamine, mannoheptulose (Salas et al., 1965), and $ZnCl_2$ (Saltman, 1953). Because glucosamine and $ZnCl_2$ caused cytotoxicity in our system (data not shown), mannoheptulose was chosen for the experiment.

Because mannoheptulose is a competitive inhibitor of HK, we took advantage of the severe repression triggered by a low concentration of 2-dG, which allows effective competition by mannoheptulose. Protoplasts electroporated with *cabZm5-cat* were treated with mannoheptulose to see whether the inhibition of HK could block repression. Figure 11 shows that the severe repression caused by 0.5 mM 2-dG was relieved (less than twofold repression) by 10 mM mannoheptulose in the incubation medium. The blockage of repression was not due to the competition of sugar transport because similar results were obtained when the same molar ratio of mannoheptulose

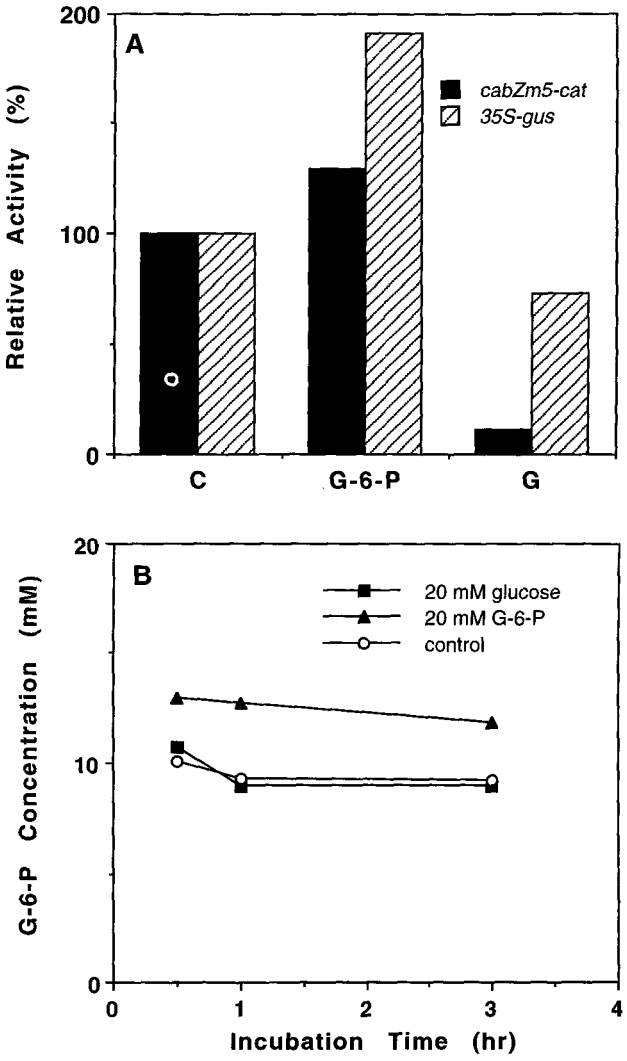


**Figure 10.** Glucose but Not G-6-P Is the Direct Signal for Sugar Repression.

**(A)** Effect of G-6-P and glucose. Reporter genes (*cabZm5-cat* and *35S-gus*) and 20 mM G-6-P or 20 mM glucose (G) were delivered into cells by electroporation. Transfected maize greening protoplasts were incubated for 4 hr before CAT and GUS assays were performed. C, control.
**(B)** Measurement of intracellular G-6-P concentration.

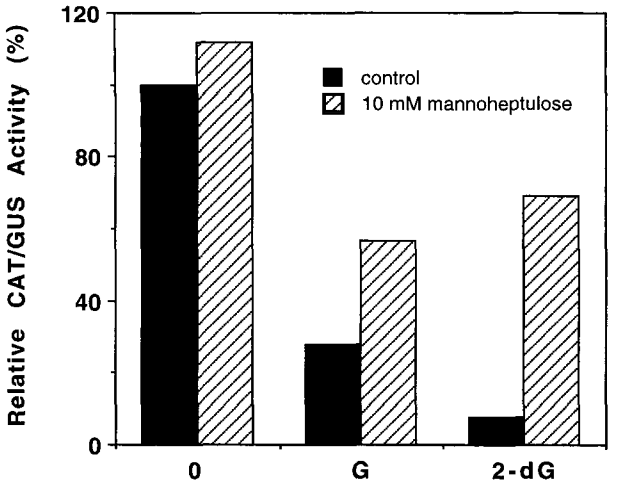


**Figure 11.** Inhibition of HK by Mannoheptulose Blocks Repression.

Transfected maize greening protoplasts were incubated in the medium with 10 mM glucose (G) or 0.5 mM 2-dG in the presence or absence (control) of 10 mM mannoheptulose. Samples were incubated for 16 hr before CAT and GUS assays were performed. The data are shown as relative *cabZm5-cat* activity that is normalized by the expression of internal control *nos-gus*. 0, sample without sugar incubation.

and 2-dG was delivered into cells by electroporation (data not shown). The effect of mannoheptulose on the derepression of *cabZm5-cat* was less significant in glucose treatment, suggesting that glucose has higher affinity to HK than mannoheptulose and 2-dG. A direct competition experiment showed that glucose and 2-dG mediated repression through the same receptor and that glucose had higher affinity (data not shown).

## DISCUSSION

### Sugar Repression in Higher Plants

Sugar repression of photosynthetic genes as a central metabolic control mechanism has now been observed in a wide range of plant species (Sheen, 1994). This study and others (Sheen, 1990; Harter et al., 1993; Krapp et al., 1993; Graham et al., 1994) demonstrate that the single cell system is not only efficient and sensitive but also reliable and versatile as a model system for elucidating the molecular mechanisms of sugar sensing and signaling and coordinated gene expression in a defined simple medium. Here, we show that hexoses, including glucose, fructose, mannose, and galactose, exert specific repression of the promoter activity of three maize photosynthetic genes. The concentration of glucose required to trigger repression was low and physiologically significant. Besides the quick and direct accessibility of glucose, it was obvious that the physiological status of the maize protoplasts is highly sensitive to the glucose signal because repression could be detected in the presence of 1 to 10 mM glucose. In transgenic plants overexpressing a yeast invertase, shaded plants and young sink leaves show less severe necrotic and stunted symptoms than nonshaded and mature source leaves (von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991), implying that physiological and metabolic status and photosynthetic capacity of the leaf cells determine the level of sugar repression.

Sucrose does not trigger the same level of repression as glucose at 10 mM. The effectiveness of sucrose in causing repression is presumably dependent on its hydrolysis to hexose sugars, which act as direct signals. This view is supported by a recent study indicating that sucrose is not a direct signal in feedback inhibition, which is correlated with high acid invertase activity in fully expanded leaves (Goldschmidt and Huber, 1992). The possibility of repression caused by osmotic change can be ruled out because the nonmetabolized glucose analogs 3-OMG and 6-dG did not cause repression. It has also been suggested that photosynthesis inhibition associated with carbohydrate accumulation is due to the increased levels of proline (Heineke et al., 1992) or the depletion of phosphate (Walker and Sivak, 1986; Brauer and Stitt, 1990). These hypotheses are not consistent with our results. Our results showed that no apparent repression of photosynthetic genes could be triggered by an excessive amount of proline delivered into cells via electroporation (data not shown). Furthermore, a sufficient amount of phosphate or ATP in the cell did not reverse the repression caused by glucose and mannose. Recently, Graham et al. (1994) showed that hexose specifically represses malate synthase and isocitrate lyase gene expression. Although these two genes are involved in the glyoxylate cycle, the sugar-sensing mechanism may be the same. Consistent with our results, they demonstrated that phosphate is not able to reverse the repression of malate synthase and isocitrate lyase caused by mannose.

Signal specificity of sugar repression in photoautotrophic plants is quite different from that in heterotrophic organisms, such as bacteria and yeasts. It seems that the priority of glucose utilization in heterotrophic microorganisms determines the repression of genes in alternative carbon source usage (Carlson, 1987; Saier, 1991; Gancedo, 1992), whereas various hexoses in higher plants represent a uniform message that represses the expression of a number of photosynthetic genes. It remains to be determined whether similar sugar-sensing and signaling pathways control other plant genes that are activated or repressed by sugars (Sheen, 1994).

### Sugar Repression Is Coupled to Environmental and Developmental Regulation

In higher plants, sugar repression is not limited to the feedback control of photosynthesis. As illustrated in Figure 12, sugar repression of photosynthetic genes can also be triggered by other extrinsic or intrinsic stimuli. For example, wounding and

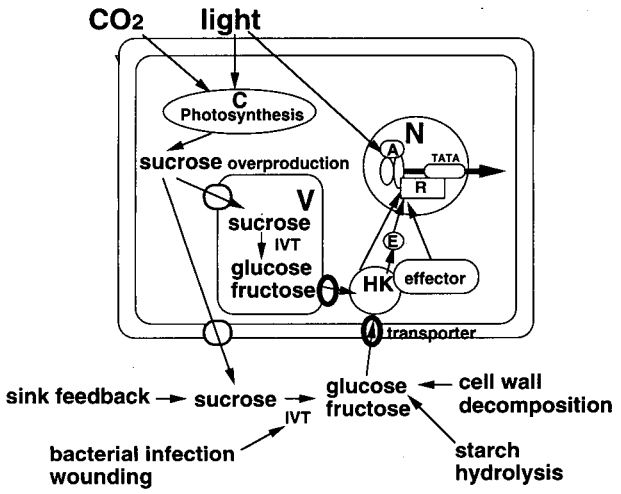


**Figure 12.** Model for Sugar Repression of Photosynthetic Gene Transcription in Higher Plants.

A, transcriptional activator; C, chloroplast; E, effector; HK, hexokinase; IVT, invertase; N, nucleus; R, transcriptional repressor; V, vacuole.

bacterial infection cause rapid induction of extracellular invertase expression (Sturm and Chrispeels, 1990). Presumably, the hydrolysis of apoplastic sucrose by this elevated invertase may lead to a higher influx of glucose and fructose, which in turn triggers the repression of photosynthetic genes. This sugar regulation mechanism may be used as a gene expression switch that facilitates the cell defense response. Indeed, it is well documented that defense-related genes such as proteinase inhibitor II (Johnson and Ryan, 1990; Kim et al., 1991) and chalcone synthase (Tsukaya et al., 1991) are induced by sugars, whereas photosynthetic gene expression is inhibited upon wounding (Peña-Cortes et al., 1988).

Leaf development is also associated with profound carbohydrate metabolism changes (Turgeon, 1989), especially in monocots whose large starch-rich seeds support leaf cell division and elongation before the commitment of terminal differentiation into photosynthetic cells. Previous studies have shown that photosynthetic gene expression is low in younger maize (Nelson et al., 1984; Loza-Tavera et al., 1990) and sugarbeet (Harn et al., 1993) leaves. Using protoplast transient assays, we observed that the promoter activities of several photosynthetic genes are very low during early maize seedling development (J.-C. Jang and J. Sheen, unpublished data). The low expression of photosynthetic genes is probably due, in part, to the high exogenous glucose/sucrose imported from the hydrolysis of starch in the endosperm. It was proposed by Graham et al. (1992, 1994) that sugar repression of malate synthase and isocitrate lyase gene transcription is tightly linked to the seedling development program in the transition of heterotrophic to autotrophic. This evidence fully supports our hypothesis in that sugar repression is also coupled to developmental regulation. As illustrated in Figure 12, a complex regulatory circuitry might link sugar repression to a variety of stimuli.

## Hexokinase and Sugar Sensing

Although sugars have been recognized as essential regulatory molecules in controlling gene expression, virtually nothing is known about sugar sensing and signal transduction in higher plants (Sheen, 1994). We have initiated the dissection of a sugar-sensing mechanism mediating the repression of photosynthetic genes. To learn whether the sugar sensor is located on the cell surface, we examined the effect of various glucose analogs. We showed that L-glucose, which cannot be transported, does not cause repression, indicating that the sensor is most likely intracellular. This conclusion is supported by experiments in which sugars, such as glucose or 2-dG, pulse-delivered into cells directly by electroporation resulted in the same levels of repression as that caused by prolonged incubation. In addition, it has also been demonstrated that glucose transport per se cannot trigger repression because 3-OMG and 6-dG are both taken up by cells but do not cause repression.

Based on several lines of evidence, we propose that HK is a putative sensor that directly mediates the first step of the sugar signal transduction pathway. First, various hexoses and a glucose analog that can be phosphorylated by HK are able to trigger repression. Second, further metabolism of sugar phosphates is not necessary to cause repression, because 2-dG and mannose that cannot be metabolized after phosphorylation cause severe repression. To eliminate the possibility that the effect of 2-dG is due to a general inhibition of *N*-glycosylation, we treated transfected maize protoplasts with tunicamycin, another widely used inhibitor of *N*-glycosylation (Pelham, 1989). No specific repression was triggered by tunicamycin (data not shown), suggesting that the repression caused by 2-dG is not due to *N*-glycosylation. The ineffectiveness of various metabolic intermediates in causing repression further strengthens the view that the sugar signaling pathway does not overlap with downstream glucose metabolic pathways. Third, it is unlikely that glucose is converted to other derivatives that trigger repression without going through phosphorylation. We treated transfected maize protoplasts with sugar alcohols (mannitol, glycerol, and inositol) and sugar acids (D-glucuronic acid and ascorbic acid) and observed no similar repression of the photosynthetic fusion genes (data not shown). Fourth, in contrast to a popular proposal that suggests that G-6-P is the repression signal in yeast and mammals (Brun et al., 1993), we showed that direct delivery of sugar phosphates into maize cells via electroporation did not trigger repression. Results of the measurement of metabolites also suggest that the direct signal is glucose, because intracellular G-6-P concentration does not increase upon the treatment of glucose. Finally, a competitive inhibitor of HK, mannoheptulose, was able to block the severe repression triggered by

2-dG, suggesting that HK is the sensor in mediating the repression signal. This view is further supported by the observation that 3-OMG could not relieve the strong repression caused by 2-dG (data not shown), because 3-OMG cannot be phosphorylated by HK (Dixon and Webb, 1979).

### Conserved Sugar-Sensing Mechanism in Prokaryotes and Eukaryotes

In yeast, molecular, biochemical, and genetic studies support the notion that HK controls the bottleneck step in glucose metabolism and acts as a sensor for glucose-mediated gene regulation. Hexokinase PII is proposed as a bifunctional enzyme with catalytic and regulatory domains (Entian and Frölich, 1984; Entian et al., 1985). Although the regulatory domains have not been defined physically, catalytic activity is required for gene repression (Ma and Bostein, 1986; Ma et al., 1989; Rose et al., 1991).

Currently, the mechanism for the HK regulatory function in plants is not clear. As shown in Figure 12, one possible explanation is that the conformational change (Bennett and Steitz, 1978) of HK upon binding and/or phosphorylation of hexoses modulates its interaction with putative downstream regulatory effectors and triggers the signaling cascade for the repression. In yeast, the conformational change of HK upon binding of glucose was proposed in the signal transduction pathway (Entian et al., 1985). Recently, a glucose-sensing complex composed of glucose transporter, hexokinase, and the gene product of *GGS1* was proposed. Additional putative glucose repressible protein might also be a component of the same complex (Thevelein, 1992). In *E. coli*, the transport of sugars across the cell membrane mediated by the phosphotransferase system is tightly coupled to sugar phosphorylation and sugar signaling (Stock, 1993). Therefore, it is likely that a glucose-sensing mechanism is conserved between prokaryotes and eukaryotes. The severe repression elicited by 2-dG and mannose that we have observed here may be due to their ability to activate the signal transmission more effectively or constitutively. In yeast, 2-dG also causes strong repression at a low concentration (Zimmermann and Scheel, 1977; Ma et al., 1989).

In summary, using an efficient transient gene expression system, we demonstrated that glucose and other hexoses are direct signals mediating photosynthetic gene repression. Glucose transport and glucose phosphorylation are both required in the generation of repression signals. HK is likely to be the sensor in the signaling pathway. Further studies are required to directly prove the hypothesis that is illustrated in Figure 12. Because glucose repression is conserved in other plants, including Arabidopsis (Cheng, 1992; P. Leon and J. Sheen, unpublished data), more genetic and biochemical mechanisms can probably be revealed by the screening and characterization of repression and derepression mutants in Arabidopsis. Moreover, we have recently cloned two plant hexokinase genes (J.-C. Jang and J. Sheen, unpublished data). The study of their functions in transgenic plants and their interaction with other regulatory proteins may provide valuable new insights into the sugar signal transduction pathway in higher plants.

## METHODS

### Plant Growth and Protoplasts Isolation

Twelve-day-old green and greening maize seedlings were obtained as described previously (Sheen, 1991). The procedures for the isolation of green and greening leaf mesophyll protoplasts were described previously (Sheen, 1991).

### Chimeric Gene Constructs

The chimeric constructs of chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) fusion genes were described previously (Sheen, 1990, 1993).

### Electroporation and Protoplasts Incubation

Electroporation was performed with $3 \times 10^5$ protoplasts in 300 μL of electroporation solution with 50 μg of reporter CAT plasmid DNA and 10 μg of internal control GUS plasmid DNA. The electroporation condition was 200 μF, 400 V/cm, 10 msec, and a single pulse (unless specified) with an X-Cell 450 apparatus (Promega). The electroporation solution was 0.6 M mannitol, 15 mM KCl, 5 mM Mes, pH 5.8. Electroporated protoplasts, $7.5 \times 10^4$ cells per sample, were incubated in solution containing 0.6 M mannitol, 5 mM Mes, pH 5.8, and 10 mM KCl at 23°C for 16 hr or as otherwise specified.

### CAT and GUS Assays

CAT and GUS assays were described previously (Sheen, 1991). CAT assay was performed using cell extract from $7.5 \times 10^3$ protoplasts electroporated with a single pulse or from $3.75 \times 10^3$ protoplasts electroporated with double pulses. GUS assay was performed with the same amount of cell extract as for CAT assay in 100 μL of 10 mM Tris-Cl, pH 8, 2 mM $MgCl_2$, and 1 mM 4-methylumbelliferyl β-D-glucuronide for 90 min at 37°C. The fluorescence generated by GUS activity was measured by a Hoefer Fluorometer at $\lambda_{excitation}$ 365 nm and $\lambda_{emission}$ 460 nm.

### Reverse Transcriptase–Polymerase Chain Reaction Assay

Total RNA was isolated from electroporated protoplasts within 3 hr after incubation. Protoplasts ($3 \times 10^5$ cells) were concentrated by centrifugation and lysed by repeated freeze-thaw. The cell pellet was resuspended and extracted for 20 sec with an equal volume of extraction buffer (0.1 M Tris-Cl, pH 8.4, 25 mM EDTA, 0.1 M 2-mercaptoethanol) and phenol. The mixture was then centrifuged at 10,000 rpm at room temperature in a microcentrifuge for 1 min. The supernatant was transferred to a fresh tube and reextracted with 300 μL of phenol and centrifuged for 1 min. Supernant was extracted once more with phenol-

chloroform. Total nucleic acid was precipitated with sodium acetate and ethanol at −20°C for at least 2 hr. RNA was then selectively precipitated twice by using 2 M LiCl, first at 4°C overnight and later at 0°C for 2 hr. RNA sample was then treated with RNase-free DNase I (Promega) to remove residual plasmid DNA. A polymerase chain reaction (PCR) was first performed by using the same set of primers to ensure that no plasmid DNA was left and could be amplified from DNase I–treated RNA samples. The CAT primers are two 24-bp oligonucleotides within the CAT coding sequences: CAT1, 5′-TCACTGGATATACCACCGTTGATA-3′, and CAT5, 5′-CGAAGAAGTTGTCCATATTGGCCA-3′. The GUS primers are 24-bp oligonucleotides within the GUS coding region: GUS3, 5′-CTGTGGGCATTCAGTCTGGATCGC-3′, and GUS5, 5′-GCGTGACATCGGCTTCAAATGGCG-3′. For reverse transcriptase (RT) reaction, total RNA (1 to 5 μg) was mixed with primers (50 ng each), heated in a 70°C water bath for 5 min, and quickly chilled on ice. The primers for the RT reaction are CAT2 and GUS2 from the coding sequences of CAT and GUS, respectively. The CAT2 sequence is 5′-TGC-CACTCATCGCAGTACTGTTGT-3′ and the GUS2 sequence is 5′-GACATGCGTCACCACGGT-GATATC-3′. RT reaction was performed by using avian myeloblastosis virus (AMV) RTase from Life Science (St. Petersburg, FL) at 41°C (50 mM Tris.Cl, pH 8.4, 50 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 40 μg/mL actinomycin D, 0.5 mM deoxynucleotide triphosphates, and 20 U of AMV for 5 μg of total RNA) for 1 hr. The reaction was then treated with RNase-A to remove residual RNA and extracted with phenol-chloroform before ethanol precipitation of the cDNA. An aliquot of cDNA product (2 to 5 ng) was then used for PCR with CAT1, CAT5, GUS3, and GUS5 primers. PCR was conducted for 20 cycles at 94°C for 30 sec, 50°C for 45 sec, and 72°C for 60 sec.

### Glucose and Glucose-6-Phosphate Measurements

Protoplasts were collected by centrifugation at 0°C for 30 sec and resuspended and washed with 100× volume of 0°C 0.6 M mannitol solution. Protoplasts were then concentrated by centrifugation at 0°C and immediately frozen with liquid nitrogen. Glucose and glucose-6-phosphate concentrations were measured by an enzymatic assay (Stitt et al., 1989). Intracellular metabolite concentration was calculated based on the measurement of packed cell volume.

### Phosphate Uptake Assay

Protoplasts were incubated with standard incubation medium containing 20 mM $KH_2PO_4$ and 4 μCi (148 KBq) $KH_2{}^{32}PO_4$ (1000 mCi/mmol; Du Pont), with or without 1 mM glucose for 1, 2, 4, and 16 hr. Because cell volume changed slightly during incubation, a control sample was included at each time point. Protoplasts were collected by centrifugation (1000 rpm, 1 min at 0°C) and resuspended and washed in 0°C incubation medium. Cell volume from the packed cells was estimated. The uptake of phosphate was estimated by counting the retained $^{32}PO_4$. To eliminate the background from nonspecific binding, 4 μCi (148 KBq) of $KH_2{}^{32}PO_4$ was added to the control samples immediately before harvest at 0°C. The radioactivity of control samples was subtracted from that retained in each sample.

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