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Diauxic Growth in Rice Suspension Cells Grown on Mixed Carbon Sources of Acetate and Glucose[1]

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Diauxic growth was observed in rice (*Oryza sativa* L.) suspension cells growing on acetate (10 mM) and glucose (10 mM). Cells used acetate during the first growth phase and the acetate level in the medium was rapidly decreased, whereas the level of glucose remained essentially unchanged. After acetate was depleted from the medium, cells started to use glucose, forming the second growth phase. It appears that uptake of [^{14}C]glucose was repressed during the first growth phase and became active during the second growth phase. In contrast, uptake of [^{14}C]acetate occurred actively throughout the diauxic growth. By further demonstrating the specific induction of isocitrate lyase (EC 4.1.3.1), a glyoxylate cycle enzyme, and hexokinase (EC 2.7.1.1), a glycolysis enzyme, during the first and second growth phases, respectively, it was clearly shown that rice cells use acetate first and do not use both carbon sources simultaneously. This kind of diauxic growth pattern has been observed in bacteria. To our knowledge, this study is the first report demonstrating the presence of diauxic growth in plant cells.

Diauxic growth is characterized by two different growth rates when two different carbon sources are present in the culture medium. The first growth phase occurs when cells use a preferred carbon source, and the second growth phase begins when the less-preferred carbon source is used after the preferred carbon source has been depleted. This growth pattern was originally observed in *Escherichia coli* grown in a medium containing Glc and lactose as carbon sources (Monod, 1947). Glc was used first, and lactose utilization followed upon exhaustion of Glc. During the first phase of growth, lactose metabolism was inhibited by Glc. This phenomenon, called catabolite repression, is at least partly responsible for the diauxic growth. Catabolite repression can be defined as the inhibition of the synthesis of a number of enzymes involved in the utilization of the less-preferred carbon source by the presence of the preferred carbon source. In addition to catabolite repression, inhibition of the uptake of the less-preferred carbon source by the preferred carbon source may also be responsible for the diauxic growth (Mukherjee and Ghosh, 1987). The uptake of lactose is also inhibited by the presence of Glc in the medium, and this phenomenon is called inducer exclusion. Both catabolite repression and/or inducer exclusion are likely to be important causes of diauxic growth, but the mechanism of diauxic growth is poorly understood.

Diauxic growth has been observed in microorganisms other than *E. coli* and yeast, e.g. in *Rhizobium meliloti*, a symbiotic nitrogen-fixing bacterium, grown on succinate and lactose (Ucker and Signer, 1978). In the first phase, succinate was actively metabolized, whereas lactose was not used until the initiation of the second phase of growth. In *Pseudomonas fluorescens* cells grown in a mixture of malate and Glc, malate was used first and was inhibitory to Glc utilization during the first growth phase (Lynch and Franklin, 1978). Malate was found to repress the Glc uptake and the synthesis of enzymes needed for Glc metabolism. The yeast *Candida tropicalis* exhibited Glc repression of cellobiose utilization and produced a diauxic growth (Bajpai and Ghose, 1978). A carbon source that allows the highest growth rate is generally used as the preferred carbon source. Once the preferred carbon source is depleted, there is a lag phase during which the cells prepare themselves for the utilization of the less-preferred carbon source. In this paper we report that plant cells also have a diauxic growth pattern, similar to those observed in bacteria and yeast. Using suspension-cultured rice (*Oryza sativa* L.) cells grown on acetate and Glc, we demonstrate a diauxic growth pattern with acetate as the preferred carbon source.

MATERIALS AND METHODS

Maintenance of Rice Suspension Culture

Rice (*Oryza sativa* L.) suspension cells, originated from callus, were kindly provided by the Korean Agricultural Science-Technology Institute (Suwon). Suspension cultures were maintained by weekly subculturing. The culture medium (1 L) consisted of Murashige and Skoog basal medium (Murashige and Skoog, 1962) with 2.94 g of KCl, 75 mg of Gly, 877 mg of L-Gln, 266 mg of L-Asp, 228 mg of L-Arg, 2 mg of 2,4-D, 0.2 mg of kinetin, 0.1 mg of GA_3, and 20 g of Glc. After the pH was adjusted to 5.8 with KOH, the medium was sterilized by membrane filtration (0.2 μm pore size). The cultures were maintained at 25°C with constant agitation at 100 rpm in the dark. For the growth experiments, the carbon sources of the culture medium were adjusted to final concentrations of 10 mM Glc and/or 10 mM potassium acetate.

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Abbreviation: ICL, isocitrate lyase.

Measurements of Growth Rates

Growth of the rice suspension cells was determined according to Street (1977). At 24-h intervals, 3 mL of the culture were aseptically harvested and placed on a membrane filter. The fresh weight of the cell mass was measured. The measurements were repeated three times and averaged.

Determination of Glc and Acetate Levels in the Medium

Cell cultures were harvested and immediately centrifuged (12,000*g*) for 20 min to collect the supernatant. The supernatants were kept at −20°C until use. The concentration of Glc was determined according to Alexander and Griffiths (1993). One milliliter of alkaline copper solution (40 g of anhydrous Na_2CO_3, 7.5 g of tartaric acid, 4.5 g of $CuSO_4 \cdot 5H_2O$ per L) was added to 1 mL of the supernatants. The mixtures were heated for 8 min in a boiling water bath and then cooled on ice. One milliliter of phosphomolybdic acid solution (70 g of molybdic acid, 10 g of sodium tungstate, 40 g of NaOH, 250 mL of concentrated H_3PO_4 per L) was added to the mixtures along with 7 mL of H_2O. The A_{420} was determined spectrophotometrically (Shimadzu [Kyoto, Japan] UV-240).

The acetate concentration was determined by HPLC (Knauer, Berlin, Germany) using a fermentation monitoring column (Bio-Rad, 150 × 7.8 mm). Acetate-containing supernatants were mixed with the same volume of 0.005 N H_2SO_4. The mixture was centrifuged (12,000*g*), and the supernatants were filtered through a membrane (Gelman [Ann Arbor, MI], 0.2 μm pore size). Twenty microliters of the filtered sample were injected, and the flow rate was 0.8 mL/min. The temperature of the column was 65°C, and the retention time of acetate was about 5.6 min.

Uptake of ^{14}C-Labeled Acetate and Glc

Aliquots of cells were harvested and filtered through stainless-steel mesh. The harvested cells were washed with an excess of carbon-free medium to remove the residual carbon sources. Approximately 1 g of cells was resuspended in 50 mL of carbon-free culture medium. Uptake of each carbon source was initiated by adding [U-^{14}C]Glc (293 mCi/L) (Amersham) or [1-^{14}C]acetate (60 mCi/L) (Amersham) to the medium at the final concentrations of 6.83 or 33.33 μM, respectively. The mixtures was shaken on a rotary shaker and aliquots were withdrawn at 10-min intervals. The cells with labeled Glc or acetate were rapidly filtered through a membrane (GF/A Whatman, 0.45 μm pore size) attached to a vaccum pump (Hoefer [San Francisco, CA] PV100) and washed with an excess of carbon-free medium. The filters were dried completely under a high-illumination light bulb and placed in a scintillation vial containing 4 mL of scintillation fluid [666 mL of toluene, 333 mL of Triton X-100, 5.5 g of 2,5-diphenyloxazole, and 0.15 g of 1,4-bis(5-phenyl-2-oxazoyl)-benzene]. Radioactivity was measured with a scintillation counter (RackBeta 1211, Turku, Finland). Uptake was expressed as nmol of the radioactive carbon source per g (fresh weight) of cells.

Enzyme Assays

Cells (1 g) were ground in 2 mL of homogenization buffer (170 mM Tricine-NaOH, pH 7.5, 10 mM KCl, 1 mM EDTA, and 10 mM DTT) with a prechilled mortar and pestle. After centrifugation of the homogenates at 12,000*g* for 20 min, the supernatants were collected and used for enzyme assays. These procedures were performed at 0 to 4°C.

ICL activity was determined according to Franzisket and Gerhardt (1980). The assay mixture contained 170 mM Tricine-NaOH (pH 7.5), 3 mM phenylhydrazine-HCl (freshly prepared), 5 mM $MgCl_2$, 2 mM EDTA, and 100 μL of crude extract in a total reaction volume of 1.1 mL. The reaction was initiated by adding 10 μL of 0.33 M DL-isocitrate and the reaction rates were determined at 324 nm in a spectrophotometer (Shimadzu UV-240). The reaction mixture for hexokinase activity included 170 mM Tricine-HCl (pH 7.5), 5 mM Glc, 1 mM ATP, 0.05 mM NADP, 0.3 unit of Glc-6-P dehydrogenase, and 100 μL of crude extract in a reaction volume of 1.5 mL. The reaction rates were measured at 340 nm. The protein concentration was measured using a Bio-Rad protein assay kit with BSA as the standard.

RESULTS

Diauxic Growth of Rice Cell Suspensions on Medium Containing Acetate and Glc

Rice suspension cells were grown in Murashige and Skoog medium containing 10 mM Glc and 10 mM potassium acetate and their growth kinetics determined. At 24-h intervals, cells were harvested and cell weight was plotted against time to construct a growth curve (Fig. 1). The experiments were repeated three times. The initial growth for the first 3 d was slow and then the cells started to grow more rapidly, exhibiting two apparently different growth rates on this medium with mixed substrates. Cells used to initiate the cultures were pregrown on acetate for longer

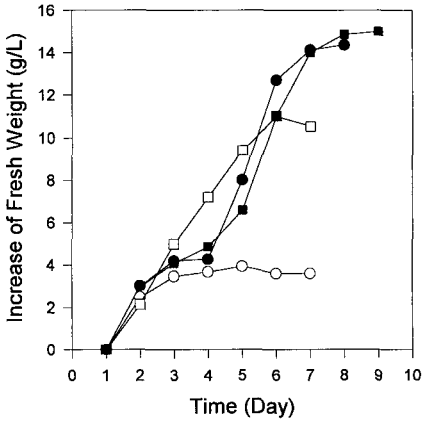

Figure 1. Growth of rice suspension cells in modified Murashige and Skoog medium containing 10 mM Glc (□), 10 mM Glc plus 10 mM potassium acetate (●, acetate-pregrown cells; ■, Glc-pregrown cells), and 10 mM potassium acetate (○). Fresh weights of cultures were determined according to Street (1977). Each fresh weight represents the mean of three determinations.

than 1 d. Growth rates were also determined for acetate-only and Glc-only cultures, and these corresponded to the slower growth rate during the first growth phase and faster growth rate during the second growth phase, respectively, on the mixed-substrate culture (Fig. 1). These observations suggest that the suspension cells may have used acetate during the first phase and Glc during the second phase. The culture on the mixed-carbon sources produced a diauxic growth curve with a lag phase of about 1 d between the two growth phases.

Rice cells may have used acetate during the first growth phase because the cells had been adapted to acetate metabolism during the pregrowth. This was not the case, however. When cells used to initiate the culture were pregrown on Glc, the diauxic growth pattern was largely similar to that of the culture with acetate-pregrown cells (Fig. 1). These results suggest that preadaptations to either acetate or Glc metabolism do not significantly affect the observed growth on acetate and Glc.

Residual Concentrations of Acetate and Glc in Culture Medium

To determine at what point during the growth period acetate and Glc were used, residual concentrations of both carbon sources in the culture medium, initiated with the acetate-pregrown cells, were monitored (Fig. 2A). The acetate concentration dropped rapidly during the first phase of growth, and acetate was not detected after the 3rd d of the culture. The Glc level remained virtually unchanged during the first 3 d and decreased rapidly as acetate became undetectable and the second phase of growth commenced. Glc was not detected after the 5th d of culture, whereas the cells continued to grow until the 7th d. These observations clearly indicate that rice cells use acetate first and that Glc is utilized after acetate is depleted, initiating the second phase of diauxic growth. Since acetate was used as a preferred carbon source, it is very likely that Glc utilization during the first growth phase was repressed by acetate and/or acetate-derived metabolites.

When the culture medium initiated with Glc-pregrown cells was analyzed for the residual concentrations of acetate and Glc, both carbon sources were found to have been consumed at rates similar to those of the culture with acetate-pregrown cells (Fig. 2B). In the culture with the Glc-adapted cells, the use of acetate was delayed about 1 d as Glc was used during the 1st d of the culture. These results further support the previous suggestion that the growth pattern of the culture with Glc-pregrown cells is not significantly different from that with acetate-pregrown cells.

Uptake of Acetate and Glc during the Diauxic Growth

Catabolite repression might be responsible for the observed diauxic growth, in which acetate or metabolites derived from acetate repressed Glc-metabolizing enzymes. To document that catabolite repression is actually occurring in this system is beyond the scope of the present studies. It is also possible that the uptake of Glc is inhibited by the presence of acetate in the medium during the first growth phase. Using cells preadapted to acetate, the uptake of acetate and Glc were determined throughout the diauxic growth period (Fig. 3, A and B). The cells were harvested 1 d (first growth phase) and 5 d (second growth phase) after the initiation of the culture and used for uptake experiments. The cells harvested were mixed with [^{14}C]acetate or [^{14}C]Glc to measure the incorporation of the labeled carbon sources for 60 min at 10-min intervals. In the cells harvested during the first growth phase, the uptake of [^{14}C]acetate was significantly greater than that of [^{14}C]Glc (Fig. 3, A and B). After the onset of the second growth phase, the rate of Glc uptake increased and acetate uptake remained high throughout the second growth phase (Fig. 3, A–D). In the culture with cells pregrown in Glc, the rate of Glc uptake was still high in d 1 of the culture but dropped significantly on d 2 (first growth phase) (Fig. 3D), indicat-

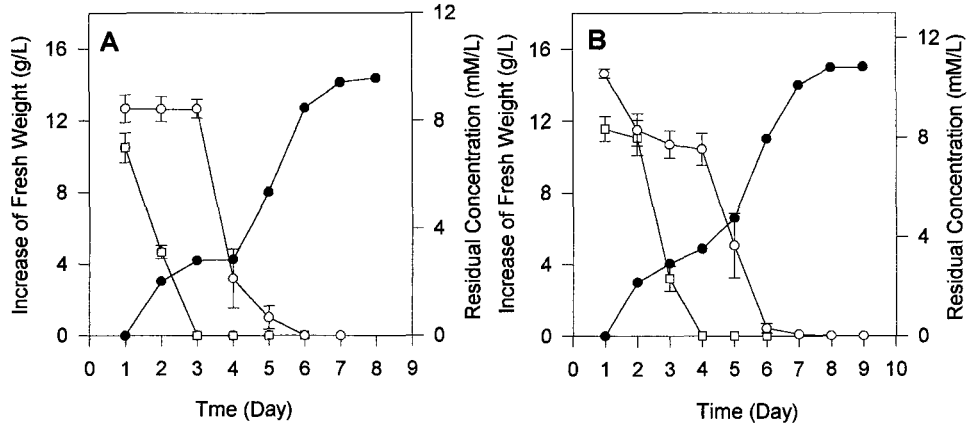

Figure 2. Residual Glc (○) and acetate (□) concentrations in medium of diauxic growth culture. Growth curve (●) is the same as in Figure 1. Residual Glc concentrations were determined by the modified method of Alexander and Griffiths (1993). Residual concentrations of acetate were determined using HPLC equipped with a fermentation monitoring column. Bars indicate SE values from three determinations. A, Acetate-pregrown cells; B, Glc-pregrown cells.

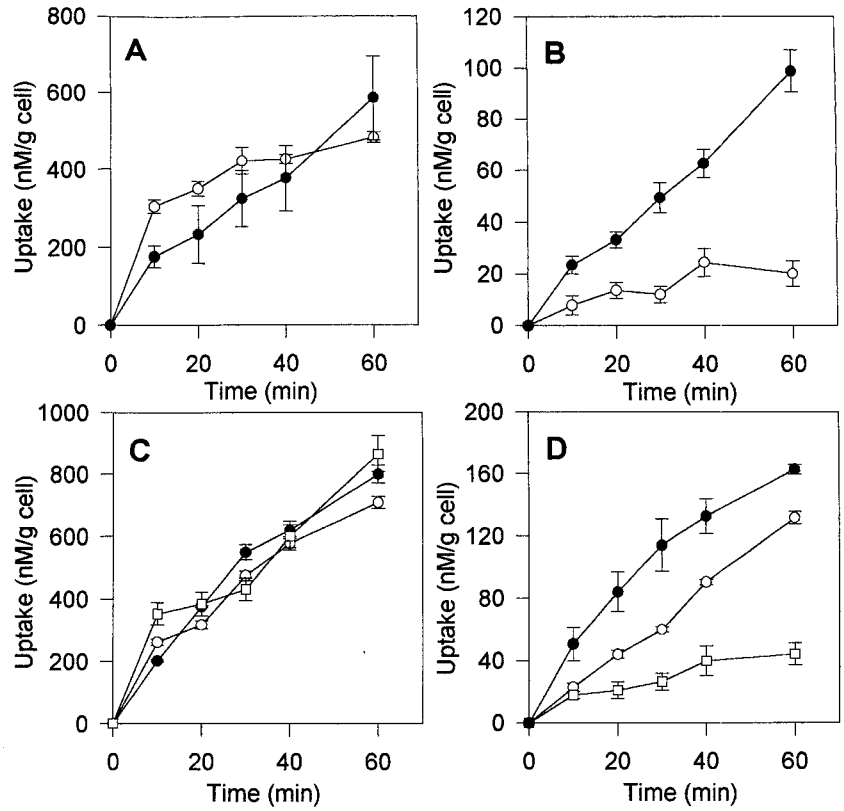

Figure 3. Uptake of [^{14}C]acetate and [^{14}C]Glc at the d 1 (○), 2 (□), and 5 (●) of the diauxic growth. Labeled substrates were added in the harvested cells at the designated times. The mixture was withdrawn and filtered through a membrane at 10-min intervals. The filters were dried, and amounts of cell-carrying labels were determined by a liquid scintillation counter. Bars indicate SE values from three determinations. A, [^{14}C]Acetate uptake of acetate-pregrown cells; B, [^{14}C]Glc uptake of acetate-pregrown cells; C, [^{14}C]acetate uptake of Glc-pregrown cells; D, [^{14}C]Glc uptake of Glc-pregrown cells.

ing that the Glc uptake system established during the pregrowth with Glc was efficiently inhibited by acetate. These results suggest that the inhibition of the Glc uptake by acetate is, at least in part, responsible for the observed diauxic growth.

Inductions of ICL and Hexokinase on the Different Carbon Sources

The selective uptake of acetate during the first growth phase suggests that rice suspension cells may utilize acetate rapidly, probably with the concurrent induction of acetate-metabolizing enzymes. To find out if the induction of acetate-induced metabolism took place in these cells, a glyoxylate pathway-specific enzyme, ICL, was chosen for study (Fig. 4). The glyoxylate cycle is activated in the presence of acetate (Kornberg, 1966; McCullough and John, 1972; Kudielka and Theimer, 1983a). Therefore, it is likely that once acetate is taken up it will be converted into acetyl-CoA by acetyl-CoA synthetase, and acetyl-CoA may then enter the glyoxylate cycle. During the first growth phase, ICL activity was high and it decreased rapidly to an undetectable level before the onset of the second growth phase. During the second growth phase, the activity of ICL remained low. Activities of malate synthase, another glyoxylate cycle-specific enzyme, were also monitored, and the induction pattern was very similar to that of ICL (data not shown). Increase of ICL activity was observed at d 7 and 8 after culture initiation (Fig. 4), at which time Glc was depleted and the cells underwent carbon starvation. A similar result was found in cucumber cell suspension cultures in which ICL was induced as Suc or Glc disappeared from the medium (Graham et al., 1994a, 1994b).

To further demonstrate the cellular response toward Glc during the second growth phase, hexokinase was monitored for its activity throughout the diauxic growth phase (Fig. 4). Hexokinase, which catalyzes the conversion of Glc to Glc-6-P, is a glycolysis enzyme that can be expected to be induced in the presence of Glc (Jang and Sheen, 1994). In cultured rice cells, the activity was low during the first

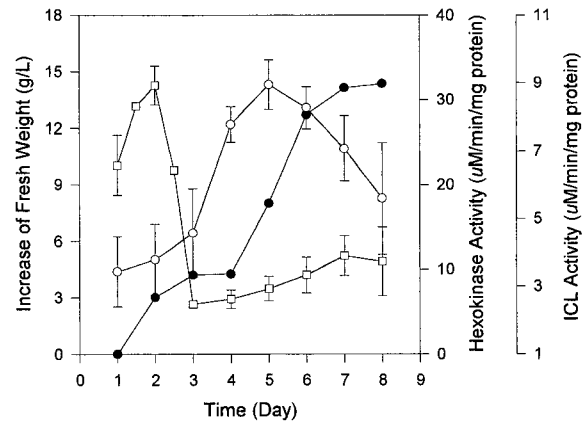

Figure 4. Changes in activities of ICL (○) and hexokinase (□) during the diauxic growth on mixed carbon sources. Growth curve (●) is the same as in Figure 1. Preparation of crude enzyme extracts and ICL assay procedure were based on those described by Franzisket and Gerhardt (1980). Hexokinase activities were determined spectrophotometrically (340 nm). Bars indicate SE values from three determinations.

growth phase and increased significantly as the cells used Glc during the second growth phase. This activity decreased as Glc was depleted after d 5 of the culture. This clear metabolic switching between the acetate- and Glc-utilizing growth phases further substantiates the presence of a diauxic growth pattern in this rice cell culture system.

DISCUSSION

Our results clearly demonstrate that rice suspension cells growing on mixed carbon sources (acetate and Glc) use acetate first and start to use Glc after acetate is consumed. Our results also show that acetate inhibits the uptake of Glc. Similar results have been obtained with bacteria and bacterial membrane preparations. Banes (1974) noted that acetate can reduce Glc uptake as much as 93% in *Azotobacter vinelandii* membrane vesicles. In membrane vesicles prepared from *Bacillus subtilis*, acetate has also been shown to repress the uptake of Ser (Sheu et al., 1972). It is possible that Glc accumulated intracellularly, because of catabolite repression of Glc utilization in the presence of acetate, thereby repressing further Glc uptake. Alternatively, acetate may have directly repressed the synthesis of proteins necessary for Glc uptake. Whatever the exact causes, the inhibition of Glc uptake by acetate is responsible for the observed diauxic growth. The acetate uptake system appears to be constitutive, since the uptake rate was active throughout the diauxic growth (Fig. 3, A and C). In contrast, the uptake of Glc was very sensitive to the presence of acetate in the culture medium. It has been suggested that Glc uptake is carrier mediated and requires energy (Reinhold and Kaplan, 1984). The preferred uptake of acetate over Glc by rice suspension cells was unexpected, since plant cells generally grow better on Glc than on acetate. In bacteria, a carbon source that permits a higher growth rate is the preferred carbon source and it is used first. In our system the situation is reversed: Glc, which allows a higher growth rate, is used after acetate. Rice suspension cells may prefer acetate over Glc because acetate can readily diffuse into cells without a specific transporter. It is also possible that the rice cells use acetate first for some other reasons that are not immediately understood.

The increase in ICL activity further supports the preferential use of acetate during the first growth phase. It has been reported that the glyoxylate cycle is induced on acetate-grown anise suspension cells (Kudielka and Theimer, 1983a, 1983b). The glyoxylate cycle is also induced in detached or senescing leaves (Gut and Matile, 1988; DeBellis et al., 1990; Graham et al., 1992) or in nutrient-starved cells (Graham et al., 1992, 1994a, 1994b). The induction of glyoxylate-cycle enzymes in acetate-utilizing cells results in a channeling of acetate away from the citric acid cycle in which two carbon atoms are removed by CO_2 evolution during the isocitrate dehydrogenase and 2-ketoglutarate dehydrogenase steps. It is known that Suc is actively synthesized via the glyoxylate cycle and through continuous gluconeogenesis in young seedlings. Suc is an important nutrient for the young leaves before they acquire the capability for photosynthesis (Kornberg and Beevers, 1957). However, an active gluconeogenetic pathway is not expected in this suspension culture system. It was assumed that succinate, a product of the glyoxylate cycle, would be used for anaplerotic purposes (Graham et al., 1994a). The fate of malate and succinate in these rice cells is under investigation.

It is known that Glc represses malate synthase and ICL gene expression in a cucumber cell culture system (Graham et al., 1994b). These results suggest that there should not be any significant level of intracellular Glc in the first growth phase of the rice culture, since the glyoxylate cycle operates actively. At the onset of the second growth phase, the intracellular Glc level may have increased and Glc repressed the synthesis of the glyoxylate cycle-specific enzymes, with the simultaneous induction of hexokinase. Recently, Jang and Sheen (1994) showed that intracellular Glc was sensed by hexokinase in a maize protoplast system. We speculate that acetate may have inhibited Glc utilization during the first growth phase simply by repressing the Glc-metabolizing enzymes. The exact mechanism underlying the diauxic growth in this rice culture is not understood.

Most living cells, prokaryotic or eukaryotic, are capable of controlling cellular metabolism for physiological economy. It is probable that cells are frequently exposed to more than one carbon source in nature. It appears that bacteria can choose a preferred carbon source, delaying the use of a less-preferred source. This growth strategy may represent the best adaptation to the growth environments with multiple carbon sources. The metabolic strategies of plant cells are very different from those of bacteria or low eukaryotic cells. Plant cells have a well-developed cellular compartmentation system that divides metabolic demands between different cellular organelles. Therefore, it was reasonable to expect that plant cells would have the capacity to utilize multiple carbon sources simultaneously. Instead, our studies with rice suspension cells demonstrate that plant cells, much like bacteria, use the preferred carbon source first and then use the less-preferred carbon source. The presence of the diauxic growth in rice cell culture suggests that plant cells, in general, may adopt a diauxic growth in the presence of two different carbon sources. Considering that more than one carbon source exists around the plant, it may be important for plant cells to have a metabolic strategy in which a preferred carbon source is utilized first. The presence of diauxic growth using other carbon sources in rice and other plant cells is being investigated. The diauxic growth demonstrated here can be used to study metabolic regulation, such as induction/repression of the glyoxylate cycle.

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