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Carbon Catabolite Repression Regulates Glyoxylate Cycle Gene Expression in Cucumber

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We have previously proposed that metabolic status is important in the regulation of cucumber malate synthase (MS) and isocitrate lyase (ICL) gene expression during plant development. In this article, we used a cell culture system to demonstrate that intracellular metabolic status does influence expression of both of these genes. Starvation of cucumber cell cultures resulted in the coordinate induction of the expression of MS and ICL genes, and this effect was reversed when sucrose was returned to the culture media. The induction of gene expression was closely correlated with a drop in intracellular sucrose, glucose, and fructose below threshold concentrations, but it was not correlated with a decrease in respiration rate. Glucose, fructose, or raffinose in the culture media also resulted in repression of MS and ICL. Both 2-deoxyglucose and mannose, which are phosphorylated by hexokinase but not further metabolized, specifically repressed MS and ICL gene expression relative to a third glyoxylate cycle gene, malate dehydrogenase. However, the addition of 3-methylglucose, an analog of glucose that is not phosphorylated, did not result in repression of either MS or ICL. It is proposed that the signal giving rise to a change in gene expression originates from the intracellular concentration of hexose sugars or the flux of hexose sugars into glycolysis.

INTRODUCTION

Until recently, studies of metabolic control in higher plants have focused on the biochemical regulation of enzyme activity. It is now becoming apparent that metabolite concentration also regulates transcription of genes encoding proteins of metabolic importance, as is the case in prokaryotes and lower eukaryotes. This "coarse" control of plant metabolism at the level of transcription will be most apparent when there is a significant change in the intracellular nutritional status of the plant cell, for example during a developmental transition or as a result of environmental stress such as drought. To understand the mechanisms involved in this control, it will be necessary to identify the regulatory metabolites and establish how their levels are sensed and transduced to the transcription machinery of the genes involved. This article demonstrates that expression of the glyoxylate cycle genes malate synthase (MS) and isocitrate lyase (ICL) from cucumber is under metabolic control and describes the changes in intracellular metabolite concentrations that result in induction and repression of these genes.

A good example of the complexity of metabolic control is the carbon catabolite repression response of microorganisms whereby the synthesis of a great variety of enzymes including those required for the utilization of alternate carbon sources is inhibited in the presence of the preferred carbon source. Work using *Saccharomyces cerevisiae* mutants has allowed many of the components of the carbon catabolite repression response to be identified, but it is still not clear how all of these interact to regulate transcription (for reviews, see Gancedo, 1992; Trumbly, 1992). The mechanism that triggers the response involves glucose and hexokinases PI and PII (Ma et al., 1989a, 1989b; Rose et al., 1991), and although not fully understood, it would appear that either the production or transduction of the repression signal is directly associated with hexose phosphorylation by the hexokinases.

Among the many genes regulated by the carbon catabolite repression response in *S. cerevisiae* are those encoding the glyoxylate cycle enzymes MS and ICL (Witt et al., 1966). Metabolic control of the glyoxylate cycle has been demonstrated in a range of microorganisms, including *Escherichia coli* (Kornberg, 1966), *Neurospora crassa* (Sjogren and Romano, 1967), and *Chlorella* (McCullough and John, 1972). In microorganisms, the glyoxylate cycle allows growth on acetate or other two-carbon compounds, and these are utilized when glucose and other sugars are depleted from the growth media.

In animals, a variety of genes appear to be directly regulated by metabolite concentrations. For example, in *Drosophila melanogaster*, an amylase gene is subject to glucose repression (Benkel and Hickey, 1987), and the synthesis of a specific set of proteins localized within the endoplasmic reticulum of animal cell cultures is induced under glucose starvation conditions (Lee, 1987). Homologs of several of these glucose-

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regulated proteins have recently been identified in higher plants on the basis of amino acid or nucleotide sequence similarity (Shorrosh and Dixon, 1992; D'Amico et al., 1992). It has not yet been determined whether genes encoding these plant homologs are induced by glucose starvation.

A number of recent reports demonstrate that provision of sugars to plant tissues or protoplasts results in either induction or repression of various genes. Studies of carbohydrate assimilation in potato tubers have revealed that genes encoding patatin (Rocha-Sosa et al., 1989; Wenzler et al., 1989), ADP-glucose pyrophosphorylase (Müller-Röber et al., 1990), and sucrose synthase (Salanoubat and Belliard, 1989) are all induced by elevated levels of sucrose. Similarly, the tuberous root storage protein genes of sweet potato are induced by sucrose (Hattori et al., 1990). Putative regulatory elements involved in the sugar response have been identified in the 5′ upstream region of these genes (Hattori and Nakamura, 1988), and similar sequences are present in the promoter region of the petunia chalcone synthase gene, which is induced by elevated sugar levels in transgenic Arabidopsis (Tsukaya et al., 1991).

In Arabidopsis, sucrose mimics the light induction of nitrate reductase gene transcription (Cheng et al., 1992), and in maize, elevated levels of sucrose differentially regulate the sucrose synthase genes (*Sus1* and *Sh1*) such that *Sus1* is induced and *Sh1* is repressed (Maas et al., 1990; Koch et al., 1992). Repression by sugars has been demonstrated for a number of other plant genes including seven maize photosynthetic genes (Sheen, 1990), tobacco chlorophyll *a/b* binding protein and Rubisco small subunit genes (Criqui et al., 1992), and the α-amylase gene from suspension cultured cells of rice (Yu et al., 1991). Recently, Krapp et al. (1993) demonstrated that photosynthetic genes are regulated by carbohydrates in both cell cultures and whole plants and proposed that photosynthetic gene expression is inhibited by metabolic factors related to high carbohydrate content. It is not known what metabolite is responsible for signaling nutritional status or how such a signal is perceived in any of the above examples.

Previously, we suggested a model for the transcriptional control of a gene encoding the glyoxylate cycle enzyme MS that was based on gene expression patterns during plant development (Graham et al., 1992). This model proposed that intracellular metabolic status, either in addition to or as an alternative to specific developmental signals, regulates MS gene transcription. In higher plants, the glyoxylate cycle plays an important role in the mobilization of lipid reserves during early postgerminative growth (Kornberg and Beevers, 1957). In this cycle, two enzymes, MS and ICL, allow the decarboxylation steps of the citric acid cycle to be bypassed, thereby allowing the net conversion of 2 M of acetyl Coenzyme A into one of succcinate. MS and ICL are coordinately synthesized during postgerminative growth in a wide range of higher plant seeds when lipid is undergoing rapid mobilization to yield acetyl Coenzyme A (Weir et al., 1980; Allen et al., 1988; Comai et al., 1989; Turley and Trelease, 1990). Sucrose is ultimately produced from the products of the glyoxylate cycle by way of gluconeogenesis and is used to support early seedling growth.

The metabolism of the young seedling changes dramatically during the transition from heterotrophic to autotrophic growth as it becomes photosynthetically competent. During this transition, glyoxylate cycle enzyme synthesis stops and MS and ICL transcript levels and enzyme activities decrease to undetectable levels. MS gene expression is induced again in the cotyledons as they senesce a few weeks later and also in senescing leaves and petals of cucumber plants. Dark treatment of whole plants and detached leaves also results in the accumulation of MS mRNA (Graham et al., 1992). Glyoxylate cycle activation in senescent tissue has also been demonstrated in a number of other plant species, including barley, rice, leaf-beet, pumpkin, and wheat (Gut and Matile, 1988; De Bellis et al., 1990; De Bellis and Nishimura, 1991; Pistelli et al., 1991). The role of the glyoxylate cycle during senescence has not been clearly defined, but the most obvious possibility is that it acts as a salvage pathway for the conversion of membrane lipids into sucrose, which can be transported from the senescing tissue. Carbon-14 tracing experiments in senescent barley leaves support this assumption, as they show that gluconeogenesis from acyl residues of galactolipids results in newly labeled sugars (Wanner et al., 1991).

To test the significance of metabolic control in regulating glyoxylate cycle gene expression in higher plants, we have used cucumber cell cultures to investigate the change in steady state levels of MS and ICL mRNA under different growth conditions. We show that expression of these genes responds to changes in intracellular metabolite concentration in a manner remarkably similar to that found in microorganisms.

RESULTS

Effect of Sucrose Starvation on Cell Cultures

The effects of prolonged sucrose starvation on the rate of O_2 consumption in heterotrophic cucumber cells is shown in Figure 1. In this and subsequent experiments, starvation conditions were initiated by replacement of 20 mM sucrose by 20 mM mannitol in the culture medium to avoid possible osmotic effects. The respiration rate remained constant until 20 hr of starvation and then gradually fell to about half its original value after 72 hr. Addition of 20 mM sucrose to the culture media after 72 hr resulted in the rapid recovery of respiration rate to prestarvation values.

If nutritional status is important in regulating expression of the genes encoding glyoxysomal MS and ICL in higher plants, then the treatment described above should have a dramatic effect on transcript levels. After 48 hr (2 days) of starvation, there was a massive increase in steady state levels of both MS and ICL transcripts, and levels remained elevated until 96 hr (4 days) (Figure 2). Immediately after the 96-hr sampling

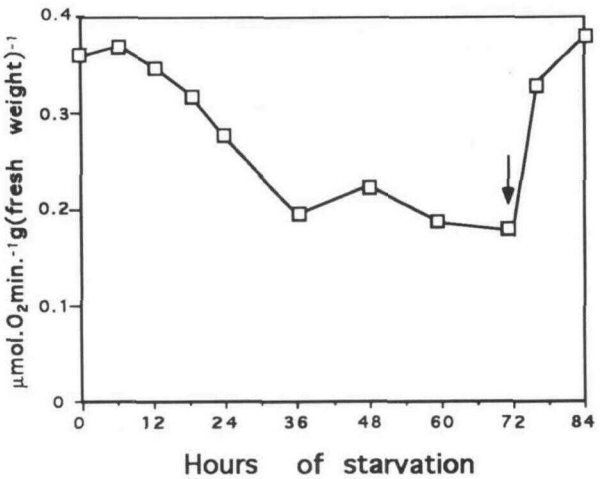

Figure 1. Respiration Rate in Starved Cell Cultures.

Cucumber cells cultured for 24 hr in 20 mM sucrose were transferred into 20 mM mannitol to initiate starvation conditions at time 0. The arrow at 72 hr starvation represents the time at which 20 mM sucrose was added back to the culture media. Respiration rate measurements were performed in duplicate, and the average was taken. Three repeats of the experiment gave the same pattern of change in respiration rate.

point, 20 mM sucrose was added back to the culture media; this resulted in a fall to undetectable levels of both MS and ICL transcripts after a further 48 hr.

To establish if acetate has an induction effect on transcription of MS and ICL as proposed in the model by Graham et al. (1992), a parallel experiment was performed using 0.2 mM acetate in the culture media (Figure 2). Higher concentrations of acetate could not be used as they are toxic and result in rapid cell death. The presence of 0.2 mM acetate did not affect the steady state levels of MS and ICL transcripts during the starvation period or after sucrose was added back to the cells, suggesting that acetate does not play a role in regulating MS and ICL gene expression.

The amount of acetate in the culture media was significantly less than the reported physiological concentrations of ~1 mM for various plant tissues (Kuhn et al., 1982). Furthermore, since the ionization constant of acetate (pK_a = 4.75) is significantly less than the pH of the culture media (pH 5.6), then the acetate will be predominantly ionized and not available for passive diffusion. It is also possible that endogenous levels of acetate, or an associated metabolite, increase as a consequence of sucrose starvation, resulting in an effect on gene expression.

Correlation of a Fall in Metabolite Concentrations with Induction of MS and ICL

Samples of cultured cells were taken at intervals following the removal of sucrose from the culture media, and MS and ICL transcript levels, respiration rate, and intracellular metabolite concentrations were measured (Figures 3 and 4). MS and ICL transcript levels were coordinately induced during the early stages of starvation. The timing of this induction was dependent on the concentration of sucrose in the culture media prior to starvation. In those cultures maintained on 50 mM sucrose prior to starvation, MS and ICL transcripts were detected after 6 hr and reached maximal levels after 12 hr, whereas in the 5 mM pretreated cultures, transcripts were detected after 2 hr and reached maximal levels after 4 hr. The respiration rate, which is dependent on glycolysis and the tricarboxylic acid cycle, was constant during this period, thus indicating that levels of glycolytic and tricarboxylic acid cycle intermediates were maintained. However, the increase of MS and ICL transcript levels was closely correlated with a decrease in the intracellular concentrations of sucrose, glucose, and fructose. The intracellular concentrations of these sugars were remarkably similar in the two treatments when the transcripts were first detected and when they reached their maximal amounts.

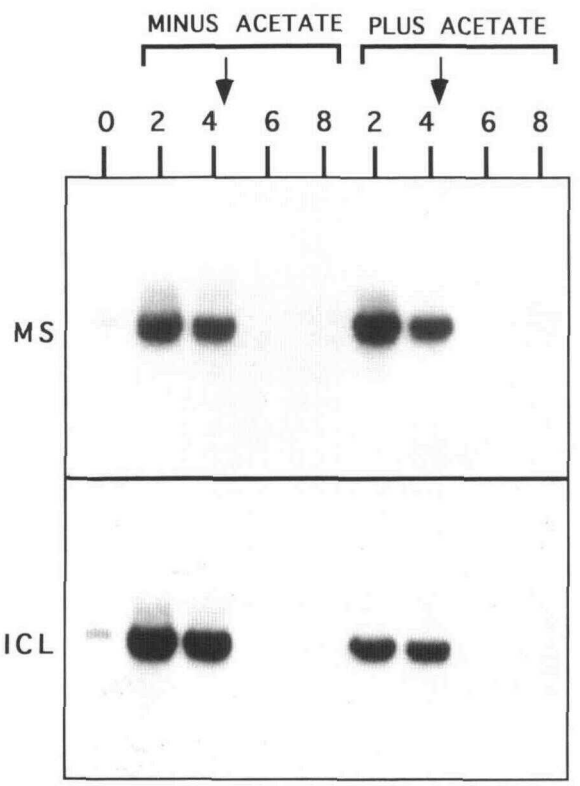

Figure 2. RNA Gel Blot Analysis Showing Induction and Repression of MS and ICL in Cucumber Cell Cultures.

Numbers above lanes represent time in days after transfer into 20 mM mannitol following 24-hr culture in 20 mM sucrose. Arrows indicate time at which 20 mM sucrose was added back to the culture media. Two-tenths millimoles acetate was used in the plus acetate treatment. Ten micrograms total RNA was loaded per track.

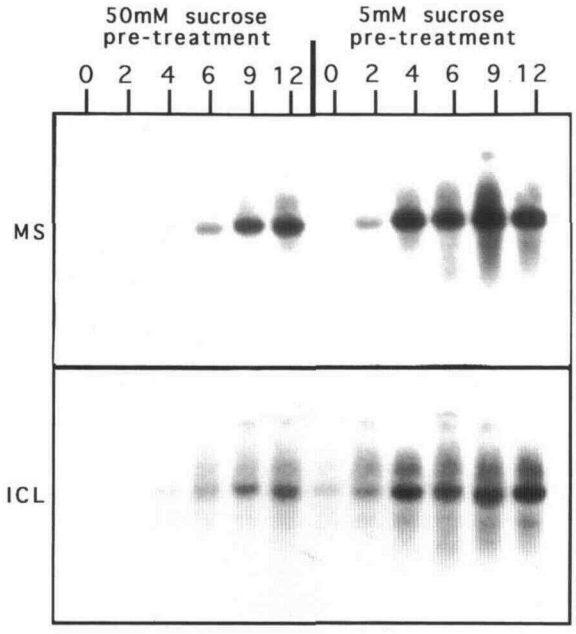

Figure 3. RNA Gel Blot Analysis Showing Time Course of Induction of MS and ICL in Cell Cultures after Removal of Sucrose from the Culture Media.

Cells were precultured in either 50 mM or 5 mM sucrose for 24 hr. Numbers above lanes represent time in hours after transfer into 20 mM mannitol. Ten micrograms total RNA was loaded per track.

The intracellular threshold concentration of sucrose below which MS and ICL transcripts were first detectable was ~3 mM. This value is in agreement with the observation that MS and ICL are induced when grown on 2 mM sucrose (data not shown) and repressed when grown on 5 mM sucrose (Figure 4).

The close correlation between an increase in steady state levels of MS and ICL transcripts with a decrease in sugars below an intracellular threshold concentration suggests that a sugar is the active metabolite initiating the response leading to a change in gene expression.

Effect of Addition of Sucrose to Starved Cells

Cells were cultured in 20 mM mannitol for 24 hr and subsequently transferred into 20 mM sucrose. Respiration rates, intracellular sugar concentrations, and the steady state level of MS and ICL transcripts were determined. The 24-hr mannitol pretreatment resulted in a significant decrease in the respiration rate of the cells, which was restored upon addition of sucrose (Figure 5). The intracellular sugar concentration increased above the threshold concentration established in Figure 3 (Figure 5), and within 4 hr, MS and ICL transcripts could not be detected (Figure 6).

Effect of Carbon Source on Steady State Levels of MS and ICL Transcripts

Cell cultures were transferred from plates into media containing a range of individual carbon compounds (20 mM in each treatment), and after 48 hr, respiration rate, metabolite concentrations, and MS and ICL transcript levels were measured. Culture in media containing glucose, fructose, or raffinose resulted in the respiration rate and metabolite concentrations remaining elevated, as was the case with cells grown on sucrose (Figure 7). Accumulation of MS and ICL transcript levels was repressed in cells maintained on any of these sugars (Figure 8). Raffinose is one of the major translocated sugars in cucurbits (Richardson et al., 1984). Although detectable by gas chromatography analysis, this sugar was not detected in cell extracts, which suggests that it was hydrolyzed extracellularly

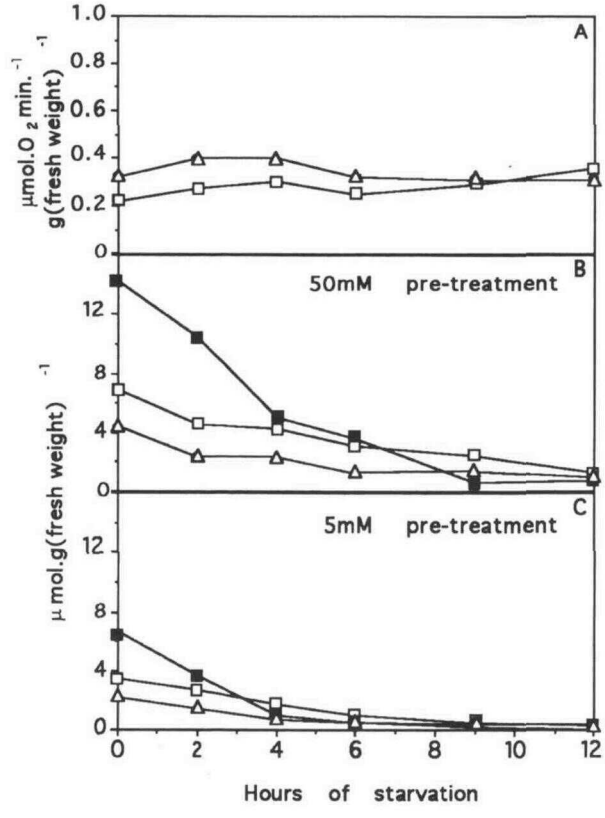

Figure 4. Respiration Rate and Intracellular Sugar Concentrations during Early Stages of Starvation of Cucumber Cell Cultures.

Cells were precultured in either 50 mM or 5 mM sucrose for 24 hr. Respiration rate, intracellular sugars, and RNA transcript levels (as shown in Figure 3) were determined from aliquots of the same sample at each time point.

(A) Respiration rate. △, cells precultured in 50 mM sucrose; □, cells precultured in 5 mM sucrose.

(B) and **(C)** Intracellular sugar concentrations. ■, sucrose; △, glucose; □, fructose.

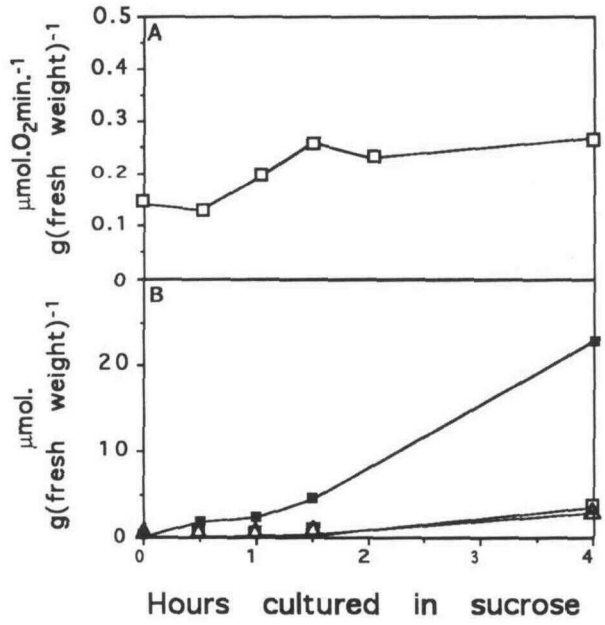

Figure 5. Respiration Rate and Intracellular Sugar Concentrations during the Time Course of Repression of MS and ICL in Cucumber Cell Cultures after Transfer into 20 mM Mannitol following 24-hr Culture in 20 mM Sucrose.

(A) Respiration rate.
(B) Intracellular sugar concentrations. ■, sucrose; △, glucose; □, fructose.

to sucrose and galactose before being taken up by the cells. This observation also demonstrates that only intracellular metabolites are being measured by the gas chromatography procedure.

Although taken up by the cells, 3-methylglucose, a non-metabolizable analog of glucose, did not result in repression. MS and ICL transcripts accumulated to levels similar to those present in cultures maintained on mannitol. This was also the case with cultures maintained on succinate or malate. After 48 hr, respiration rate and metabolite concentrations in the 3-methylglucose, succinate, and malate treatments all decreased to the same extent as in the mannitol treatment. These results indicate that when cells are cultured in malate or succinate, intracellular sugar concentrations cannot be maintained by gluconeogenesis. Malate and succinate are transported into the cells as indicated by an elevated intracellular concentration in the appropriate treatment (I.A. Graham, K.J. Denby, and C.J. Leaver, unpublished data).

Effect of Glucose Analogs on Glyoxylate Cycle Gene Expression

To investigate the importance of phosphorylation of hexose sugars in initiating the metabolic response that results in a change in glyoxylate cycle gene expression, a series of experiments was conducted using glucose analogs. Cultures were grown in 3-methylglucose, which is taken up by cells but is not phosphorylated, and 2-deoxyglucose, which is phosphorylated (Dixon and Webb, 1979). Initial experiments demonstrated that 2-deoxyglucose is toxic to plant cells at concentrations as low as 0.2 mM over a 24-hr period, as indicated by a drop to 0 of the respiration rate. The toxicity of 0.2 mM or 2 mM 2-deoxyglucose could be alleviated by coculturing the cells in 20 mM glucose but not by coculturing in 20 mM raffinose. This phenomenon is also observed in *S. cerevisiae* where, in addition to a range of detrimental effects on metabolism (Herve et al., 1992), 2-deoxyglucose also exerts carbon catabolite repression on a number of enzymes including MS and ICL (Witt et al., 1966). In yeast, carbon sources other than glucose are not utilized in the presence of 2-deoxyglucose as the genes necessary for their conversion to glucose are repressed. To establish if 2-deoxyglucose has a repression effect on the cucumber glyoxylate cycle genes MS and ICL, distinct from the toxic effect on the cell cultures, experiments with glucose analogs were performed over a 4-hr period to minimize the detrimental effects on metabolism. The respiration rate of cell cultures treated with 2-deoxyglucose was not significantly different from those maintained in mannitol during this 4-hr period (see below).

The effects of 2 mM 2-deoxyglucose, 20 mM 3-methylglucose, and 20 mM glucose on steady state levels of MS, ICL, and glyoxysomal malate dehydrogenase (MDH) transcripts in cells precultured in 20 mM mannitol (i.e., MS and ICL already induced) are shown in Figure 9. Steady state levels of

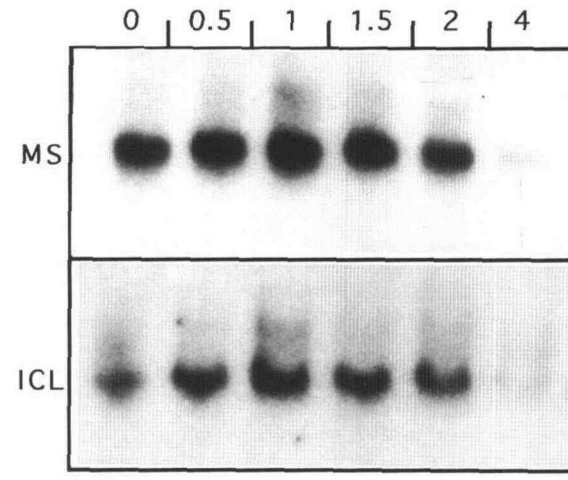

Figure 6. RNA Gel Blot Analysis Showing Time Course of Repression of MS and ICL in Cucumber Cell Cultures.

RNA was extracted from aliquots of the cultures used in the experiments shown in Figure 5. Numbers above lanes represent time in hours after the cells were transferred to media containing 20 mM sucrose. Ten micrograms total RNA was loaded per track.

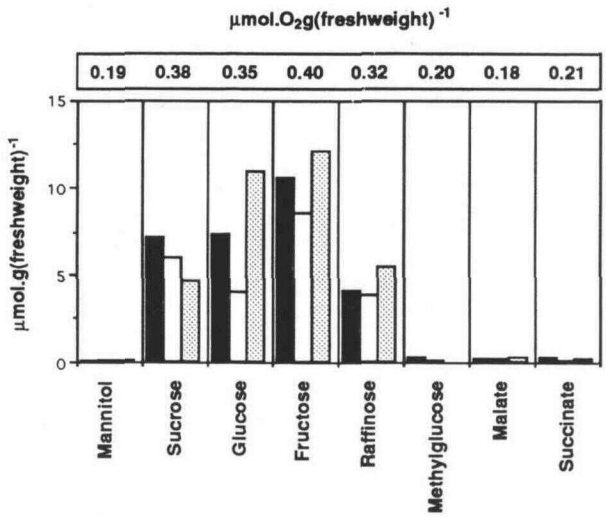

Figure 7. Respiration Rate and Intracellular Sugar Concentrations of Cucumber Cell Cultures Maintained on Different Carbon Sources for 48 hr.

The respiration rate of each culture after 48-hr treatment is shown above the bar graph. ▨, sucrose; □, glucose; ■, fructose.

glyoxysomal MDH transcript were not regulated by nutritional status in the same manner as MS and ICL. MDH is therefore a useful marker to demonstrate the specificity of the repression effect on MS and ICL expression. Inclusion of 2-deoxyglucose in the cell culture medium resulted in a significant decrease in the steady state levels of both MS and ICL transcripts over the 4-hr period but had no effect on the MDH transcript levels. In the glucose treatment, the same pattern of repression was observed, and MS and ICL transcript levels were undetectable after 4 hr. As described earlier, 3-methylglucose did not affect the steady state levels of MS, ICL, or MDH transcripts (Figure 9). Sugar concentrations fell to the same extent in the 2-deoxyglucose and 3-methylglucose treatments, whereas they increased rapidly in the glucose treatment (Figure 9). Under normal starvation conditions, when intracellular sugar concentrations fell to the same concentration as those in the 2-deoxyglucose treatment, the MS and ICL genes were induced (see Figures 3 and 4). It appears then that 2-deoxyglucose, while not preventing the metabolism of other sugars, is sufficient to cause specific repression.

The effect of 2 mM 2-deoxyglucose, 20 mM 3-methylglucose, and 20 mM mannitol on steady state levels of MS, ICL, and MDH transcripts from cell cultures that were precultured in 20 mM glucose (i.e., MS repressed) are shown in Figure 10. In this case, the 2-deoxyglucose treatment did not result in any increase in the steady state level of MS or ICL transcripts, whereas the 3-methylglucose treatment resulted in a dramatic increase that was greater than that of the mannitol treatment. MDH transcript levels did not appear to be affected by any of these treatments. Sugar concentrations in the 2-deoxyglucose treatment fell to a greater extent than either of the other two treatments. These observations further confirm that 2-deoxyglucose has a repression effect on MS and ICL expression when the intracellular concentration of other sugars are below the threshold that is normally associated with induction.

Effect of Mannose on Glyoxylate Cycle Gene Expression

In many plant species, mannose is phosphorylated to mannose phosphate and is not further metabolized (Sheu-Hwa et al., 1975). Cells were cultured in 20 mM mannose, 20 mM mannose plus 20 mM Pi, and 20 mM glucose after preculture in 20 mM mannitol. Respiration rates, which remained low in the mannose treatments, indicated that mannose is not further metabolized in cucumber cell cultures. However, mannose

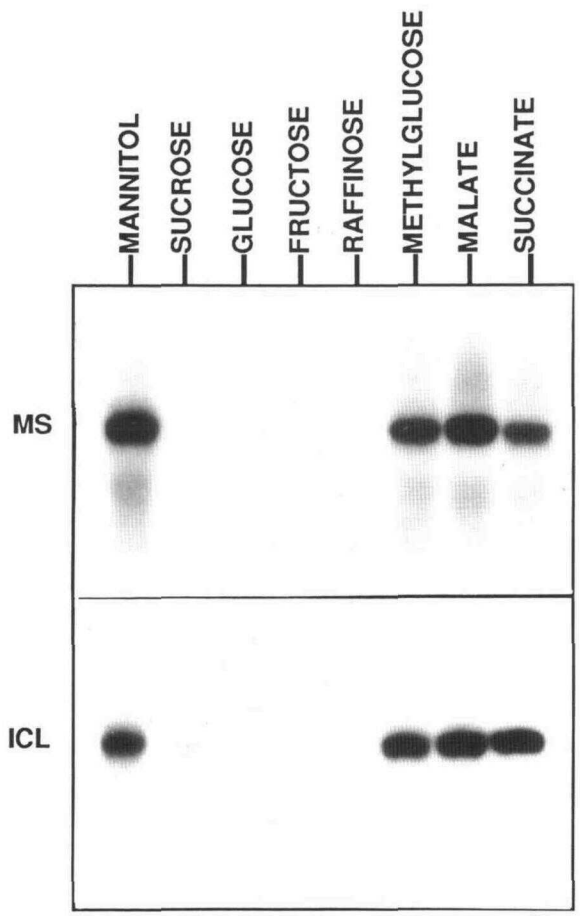

Figure 8. RNA Gel Blot Analysis Showing the Effect of Different Carbon Sources on Expression of MS and ICL in Cucumber Cell Cultures.

RNA was extracted from aliquots of the cultures used in the experiment shown in Figure 7. Ten micrograms total RNA was loaded per track.

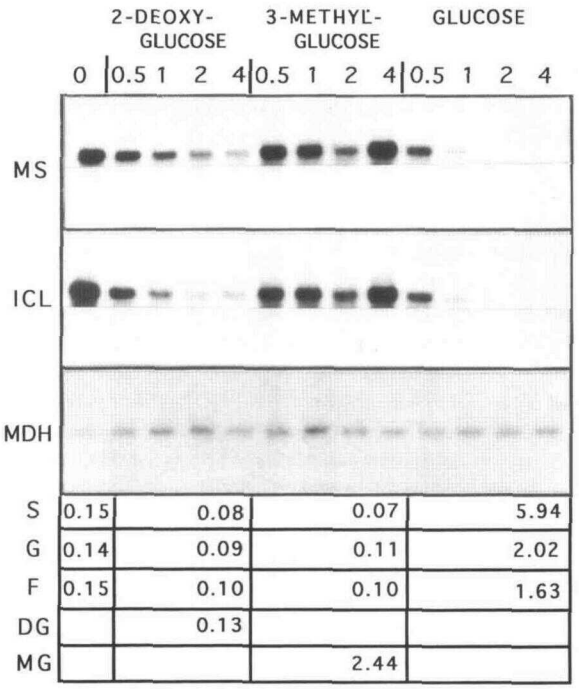

Figure 9. Effect of Glucose Analogs on Glyoxylate Cycle Gene Expression and Intracellular Sugar Concentrations in Cucumber Cell Cultures.

RNA gel blot analysis and intracellular sugar concentrations. Cells were precultured in 20 mM mannitol for 24 hr (MS and ICL induced) prior to transfer into 2 mM 2-deoxyglucose, 20 mM 3-methylglucose, or 20 mM glucose. Numbers above lanes represent time in hours in each treatment. Ten micrograms total RNA was loaded per track. The sugar concentrations (μmol g[freshweight]$^{-1}$) shown below the lanes correspond to intracellular concentrations after 24-hr culture in mannitol (i.e., time 0) and 4-hr culture in each treatment. S, sucrose; G, glucose; F, fructose; DG, 2-deoxyglucose; MG, 3-methylglucose.

does specifically repress MS and ICL transcript levels in the same manner as glucose (Figure 11). MDH transcript levels remained constant, again demonstrating the specificity of the repression effect. Addition of 20 mM Pi did not abolish the repression caused by inclusion of mannose in the medium, demonstrating that the response is not the result of sequestration of Pi due to accummulation of mannose-6-phosphate.

It has also been shown that sequestration of Pi by 2-deoxyglucose is not responsible for the repression of MS and ICL expression shown in Figures 9 and 10 (I.A. Graham, K.J. Denby, and C.J. Leaver, unpublished data). These results demonstrate that 2-deoxyglucose and mannose, which are phosphorylated by hexokinase, can repress MS and ICL gene expression in a manner similar to that of metabolizable sugars. This is not a general effect, as the transcript levels of another glyoxylate cycle gene, MDH, remain unaffected by these treatments. MS and ICL gene expression is not repressed by 3-methylglucose, which is taken up by plant cells but is not known to be phosphorylated by hexokinase.

DISCUSSION

The results described provide convincing evidence that MS and ICL gene expression, as measured by steady state transcript levels, is under metabolic control in cucumber cell cultures. The observed changes in steady state transcript levels

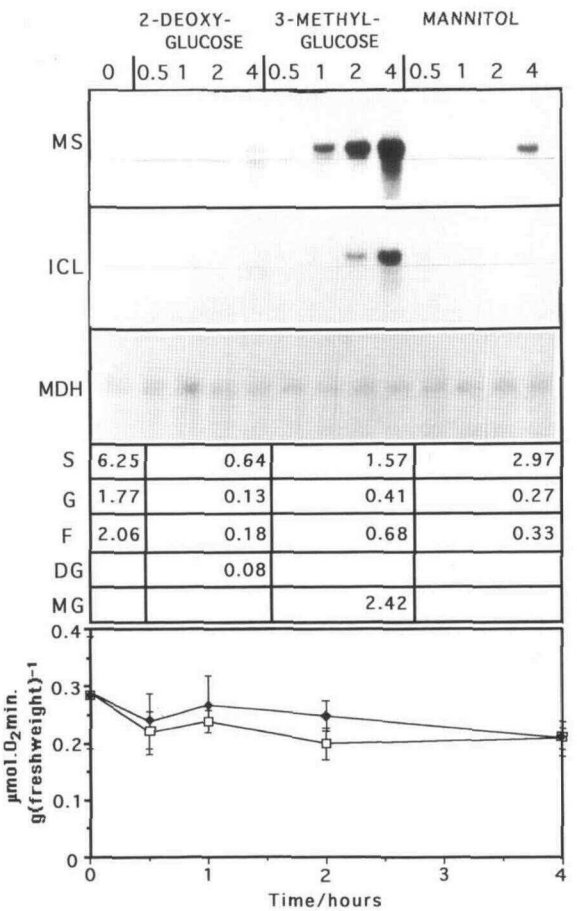

Figure 10. Effect of Glucose Analogs on Glyoxylate Cycle Gene Expression and Intracellular Sugar Concentrations in Cucumber Cell Cultures.

RNA gel blot analysis, intracellular sugar concentrations, and respiration rate of the cells are shown. The cells were precultured in 20 mM glucose for 24 hr (MS and ICL repressed) prior to transfer into 2 mM 2-deoxyglucose, 20 mM 3-methylglucose, or 20 mM mannitol. Numbers above lanes represent time in hours in each treatment. Ten micrograms total RNA was loaded per track. The intracellular sugar concentrations (μmol g[freshweight]$^{-1}$) shown below the RNA gel blot analysis correspond to concentrations after 24-hr culture in glucose (i.e., time 0) and 4-hr culture in each treatment. S, sucrose; G, glucose; F, fructose; DG, 2-deoxyglucose; MG, 3-methylglucose. The respiration rates of cell cultures maintained for 24 hr in 20 mM glucose and transferred to 2 mM 2-deoxyglucose or 20 mM mannitol are shown over a 4-hr period in the graph below. ♦, 2-deoxyglucose; □, mannitol.

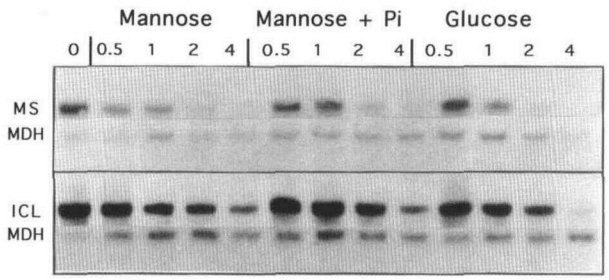

Figure 11. RNA Gel Blot Analysis Showing the Effect of Mannose on Glyoxylate Cycle Gene Expression in Cucumber Cell Cultures.

Cells were cultured in 20 mM mannitol for 24 hr (MS and ICL induced) prior to transfer into 20 mM mannose, 20 mM mannose plus 20 mM Pi, or 20 mM glucose. Numbers above lanes represent time in hours in each treatment. Ten micrograms total RNA was loaded per track. MDH tracks are from the same blot but exposed for different lengths of time.

could be due to changes in the rate of transcription and/or the rate of degradation of specific RNA species. Transient expression analysis of MS promoter–β-glucuronidase reporter gene constructs in cucumber mesophyll protoplasts demonstrates that metabolic control of MS gene expression is at the level of transcription (I.A. Graham, C.J. Baker, and C.J. Leaver, manuscript submitted). That changes in steady state levels of MS and ICL mRNA reflect changes in enzyme activities in cell cultures is supported by previous work that demonstrated the induction and repression of glyoxysomal enzyme activities in anise (*Pimpinella anisum* L.) suspension cultures (Kudielka and Theimer, 1983a, 1983b) under conditions similar to those described in this article.

Role of the Glyoxylate Cycle in Carbohydrate-Starved Cell Cultures

In postgerminative storage tissue, the glyoxylate cycle along with gluconeogenesis is responsible for the net conversion of acetyl-CoA derived from lipid breakdown into sucrose, which can be transported to other parts of the seedling. In starved cell cultures, there is no obvious requirement for gluconeogenesis to produce sugars for export. The observation that cell cultures maintained on malate or succinate for 48 hr are depleted in their sugar reserves suggests that gluconeogenesis is not operating even though the glyoxylate cycle genes encoding MS and ICL are induced. This apparent dissociation of MS and ICL expression from gluconeogenesis may be due to the glyoxylate cycle having an anapleurotic role in starved cell cultures, whereby it provides succinate to replenish the carbon skeletons of the tricarboxylic acid cycle. In nonstarved cells, phosphoenolpyruvate carboxylase plays an anapleurotic role, converting phosphoenolpyruvate to oxaloacetate, but in the absence of glycolytic intermediates this reaction will not occur.

During prolonged sucrose starvation, the acetyl-CoA necessary for operation of the glyoxylate cycle is probably derived from breakdown of cytoplasmic contents. Support for this idea comes from detailed studies of the biochemical changes that occur during the course of sucrose starvation in sycamore (*Acer pseudoplatanus*) cells (Rebeille et al., 1985; Journet et al., 1986; Roby et al., 1987). This work shows that in addition to a decrease in respiration rate and carbohydrate pools, intracellular fatty acid content also changes dramatically with phospholipid and galactolipid (endomembrane components) levels decreasing and sterol compounds (tonoplast and plasmalemma component) remaining constant. Along with other observations (for a review, see Douce et al., 1990), these observations led to the hypothesis that higher plant cells have the ability to conduct controlled autophagy of their cytoplasmic contents during periods of sucrose deprivation. Similar biochemical changes to those reported in cell culture have been shown to occur during glucose starvation in excised maize root tips (Brouquisse et al., 1991; Dieuaide et al., 1992). Therefore, it is feasible that the glyoxylate cycle has an important anapleurotic role that aids survival during periods of carbohydrate starvation in higher plants.

Control of Glyoxylate Cycle Gene Expression in Carbohydrate-Starved Cucumber Cell Cultures

The main point to be addressed from the current work is the nature of the metabolic signal that gives rise to a change in gene expression. Various lines of evidence have been presented that indicate that either concentrations of hexose sugars or the flux of hexose sugars into glycolysis by way of hexokinase may be important in signaling intracellular nutritional status. This evidence includes (1) a close correlation between the increase in MS and ICL gene expression with a drop in intracellular concentration of glucose, fructose, and sucrose below critical threshold concentrations; (2) the ability of glucose, fructose, and raffinose to repress gene expression to the same extent as sucrose; (3) no correlation between MS and ICL induction with changes in respiration rate; and (4) the ability of 2-deoxyglucose and mannose, but not 3-methylglucose, to mimic the repression effect of glucose on MS and ICL gene expression.

Induction of the MS and ICL genes is dependent on the intracellular concentration of sugars falling below a critical threshold concentration, and the time taken for this induction depends on the intracellular sugar concentration at the start of starvation. One important consideration regarding the nature of the signal and the sensing mechanism that gives rise to a change in gene expression is that significant amounts of intracellular carbohydrates are still present when induction of the MS and ICL genes occurs. This poses the question as to how a signal transduction response can be initiated by hexose

sugars when their intracellular concentration, although decreased, is still considerable. One explanation is that the concentration of sugars in the cytoplasm, where the response is most likely initiated, is being overestimated due to subcellular compartmentation of carbohydrates. However, induction of MS and ICL is still seen when cells are maintained on 2.5 mM glucose or sucrose, and cytoplasmic concentrations of these sugars are likely to at least reflect those of the external media, because they are readily transported.

The observation that glucose, fructose, and raffinose are as effective as sucrose in repressing MS and ICL gene expression suggests that some common metabolite, or step in the metabolism of these sugars, initiates the repression response. The specific repression effect of 2-deoxyglucose and mannose but not 3-methylglucose on expression of MS and ICL compared to glyoxysomal MDH further suggests that hexose sugars are important in initiating the repression response. The possibility that the transport of hexose sugars can give rise to the signal that causes the repression response can be ruled out because 3-methylglucose accumulates in the cultures to a similar extent as other sugars after 4 hr but does not cause repression (Figures 9 and 10). The experiments described here demonstrate that 2-deoxyglucose has a specific repression effect on MS and ICL gene expression over a period in which the intracellular concentration of other sugars falls to a concentration normally correlated with induction of these genes. Furthermore, inclusion of mannose, which is also phosphorylated, in the culture medium results in repression of MS and ICL gene expression. The fact that 3-methylglucose is not phosphorylated and does not repress MS and ICL gene expression implicates the hexokinase reaction in the initiation of the repression signal. It is possible that the substrates for hexokinase, the phosphorylated products, the flux through the hexokinase reaction, or an interaction of these could somehow give rise to a signal that reflects the metabolic status of the cell.

It is not obvious how such a low concentration of 2-deoxyglucose (~0.1 mM internal concentration; Figures 9 and 10) can be effective in causing repression compared to much higher concentrations of other hexose sugars. It is possible that the analog itself or the accumulating phosphorylated product interferes with the hexokinase activity implicated in the response.

The effect of 2-deoxyglucose is similar to that observed in *S. cerevisiae* where glucose analogs such as 2-deoxyglucose, which are phosphorylated by hexokinase but not further metabolized to F-6-P, act as gratuitous repressors of genes such as MS and ICL, which exhibit carbon catabolite repression (Witt et al., 1966). In *S. cerevisiae*, hexokinase has been implicated in initiating the signal leading to the repression response because a number of point and null mutations in the structural gene encoding hexokinase PII (*HXK2*) result in constitutive expression of glucose-repressible genes (Zimmerman and Scheel, 1977; Entian et al., 1985). The exact mode of action of HXK2 is still not understood, but analysis of numerous mutations in the *HXK2* gene indicates a strong correlation between catalytic activity and the capacity to mediate catabolite repression (Ma et al., 1989a, 1989b; Rose et al., 1991).

Hexokinases with different specificities for hexose sugars are also found in higher plants (Schnarrenberger, 1990). Nothing is known regarding a role for any of these proteins in the repression response described in this article, but the similarity of this response with that described in microorganisms is sufficiently great to merit further investigation.

It is possible that in higher plants as in microorganisms there are general mechanisms controlling expression of a variety of genes encoding enzymes from different biochemical pathways. For example, a number of reports have demonstrated that photosynthetic gene expression is affected by metabolic status in a manner similar to MS and ICL gene expression (Sheen, 1990; Krapp et al., 1993). In the work of Krapp et al. (1993), analogs of glucose that are not phosphorylated by hexokinase do not have a repression effect on gene expression, which is in full agreement with our hypothesis that phosphorylation of hexose sugars by hexokinase is important in initiating the repression response. Even if metabolic control of gene expression is a general phenomenon, the metabolic response of different genes will not necessarily be identical. Environmental and developmental signals will have an influence and, depending on the role of the gene product, the sensitivity and degree of the response will also vary.

METHODS

Cucumber Callus and Suspension Culture

Rapidly dividing cucumber callus derived from leaf explants of *Cucumis sativus* cv Hokus (Bergervoet et al., 1989) were cultured on plates of media (pH 5.6) containing MS inorganic and organic components (Murashige and Skoog, 1962), 0.1% (w/v) Oxoid tryptone, 5 μM 6-benzylaminopurine, 5 μM 2,4-D, and 0.6% (w/v) Oxoid bacteriological agar, supplemented with 3% (w/v) sucrose. The plates were maintained at 25°C in the dark, and the calli were subcultured every 14 days. For experimental work, 14-day-old callus was transferred into liquid media supplemented with different carbon sources. These liquid cultures were shaken at 100 rpm at 25°C in the dark. The dispersed cells were harvested by filtration through Whatman Grade 1 filter paper and rinsed with distilled water prior to freezing 100 mg fresh weight samples in liquid nitrogen.

Respiration Rate

The respiration rate of the liquid cell cultures was determined in an oxygen electrode (Hansatech, Kings Lynn, UK) using 1 mL culture media containing 100 mg fresh weight of cells.

Extraction of Total RNA

To isolate total RNA, frozen cells (100 mg fresh weight) were ground to a fine powder with a mortar and pestle in liquid nitrogen and

immediately transferred into 4 mL of extraction buffer (5 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 2 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM Tris-Cl, pH 7.6) in a 30-mL Sarstedt tube. Each tube was vortexed for 10 sec, 4 mL phenol and 4 mL chloroform:isoamylalcohol (24:1 v/v) were added, and the tube was vortexed again. After centrifugation at 10,000*g* for 10 min at 4°C, the upper aqueous phase was transferred to a fresh tube. Ethanol (2.5 volumes) was added, and the nucleic acids were left to precipitate at −20°C overnight. Each tube was centrifuged at 10,000*g* for 10 min, the pellet was washed in 15 mL 70% (v/v) aqueous ethanol, and the tube was recentrifuged at 15,000*g* for 10 min. The nucleic acid pellet was dried and resuspended in 100 μL of sterile distilled water containing 0.1% (w/v) SDS.

RNA Gel Blot Analysis

RNA was fractionated by electrophoresis in a 1.3% (w/v) agarose gel containing formaldehyde and blotted onto NYTRAN-N membrane (Schleicher and Schuell). Filters were prehybridized at 65°C in 3 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), and 200 μg mL^{-1} salmon sperm DNA. Hybridization (16 hr) was carried out under the same conditions with the addition of 10% (w/v) dextran sulphate. cDNA fragments for use as probes were labeled by random priming incorporation of α-^{32}P-dCTP (Feinberg and Vogelstein, 1984). Filters were washed twice in 1% (w/v) SDS, 3 × SSC, and 0.1% (w/v) sodium pyrophosphate at 65°C for 15 min and once in 1% (w/v) SDS, 1 × SSC, and 0.1% (w/v) sodium pyrophosphate again for 15 min at 65°C. Dried filters were exposed to Kodak x-ray film.

Extraction of Metabolites

The frozen cells (100 mg fresh weight) were boiled in ethanol at 80°C for 30 min, ensuring that there was sufficient ethanol to cover the cell pellet throughout this period. The extract was removed, and the cells were reextracted by boiling in 20% (v/v) aqueous ethanol for a further 30 min. These supernatants were pooled, and an internal standard of 150 nm of adonitol, a compound not present in higher plants, was added.

Gas Chromatography

Methoxime-trimethylsilyl ether derivatives of compounds in the metabolite extracts were prepared essentially using the method of Dunstan (1985) and freeze-dried overnight. Water must be excluded from both the sample and the derivatizing agents. Derivatization is carried out in sealed tubes. Fifty microliters of methoxyamine hydrochloride in pyridine (20 mg ml^{-1}) was added, and the tubes were heated to 60°C for 30 min with shaking every 10 min. After cooling, 100 μL of bis(trimethylsilyl)trifluoroacetamide was added, and the samples were heated to 100°C for 1 hr. After derivatization, 1 μL of the sample was used for gas chromatography. The analysis was performed in a gas chromatograph (model 8900 Series II; Hewlett-Packard, Palo Alto, CA) fitted with an air-hydrogen flame ionization detector and an integrator (Spectra-Physics, Mountain View, CA). The gas chromatography system was fitted with a 35 m, 0.3 mm internal diameter silica column coated with 0.5 mm bonded phase OV-1 and a splitless injector. The initial temperature of the column was 80°C. After injection of the sample, the temperature was held at 80°C for 5 min and then increased to 300°C at a rate of 2°C/min to separate different compounds. Both the injection and detection temperatures were 250°C. Peaks were identified by running standards on the same temperature program and obtaining retention times for the individual compounds. Quantitative analysis was performed using the values obtained from these standards relative to the internal adonitol standard added to all samples.

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