Metabolic turnover analysis by a combination of in vivo $^{13}$C-labelling from $^{13}$CO$_2$ and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the C$_3$ photosynthetic pathway in Nicotiana tabacum leaves

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Abstract

Understanding of the control of metabolic pathways in plants requires direct measurement of the metabolic turnover rate. Sugar phosphate metabolism, including the Calvin cycle, is the primary pathway in C$_3$ photosynthesis, the dynamic status of which has not been assessed quantitatively in the leaves of higher plants. Since the flux of photosynthetic carbon metabolism is affected by the CO$_2$ fixation rate in leaves, a novel in vivo $^{13}$C-labelling system was developed with $^{13}$CO$_2$ for the kinetic determination of metabolic turnover that was the time-course of the $^{13}$C-labelling ratio in each metabolite. The system is equipped with a gas-exchange chamber that enables real-time monitoring of the CO$_2$ fixation rate and a freeze-clamp that excises a labelled leaf concurrently with quenching the metabolic reactions by liquid nitrogen within the photosynthesis chamber. Kinetic measurements were performed by detecting mass isotopomer abundance with capillary electrophoresis-tandem mass spectrometry. The multiple reaction monitoring method was optimized for the determination of each compound for sensitive detection because the amount of some sugar phosphates in plant cells is extremely small. Our analytical system enabled the in vivo turnover of sugar phosphates to be monitored in fresh tobacco (Nicotiana tabacum) leaves, which revealed that the turnover rate of glucose-1-phosphate (G1P) was significantly lower than that of other sugar phosphates, including glucose-6-phosphate (G6P). The pool size of G1P is 12 times lower than that of G6P. These results indicate that the conversion of G6P to G1P is one of the rate-limiting steps in the sugar phosphate pathway.

Key words: Capillary electrophoresis-tandem mass spectrometry (CE-MS/MS), $^{13}$CO$_2$, in vivo labelling, metabolic turnover, photosynthesis, sugar phosphate.

Introduction

To determine the limiting steps of photosynthesis and factors that influence carbon allocation, studies of photosynthetic CO$_2$ assimilation in plant leaves have focused on the regulation of carbohydrate metabolism. In higher plants, starch and sucrose, which are the major products of photosynthetic carbon metabolism, are biosynthesized from CO$_2$ via sugar phosphates and sugar nucleotides (Fig. 1). The biosynthetic pathway is regulated by metabolic reactions involving sugar nucleotides (Dennis and Blakeley, 2000; Zeeman et al., 2007); that is, ADP-glucose...
pyrophosphorylase, which converts glucose-1-phosphate (G1P) into ADP-glucose, regulates starch content, and sucrose phosphate synthase, which converts UDP-glucose into sucrose-6-phosphate, regulates sucrose content. However, much controversy exists over the rate-limiting step of the metabolic pathway from CO₂ to G1P (Fig. 1).

Mathematical modelling of the metabolic pathway has suggested the possibility that several enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase), and fructose-1,6-bisphosphate aldolase, control the flux of C₃ photosynthetic carbon metabolism (Giersch, 2000; Poolman et al., 2000; Zhu et al., 2007). On the other hand, modifications of the activities of these enzymes through genetic engineering have demonstrated the presence of other regulatory steps of carbon flow in plants (reviewed in Tamoi et al., 2005). For example, in antisense plants for plastidial fructose-1,6-bisphosphatase (FBPase) and SBPase, the rate of photosynthesis was significantly diminished in proportion to the decrease in the ribulose-1,5-bisphosphate (RuBP) regeneration capacity in the Calvin cycle (Koßmann et al., 1994; Harrison et al., 1998, 2001; Ölander et al., 2001). This suggests that the photosynthetic carbon flow is more sensitive to a decrease in FBPase and SBPase activity. Also, by using a decreased-activity mutant of phosphoglucone isomerase (PGI) that catalyses the reversible conversion of fructose-6-phosphate (F6P) to glucose-6-phosphate (G6P) in the cytosol and chloroplasts, Kruckeberg et al. (1989) showed that the chloroplast isoenzyme exerted control over the rate of starch synthesis in saturating light intensity and CO₂, but not at low light intensity. By contrast, in transgenic plants with reduced levels of glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase and aldolase had little effect on photosynthesis (Paul et al., 1995; Price et al., 1995; Haake et al., 1998). These data indicate that these enzymes are present at levels well in excess of that required to sustain a continued rate of CO₂ assimilation. Thus, estimates based on metabolic simulation have not necessarily been the same as the results of metabolic engineering. In mathematical modelling, enzyme kinetics is used as one of the parameters which is determined in vitro and does not always reflect the turnover of in vivo metabolites. On the other hand, the result of changes in the accumulation levels of major products such as hexose, sucrose, and starch in transgenic plants are also not enough to account for the dynamic metabolic flux.

When metabolism is in a dynamic steady-state, in vivo metabolites are replaced with newly synthesized compounds at a constant rate and the total amount remains unchanged. In order to discuss metabolic flux and the rate-limiting step in the pathway, direct measurement of metabolic turnover is required (defined as the change of the newly incorporated carbon to total carbon ratio in a metabolite with respect to time). Thus, the aim was to develop a system to analyse in vivo turnover of metabolic intermediates involved in the sugar phosphate pathway and in sucrose and starch synthesis quantitatively in higher plants.

So far, the labelling of metabolites using stable isotope tracing has been used for the determination of in vivo metabolic turnover by detection with mass spectrometry (MS) or nuclear magnetic resonance (NMR) (reviewed in

Fig. 1. The path of carbon in photosynthesis in a tobacco leaf. Abbreviations: ADP-Glc, adenosine-5′-diphosphate glucose; BPGA, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; R5P, ribose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; Ru5P, ribulose-5-phosphate; S6P, sucrose-6-phosphate; SBP, sedoheptulose-1,7-bisphosphate; UDP-Glc, uridine-5′-diphosphate glucose; Xu5P, xylulose-5-phosphate. Numbers in circles denote enzymes as follows: (1) RuBP carboxylase/oxygenase (Rubisco); (2) GAP dehydrogenase; (3) FBP aldolase; (4) fructose-1,6-bisphosphatase (FBPase); (5) SBP aldolase; (6) sedoheptulose-1,7-bisphosphatase (SBPase); (7) phosphoribulokinase; (8) phosphoglucone isomerase (PGI); (9) phosphoglucomutase (PGM); (10) ADP-glucose pyrophosphorylase; (11) sucrose phosphate synthase.
Schwender, 2008). Since MS detects ionized compounds separated by their mass to charge ratio (m/z), the m/z of 13C-labelled compounds is increased by an amount equal to the number of stable isotopes incorporated. Therefore, by determining the ratio of intensity of the monoisotopic ion and its isotopic ions, the ratio of stable isotope labelling can be quantified. As experimental materials for the labelling, tissue cultures such as tomato suspension cells, developing seeds of rapeseed, Catharanthus roseus hairy root, and potato tuber have the advantages that metabolites can be labelled from 13C-sugar under strictly controlled culture conditions (Schwender, 2008). However, for the analysis of biosynthetic pathways of the photosynthetic products, it is necessary to feed labelled CO2 as a sole carbon source.

In the case of labelling photosynthetic tissues such as leaves in higher plants, highly standardized photosynthesis conditions should be required for accurate kinetic measurements. Recently, Huege et al. (2007) labelled whole plants such as Arabidopsis thaliana and Oryza sativa with 13CO2 as the carbon source in an atmospherically controlled, air-tight growth chamber where the average 13C-enrichment ± standard deviation (SD) was 91.5±10.5% for shoots and 90.2±9.7% for roots of A. thaliana. Subsequently, the authors performed dynamic isotope dilution by unlabelled CO2 to determine the 13C half-life of soluble metabolites such as organic acids, amino acids, and sucrose using GC-MS. The authors succeeded in determining the metabolite and organ-specific 13C-half-life. Nevertheless, variability among experiments was observed for some compounds, which may have resulted from slight environmental changes.

Since the flux through the photosynthetic metabolic pathway is influenced by photosynthetic rate (Farquhar et al., 1980), factors affecting photosynthesis should be strictly controlled in 13C-labelling for accurate measurement of in vivo turnover. In the present study, in vivo labelling of tobacco (Nicotiana tabacum) leaves by 13CO2 was carried out in a gas-exchange chamber that enabled real-time monitoring of the CO2 assimilation rate. Thus, the photosynthetic environmental conditions such as light intensity, CO2 concentration, relative humidity, and temperature were controlled in the chamber. The experiment under different CO2 concentration would reveal the influence of photosynthesis rate on the turnover rate of intermediates in the photosynthetic metabolic pathway. Also, since metabolic intermediates involved in photosynthesis turn over extremely fast (Sharkey et al., 1986), the labelling time must be accurate. Thus, a freeze-clamp was used that excised a labelled segment concurrently with quenching the metabolic reaction by liquid nitrogen in the photosynthesis chamber.

Metabolic intermediates such as sugar phosphates and sugar nucleotides are highly polar molecules and are present as minor components in plant cells, which require efficient separation and a sensitive detection system for qualitative and quantitative analysis. Thus, an analytical system was developed that determines the 13C-labelling rate of these compounds in plant leaves by using capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) with multiple reaction monitoring (MRM) detection.

In the present study, a novel system was developed for dynamic metabolic analysis of the C3 photosynthetic pathway in leaves by the combination of in vivo 13C-labelling from 13CO2 and metabolic profiling with CE-MS/MS. The system enabled monitoring of the in vivo turnover of sugar phosphates, which revealed that the turnover rate of G6P was significantly lower than that of other sugar phosphates including G6P. This result indicates that the conversion of G6P into G1P would be a rate-limiting step in sugar phosphate metabolism.

**Materials and methods**

**Plant growth conditions**

Wild-type tobacco plants (Nicotiana tabacum L. cv. Xanthi) were grown at 16/8 h day/night cycles at 25 °C and 50–60% relative humidity. Photon flux density was 300 μmol photons m⁻² s⁻¹. Seedlings were kept in pots (0.7 dm³ plant⁻¹) containing Metro-Mix350 (Sun Gro Horticulture, British Columbia, Canada), and were watered daily. Plants were fertilized with 500-fold diluted Hyponex solution (Hyponex Japan, Osaka) three times a week. Tobacco plants at 6–8 weeks old were used for 13CO₂-feeding experiments.

**13CO₂-feeding experiment**

One fully expanded leaf was clamped in a 0.067 dm³ aluminium gas-exchange chamber equipped with a freeze-clamp (Kohshin Rigakaku Seisakusho, Tokyo, Japan) that had a glass window to admit light. A photon flux of 1000 μmol m⁻² s⁻¹ was provided by a PCX-UHX-150 light source (Nippon PL, Tokyo, Japan). The CO2 concentration of the air containing a 20% O2 stream was regulated using a gas blender (Ollie, Osaka, Japan) to mix nitrogen, CO2 (either 12CO2 or 13CO2) and oxygen from gas cylinders. The mixture of gases was saturated with water vapour at 16 °C using an LI-610 portable dew point generator (Li-Cor, Lincoln, NE, USA). The chamber temperature was controlled at 25 °C by a flow of water from an EL-15 refrigerated water bath (TATTEC, Saitama, Japan). The chamber contained sensors for chamber air and leaf temperature with a thermocouple probe and an NR-250 data collection system (Keyence, Osaka, Japan).

Net CO2 assimilation rates were measured using an LI-7100 infrared CO2/H2O gas analyser (Li-Cor) attached to the gas-exchange chamber. At the beginning of the experiment, the leaf was exposed to a 9.17×10⁻⁸ dm³ s⁻¹ flow of 20% O2 air containing 200 or 1000 ppm CO2. After 30 min of stabilization of photosynthesis, CO2 was switched to 99% 13CO2 (Taiyo Nippon Sanso, Tokyo, Japan) at the same level (200 or 1000 ppm). After 13CO₂-feeding, a leaf disc (diameter 30 mm) was immediately freeze-clamped by liquid nitrogen in the gas-exchange chamber. The leaf disc was exposed to liquid nitrogen for 10 s and stored at -80 °C prior to the extraction of metabolites in the disc. Since the infrared absorption spectrum of 13CO2 is different from that of 12CO2, the infrared gas analyser is much less sensitive to 13CO2 than to 12CO2. The chlorophyll fluorescence of each sample leaf was measured before and after gas switching with a MINI-PAM photosynthesis yield analyser (Heinz Walz GmbH, Effeltrich, Germany) attached to the chamber.

**Extraction of metabolites and measurement of 13C-labelled fractions**

The frozen leaf disc was homogenized by a Retsch ball-mill (Haan, Germany) at 20 Hz for 1 min. Hydrophilic tobacco metabolites were extracted using a solvent mixture of 500 μl of methanol and 20 μl of internal standard solution (100 μM of ribitol and PIPES).
by shaking at 37 °C for 5 min. After the addition of 500 μl of chloroform and 180 μl of water, the extracts were centrifuged at 10,000 g for 3 min. The supernatant was transferred to a new 1.5 ml tube, followed by the addition of 200 μl of water. After the mixture was centrifuged, the resultant polar phase was centrifugally filtered through an Ultrafree MC 5 kDa cutoff filter (Millipore, Billerica, MA, USA) at 10,000 g for 60 min. The filtrate was dried in a vacuum centrifugal concentrator. The residue was dissolved in 50 μl of water and analysed by CE-MS/MS.

All CE-MS/MS analyses were performed using a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) and 4000QTRAP hybrid triple quadrupole linear ion-trap mass spectrometer with Turbo V ion source and CE-MS kit (Applied Biosystems, Foster City, CA, USA). An MP-711 micro flow pump (GL Sciences, Tokyo, Japan) was used to deliver the sheath liquid. Syringe pump Model 11 plus (Harvard Apparatus, Holliston, MA, USA) was used for infusing standard solutions for optimization of MRM detection. 32 Karat software 1.4.1 (Applied Biosystems) controlled CE performance. MS/MS data acquisition, and data evaluation were controlled by Analyst software (Beckman Coulter) controlled CE-MS/MS. CE separations were carried out on a FunCap-CE type S capillary (GL Sciences). The capillary dimensions were 50 μm i.d. and 80 cm in length. The electrolyte for the CE separation was 50 mM ammonium acetate adjusted to pH 9.0 by the addition of ammonium hydroxide. Prior to the first use, each new capillary was washed with running electrolyte for 60 min with the application of 30 psi pressure. Before injection in each analysis, the capillary was pretreated with running electrolyte for 5 min by applying a pressure of 30 psi. The sample was injected at a pressure of 2.0 psi for 5.0 s (6 nl). The CE polarity was such that the electrospray volt (inlet) was at the anode, and the ESI probe (outlet) was at the cathode. The applied voltage to the CE capillary was set at 30 kV with 0.30 min of ramp time. Electrophoresis was performed for 0–12 min. After stopping the application of voltage to CE at 12 min, the electrolyte was delivered through the capillary at 4.5 psi with an air pump for between 12 min and 16 min. The capillary temperature was maintained at 20 °C. The sheath fluid, 5 mM ammonium formate in 50% (v/v) acetonitrile/water was delivered to the electrospray probe at a rate of 10 μl min⁻¹. ESI-MS/MS was conducted in the negative ion mode. Ion spray voltage was applied at −4.5 kV only after 1 min of voltage application to the CE had passed. The setting for CE parameters were: curtain gas, 15.0 psi, collision gas, 5.0, temperature, 0.0, ion source gas 1, 20.0 psi, ion source gas 2, 0.0 psi, entrance potential, −10.0 V. Q1 and Q3 resolution were set as unit and low, respectively. CE-ESI-MS/MS analyses were performed as described previously (Harada et al., 2008) except for the following conditions. All isotopomers of the analytes were quantified by MRM. Three types of runs of CE-ESI-MS/MS were performed per sample as shown in Table 1. The MRM parameters, Q1 (m/z of

### Table 1. Method settings for isotopomer monitoring

| Period | Analyte | Q1 (m/z) | Q3 (m/z) | DP (V) | CLE (V) | CXP (V) | Total number of MRM | Scan time |
|--------|---------|----------|----------|--------|---------|---------|---------------------|-----------|
| Run 1  | None    |          |          |        |         |         |                     |           |
| 1      | HP ²a   | 269.0, 260.0, 261.0, 262.0, 263.0, 264.0, 265.0 | 97.0 | −50 | −22 | −5 | 15 | 0.825 s |
| 2      | PiP²b   | 228.9, 229.9, 230.9, 231.9, 232.9, 233.9 | 97.1 | −55 | −18 | −5 | 14 | 0.880 s |
| 3      | Ribitol | 151.0    | 89.0     | −55 | −16 | −3 | 13 | 0.770 s |
| 4      | PIPES   | 301.0    | 193.1    | −85 | −38 | −13 | 14 | 0.770 s |
| Run 2  | None    |          |          |        |         |         |                     |           |
| 1      | Ribitol | 151.0    | 89.0     | −55 | −16 | −3 | 9 | 0.600 s |
| 2      | S7P     | 289.0, 290.0, 291.0, 292.0, 293.0, 294.0, 295.0, 296.0 | 97.0 | −55 | −30 | −5 | 16 | 0.880 s |
| 3      | TP ³α   | 168.9, 169.9, 170.9, 171.9 | 97.0 | −40 | −14 | −5 | 15 | 0.825 s |
| 4      | PIPES   | 301.0    | 193.1    | −85 | −38 | −13 | 15 | 0.825 s |
| Run 3  | None    |          |          |        |         |         |                     |           |
| 1      | Ribitol | 151.0    | 89.0     | −55 | −16 | −3 | 13 | 0.880 s |
| 2      | ADP-Glc | 588.1, 589.1, 590.1, 591.1, 592.1, 593.1, 594.1 | 346.1 | −95 | −34 | −3 | 16 | 0.880 s |
| 3      | UDP-Glc | 565.1, 566.1, 567.1, 568.1, 569.1, 570.1, 571.1 | 323.1 | −85 | −34 | −1 | 16 | 0.880 s |
| 4      | PIPES   | 301.0    | 193.1    | −85 | −38 | −13 | 16 | 0.880 s |

*²a Hexose monophosphate (G6P, F6P, G1P).
²b Pentose monophosphate (R5P, Ru5P/Xu5P).
³c Triose monophosphate (GAP, DHAP).
deprotonated precursor ion), Q3 (m/z of product ion), declustering potential (DP), collision energy (CLE), and collision cell exit potential (CXP) are listed in Table 1. The DP, CLE, and CXP of all isotopomers were set to the same values.

Calculation of metabolic turnover
Relative isotopomer abundance ($m_i$) for each metabolite in which $i^{13}$C atoms are incorporated is calculated by the following:

$$m_i(\%) = \frac{M_i}{\sum_{j=0}^{n} M_j} \times 100$$

where $M_i$ represents the isotopomer abundance for each metabolite in which $i$ $^{13}$C atoms are incorporated.

The $^{13}$C fraction of the metabolite possessing $n$ carbon atoms is calculated by the following:

$$^{13}$C fraction (\%) = \sum_{i=1}^{n} i \times m_i \div n$$

Metabolic turnover rate was calculated from the initial slope of the $^{13}$C-fraction versus time curve, i.e. the change in the $^{13}$C-fraction with respect to time from 0.5 min to 2 min because the highest value of correlation coefficient was shown at the time interval. In order to discuss the distribution of assimilated $^{13}$C, the carbon turnover rate was estimated by dividing the initial slope by carbon number.

Results

Construction of $^{13}$C-enrichment system
To analyse carbon metabolic turnover in fresh leaves, a system was developed for in vivo $^{13}$C-labelling from $^{13}$CO$_2$ as shown in Fig. 2. The photosynthesis rate of an individual leaf was monitored in real time with a gas-exchange technique with light intensity, CO$_2$ concentration, relative humidity, and chamber temperature controlled. At the beginning of the experiment, the leaf carried out photosynthesis with unlabelled CO$_2$. After 30 min of stabilization of photosynthesis, $^{13}$C-labelling was initiated by switching the CO$_2$ to $^{13}$CO$_2$ (Fig. 2B).

In photosynthesis, sugar phosphates turn over extremely fast. Theoretical calculations suggest that ribulose-1,5-bisphosphate (RuBP) pool turnover times of 0.5 s may occur [pool turnover rate (s)=$\text{pool size (}\mu\text{mol m}^{-2}\text{s}^{-1})/\text{assimilation rate (}\mu\text{mol m}^{-2}\text{s}^{-1})$] (Sharkey et al., 1986). Therefore, it is important to quench metabolic reactions punctually and quickly for accurate kinetic analysis. In our system, a liquid nitrogen reservoir was mounted on the photosynthesis chamber. Photosynthetic reactions were quenched by flowing liquid nitrogen into the chamber. The labelled segment of the leaf was simultaneously cut in the chamber. A cutter was inserted from an access plate lying on the undersurface of the chamber (Fig. 2A). A PAM photosynthesis yield analyser was also attached to the chamber. The quantum yield of photosystem II (\(\text{PSII}\)) of the tobacco leaf was not changed by switching gas from unlabelled CO$_2$ to $^{13}$CO$_2$ (data not shown), supporting the assumption that photosynthetic rate remains constant before and after labelling.

Determination of mass distribution of metabolites by CE-MS/MS
Sugar phosphates were separated by CE, and detected by MS/MS in MRM mode. The MRM method was optimized for the determination of each compound (Table 1) for the most sensitive detection because the amount of some sugar phosphates in plant cells is extremely small. Figure 3 shows the incorporation of $^{13}$C in RuBP in tobacco leaves at various time points after the initiation of $^{13}$C-labelling. The major precursor ion of RuBP was shifted from an unlabelled form (m/z 309) to the fully labelled form (m/z 314) within 10 min. Figure 4 shows the time-course of relative isotopomer abundance of each metabolite. Labelling started immediately after switching the gas to $^{13}$CO$_2$. The mass distribution of all metabolites determined was shifted to higher m/z with time. The most abundant isotopomer of phosphoglycerate (PGA), dihydroxyacetone-phosphate (DHAP), ribulose-5-phosphate (Ru5P), and sedoheptulose-7-phosphate (S7P), respectively, was shifted with time from $m_0$ (unlabelled form) to the fully labelled form in order of increasing incorporated $^{13}$C number. Although the position of the labelled carbon could not be demonstrated by MS, $^{13}$C seemed to be incorporated in turn. Our analytical system enabled kinetic determination of mass isotopomer distribution during photosynthetic dark
reactions occurring in a short time. The variability in three independent experiments was narrow.

Metabolic turnover analysis of sugar phosphates in fresh tobacco leaves

The ratio of $^{13}$C to total carbon in each metabolite, i.e. the $^{13}$C-fraction (%) was calculated from mass isotopomer distributions (Fig. 5). The $^{13}$C-fraction of sugar phosphates increased with time and reached a plateau at a maximum value within 5–10 min under 1000 ppm CO$_2$. The time-course for the fraction of C as $^{13}$C, i.e. the turnover rate of metabolites was determined under CO$_2$ concentrations of 1000 ppm and 200 ppm, which demonstrated an increase in the $^{13}$C-fraction with an increase in CO$_2$ concentration. The carbon turnover rate of UDP-glucose was lower than that of ADP-glucose, which was comparable to that of G1P. The rate of $^{13}$C-labelling depends on the pool size of each metabolite in the cells. As shown in Table 2, the level of G1P in tobacco leaves was 12 times less than that of G6P. These results suggest that the conversion of G6P into G1P is a rate-limiting step in the photosynthetic sugar biosynthesis pathway (Fig. 1).

Discussion

A novel system was developed for the determination of turnover rate during C$_3$ photosynthetic metabolism in the leaves of higher plants by the combination of an in vivo $^{13}$C-labelling technique with $^{13}$CO$_2$ and mass isotopomer analysis using a CE-MS/MS profiling method. As shown in Fig. 4, the system enabled monitoring of the de novo synthesis of photosynthetic carbon metabolites at the carbon atom level and quantitative assessment of the turnover rate (Fig. 6).

The $^{13}$C-labelling ratio of sugar phosphates was significantly higher than that of amino acids and organic acids. As for sugar phosphates involved in the Calvin cycle, a large fraction of carbons were labelled within 5 min after the initiation of $^{13}$C-labelling under 1000 ppm CO$_2$ conditions. However, the $^{13}$C-labelling ratio of G1P was only 41% at 5 min, which was a distinctly lower percentage than that of other sugar phosphates. The labelling ratio of G6P was 76% at the same time point. The pool size of G1P was one-twelfth that of G6P. The turnover rate of G1P was 38% of G6P under 1000 ppm CO$_2$ conditions (Fig. 6). Also, comparing the decay of $m_0$ for G1P and G6P showed a slower decay for G1P than G6P (Fig. 4). These results
indicate that conversion from G6P to G1P is a rate-limiting step of the sugar phosphate pathway. The conversion of G6P to G1P is catalysed by phosphoglucomutase (PGM) in plant cells (Periappuram et al., 2000), that are considered to play a role in the allocation of carbon between sucrose and starch in leaves based on the analysis of a Nicotiana sylvestris mutant and a transgenic potato deficient in plastidial PGM (Huber and Hanson, 1992; Lytovchenko et al., 2002a, b, 2005). However, the role of PGM in the control of carbon metabolic flux is not clear. PGM isoforms are present in high abundance in plant cells, and the reactions they catalyse are thought to operate close to equilibrium. Earlier ideas presented on metabolic regulation stated that near-equilibrium reactions could not exert much influence on the control of pathway flux (Rolleston, 1972). However, several recent reports have shown that other near-equilibrium reactions including plastidial aldolase (Haake et al., 1998) and transketolase (Henkes et al., 2001) can exert significant metabolic control in vivo. The expression of foreign PGM in the leaf cytosol or the repression of the activity of either cytosolic or plastidial PGM in potato leaves through genetic engineering led to alteration in the level of end-products such as starch and sucrose (Lytovchenko et al., 2002a, b, 2005). However, the altered amount of the products was not proportional to the change in PGM activity in transgenic plants, lacking a sufficient explanation for rate control by the PGM reaction. Our results support that PGM controls the metabolic flux of sugar biosynthetic pathway based on direct measurement of the turnover rate of both substrate (G6P) and product (G1P), although it is difficult to discriminate among subcellular localization. As shown in Fig. 6, the metabolic turnover rate of UDP-glucose was lower than that of ADP-glucose (Fig. 6). PGM localized in the cytosol may contribute more to the control of metabolic flux than plastidial PGM.

In our analytical system, the variability in $^{13}$C fraction (%) of metabolites is lower compared with the data in a previous report (Huege et al., 2007) (Fig. 5). Since mass isotopomer distribution would be unaffected by matrix
effects that inhibit analyte ionization in the ion source of the mass spectrometer, the turnover rate would be precisely determined. In Table 2, some metabolites showed variation in the concentration. Since the amount of some sugar phosphates and sugar nucleotides is extremely small, the resolution of the MS data that is sometimes acquired around the detection limits is not necessarily high (Cruz et al., 2008). However, in the present study, the metabolic turnover and carbon distribution during photosynthetic dark reactions occurring in a short time are discussed.

Although it is possible to observe rapid metabolic turnover in cells by using our analytical system, it is impossible to distinguish subcellular localization of metabolite pools. Practically, after quenching of metabolic reactions by liquid nitrogen, fractionation of organelle cannot be accomplished. Thus, the localization of rate-limiting steps could not be demonstrated. However, a possible distinction between compartments could be indicated. As shown in Fig. 4, while the decay of $m_0$ for the RuBP that exists only in plastids is fast, the decay of $m_0$ for other metabolites such as FBP, F6P, and R5P that exist in both plastids and cytosol shows at least a two-phase alteration. Multi-phase alteration of $m_0$ decay might result from the cellular compartmentation of metabolic reactions. Also, the $m_0$ decay of UDP-glucose changes from a low rate, like the $m_0$ decay of G1P, to a high rate, like that of G6P, 2 min after the start of $^{13}$C-labelling. The turnover of UDP-glucose might be related to the rate of conversion from G6P to G1P in the cytosol.

An increase in CO$_2$ concentration from 200 ppm to 1000 ppm led to a rise in the turnover rate of intermediate compounds (Fig. 6) as well as the photosynthesis rate, indicating that the increase in the ambient CO$_2$ led to the acceleration of carbon metabolic flux. The alterations in the turnover rate were different among compounds. For example, phosphoenolpyruvate (PEP) and G6P, respectively, showed 2.8- and 3.1-fold increases in carbon turnover on changing CO$_2$ concentration from 200 ppm to 1000 ppm, whereas DHAP, PGA, and G1P, respectively,
showed only 1.9-, 1.6-, and 1.8-fold increases. Therefore, the responsiveness of turnover to CO₂ concentration is different among the metabolic reactions. The turnover rate of G1P showed lower responsiveness to CO₂ concentration than that of G6P, which also supports the hypothesis of PGM being a rate-limiting step of photosynthetic sugar biosynthesis pathway.

Surprisingly, the turnover of PEP was as fast as that of sugar phosphates (Fig. 6). Phosphoenolpyruvate dehydrogenase (enolase) catalyses the conversion of PGA to PEP during glycolysis; its plastid isoform has not been found in Arabidopsis thaliana (Van der Straeten et al., 1991). PEP is a precursor of pyruvate, which is used for the biosynthesis of amino acids and organic acids via the tricarboxylic acid (TCA) cycle, and of fatty acids from acetyl-CoA. Also, PEP is a substrate of PEP carboxylase and PEP carboxykinase to produce oxaloacetate, which is concerned with CO₂ assimilation in C₄ photosynthesis. Therefore, it is of interest to analyse the turnover of metabolites that are downstream of PEP as a next step for research.

In order to discuss the distribution of assimilated ¹³C, carbon turnover rate was estimated by dividing the metabolic turnover rate by the number of carbons that constitute each metabolite. As shown in Fig. 6, the response of the carbon turnover rate to CO₂ assimilation rate is different among metabolites. The difference should be explained by the ¹³C distribution. Although it is not certain that all carbons of metabolites are equally replaced into ¹³C by turnover, the estimation of carbon turnover rate would enable us to consider the flux of carbon distribution. Thus, the ratio of the carbon turnover rate to the CO₂ assimilation rate (T/A ratio) under different CO₂ conditions was shown in Table 3. To estimate the T/A ratio, net photosynthesis rate was used because the rate of CO₂ evolution would be negligible under the condition of high light intensity (1000 µmol photons m⁻² s⁻¹) as described in a previous paper (Brooks and Farquhar, 1985). The T/A ratio is lower under 1000 ppm CO₂ than 200 ppm CO₂ conditions. The data indicate that the efficiency of the use of carbon from CO₂ for metabolism became lower at higher CO₂ concentration. On the basis of Farquhar’s photosynthesis models (Farquhar et al., 1980, 2001; Sharkey, 1985), the assimilation rate of CO₂ in leaves is limited by Rubisco activity under low CO₂, and is limited by RuBP regeneration, which is dependent on the energy (ATP and NADPH) supplied by electron transport in photosystem complexes at above ambient (380 ppm) CO₂ concentration. Therefore, the decrease in the carbon use efficiency under 1000 ppm CO₂ (Table 3) might be limited by the supply of the energy substrates.

As shown in Fig. 5, the ¹³C-fraction of metabolites did not reach 100% under the 1000 ppm CO₂ conditions that maximized the CO₂ assimilation rate of tobacco leaves. Another report found that, in leaves of Quercus rubra exposed to 35 Pa ¹³CO₂ under 500 µmol m⁻² s⁻¹ light conditions, the ¹³C-labelling ratio of PGA finally reached 84% after 18 min of ¹³C-treatment (Delwiche and Sharkey, 1993). Although the cause of peaking below 100% is still unknown, one of the likely explanations is that newly assimilated carbon competes with carbon derived from internal stores and an equilibrium phase is reached after the initial linear enrichment phase. Although transitory starch degradation could be regulated by light, circadian rhythms, or carbon balance, under photorespiratory conditions, transitory starch breakdown occurs in the light and G6P is elevated (Weise et al., 2006). G6P is the product of phosphorolytic breakdown of transitory starch, which may be used to regenerate

| Table 2. Comparison of metabolite pools in tobacco leaves |
|----------------------------------------------------------|
| The value for Ru5P/Xu5P represents the sum of Ru5P and Xu5P content. Values are averages from measurements of three different tobacco plants, ±SEM. Leaf discs were exposed to an air stream containing 1000 ppm CO₂ for 30 min in a gas exchange chamber before sampling. |
| Metabolite | Concentration (µmol m⁻²) |
|------------|--------------------------|
| PGA        | 3.5±0.8                  |
| DHAP       | 10.5±6.6                 |
| PEP        | 1.0±0.2                  |
| FBP        | 0.6±0.0                  |
| F6P        | 16.8±1.1                 |
| SBP        | 0.1±0.0                  |
| S7P        | 70.5±3.3                 |
| RSP        | 4.5±4.3                  |
| Ru5P/Xu5P  | 14.1±8.1                 |
| RuBP       | 10.0±1.4                 |
| G6P        | 31.0±19.0                |
| G1P        | 2.5±1.7                  |
| ADP-Glc    | 0.9±0.2                  |
| UDP-Glc    | 9.3±2.5                  |

| Table 3. Ratio of carbon turnover rate to CO₂ assimilation rate under different CO₂ conditions |
|---------------------------------------------------------------------------------------------|
| Values are averages from measurements of three different tobacco plants, ±SEM. |
| Compound | T/A ratio (carbon turnover/CO₂ assimilation) | Rate of decrease in T/A ratio |
|---------|-------------------------------------------|-----------------------------|
|         | Cₐ=200 ppm (A=4.5±1.3 µmol m⁻² s⁻¹) | Cₐ=1000 ppm (A=18.3±2.5 µmol m⁻² s⁻¹) |
| PGA     | 1.08±0.07 | 0.52±0.01 | 0.48 |
| DHAP    | 1.20±0.11 | 0.47±0.02 | 0.39 |
| PEP     | 0.76±0.07 | 0.52±0.02 | 0.68 |
| FBP     | 0.43±0.07 | 0.21±0.03 | 0.50 |
| F6P     | 0.37±0.02 | 0.21±0.02 | 0.55 |
| SBP     | 0.42±0.09 | 0.22±0.02 | 0.51 |
| S7P     | 0.41±0.01 | 0.27±0.00 | 0.65 |
| RSP     | 0.50±0.05 | 0.27±0.04 | 0.55 |
| Ru5P/Xu5P | 0.49±0.04 | 0.26±0.03 | 0.52 |
| RuBP    | 0.52±0.01 | 0.34±0.00 | 0.65 |
| G6P     | 0.22±0.01 | 0.17±0.04 | 0.77 |
| G1P     | 0.14±0.05 | 0.08±0.00 | 0.46 |
| ADP-Glc | 0.37±0.03 | 0.21±0.07 | 0.55 |
| UDP-Glc | 0.09±0.00 | 0.06±0.03 | 0.69 |
Calvin cycle intermediates. Sucrose cleavage is catalysed by invertase (sucrose+H$_2$O $\rightarrow$ glucose+fructose) or sucrose synthase (sucrose+UDP $\rightarrow$ fructose+UDP-glucose) (Baroja-Fernández et al., 2001; Koch, 2004). Glucose and fructose could be phosphorylated to produce G6P and F6P, respectively by hexokinase. UDP-glucose could be a substrate of UDP-glucose pyrophosphorylase to produce UTP and G1P. However, it remains to be seen whether degradation products of storage saccharides flow in the opposite direction to carbon assimilation in leaves under photosynthetic conditions. Another possibility for the plateau in the $^{13}$C-labelling ratio is intracellular compartmental flux. In plant cells, most metabolites are present in two or more pools, which exhibit slow exchange rates. One of these pools would be required to be metabolically inert, for example, the vacuole or possibly the apoplast. Although Winter et al. (1994) estimated the subcellular metabolite concentrations in spinach leaves based on the measurement of the volume of cellular and subcellular compartments, the concentration of sugar phosphate was much lower in the vacuole than in both the stroma and the cytosol. Another explanation for lower than 99% labelling is that not all cells are equally photosynthetically active. The leaf is a mixture of many different cell types since some may be old and senescent and act as storage cells. Certain cell types would not be metabolically active. However, in the present study, when extending the labelling time to 36 h, the $^{13}$C-labelling ratio of metabolites still did not reach 100%.

So far, metabolic profiling techniques (Fiehn et al., 2000; Roessner et al., 2000; Sato et al., 2004; Cruz et al., 2008; Harada et al., 2008) have enabled the analysis of a large number of metabolites, including minor intermediates, simultaneously by using MS, which has high sensitivity, high mass resolution, and high scan speed. However, the information available from this technique is a snapshot at the time of sampling the metabolites. To estimate dynamic metabolic flux, a combination with in vivo labelling by means of a stable isotope is required. Thus, a novel $^{13}$C-enrichment system was developed with $^{13}$CO$_2$ to analyse the metabolic flux of the C$_3$ photosynthetic pathway. Detection of mass isotopomer abundance by CE-MS/MS enabled quantification of the metabolic turnover rate of minor intermediates such as sugar phosphate and sugar nucleotides in tobacco leaves for the first time. Our analytical system would also be useful for the elucidation of the mechanism of metabolic responses to stress and other alterations in ambient conditions such as temperature and CO$_2$ influx as well as for the identification of rate-limiting steps in metabolic pathways. In aiming to produce useful compounds in transgenic plants through metabolic engineering, the enhancement of the activity of a rate-limiting enzyme in a pathway could be an efficient means to increase the metabolic flux of target compounds. Plants assimilate CO$_2$ as a carbon source and produce a large number of substances. Starch, sucrose, and cellulose are major forms of biomass. Among secondary metabolites such as fatty acids, isoprenoids, and flavonoids, there are a number of compounds used as industrial products. This study’s approach should provide a powerful tool for the evaluation of the carbon allocation and turnover of carbon metabolism in higher plants.

### Supplementary data

Supplementary data are available at JXB online.

**Supplementary Fig. S1.** Time-course of $^{13}$C fraction of amino acids and organic acids in tobacco leaves.

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