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Preferential Use of Glucose during Diauxic Growth of Carrot Cells Growing on Glucose and Malate

Jung Bin Suh† and Woo Sung Lee*
Department of Biological Science and The Basic Science Research Institute, Sungkyunkwan University, Suwon 440-746, Korea

Suspension cells of carrot plants grown on mixed carbon sources of glucose (Glc) and malate preferentially used Glc. The cells started to utilize malate only after Glc was depleted from the medium, thus exhibiting a diauxic growth. The residual concentration of Glc decreased rapidly during the first growth phase, and that of malate decreased only during the second growth phase. Malate uptake was negligible throughout the diauxic growth, suggesting that malate was being utilized via another metabolite. An active metabolic flow from fumarate to pyruvate and oxaloacetate via malate was induced in cells during the second growth phase. These results strongly suggested that malate remained unused in the medium in the first phase, and in the second phase it was converted extracellularly into fumarate, which was subsequently transported into cells and metabolized into malate and further into pyruvate and oxaloacetate. This study presents the second case of diauxic growth in plants and the peculiar mode of malate utilization.

Keywords: Diauxic growth, fumarase, glucose, malate

Diauxic growth represents a biphasic growth in which a preferred carbon source is utilized at the first growth phase, and a less-preferred one is utilized only after the preferred one is depleted from the medium, thus forming the second growth phase (Monod, 1947). This phenomenon has been widely observed in bacteria and yeast (Bajpai and Ghose, 1978; Ucker and Signer, 1978; Mukherjee and Ghose, 1987). Between the two different growth phases, there is ususally a lag phase during which cells prepare themselves for the synthesis of the enzymes needed for the utilization of the less-preferred carbon sources. Studies on diauxic growth in plants have been largely neglected to the present. Considering that plant cells are exposed to multiple carbon sources which originate from photosynthesis and phloem translocation, it must be important to understand the mechanism as to how cells utilize the multiple carbon sources.

In the previous report, we demonstrated the presence of diauxic growth in plants for the first time (Lee and Lee, 1996). When rice suspension cells were grown on Glc and acetate, acetate was used preferentially, and Glc was utilized only subsequently at the second growth phase. During the acetate-utilizing first growth phase, Glc uptake and hexokinase, which catalyzes Glc into Glc-6-phosphate, remained repressed until the onset of the second growth phase. Acetate-mediated changes of extracellular and intracellular pH changes appeared to be responsible for the abolition of Glc utilization during the first growth phase (paper in press). To extend the observation of diauxic growth in plants, this study employed carrot cells which were grown on Glc and malate. Malate is frequently observed in phloem exudates (Hall and Baker, 1972; Richardson et al., 1982), and a co-presence of Glc and malate is likely to occur in sink tissues. Therefore, there must be an inherent strategy for cells to effectively use these two carbon sources. It would also be interesting to see whether malate, a weak acid like acetate, has the ability to inhibit Glc utilization. This study demonstrates that, unlike in the operation of Glc/acetate diauxic growth, Glc was the preferred carbon source and malate was used only at the second growth phase. It was also demonstrated that malate was used after its extracellular conversion into fumarate in the growth medium. Fumarate was subsequenly transported into cells and metabolized for cell growth. Here, we report yet another case of diauxic growth in plants, along with the unique mode of malate utilization during the second growth phase.

*Corresponding author; fax 82-331-290-7015
email wslee@yurim.skku.ac.kr
†Present Address (J.B.S): Rural Developmental Cooperation, Ansan City, Kyungki Do, Korea

Abbreviations: DNP, dinitrophenol; Glc, glucose, MDH, malate dehydrogenase; ME, malic enzyme

MATERIALS AND METHODS

Organism, Maintenance, and Growth Measurements

Carrot (*Daucus carota* L.) suspension cells, originated from tap roots, were maintained by weekly subculturing. The culture medium (1 L) consisted of Murashige and Skoog's basal medium (Murashige and Skoog, 1962) supplemented with 2,4-D (2 mg), kinetin (0.1 mg) and Glc (30 g), and the pH was adjusted to 5.8 with KOH. The cultures were maintained at 25°C with constant agitation at 100 rpm in the dark. For the experiments, L-Glc and/or malate were supplemented aseptically to the medium to final concentrations of 10 mM each. Growth was measured according to Street et al. (1977). Briefly, 3 mL of suspension cells were harvested daily, and the liquid was removed through a membrane filter. Each measurement of the fresh weight was the mean of five different determinations.

Determination of Residual Glc, Malate, and Fumarate

Daily harvested cells were centrifuged (12,000g for 20 min) and the supernatants were stored at −20°C until use. The determination of Glc was performed according to the method of Alexander and Griffiths (1993). 1 mL of the alkaline copper solution (40 g Na_2CO_3, 7.5 g Tartaric acid, 4.5 g $CuSO_4 \cdot 5H_2O$ in 1 liter of H_2O) was added to 1 mL of the supernatant, and the assay mixture was boiled for 8 h and cooled on ice. With this assay mixture, 1 mL of the phosphomolybdic acid solution (70 g molybdic acid, 10 g sodium tungstate, 40 g NaOH, 250 mL of concentrated H_3PO_4 in 1 L of H_2O) was mixed and 7 mL H_2O was added. The concentration of Glc was determined at 260 nm (Shimadzu UV-240, Japan).

Determination of malate and fumarate was carried out by HPLC (Knauer, Germany) with a fermentation column (Bio-rad, 150 × 7.8 mm). The supernatants containing malate and fumarate were mixed with the same volume of 0.0005N H_2SO_4, and the mixture was centrifuged and the supernatants were filtered through the membrane (Gelman, 0.2 μm pore size, USA). 20 μL of the filtrated solution was injected into the HPLC with the flow rate of 0.8 mL/min. The retention times for malate and fumarate were 3.74 min and 5.38 min, respectively (column temperature : 65°C).

Biochemical Studies

The experimental procedures for the determinations of growth rates and uptake rates were essentially the same as described by Lee and Lee (1996). The enzyme assays for HK/isocitrate lyase (ICL) and the uptake experiments for Glc/acetate were also carried out as described by Lee and Lee (1996). Malate dehydrogenase activity was assayed by using the Malic Acid kit (Boehringer Mannheim). Malic enzymes (ME) were assayed in a reaction mixture containing 50 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 10 mM EDTA, 5 mM malate-NaOH (pH 7.5), and 5 mM Malate-NaOH in a final volume of 1.5 mL. The reaction was initiated by adding 100 μL of the cell crude extract. Fumarase was assayed in a reaction mixture containing 0.5 mM Kpi buffer (pH 7.3) and 0.5 mM malate-NaOH (pH 7.3). The Reaction was initiated by the addition of cell extracts.

RESULTS

Actively growing suspension cells of carrot plants which were grown on Glc were tranferred to a medium containing both Glc and malate at 10 mM each (pH 5.8) at time zero to initiate diauxic growth for the study. Cultures grown previously either on Glc or malate did not make any significant differences in the results to be presented (data not shown). Cells and media were collected daily, and the various experimental parameters were determined. The growth curve of these cultures showed a typical diauxic growth characterized by two different growth phases (Fig. 1). The first and second growth phases

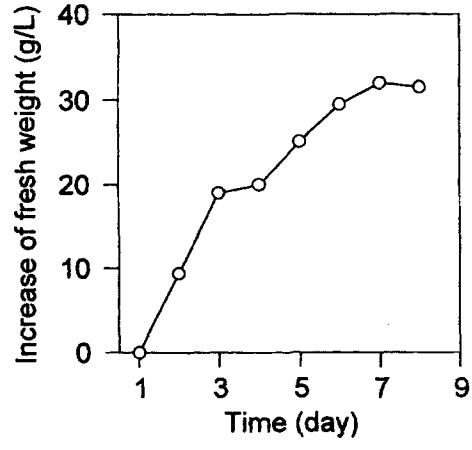

Figure 1. Growth of suspension cells of carrot plants grown on Glc and malate at 10 mM each. The fresh weights of the cells harvested daily was determined after filtering out the excessive culture liquids on a membrane filter. Each fresh weight is the mean of the five determinations.

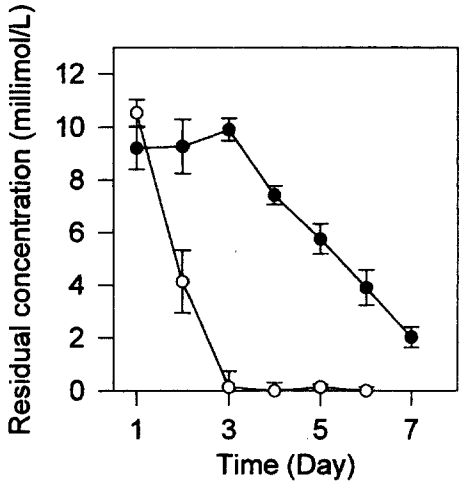

Figure 2. Residual concentration of Glc (○) and malate (●). Cell cultures were harvested daily and were immediately centrifuged to pellet down the cells. The concentrations of Glc and malate in the supernatants were determined as described in Materials and Methods. Bars represent the mean ± SE of the three independent measurements.

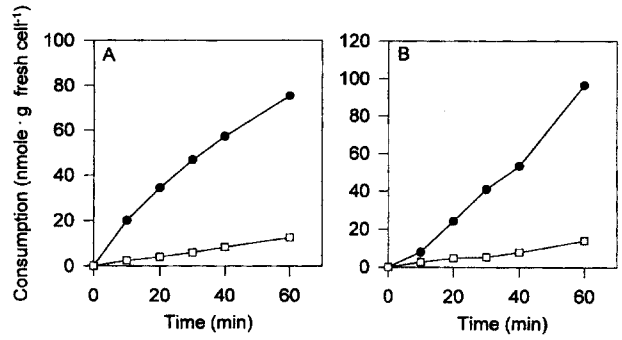

Figure 3. Uptake of [^{14}C] Glc and [^{14}C] malate in cells harvested during the first (A) and second growth phases (B). Cells were harvested at d 2 (first growth phase) and d 6 (second growth phase). The radiolabelled substrates were added to the aliquots of the cells. The mixture was withdrawn at 10 min intervals and was filtered through a membrane to collect cells by removing residual radiolabelled substrates by washing with excessive amount of the culture medium. The retained cells were dried and the amount of the radioactivity in the cells was determined by a liquid scintillation counter. (●), uptake of [^{14}C] Glc; (□), uptake of [^{14}C] malate.

have doubling times of about one and six days, respectively. Prior to the initiation of active malate utilization at d 4, there was a lag phase of one day between d 3 and d 4. To determine which of the two carbon sources was used earlier, residual concentrations of Glc and malate in the culture medium were determined. It was shown that Glc was rapidly depleted during the first growth phase, while malate remained little changed during this period and was found to rapidly disappear during the second growth phase (Fig. 2). These results strongly suggested that Glc was utilized as a preferred carbon source and malate was utilized only after Glc was depleted from the medium.

In order to examine the uptake characteristics of Glc and malate during the diauxic growth, uptake rates of [^{14}C] Glc and [^{14}C] malate were measured in the cells harvested at d 2 (first growth phase) and d 6 (second growth phase) (Fig. 3). Uptake of [^{14}C] Glc was active in cells of the both growth phases, whereas that of [^{14}C] malate was negligible in the both phases. These results suggest that Glc uptake is constitutive throughout the diauxic growth. The results also showed that cells were not able to transport malate even in the d 6-cells of the second growth phase, during which it is being utilized by the cells. Subsequently, the malate uptake was further examined at various pHs (pH 2 pH 8), since ionization status of malate might be important for its uptake. Results showed that malate uptake was found to be insignificant at all of the pHs tested (data not shown). This observation was very puzzling, since malate was clearly used for cell growth during the second growth phase.

In order to trace the fate of malate in the medium, all the acid components in the medium were collected at d 6 and were identified by GC-MS (data not shown). The results showed that a significant amount of fumarate appeared along with malate. To further

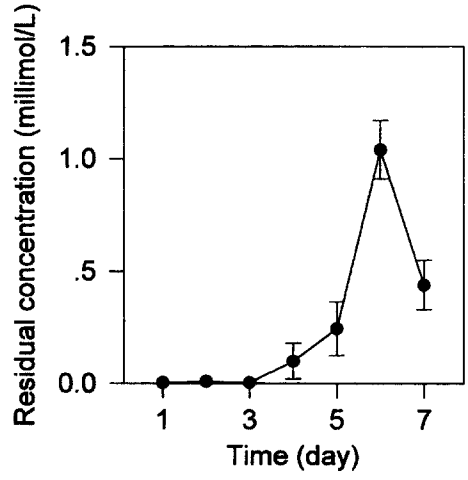

Figure 4. Residual concentration of fumarate. Residual concentration of fumarate in the culture medium collected daily was determined by HPLC as described in Materials and Methods. Bars represent the mean ± SE of the three independent measurements.

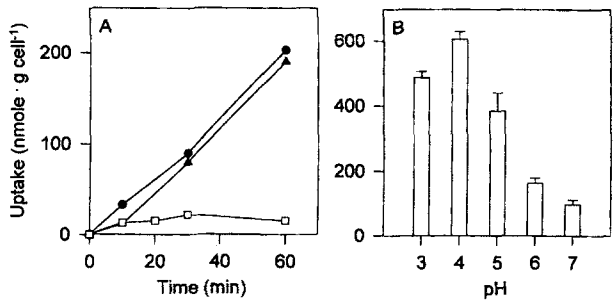

Figure 5. Uptake characteristics of [^{14}C] fumarate. Cells were harvested at d 2 and d 6 for the uptake experiments. (A) Uptake rates of [^{14}C] fumarate in cells harvested at d 2 (▲) and d 6 (●) were determined, and the same experiment with d 6-cells was carried out in the presence of 0.1 mM DNP (□). (B) Uptake of [^{14}C] fumarate at various pHs. Cells were transferred into the culture medium whose pH had been adjusted to the various values and were incubated for 1 h in the presence of [^{14}C] fumarate. Radioactivity remaining in the cells was determined after removing the culture medium.

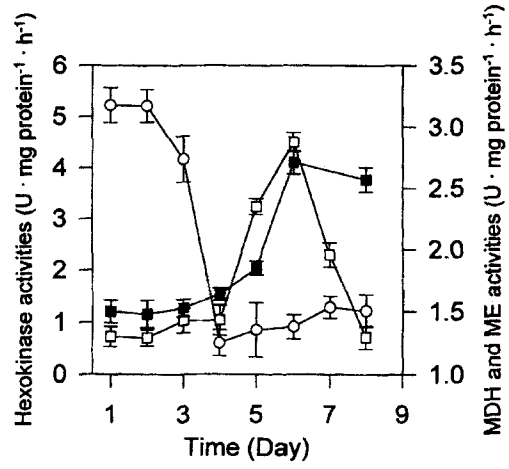

Figure 6. Temporal changes of the activity of the enzymes involved in the diauxic growth. Using the crude extracts prepared from the cells harvested daily, the activity of HK (○), MDH (■), and ME (□) was determined as described in Materials and Methods.

xamine the pattern of fumarate production, residual concentrations of fumarate were determined from the medium after pelleting down the daily harvested cells (Fig. 4). It was found that fumarate was not detected until d 3 in the medium, and it was found to increase thereafter, peaking at d 6. These observations strongly suggested that malate was getting converted extracellularly into fumarate, which was subsequently utilized as the carbon source during the second growth phase. To confirm the fumarate utilization by cells, uptake of [^{14}C] fumarate was examined, and the uptake rate was found to be high at both d 2 and d 6, indicating that fumarate uptake is also constitutive, comparable to that of Glc, throughout the diauxic growth (Fig. 5A). It was also found that fumarate uptake was sensitive to the protonophore, dinitrophenol (DNP) (Fig. 5A), and the pH of the medium (Fig. 5B), suggesting that proton gradient across the plasma membrane is important for the fumarate uptake.

In order to further confirm and analyse the utilization pattern of both carbon sources at their respective growth phases, temporal changes in the activity of several enzymes were examined using cells harvested daily (Fig. 6). Hexokinase was highly active during the first growth phase, while fumarase, malate dehydrogenase (MDH), and malic enzyme (ME) were activated only during the second growth phase. Significant activations of these enzymes suggest that in cells fumarate are actively metabolized into malate, which is further converted into oxaloacetate and pyruvate (Fig. 6).

It is of interest to find out how Glc could cause the inhibition of malate utilization during the first growth phase. Accordingly, it was examined to find out whether Glc could inhibite the fumarate uptake system (Fig. 7). The saturation kinetics for [^{14}C] Glc (Fig. 7A) and [^{14}C] fumarate (Fig. 7B) uptake were determined in the presence of cold fumarate and Glc at 10 mM each, as the competing carbon sources. These results clearly showed that the uptake of Glc and fumarate was not affected in the presence of the competing fumarate and Glc. It remains to be studied

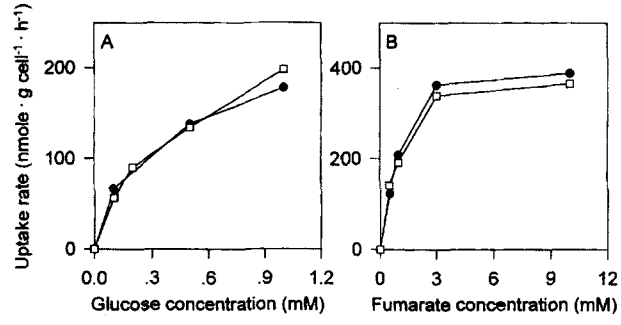

Figure 7. Competition kinetics between Glc and fumarate uptakes. Cells used in this study were harvested at d 6 at which both Glc and fumarate uptake are active. To observe the uptake kinetics, the uptake rate of the radiolabelled substrates were determined at increasing concentrations of the corresponding substrate. The same experiment was carried out in the presence of the competing substrate at 10 mM. (A) Uptake of [^{14}C] Glc in the absence (●) or presence (□) of fumarate. (B) Uptake of [^{14}C] fumarate in the absence (●) or presence (□) of Glc.

if Glc could inhibit the extracellular conversion of malate into fumarate to inhibit the utilization of malate until the second growth phase. In summary, this study provides yet another case of diauxic growth in which Glc is the preferred carbon source over malate. In addition, the results of the present study suggest that malate utilization involves extracellular conversion of malate into fumarate and subsequent fumarate metabolism involving fumarase, MDH and ME. The presence of fumarase polypeptide in medium is under investigation.

DISCUSSION

Combined with our previous findings in which acetate was preferentially utilized over Glc in rice cells (Lee and Lee, 1996), the present study provides evidence that diauxic growth can occur both in monocot and dicot plants. It is also noted that Glc can serve either as a preferred or a less-preferred carbon source, depending on the availability of the co-presenting carbon source. It appears conclusive that plant cells do not utilize Glc and organic acids simultaneously. It is speculative that organisms in general, prokaryotes and eukaryotes, may have the capability and necessity to select the preferred carbon source, which in turn generates a peculiar signal transduction system to repress the utilization of the less-preferred carbon sources. Except under specific circumstances, cells may have an advantage in using just one carbon source, so that they do not have to synthesize diverse kinds of enzymes needed for the simultaneous utilization of different carbon sources. In this study, Glc was found to be the preferred carbon source which prevented malate from being used until the second growth phase. In contrast, malate was the preferred carbon source over Glc in bacteria, *Pseudomonas fluoresens* (Lynch and Franklin, 1978).

By identifying the organic acids in the medium and tracing the fate of externally added [^{14}C] malate, it was clearly demonstrated that fumarate was actively produced extracellularly from malate. Malate uptake was found to be negligible and not induced even at the malate-utilizing second growth phase. These results strongly suggest that there is malate-inducible mechanism responsible for the extracellular conversion of malate to fumarate. It is highly speculative that fumarase could be released into the medium or be associated with plasma membrane or cell wall. Fumarase is a citric cycle enzyme which catalyzes the reversible reactions between fumarate and malate, and is usually present in mitochondia. Our preliminary experiments showed that antibodies raised against *Arabidopsis* fumarase provided a strong indication of the presence of fumarase in the medium during the second growth phase (data not shown).

It was also noted that fumarate originated from malate was transported into cells to be further metabolized into malate which in turn gets metabolized either into pyruvate by ME or into oxaloacetate by MDH (Fig. 6). These results show that forward (fumarate into malate) and backward (malate into fumarate) reactions of fumarase activity could occur in cells and in the medium, respectively. Based on the results that fumarate uptake is sensitive to DNP and pH (Fig. 5), it is strongly suggested that the reversible fumarase activities across the plasma membrane play an important role in utilizing fumarate by increasing the proton gradient across the plasma membrane. The forward reaction (fumarate into malate) consumes a proton, whereas the backward reaction generates a proton. The specific increases of MDH and ME are likely to accerelate intracellular fumarase activity by removing the reaction product, malate. It is likely that the metabolic pathways operating at either sides of the plasma membrane are coupled to increase the proton gradient.

Malate may not be a favorable carbon source, based on the observation that it is not transported across the plasma membrane throughout the diauxic growth. It is speculative that synthesis of fumarase and directing it to the proper location may be too demanding for cells, thus the favoring of the utilization of Glc earlier. In any case, it is logical to believe that Glc inhibited the activity of fumarase in the conversion of malate into fumarate extracellularly, since this enzyme is very likely the first one to mobilize the otherwise inert malate present in the medium. Fumarate uptake, the probable next step following the extracellular conversion from malate, did not appear to be the point of Glc inhibition (Fig. 7). This study may represent the real (in-situ) phenomenum by which plant cells use phloem exudates containing Glc and malate. The diauxic growth observed in the present study may be a valuable experimental system to investigate Glc-mediated repression of the enzymes involved in the utilization of the other carbon source.

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