Plant Cell Physiol. 40(10): 1046-1052 (1999)

Preferential Use of Acetate over Glucose Involves Acetate-Mediated Inhibition of Glucose Uptake during Diauxic Growth of Carrot Cells

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It has been reported that suspension-cultured rice cells grown on mixed carbon sources of glucose (Glc) and acetate exhibited diauxic growth in which acetate was the preferred carbon source (Lee and Lee 1996). Carrot (*Daucus carota* L.) suspension cells, showing a diauxic growth very similar to that of rice cells, were used to delineate the mechanisms underlying this preferential use of acetate over Glc. Uptakes of both Glc and 3-*O*-methylglucose (3-OMG), a non-metabolizable Glc analogue, were similarly inhibited when acetate or butylate, weak acids which are capable of transporting protons into the cytosol, were present in the uptake assay mixture containing cells harvested during the Glc-utilizing second growth phase. Inhibition of Glc uptake by these weak acids was similar when equivalent experiments were carried out with isolated plasma membranes. It was further shown that Glc uptake, which requires a proper proton gradient across the plasma membranes, was inhibited during the first growth phase by acetate-mediated alkalization of growth medium and/or simultaneous acidification of cytosol. This study strongly suggests that Glc utilization in plant cells is inhibited by co-presenting carbon source(s) which can alter the proton gradient across the plasma membrane.

Key words: Acetate inhibition — Carrot — Diauxic growth — Glucose uptake.

Carbon sources in plants are redistributed mostly through the phloem, which often contains more than one carbon source, including sucrose, Glc, organic acids, and amino acids. A variety of carbon sources synthesized by photosynthesis and/or other metabolic processes are unloaded at sink tissues. It is important to understand how these multiple carbon sources are taken up and utilized by these tissues. However, studies of these aspects of cellular behavior have largely been neglected by plant scientists. To understand how plant cells respond to the multiple carbon sources available to them, we have investigated diauxic growth using suspension-cultured cells growing on acetate and Glc simultaneously present in the growth medium. In diauxic growth, the preferential carbon source is used first and the less-preferred one is not used until the preferred one is consumed. We reported diauxic growth in rice suspension cells, similar to that in bacteria and yeast, in which acetate and Glc are utilized exclusively during the first and second growth phases, respectively (Lee and Lee 1996). The mechanisms underlying diauxic growth are in general not well understood, and they have not been explored in higher eukaryote cells to the best of our knowledge.

Extensive studies with microbial systems has shown that catabolite repression and/or inducer exclusion are responsible for the initial use of the preferred carbon source over the less-preferred one (Magasanik 1970). Catabolite repression involves the preferred carbon source-mediated repression of the enzymes required for the use of the less-preferred one. In the well-known experiments by Jacob and Monod, Glc repressed the enzymes required for lactose utilization in *E. coli* grown on Glc and lactose, establishing a diauxic growth pattern in which Glc was used before lactose. Inducer exclusion, a term that describes inhibition of the uptake of the less-preferred carbon source by the preferred one, is also observed in several diauxic growth patterns. Plants might have different mechanisms for utilizing two carbon sources, because that they have a well-developed compartmentation system in which metabolic capacities are divided into several subcellular organelles.

It has been widely accepted that Glc is a preferred carbon source in most organisms. In plants, Glc is known to repress proteins involved in photosynthesis (Jang and Sheen 1994) and other metabolic processes, such as the glyoxylate cycle (Graham et al. 1994, Lee and Lee 1996). There have been a few reports that hexokinase plays a pivotal role in expressional control of the Glc-sensitive genes (Graham et al. 1994, Jang and Sheen 1994, Jang et al. 1997). In diauxic growth in bacteria, Glc is usually the preferred carbon source, but in certain cases, it becomes less-preferred when it competes with several organic acids, including acetate, malate and succinate (George et al. 1985, Mukherjee and Ghose 1987). It was observed that Glc was utilized before malate in carrot cells (Suh et al. paper in preparation), suggesting that organic acids are not always the preferred carbon source in plants as they are in bacteria. It is not understood how organic acids inhibit Glc utilization during the first growth phase of diauxic growth.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyl [2-^{14}C]oxazolidine-2,4-dione; DNP, 2,4-dinitrophenol (DNP); FC, fusicoccin; Glc, glucose; ICL, isocitrate lyase; HK, hexokinase; OMG, *O*-methylglucose.

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This study demonstrates that an acetate-mediated change in the proton gradient across the plasma membrane (alkalization in medium and acidification in cytosol) is a major cause of the inhibition of Glc utilization during the first growth phase.

Materials and Methods

Chemicals—D-[U-^{14}C]Glc, [1-^{14}C]acetic acid, 3-*O*-methyl-D-[U-^{14}C]Glc (OMG), and 5,5-dimethyl [2-^{14}C]oxazolidine-2,4-dione (DMO) were purchased from Amersham Chemical Co., and the respective cold chemicals were obtained from Sigma. 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and fusicoccin (FC) were also purchased from Sigma.

Plant material and growth experiments—Carrot (*Daucus carota* L.) suspension cells originating from tap root were kindly provided by Dr. Cho, Bong-Huey (Suwon University, Suwon, Korea) and maintained by weekly subculturing. The culture medium (1 liter) consisted of Murashige and Skoog basal medium (1962) supplemented with 2,4-D (2 mg), kinetine (0.1 mg), and Glc (30 g). Its 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 diauxic growth with Glc and acetate, Glc and potassium acetate were aseptically added to the medium at time zero. Their final concentrations were adjusted to 10 mM. Determinations of growth rate and residual concentrations of Glc/acetate and the enzyme assays for HK and ICL have been described by Lee and Lee (1996).

Uptake experiments—Cells were harvested and rinsed twice with ample amounts of carbon source-free medium to remove any residual carbon sources. Approximately 1 g of cells were used to determine the uptake rates of [^{14}C]Glc and [^{14}C]acetate at room temperature. Uptake was initiated by addition of the respective radiolabelled carbon source to the cells suspended in 50 ml carbon source-free medium solution at the final concentrations of 6.83 (Glc) or 33.33 (acetate) μM. The mixtures were shaken on a rotary shaker and aliquots were withdrawn at 10-min intervals. Detailed procedures have been described by Lee and Lee (1996).

For the experiments examining the pH-dependence of Glc uptake, the uptake medium was adjusted to pH 4.0, 5.0, 6.0, 7.0, or 8.0 with 1 M HCl or KOH. For the experiments characterizing Glc uptake, DNP (10 μM) was freshly prepared in absolute ethanol. CCCP (5 μM) and FC (2 μM) were also prepared in absolute ethanol and stored at −20°C until use. Assays conducted in the absence of these compounds contained an equal volume of absolute ethanol.

Isolation of plasma membrane vesicles—Cells harvested at d 5 (five day after initiation of diauxic growth) were used in preparation of plasma membrane vesicles by two-phase partitioning (Buckhout 1989, Sandrine et al. 1995). All procedures were performed at 4°C. Aliquots of 100 g of carrot suspension cells were ground with a polytron in homogenization buffer (50 mM K-phosphate buffer, pH 7.5, 0.5 M sorbitol, 0.6% polyvinylpolypyrrolidone, 5 mM ascorbic acid, and 3.6 mM cysteine). The homogenates were filtered through eight layers of cheesecloth, and the resulting suspension was centrifuged at 10,000 × *g* for 10 min. The supernatant was recentrifuged at 50,000 × *g* for 45 min. The pellet was resuspended in 5–6 ml of PSK buffer (20 mM K-phosphate buffer, pH 7.8, 1 M sorbitol, 8 mM KCl) and used for preparation of the plasma membrane fraction. Detailed procedures of the two phase partitioning were essentially as described by Buckhout (1989).

Measurements of external and cytoplasmic pH—Cells were harvested at d 5 by filtration, and approximately 2 g were resuspended in 50 ml of carbon-free medium. The designated carbon source(s) was added into culture medium at the final concentration of 10 mM. External pH (pHe) was measured by pH electrode (ATI Orion 420A, U.S.A.). Three ml of culture medium were withdrawn into a test tube at designated intervals, and changes in pHe were monitored.

Cytoplasmic pH (pHc) was determined according to Kurkdjian et al. (1978) with minor modifications. Briefly, two aliquotes (for replicate experiments) of 1 ml culture medium containing actively growing cells were harvested and centrifuged at 10,000 × *g* for 2 min. The resulting pellets were washed with 500 μl of 0.1 M K-phosphate, pH 6.0 and resuspended in 760 μl of K-phosphate, pH 6.0. Each mixture was then transferred into a test tube and placed in a shaking incubator (at 200 rpm) set at 25°C. One μl of [^{14}C]DMO (about 10^5 cpm) and 8 μl of cold DMO were then added into the mixture at the final DMO concentration of 10 μM to initiate an experiment. At 10 min intervals, three aliquotes (for triplicate experiments) of 200 μl of cell suspensions were filtered through a nitrocellulose filter (0.2 μM pore size), and the filters were washed with 2 ml of ice-cold distilled water. Each filter carrying cells and each filtered solution was separately counted in a 10 ml scintillation cocktail. pHc was calculated by the formula.

$$pHc = 6.3 \pm \log_{10}[(cpm_i \times 760)/(cpm_a \times 166)]$$

cpm_i, total cpm of the filter; cpm_a, total cpm of filtered solution.

Results and Discussion

Diauxic growth of carrot suspension cells grown on Glc and acetate—Our primary objective was to understand how acetate inhibited Glc utilization during the first growth phase in which acetate is exclusively utilized. This study utilized a carrot suspension culture which had a growth pattern (Fig. 1) very similar to the previously reported diauxic growth of rice cells grown on 10 mM Glc and 10 mM acetate (Lee and Lee 1996). Acetate was used first and Glc only after acetate was depleted from medium, forming two different growth phases. During the first growth phase (d 1–d 3), the acetate level in the medium rapidly decreased, while the Glc level remained unchanged and then rapidly decreased at the second growth phase (d 4–d 6). Activities of ICL and HK were induced at the acetate-utilizing and the Glc-utilizing growth phases, respectively. These results confirmed that carrot cells exhibit diauxic growth in which acetate and Glc are the preferred and less-preferred carbon sources, respectively. These results also suggest that both monocot and dicot plants share a same strategy for diauxic growth of cells grown on Glc and acetate.

Inhibition kinetics of Glc uptake by acetate—During the first growth phase, acetate might have inhibited either Glc uptake (inducer exclusion) or Glc-catabolizing enzymes (catabolite repression) to block Glc utilization. In cells harvested at d 5 (second growth phase) uptake of [^{14}C]-Glc was significantly inhibited in the presence of acetate (Fig. 2A). A similar inhibition was also observed when

isolated plasma membrane vesicles were used for otherwise identical uptake experiments (Fig. 2B).

To understand if acetate inhibition of Glc uptake was linked to Glc metabolism, the uptake rate of OMG, a non-metabolizable Glc analogue, was determined. When d 5-cells were incubated with [^{14}C]OMG in the presence of 10 mM acetate, the uptake of 3-OMG was significantly inhibited (Fig. 2C).

It was also noted that the activity of HK in cells used for the uptake experiments described above remained uninhibited during the period following the addition of

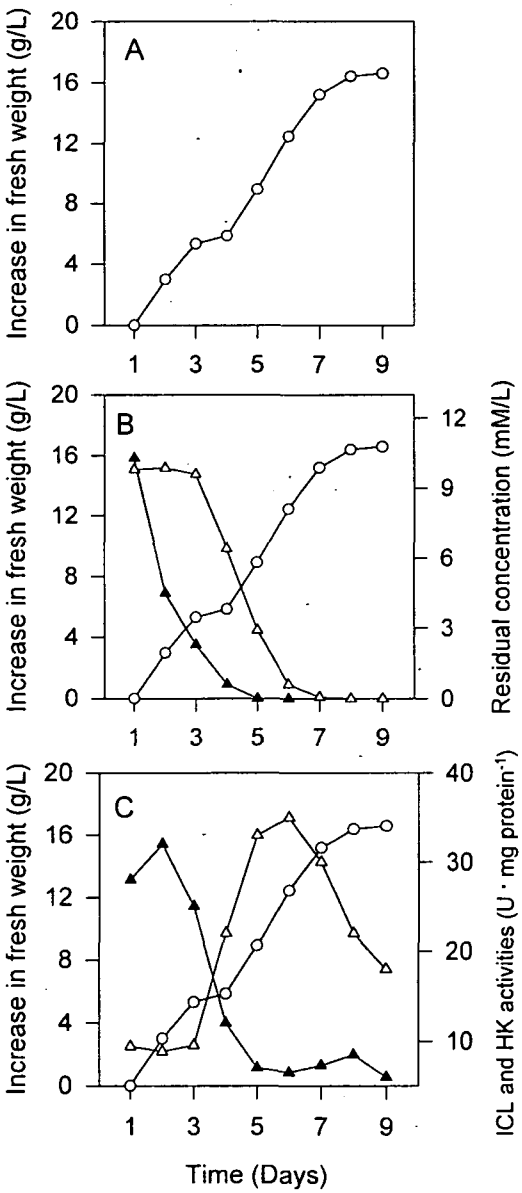

Fig. 1 Diauxic growth of carrot suspension cells on acetate and glucose. (A) Growth curve of carrot suspension cells. Cells were growing on 10 mM acetate and 10 mM glucose. At 24 h intervals, 3 ml of the culture were aseptically harvested, and the fresh weights of each cell mass were measured. The values are the average of the three independent experiments. (B) Changes in the levels of the residual acetate (▲) and glucose (□) during diauxic growth. Culture media with cells removed by centrifugation were used to determine the levels of acetate and glucose by the methods described by Lee and Lee (1996). (C) Specific inductions of isocitrate lyase (▲) and HK (□) during diauxic growth. Extracts from the cells harvested daily during diauxic growth were subjected to enzyme assays according to Lee and Lee (1996).

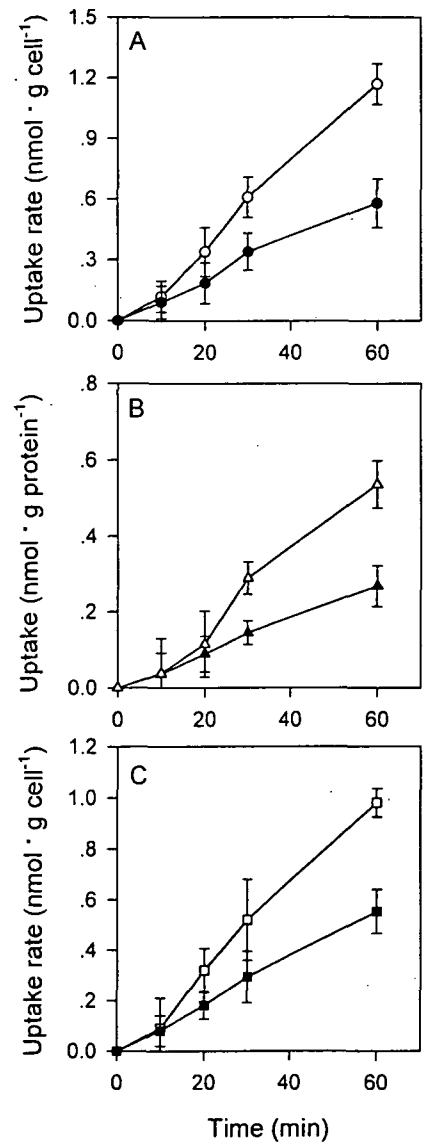

Fig. 2 Uptake of [^{14}C]Glc or [^{14}C]OMG in whole cells or plasma membrane vesicles. Uptake procedures of [^{14}C]Glc and [^{14}C]OMG are described in "Materials and Methods". Uptake experiments were carried out with d 5-cells or plasma membrane vesicles prepared from d 5-cells. Uptake was determined in the presence (●) or absence (○) of 10 mM acetate. (A) Uptake of [^{14}C]Glc in whole cells. (B) Uptake of [^{14}C]Glc in plasma membrane vesicles. (C) Uptake of [^{14}C]OMG in whole cells. Each bar represents means ± SE of three replicates.

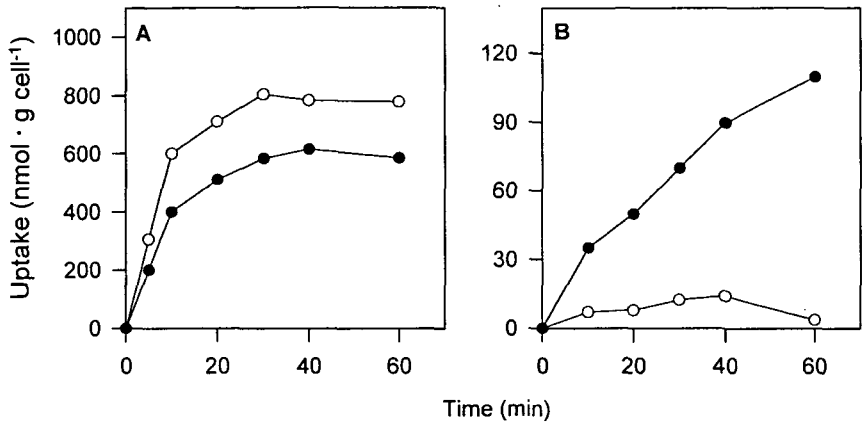

Fig. 3 Uptakes of [^{14}C]acetate and [^{14}C]Glc during the first and second growth phases. Cells harvested at d 1 (first growth phase) and d 5 (second growth phase), after initiation of diauxic growth, were mixed with [^{14}C]acetate or [^{14}C]Glc, and aliquots of mixtures were withdrawn at the 10 min intervals to measure cell-bound radioactivities. ○, cells harvested at d 1. ●, cells harvested at d 5. (A) uptake of [^{14}C]acetate. (B) uptake of [^{14}C]Glc. Two additional experimental repeats showed very similar results, so representative data are shown here.

acetate to the assay mixture (data not shown). These results suggested that the immediate target for acetate is Glc uptake, not Glc catabolism, and that acetate-inhibition of Glc uptake was responsible for the observed inhibition of Glc utilization during the first growth phase of diauxic growth. We subsequently concentrated our study on understanding how acetate inhibited Glc uptake.

To demonstrate the acetate-mediated inhibition of Glc uptake, the characteristics of Glc and acetate uptake were initially examined. Using cells harvested at d 1 (first growth phase) and d 5, uptake rates of [^{14}C]Glc and [^{14}C]acetate were measured by plotting the amounts of the radioactivities taken up by cells against the time of incubation. Acetate was taken up actively in both growth phases (Fig. 3A), whereas Glc was taken up only in the second growth phase (Fig. 3B), indicating that acetate uptake was constitutive throughout the diauxic growth, but Glc uptake was induced only in the second growth phase.

To see how these uptakes were affected by the presence of the co-presenting carbon sources, uptake-inhibition kinetics of acetate and Glc were examined in the presence of Glc and acetate, respectively, in the assay mixtures (Fig. 4). In cells harvested at d 5, at which Glc uptake was active (Fig. 3B), the uptake of [^{14}C]acetate was measured at various concentrations of acetate in the presence and absence of 10 mM Glc (Fig. 4A). The kinetics were very similar regardless of the presence of Glc, indicating that acetate uptake was not inhibited by Glc. In a parallel experiment,

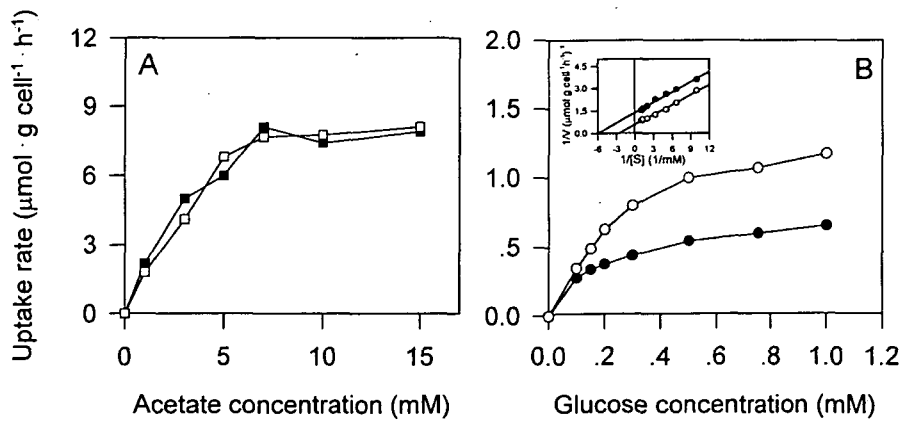

Fig. 4 Inhibition kinetics of Glc and acetate uptakes. Uptakes of [^{14}C]acetate and [^{14}C]Glc were determined in cells harvested at d 5 in the presence of 10 mMs of Glc and acetate, respectively, in the assay mixture. (A) Saturation kinetics of acetate uptake at various acetate concentrations in the absence (□) and presence (■) of 10 mM Glc. (B) Saturation kinetics for Glc at various Glc concentrations in the absence (○) and presence (●) of 10 mM acetate. In box, Lineweaver-Burk plot for the Glc uptake in the absence (○) and presence (●) of acetate. These experiments were repeated five times, and the results were very similar. Representative data are shown here.

Table 1 Influence of the proton gradient-affecting compounds on Glc uptake

	Glc uptake (% of control)
Control	100
5 μM CCCP	22.4 ± 6.0
10 μM DNP	21.8 ± 3.7
2 μM FC	153.65±13.0

Cells pregrown with Glc were incubated for 10 min in the presence of CCCP (5 μM), DNP (10 μM), and FC (2 μM). Glc uptake was then initiated by addition of [^{14}C]Glc, and the respective V_{max} values were determined. Taking that of the control (without the addition of the compounds) as 100, relative values are shown. The values are means±SE of three replicates.

acetate inhibition of Glc uptake was analysed by determining the kinetics of Glc uptake in the presence of acetate using cells harvested at d 5 (Fig. 4B). The Vmax of Glc uptake was significantly decreased in the presence of 10 mM acetate (from 1.75 to 0.72 μmol (g cell)$^{-1}$ h^{-1}), indicating that acetate effectively inhibited Glc uptake. The Lineweaver-Burk plot indicated that acetate inhibited Glc uptake in an noncompetitive manner characterized by decreases in both K_m and V_{max} values in the presence of an inhibitor (box in Fig. 4B). Both Glc and acetate uptakes appeared to be saturatable, suggesting that specific transporters are involved with the respective substrates. These kinetic data suggest that acetate does not directly compete with Glc for binding to the probable Glc transporter (competitive inhibition) and also that acetate does not function as an allosteric regulator for the Glc transporter (non-competitive inhibition). According to the determined noncompetitive inhibition, acetate inhibited Glc uptake in an indirect manner. In general, the kinetic data do not provide conclusive clues to the actual mechanism of inhibition of the transport system, because uptake systems are frequently linked to cellular metabolism. However, since acetate-mediated inhibition of Glc uptake appeared to be inde-

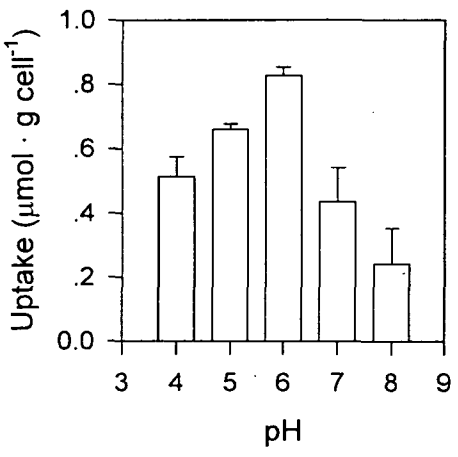

Fig. 5 pH-dependency of Glc uptake. Cells grown on 3% Glc were mixed with [^{14}C]Glc to measure the rate of Glc uptake (V_{max}). Uptake media were adjusted to pH 4.0, 5.0, 6.0, 7.0 and 8.0 with HCl or KOH. Bars represent means±SE of three replicates.

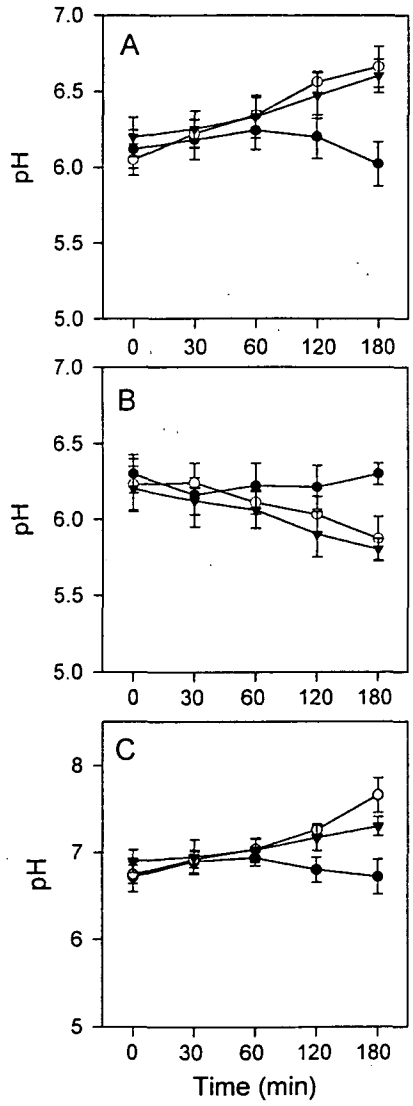

Fig. 6 Time-course of acetate and butylate effects on the external (pHe) and cytoplasmic pH (pHc). Plasma membrane vesicles prepared from d 5-cells and d 5-cells were transferred into fresh medium containing 10 mM Glc in the absence (●) or presence of acetate (○) or butylate (▼) at time zero. At the designated intervals, aliquots of the medium were withdrawn to determine pHe and pHc. Plasma membrane vesicles were equilibrated at pH 7 (inside) and suspended in growth medium (pH 6). Measurements were started after the addition of 10 mM weak acid. (A) Changes in pHe in cells. (B) Changes in pHc in cells. (C) Change in pHe in plasma membrane vesicles.

pendent of cellular metabolism, as shown in Fig. 1, the kinetic data in this study are likely to represent the actual mechanism involved. Directional inhibition of acetate over Glc uptake, not of Glc over acetate uptake (Fig. 4), further suggests that the Glc and acetate uptake systems operate independently and that acetate therefore inhibits Glc uptake indirectly.

Mechanism of acetate inhibition of Glc uptake— There have been a few reports in that Glc is co-transported into cells with H^+ in algae and plant systems (Komor 1973, Bush 1993). If such a Glc/H^+ symport also operates in carrot cells, it is logical to predict that acetate creates an unfavorable environment for Glc uptake. It is known that weak acids such as acetate and butyrate tend to change cytoplasmic (pHc) and external pH (pHe) in plant cells by taking external H^+ into cells (Reid et al. 1985, Felle et al. 1986). To confirm the probable H^+-dependence of Glc uptake, protonophores DNP and CCCP were used to monitor changes in Glc uptake (Table 1). In the presence of these compounds, uptake efficiency decreased by about 5 fold. In contrast, the fungal toxin FC, which binds to plasma membrane H^+-ATPase to stimulate H^+-extrusion into the medium (Meyer et al. 1993), enhanced Glc uptake by approximately 50% (Table 1). Glc uptake was also sensitive to pHe and was most active at pH 6 (Fig. 5). Changes in pHe and pHc were monitored in the absence and presence of acetate or butylate in both cells (Fig. 6A, B) and isolated plasma membrane vesicles (Fig. 6C) prepared from cells harvested at d 5. Results showed that presence of the Glc without the weak acids in medium caused transient alkalization followed by acidification in medium, suggesting that Glc was initially taken up with a proton, resulting in medium alkalization. It is likely that the subsequent acidification was caused by H^+-extrusion by H^+-ATPase present in plasma membrane. The observed changes in pHe were largely proportional to the concurrent changes in pHc (Fig. 6A, B), which was measured by the weak acid distribution method using DMO. These results indicated that these weak acids overwhelmed Glc in controlling the changes of pHe and pHc. In addition, Glc uptake was significantly inhibited by butylate in cells (Fig. 7A) or isolated plasma membranes (Fig. 7B), as it was by acetate (Fig. 2A, B). These results confirmed that these weak acids inhibited Glc uptake indirectly by altering pHe and/or pHc.

It was obvious that Glc/H^+ symport acidified the cytosol. In order for cells to maintain their normal pHc, they have to export protons through H^+-ATPase in plasma membrane (Palmgren 1998). It is generally accepted that the proton gradient across the plasma membrane plays a critical role in Glc uptake (Reinhold and Kaplan 1984). Komor (1973) reported that hexose transport in *Chlorella* was particularly sensitive to pHc. It is likely that active production of ATP from Glc catabolism in these cells allows H^+-ATPase to maintain an optimal proton gradient and in turn control Glc uptake. Despite the fact that cells did not grow actively with 10 mM acetate (data not shown), it was shown that they induced ICL, an acetate-metabolizing glyoxylate cycle enzyme, implying that the acidification of the cytosol imposed by acetate can be alleviated by its cellular metabolism. As indicated in Fig. 6A and C, pHe was continuously alkalized upon acetate addition, suggesting that H^+-ATPase was not properly operating. It is probable that operation of the glyoxylate cycle, bypassing several dehydrogenation steps in the citric acid cycle, does not favor ATP production, causing little stimulation of H^+-ATPase. It thus appears that this inadequate maintenance of proton gradient by acetate inhibited Glc uptake.

In summary, this study clearly showed that acetate-mediated changes in pHe and pHc were major factors in the delayed utilization of Glc during the first phase of diauxic growth. Our results strongly suggest that Glc uptake in most plant cells is inhibited by a co-presenting carbon

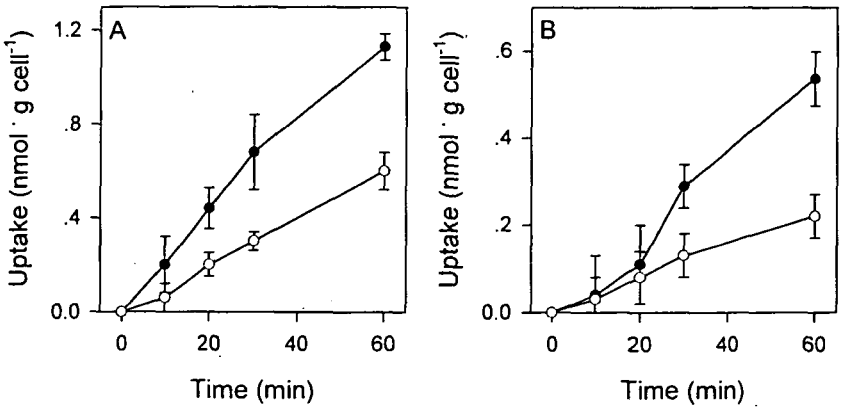

Fig. 7 Butylate-inhibition of Glc uptake in cells and plasma membrane vesicles. Cells harvested at d 5 were used to examine the uptake of [^{14}C]Glc in the absence (○) and presence (●) of 10 mM butylate. (A) Cells; (B) Plasma membrane vesicles.

source which can alter the proton gradient across the plasma membrane.

This work was supported by Faculty Research Fund, Sungkyunkwan University, 1998.

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(Received April 1, 1999; Accepted July 23, 1999)