



Title	Developmental changes of plant affecting primary photosynthate distribution in rice leaves
Author(s)	Shinano, T.; Ando, K.; Okazaki, K. et al.
Citation	Photosynthetica, 44(4), 591-598 <a href="https://doi.org/10.1007/s11099-006-0076-8">https://doi.org/10.1007/s11099-006-0076-8</a>
Issue Date	2006
Doc URL	<a href="https://hdl.handle.net/2115/17170">https://hdl.handle.net/2115/17170</a>
Rights	The original publication is available at <a href="http://www.springerlink.com">www.springerlink.com</a>
Type	journal article
File Information	PH44-4.pdf



## Developmental changes of plant on primary photosynthate distribution in rice leaves

Takuro Shinano<sup>\*</sup>, Kaori Ando<sup>\*\*</sup>, Keiki Okazaki<sup>\*\*\*</sup>, and Mitsuru Osaki<sup>\*\*</sup>

*Creative Research Initiative “Sousei”, Hokkaido University, Sapporo 001-0021, Japan<sup>\*</sup>*

*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan<sup>\*\*</sup>*

*National Agricultural Research Center for Hokkaido Region, Sapporo 062-8555, Japan<sup>\*\*\*</sup>*

### Abstract

Developmental changes of plant in the regulation of photosynthate distribution of leaves were studied in hydroponically cultivated rice by the  $^{14}\text{CO}_2$  tracer technique and analysis of the activity of the regulatory enzymes, sucrose phosphate synthase (SPS), phosphoenolpyruvate carboxylase (PEPC), and pyruvate kinase (PK). The distribution of primary photosynthates to sugars, amino acids, organic acids, sugar phosphates, proteins, and polysaccharides was determined by column chromatography. The relative primary photosynthate distribution to the sugar phosphate fraction was significantly larger in the 5<sup>th</sup> than in the 6<sup>th</sup> leaf. Correspondingly, the  $V_{\max}$  of PEPC was significantly higher in the 5<sup>th</sup> than in the 6<sup>th</sup> leaf, while no significant differences between leaves were detected in the other enzymes. As a consequence, the ratio of the  $V_{\max}$  of SPS and PEPC was lower in the 5<sup>th</sup> than in the 6<sup>th</sup> leaf. As the 5<sup>th</sup> leaf develops before panicle initiation in rice, it predominantly supports vegetative growth, while the 6<sup>th</sup> leaf develops after panicle initiation and thus contributes mainly to reproductive growth. We conclude that the physiological properties of each leaf are regulated developmentally. When the 6<sup>th</sup> leaf became fully expanded (corresponding to the panicle initiation stage of plant), the distribution pattern of  $^{14}\text{C}$  was transiently changed in the 5<sup>th</sup> leaf, indicating that individual organs that are mainly involved in vegetative development are affected to some extent by the whole-plant-level physiological transformation that occurs at the transition from the vegetative to the reproductive stage.

*Additional key words:*  $^{14}\text{CO}_2$ ; developmental change; leaf position; *Oryza sativa*; phosphoenolpyruvate carboxylase; primary photosynthate; sucrose phosphate synthase.

*Abbreviations:* PEPC - phosphoenolpyruvate carboxylase; SPS - sucrose phosphate synthase; PK - pyruvate kinase; UDP-Glc - uridine 5'-diphosphoglucose; PMSF - phenyl methyl sulfonyl fluoride; EDTA - ethylene diamine tetra-acetic acid; PVPP - polyvinylpyrrolidone; F6P -

fructose 6-phosphate; G6P - glucose 6-phosphate; DTT - dithiothreitol; MDH - malate dehydrogenase; LDH - lactate dehydrogenase; PEP - phosphoenolpyruvate; DTE - dithioerythritol; BSA - bovine serum albumin.

*Acknowledgement:* We used the Radioisotope Laboratory of the Graduate School of Agriculture, Hokkaido University. This research is supported in part by a Grant-in-Aid for Scientific Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan (17380042).

## **Introduction**

Rice plants change their physiological status dramatically when proceeding from the vegetative growth stage to the flowering and maturation stage (Osaki *et al.* 1988, 1991). In particular, nitrogenous compounds (proteins) are vigorously synthesized during the phase of vegetative growth, while saccharides (starch) are predominantly produced during maturation time. Nakamura *et al.* (1997) demonstrated that the distribution of photosynthetically assimilated  $^{14}\text{CO}_2$  differed between the vegetative and flowering stages; the proportion of  $^{14}\text{C}$  incorporated into sugars and starch was higher in the flowering period. On the other hand, different leaves are known to have different physiological roles; for instance, older leaves from the base of the rice stem tend to translocate saccharides to roots while almost all of the photoassimilates from the flag leaf are transferred to the panicle (Tanaka 1961). Generally, there is a strong linkage between leaf physiological status and the developmental stage of the rice plant. Leaves 1 to 5 are formed in the seed and are photosynthetically active during the vegetative growth stage. Leaves 6 to 9 are formed at the vegetative growth stage but function as photoassimilate sources during the flowering stage, while leaves at position 10 to 12 are formed at the flowering stage and produce photoassimilates during the maturation stage.

Mineral nutrient contents change with ontogenetic leaf development. The ratio between the contents of nitrogen and phosphorus in leaves changes greatly during the transition from the vegetative to the flowering and maturation stage irrespective of leaf position in rice and maize, but not in the dicot crops soybean, adzuki bean, potato, and sugar beet (Osaki 1995). Thus, the physiological status of gramineous leaves appears to change in accordance with the demands of carbon and nitrogen at different growth stages.

Many enzymes involved in the metabolic regulation of photosynthate distribution such as phosphoenolpyruvate carboxylase (PEPC) are known to regulate carbon flow from triose phosphates to the pools of organic acids and amino acids (Champigny and Foyer 1992, Quy *et al.* 1991, Quy and Champigny 1992, Foyer *et al.* 1994). Sucrose phosphate synthase

(SPS) regulates the carbon flow for the synthesis of sucrose (Worrel *et al.* 1991, Signora *et al.* 1998, Murchie *et al.* 1999). The expression of pyruvate kinase (PK) was decreased by enhanced nitrate contents, but increased when glutamic acid concentrations were depleted (Sheible *et al.* 1997), indicating that the regulation by PK of the carbon flow from glycolysis to the TCA cycle is itself controlled by the nitrogen content in the cell.

SPS and PEPC appear to be regulated in a similar manner. Introduction of exogenous SPS into plants leads to an increase of the ratio of sucrose to starch in rice (Takahashi *et al.* 2000), and to an enhanced dry matter accumulation in the reproductive organs of tomato (Laporte *et al.* 1997, 2001) and *Arabidopsis* (Signora *et al.* 1998). Thus, changes in the activity of SPS will directly affect carbon flow not only in leaves but also on the whole plant level. While SPS activity is down-regulated by nitrate (Quy *et al.* 1991, Quy and Champigny 1992, Foyer *et al.* 1994), PEPC is activated by PEPC protein kinase (Duff and Chollet 1995, Li *et al.* 1996) which is more active under high nitrate conditions (Quy and Champigny 1992, Mahn *et al.* 1993, Duff and Chollet 1995), probably due to increased gene expression (Mahn *et al.* 1993).

In our previous paper (Shinano *et al.* 2006), we have analyzed the effects of nitrogen nutrition on the contents of SPS and PEPC. We found that the leaf nitrogen status contributed little to the regulation of the distribution of  $^{14}\text{C}$  to primary photosynthate compounds, while the developmental status of plant on the leaf appeared to be a more decisive determinant in the regulation of the distribution of photoassimilates into the carbon or nitrogen pool. As sugar phosphates are the major intermediates in the biosynthesis of photosynthesis-derived compounds, they need to be analyzed in detail to allow an identification of regulatory anabolic steps. In the present study, we evaluated whether the demand from sinks can alter the physiological status of leaves of rice, through the analysis of  $^{14}\text{C}$  distribution patterns and SPS, PK, and PEPC enzyme activities. We especially focused on differences between leaves active during the vegetative growth stage and during the flowering stage.

## Materials and methods

**Plant material:** Rice (*Oryza sativa* L. cv. Michikogane) seeds were sown on 13<sup>th</sup> June and transplanted to 56 l vats in batches of 28 on 2<sup>nd</sup> July when the plants had reached their 3<sup>rd</sup> leaf stage; they were then kept in a greenhouse of Hokkaido University. The average day length in July is 15 hrs and average day/night temperature in the greenhouse was 24 °C/18 °C. The vats contained a nutrient solution made up of N ( $\text{NH}_4\text{NO}_3$ ) 30 mg dm<sup>-3</sup>, P ( $\text{NaH}_2\text{PO}_4$ ) 2 mg dm<sup>-3</sup>, K ( $\text{K}_2\text{SO}_4$  : KCl = 1:1) 30 mg dm<sup>-3</sup>, Ca ( $\text{CaCl}_2$ ) 50 mg dm<sup>-3</sup>, Mg ( $\text{MgSO}_4$ ) 20 mg dm<sup>-3</sup>, and trace elements including Fe, Mn, B, Zn, Cu, and Mo at concentrations of 2, 0.5, 0.5, 0.2, 0.01, and

0.005 mg dm<sup>-3</sup>, respectively. The pH of the solution was adjusted to 5.0 daily at 07:00 and 16:00, and the solution was renewed every week. Plants were sampled on 13<sup>th</sup> July (5<sup>th</sup> leaf stage), 16<sup>th</sup> July (6<sup>th</sup> leaf stage), 21<sup>st</sup> July (7<sup>th</sup> leaf stage), and 26<sup>th</sup> July.

Before sampling, whole plants were allowed to assimilate <sup>14</sup>CO<sub>2</sub>. Each plant was covered with a clear polyethylene bag (10 l) filled with air. <sup>14</sup>CO<sub>2</sub> was liberated by mixing 1 cm<sup>3</sup> 0.18 mM NaHCO<sub>3</sub>, 0.74 MBq NaH<sup>14</sup>CO<sub>3</sub>, and 1 cm<sup>3</sup> of 30 % HClO<sub>4</sub> within the bag. The ambient CO<sub>2</sub> concentration was around 0.036 % while the content of CO<sub>2</sub> generated was about 5 × 10<sup>-7</sup> %; thus, no significant CO<sub>2</sub> enrichment occurred in this experiment. After 5 min exposure to <sup>14</sup>CO<sub>2</sub> under natural light conditions (in excess of 1000 μmol photon m<sup>-2</sup>s<sup>-1</sup>; the experiments were performed between 10:00 and 12:00), leaves were removed, frozen in liquid nitrogen, and then lyophilized and stored at -80 °C for subsequent analysis. For enzyme analysis, leaves were collected between 10:00 and 12:00, and samples of leaf tissue (0.2 g) were frozen in liquid nitrogen and stored at -80 °C for analysis. The remaining leaf tissue was dried in an air-forced oven for 72 h at 80 °C for mineral analysis.

**Nitrogen content:** Dried samples were digested with sulfuric acid and hydrogen peroxide (Mizuno and Minami 1980). Total N was measured by the semi-micro Kjeldahl method (Hind 1993).

**Extraction and fractionation of photosynthates:** Two-step leaf extractions were carried out using methanol, chloroform, and water (12/5/3, v/v/v) in the first step, and 0.2 mM formic acid in 20 % ethanol in the second. The combined water-soluble supernatant was separated into organic acid, amino acid, sugar, and phosphate ester fractions by ion-exchange chromatography using *SP Sephadex C-25 (cation resin, Amersham Biosciences, Piscataway, NJ, USA)* and *QAE Sephadex A-25 (anion resin, Amersham Biosciences)* as described by Redgwell (1980). To measure the amounts of amino acids in proteins, the insoluble fraction (residue after extraction) was hydrolyzed and purified through SP Sephadex C-25 according to the method of Shinano et al. (1994). The residual fraction (= total <sup>14</sup>C – soluble <sup>14</sup>C – protein <sup>14</sup>C) was assumed to represent the starch fraction because assimilated <sup>14</sup>C in residual fraction is known to become incorporated into starch within minutes after exposure. The radioactivity of each fraction was determined by liquid scintillation counting (*Aloka, Liquid Scintillation Counter, LSC-5100, Tokyo, Japan*).

**Fractionation of sugar phosphates:** The sugar phosphate fraction was further fractionated by paper chromatography using *Whatman 31 ETCHR paper (Whatman, Kent, UK)*. The media

were 2-Propanol/ammonia/water, 6/2/1 v/v/v (first dimension) and n-propyl acetate/formic acid (90%)/water, 11/5/3 v/v/v (second dimension). The distribution pattern of radioactivity on the chromatogram was visualized using an imaging plate (*BAS-MP, Fuji Film, Tokyo, Japan*) and a *Bio Imaging Analyzer (BAS-1000, Fuji Film)*.

**Enzyme extraction:** For the analysis of SPS and PEPC activity, frozen leaf samples (0.2 g) were homogenized in 0.8 cm<sup>3</sup> extraction buffer containing 100 mM Tris-HCl pH 7.5, 5 % (m/v) glycerol, 5% (m/v) ethylene glycol, 5 mM sodium phosphate, 50 mM NaF, 10 mM EDTA, 14 mM 2-mercaptoethanol, 2 mM benzamidine-HCl, 1 mM PMSF, 10 μM leupeptin, 16 μM chymostatin, 1 μM microcystin-LR, and 5 % (m/v) PVPP. After centrifugation at 17000 g for 5 min at 4 °C, the supernatant was collected as a crude extract. For the determination of SPS activity, 100 mm<sup>3</sup> of the supernatant was desalted on a *Sephadex G-25* column equilibrated with 50 mM MOPS-NaOH pH 7.5, 10 mM MgCl<sub>2</sub>, 1mM EDTA, 25 mM DTT, 10 % glycerol, and 0.1 % (m/v) PMSF. Then the eluate was frozen in liquid nitrogen and stored at -80 °C until analysis. For the determination of the activated state of PEPC, 150 mm<sup>3</sup> of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution were mixed well with 100 mm<sup>3</sup> of the supernatant, placed on the ice for 10 min, frozen in liquid nitrogen, and stored at -80 °C until analysis. For the quantification of maximum activities of PEPC and PK, 100 mm<sup>3</sup> of the untreated supernatant were frozen in liquid nitrogen and stored at -80 °C for analysis.

**Enzyme activities:**  $V_{\max}$  and  $V_{\text{limit}}$  of SPS were determined based on Huber *et al.* (1989). After centrifugation of the desalted supernatant described above at 17000 g for 5 min at 4 °C, 25 mm<sup>3</sup> of the supernatant were added to 45 mm<sup>3</sup> reaction buffer ( $V_{\max}$ : 50 mM Mops-NaOH (pH7.5), 15 mM MgCl<sub>2</sub>, 2.5 mM DTT, 10 mM F6P, 40 mM G6P, 10 mM UDP-Glc; and  $V_{\text{limit}}$ : 50 mM MOPS-NaOH (pH7.5), 15 mM MgCl<sub>2</sub>, 2.5 mM DTT, 3 mM F6P, 12 mM G6P, 10 mM UDP-Glc, 10 mM Pi); the mixture was incubated at 37 °C for 15 min. After the reaction had been stopped by the addition of 30 % KOH, the solution was kept on a boiling water-bath for 10 min. After cooling on ice, 1 cm<sup>3</sup> of a 0.14 % (m/v) anthron/H<sub>2</sub>SO<sub>4</sub> solution was added and the reaction was allowed to proceed for 20 min at 40 °C. A sample to which anthron/H<sub>2</sub>SO<sub>4</sub> had been applied before the incubation at 37 °C served as a control. The absorbance at 620 nm was monitored by spectrophotometry (*Shimadzu UV-1600, Tokyo, Japan*); one unit of activity was defined as the amount of protein which produced 1 mol sucrose 6-phosphate in 1 min.

To measure the maximum activities of PK and PEPC, the untreated supernatant of the centrifuged crude extract was used. PK  $V_{\max}$  activity was determined according to Plaxton (1998). Thirty mm<sup>3</sup> of the supernatant were added to 928 mm<sup>3</sup> of a solution containing 50 mM

Hepes-NaOH (pH6.9), 10 mM MgCl<sub>2</sub>, 2 mM DTE, 0.15 mM NADH, 0.2 mg/cm<sup>3</sup> BSA, 2 units of LDH, and 20 mm<sup>3</sup> 100 mM ADP. The reaction was started by adding 20 mm<sup>3</sup> of 100 mM PEP, and the absorbance at 340 nm was followed at 30 °C for 15 min. PEPC V<sub>max</sub>, 25 mm<sup>3</sup> of the supernatant were combined with 908 mm<sup>3</sup> of a solution containing 100 mM HEPES-NaOH (pH 7.3), 10 mM MgCl<sub>2</sub>, 1 mM NaHCO<sub>3</sub>, 0.2 mM NADH, 2 units of MDH, and 25 mm<sup>3</sup> 200 mM G6P. The reaction was started by adding 40 mm<sup>3</sup> 100 mM PEP, and the absorbance at 340 nm was monitored at 30 °C for 15 min.

To determine the PEPC activation state, the crude extract combined with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was centrifuged at 17000 g for 5 min at 4 °C, and the supernatant was discarded. The pellet was resuspended with 100 mm<sup>3</sup> buffer (50 mM Hepes-NaOH (pH7.5), 5 % (v/v) glycerol, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 14 mM 2-mercaptoetanol, 1 mM PMSF, 16 μM chymostatin) and centrifuged at 17000 g for 10 min at 4 °C. Twenty mm<sup>3</sup> of the supernatant were added to 914 mm<sup>3</sup> of a solution containing 100 mM HEPES-NaOH (pH 7.3), 10 mM MgCl<sub>2</sub>, 1 mM NaHCO<sub>3</sub>, 0.2 mM NADH, 2 units of MDH, and the PEPC activation state was assayed by comparing the activity of PEPC in the presence or absence of 40 mM malate (20 mm<sup>3</sup>). The reaction was started by adding 40 mm<sup>3</sup> 50 mM PEP, and monitored by following the absorbance at 340 nm for 15 min.

Total soluble protein was measured by the Bradford method (Bradford 1976), using BSA as a standard.

## Results

**Growth and nitrogen nutrition:** Panicle initiation started when the 6<sup>th</sup> leaf was fully expanded, and thus we assume that photosynthesis in the 5<sup>th</sup> leaf contributed mainly to vegetative growth while the 6<sup>th</sup> and younger leaves predominately fuelled reproductive growth. Leaf nitrogen contents were similar in all leaves and growth stages examined (Fig. 1).

**Primary photosynthate distribution:** A general linear model (GLM) analysis was performed to find differences in the distribution patterns of different groups of photosynthesis products in leaves at different positions. There were significant differences between the 5<sup>th</sup> leaf on one hand the 6<sup>th</sup> and 7<sup>th</sup> leaf on the other in the sugar fraction, between the 5<sup>th</sup> and 6<sup>th</sup> leaf in the sugar phosphate fraction, and between the 7<sup>th</sup> and the other leaves in the organic acids fraction. From the 5<sup>th</sup> leaf fully expanded stage to the 7<sup>th</sup> leaf fully expanded stage, the distribution of assimilated <sup>14</sup>C to the sugar phosphate fraction was transiently increased by a factor of two in the 5<sup>th</sup> leaf (Fig. 2).

In sugar phosphate fraction, the major compounds labeled with  $^{14}\text{C}$  were 3PGA followed by hexose 6-phosphate and F1,6-BP (Fig. 3). The distribution ratio to each sugar phosphate compound was higher in the 5<sup>th</sup> than in the 6<sup>th</sup> leaf, and it was highest when the 6<sup>th</sup> leaf had fully expanded; thereafter it gradually decreased.

#### **Enzyme activities:**

**SPS activity:** Neither SPS  $V_{\max}$  and SPS  $V_{\text{limit}}$  nor the ratio of the two parameters were significantly different between leaves (Fig. 4). Similarly, no significant difference between leaves was detected by GLM analysis.

**PEPC activity:** PEPC  $V_{\max}$  generally increased during the time of observation but dropped again in the 5<sup>th</sup> leaf after the 7<sup>th</sup> leaf was fully expanded (Fig. 5). The PEPC inhibition ratio appeared constant over time (Fig. 5). Nevertheless, a significant difference in PEPC inhibition rate between the 5<sup>th</sup> and 6<sup>th</sup> leaf was found by GLM analysis.

**PK activity:** PK  $V_{\max}$  was more or less constant over time and similar in all leaves examined (Fig. 6). GLM analysis did not reveal significant differences between leaves.

#### **Discussion**

The carbon flow from photosynthesis has been proposed to be directed by SPS into the carbon pool (sugars and starch; Worrel *et al.* 1991, Signora *et al.* 1998, Murchie *et al.* 1999) or, alternatively, by PEPC into the nitrogen pool (amino acids and proteins; Champigny and Foyer 1992, Foyer *et al.* 1994, Huber *et al.* 1994, Stitt 1999). We have corroborated this idea by an analysis of photosynthate distribution in rice leaves at different levels of nitrogen nutrition (Shinano *et al.* 2006). In rice, the physiological function of each leaf changes during the course of plant development; leaves formed early in the phase of vegetative growth of plant support the development of younger leaves and the root system, while leaves formed towards the end of the vegetative phase of plant tend to translocate almost all of their assimilated carbon to the reproductive organs (Osaki *et al.* 1988, 1991, Tanaka 1961). We hypothesized that the regulation of carbon flow by the relative activities of SPS and PEPC might correspond to these differences in leaf function. Therefore, we analyzed  $^{14}\text{C}$  distribution in leaves after  $^{14}\text{CO}_2$  assimilation, and the activities of SPS and PEPC activities in different leaves and growth stages of plant. Plant growth stage-dependent effects on the distribution pattern of primary photosynthates were observed only transiently at the panicle initiation stage (6<sup>th</sup> leaf fully

expanded) in the 5<sup>th</sup> leaf, suggesting that the physiological characteristics of a leaf are determined by leaf position and therefore probably are genetically rather than environmentally regulated. When mineral nutrient status of leaves were evaluated (Osaki 1995), and it was demonstrated that nitrogen contents were regulated in dependence of the developmental stage rather than of environmental factors. The regulation by developmental stage seems to be occurred in the primary photosynthate distribution also. Because the GLM analysis revealed a significant difference in the distribution of primary photosynthates to the sugar phosphate pool between the 5<sup>th</sup> and 6<sup>th</sup> leaf. As panicles are initiated after the development of the 5<sup>th</sup> leaf, the 5<sup>th</sup> leaf is classified as mainly contributing to vegetative growth, whereas the 6<sup>th</sup> and all younger leaves support reproductive growth.

In contrast to PEPC, the activities of SPS and PK appeared constant irrespective of leaf number and growth stage of plant. As PEPC  $V_{\max}$  of the 5<sup>th</sup> leaf was high especially during the early vegetative growth stage, we plotted the ratio of the  $V_{\max}$  of SPS and that of PEPC against the growth stages (Fig. 7). The relative activity of SPS was lower in the 5<sup>th</sup> than in the 6<sup>th</sup> and 7<sup>th</sup> leaf before the 7<sup>th</sup> leaf was fully expanded. Higher proportions of <sup>14</sup>C are directed into the nitrogen metabolism-related pool (amino acids and organic acids) during the vegetative growth stage, whereas after this stage, fluxes into the carbon metabolism-related pool (sugars and starch) become dominant (Nakamura *et al.* 1997). While the enzyme activities explain the differences in the distribution patterns to some degree, the function of carbon metabolites (hexose, sucrose, etc.) in sensing and signaling the nutritional status of the plant (Halford and Paul 2003) must also be taken into account. Sucrose may control the initiation of the reproductive stage; exogenous sucrose was able to restore the normal phenotype in the late floral transition mutant of *Arabidopsis* (Ohto *et al.* 2001), and over-expression of SPS caused early flowering (Laporte *et al.* 1997, Baxter *et al.* 2003).

If the physiological characteristics of a leaf are determined mostly by the source-sink balance of the whole plant, the carbon metabolism (which we have characterized by the distribution of primary photosynthates; Fig. 2) would be expected to change at the transition from vegetative growth to the flowering stage. However, we merely observed a transient change in the primary photosynthate distribution pattern in the 5<sup>th</sup> leaf at the time when the 6<sup>th</sup> leaf had fully expanded; this effect could be explained by an increase in sugar phosphates (especially 3-phospho glycerate (3PGA)). As 3PGA is the initial product of the ribulose 1,5-bis-phosphate carboxylase reaction, it appeared that a retardation of photosynthate utilization occurred during this transient stage. The relative activities of SPS and PEPC corresponded to the distribution ratio to sugar phosphates (Figs. 2 and 7). Though we had not quantified the sugar phosphate fraction in our previous reports (Shinano *et al.* 2006), it seems

that the relative distribution of carbon to sugar phosphates is an indicator of the rate of transformation of primary photosynthates into derived compounds. In the 5<sup>th</sup> leaf, the ratio of the  $V_{\max}$  values of SPS and PEPC was lower than in the 6<sup>th</sup> and 7<sup>th</sup> leaf. This would be expected to decrease the carbon flow to sucrose synthesis, but the proportion of <sup>14</sup>C distribution to the sugar fraction did not corroborate the idea. In our previous study (Shinano *et al.* 2006), we had found a positive relationship between the ratio of the  $V_{\max}$  of SPS and PEPC on one hand and the proportion of <sup>14</sup>C distribution to sugars on the other, but we had not analyzed the sugar phosphate fraction. Sugar phosphates are metabolic intermediates and form substrates for SPS and PEPC (Champigny and Foyer 1992). The relatively higher proportion of primary photosynthate distribution to this fraction seems to indicate a retardation of carbon flow to sugar and/or organic acid synthesis through SPS or PEPC, or possibly a re-distribution of PEP into the chloroplasts (Shinano *et al.* 2005). In rice, carbon distribution between chloroplasts and cytoplasm depends on the plant phosphorus status (Shinano *et al.* 2006), and the ratio of nitrogen to phosphorus contents is greatly modified during the transition from vegetative to reproductive growth of plant (Osaki 1995). This suggests that phosphorus metabolism is another important factor that regulates carbon flow in rice, which will be addressed in future studies.

## References

- Baxter, C.J., Foyer, C.H., Turner, J., Rolfe, S.A., Quick, W.P.: Elevated sucrose-phosphate synthase activity in transgenic tobacco sustains photosynthesis in older leaves and alters development. - *J. exp. Bot.* **54**: 1813-1820, 2003.
- Bradford, M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. - *Anal. Biochem.* **72**: 248-254, 1976.
- Champigny, M.L., Foyer, C.H.: Nitrate activation of cytosolic protein kinase diverts photosynthetic carbon from sucrose to amino acid biosynthesis. - *Plant Physiol.* **100**: 7-12, 1992.
- Duff, S.M.G., Chollet, R.: In vivo regulation of wheat-leaf phosphoenolpyruvate carboxylase by reversible phosphorylation. - *Plant Physiol.* **107**: 775-782, 1995.
- Foyer, C.H., Noctor, G., Lelandais, M., Lescure, J.C., Valadier, M.H., Boutin, J.P., Horton, P.: Short-term effects of nitrate, nitrite and ammonium assimilation on photosynthesis, carbon partitioning and protein phosphorylation in maize. - *Planta.* **192**: 211-220, 1994.
- Halford, N.G., Paul, M.J. Carbon metabolite sensing and signaling. - *Plant Biotech. J.* **1**: 381-398, 2003.

- Hind, G.: Thylakoid components and processes. - In: Hall, D.O., Scurlock, J.M.O., Bolhar-Nordenkamp, H.R., Leegood, R.C., Long, S.P. (ed.): Photosynthesis and Production in a Changing Environment, a Field and Laboratory Manual. Pp. 283-298, Chapman & Hill, London – Glasgow – New York – Tokyo – Melbourne – Madras 1993.
- Huber, J.L., Redinbaugh, M.G., Huber, S.C., Campbell, W.H.: Regulation of maize leaf nitrate reductase activity involves both gene expression and protein phosphorylation. - *Plant Physiol.* **106**: 1667-1674, 1994.
- Huber, S.C., Nielsen, T.H., Huber, J.L.A., Pharr, D.M.: Variation among species in light activation of sucrose-phosphate synthase. - *Plant Cell Physiol.* **30**: 277-285, 1989.
- Laporte, M.M., Galagan, J.A., Shapiro, J.A., Boersig, M.R., Shewmaker, C.K.: Sucrose-phosphate synthase activity and yield analysis of tomato plants transformed with maize sucrose-phosphate synthase. - *Planta* **203**: 253-259, 1997.
- Laporte, M.M., Galagan, J.A., Prash, A.L., Vanderveer, P.J., Hanson, D.T., Shewmaker, C.K., Sherkey, T.D.: Promoter strength and tissue specificity effects on growth of tomato plants transformed with maize sucrose-phosphate synthase. - *Planta* **212**: 817-822, 2001.
- Li, B., Zhang, X.Q., Chollet, R.: Phosphoenolpyruvate carboxylase kinase in tobacco leaves is activated by light in a similar but not identical way as in maize. - *Plant Physiol.* **111**: 497-505, 1996.
- Mahn, C.T., Bismuth, E., Boutin, J.P., Provot, M., Champigny, M.L.: Metabolite effectors for short-term nitrogen-dependent enhancement of phosphoenolpyruvate carboxylase activity and decrease of net sucrose synthesis in wheat leaves. - *Physiol. Plant.* **89**: 460-466, 1993.
- Mizuno, N., Minami, M.: [The use of H<sub>2</sub>SO<sub>4</sub>-H<sub>2</sub>O<sub>2</sub> for the destruction of plants matter as a preliminary to determination of N, K, Mg, Ca, Fe, Mn.] - *Jap J. Soil Sci. Plant Nutr.* **51**: 418-420, 1980. [In Japanese with English Summary.]
- Murchie, E.H., Sarrobert, C., Contard, P., Betsche, T., Foyer, C., Galtier, N.: Overexpression of sucrose-phosphate synthase in tomato plants grown with CO<sub>2</sub> enrichment leads to decreased controls. - *Plant Physiol. Biochem.* **37**: 251-260, 1999.
- Nakamura, T., Osaki, M., Shinano, T., Tadano, T.: Difference in system of current photosynthesized carbon distribution to carbon and nitrogen compounds between rice and soybean. - *Soil Sci. Plant Nutr.* **43**: 777-788, 1997.
- Ohto, M., Onai, K., Furukawa, Y., Aoki, E., Araki, T., Nakamura, K.: Effects of sugar on vegetative development and floral transition in *Arabidopsis*. - *Plant Physiol.* **127**: 252-261, 2001.

- Osaki, M., Hada K., Tanaka A.: Reconstruction of the leaf-proteins into grain-proteins during ripening in the rice plant. - Jap. J. Soil Sci. Plant Nutr. **59**: 272-278, 1988. [In Japanese with English summary.]
- Osaki, M., Shinano, T., Tadano T.: Redistribution of carbon and nitrogen compounds from the shoot to the harvesting organs during maturation in field crops. - Soil Sci. Plant Nutr. **37**: 117-128, 1991.
- Osaki, M.: Ontogenic changes of N, P, and K contents in individual leaves of field crops. - Soil Sci. Plant Nutr. **41**:429-438, 1995.
- Plaxton, W.C.: (1998) Metabolic aspects of phosphate starvation in plants. – In: Lynch, J.P., Deikman, J. (ed.) Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes. Pp. 41-93. American Society of Plant Physiologists, Rockville, 1998.
- Quy, L.V., Foyer, H., Champigny, M.L.: Effect of light and NO<sub>3</sub><sup>-</sup> on wheat leaf phosphoenolpyruvate carboxylase activity. - Plant Physiol. **97**: 1476-1482, 1991.
- Quy, L.V., Champigny, M.L.: NO<sub>3</sub><sup>-</sup> enhances the kinase activity for phosphorylation of phosphoenolpyruvate carboxylase and sucrose phosphate synthase proteins in wheat leaves. - Plant Physiol. **99**: 344-347, 1992.
- Redgwell, R.J.: Fractionation of plant extracts using ion-exchange Sephadex. - Anal. Biochem. **107**: 44-50, 1980.
- Sheible, W.R., Gonzalez-Fontes, A., Lauerer, M., Muller-Rober, B., Caboche, M., Stitt, M.: Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. - Plant Cell **9**: 783-798, 1997.
- Shinano, T., Nakajima, K., Wasaki, J., Mori, H., Tei, Z., Osaki, M.: Developmental regulation of photosynthate distribution in leaves of rice. - Photosynthetica **44**: 1-10, 2006.
- Shinano, T., Nanamori, M., Dohi, M., Wasaki, J., Osaki M.: Evaluation of phosphorus starvation inducible genes relating to efficient phosphorus utilization in rice. - Plant Soil **269**: 81-87, 2005.
- Shinano, T., Osaki, M., Tadano, T.: <sup>14</sup>C-allocation of <sup>14</sup>C-compounds introduced to a leaf to carbon and nitrogen components in rice and soybean during ripening. - Soil Sci. Plant Nutr. **40**: 199-209, 1994.
- Signora, L., Galtier, N., Skot, L., Lucas, H., Foyer, C.H.: Over-expression of sucrose phosphate synthase in *Arabidopsis thaliana* results in increased foliar sucrose/starch ratios and favours decreased foliar carbohydrate accumulation in plants after prolonged growth with CO<sub>2</sub> enrichment. - J. exp. Bot. **49**: 669-680, 1998.
- Stitt, M.: Nitrate regulation of metabolism and growth. - Curr. Opin. Plant Biol. **2**: 178-186,

1999.

Takahashi, S., Ono, K., Ugaki, M., Ishimaru, K., Aoki, N., Ohsugi, R.: Ser162-dependent inactivation of overproduced sucrose-phosphate synthase protein of maize leaf in transgenic rice plants. - *Plant Cell Environ.* **41**: 977-981, 2000.

Tanaka, A.: Studies on the nutritio-physiology of leaves of rice plant. - *J. Fac. Agr. Hokkaido Univ.* **51**: 491-550, 1961.

Worrel, A.C., Brunearu, J-M., Summerfelt, K., Boersig, M., Voelker, T.A.: Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. - *Plant Cell* **3**: 1121-1130, 1991.

## Figure legends

Fig. 1. Nitrogen contents of leaves at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. The arrow indicates the panicle initiation stage. Values shown are means with SE (n=3).

Fig. 2. Primary photosynthate distribution in different leaves at different growth stages.

I, II, II, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n = 3). There were significant differences between the 5<sup>th</sup> and other leaves in the sugar fraction, between the 5<sup>th</sup> and 6<sup>th</sup> leaf in the sugar phosphate fraction, and between the 7<sup>th</sup> and other leaves in the organic acid fraction. Differences were analysed using the General Linear Model followed by Bonferoni's post hoc test with  $P < 0.05$ . As protein fraction was negligible in all the case, data were not shown in the figure.

Fig. 3. Distribution of primary photosynthates of the phosphate ester fraction in the 5<sup>th</sup> (left figure) and 6<sup>th</sup> leaf (right figure) at different growth stages.

I, II, II, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

□ ; fructose 1,6-bisphosphate, △ ; UDP glucose, ▽ ; hexose 6-phosphate, ◇ ; 3 phosphoglyceric acid, ○ ; phosphoenolpyruvate, ◦ ; glucose 3-phosphate.

Fig. 4. Sucrose phosphate synthase (SPS) activity and the ratio of  $V_{\text{limit}}$  and  $V_{\text{max}}$  at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

Fig. 5. Phosphoenolpyruvate carboxylase (PEPC) activity and the ratio of  $V_{\text{limit}}$  and  $V_{\text{max}}$  at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

PEPC  $V_{\text{max}}$  differed significantly between the 5<sup>th</sup> and 6<sup>th</sup> leaf. Differences between leaves were analysed by a General Linear Model followed by Bonferoni's post hoc test with  $P < 0.05$ .

Fig. 6. Phosphoenolpyruvate kinase (PK V<sub>max</sub>) activity at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

Fig. 7. Ratio of SPS V<sub>max</sub> and PEPC V<sub>max</sub> at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

There was a significant difference between the 5<sup>th</sup> and 6<sup>th</sup> leaf, as revealed by General Linear Model analysis followed by Bonferoni's post hoc test with  $P < 0.05$ .

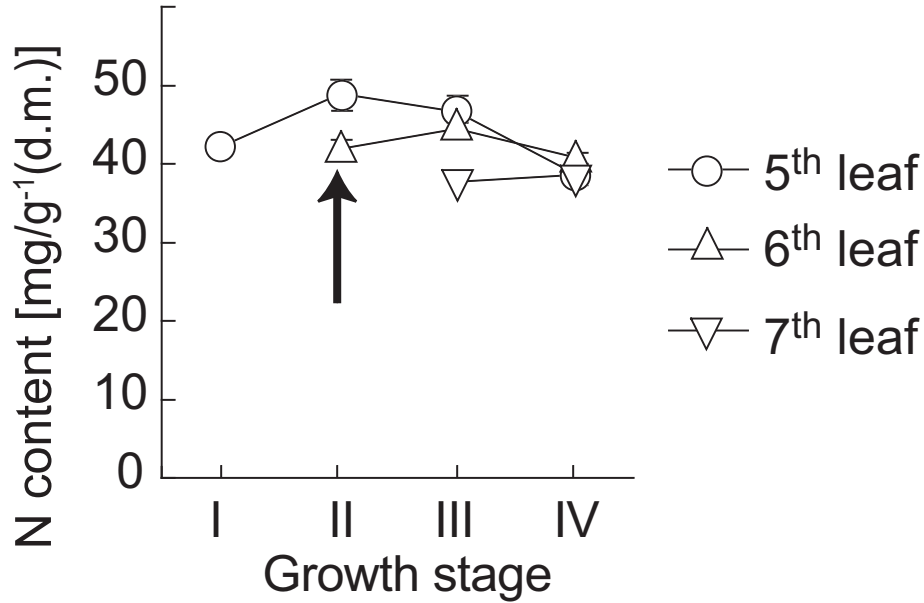


Fig. 1. Nitrogen contents of leaves at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. The arrow indicates the panicle initiation stage. Values shown are means with SE (n=3).

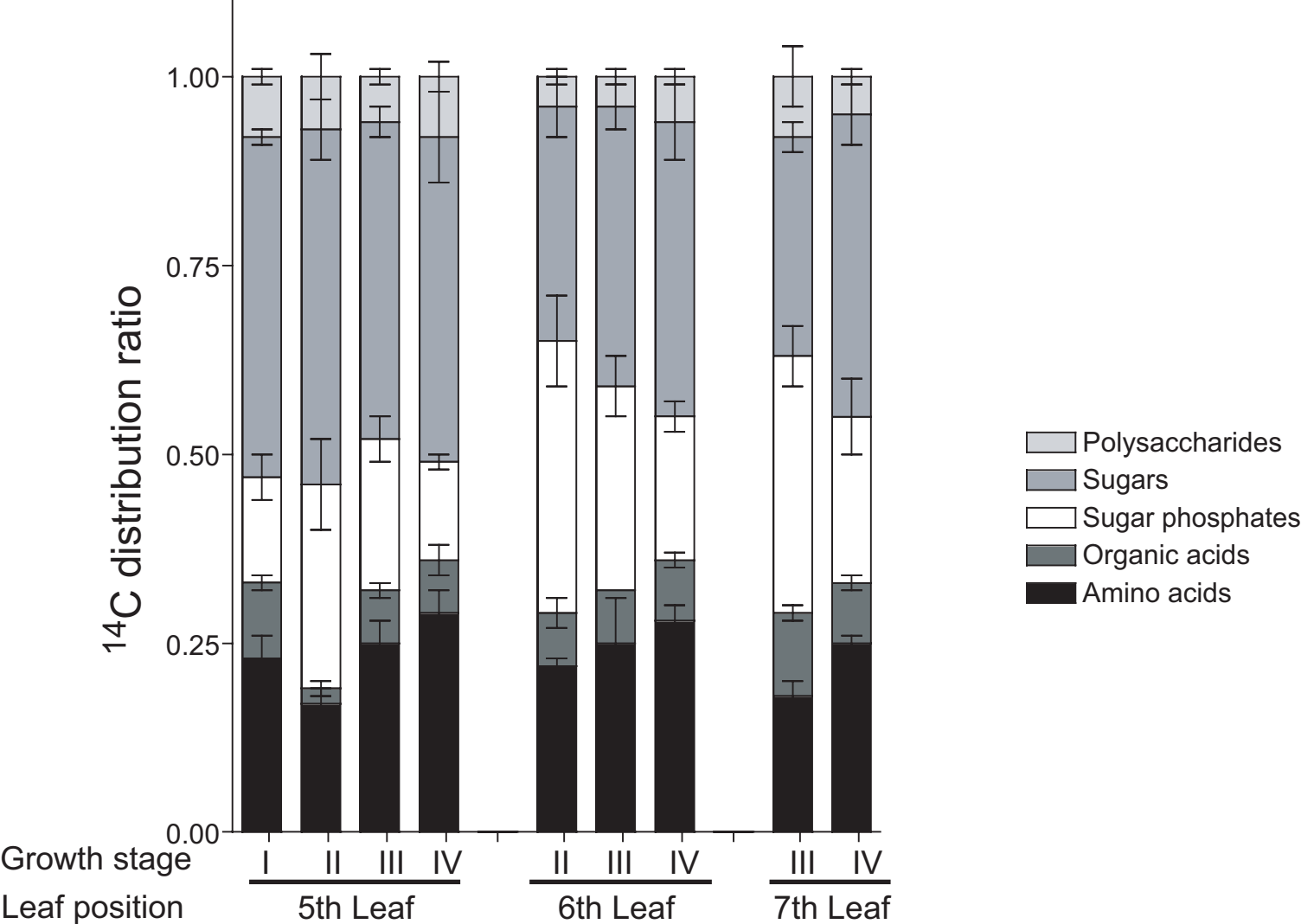


Fig. 2. Primary photosynthate distribution in different leaves at different growth stages. I, II, II, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n = 3). There were significant differences between the 5<sup>th</sup> and other leaves in the sugar fraction, between the 5<sup>th</sup> and 6<sup>th</sup> leaf in the sugar phosphate fraction, and between the 7<sup>th</sup> and other leaves in the organic acid fraction. Differences were analysed using the General Linear Model followed by Bonferoni's post hoc test with P < 0.05. As protein fraction was negligible in all the case, data were not shown in the figure.

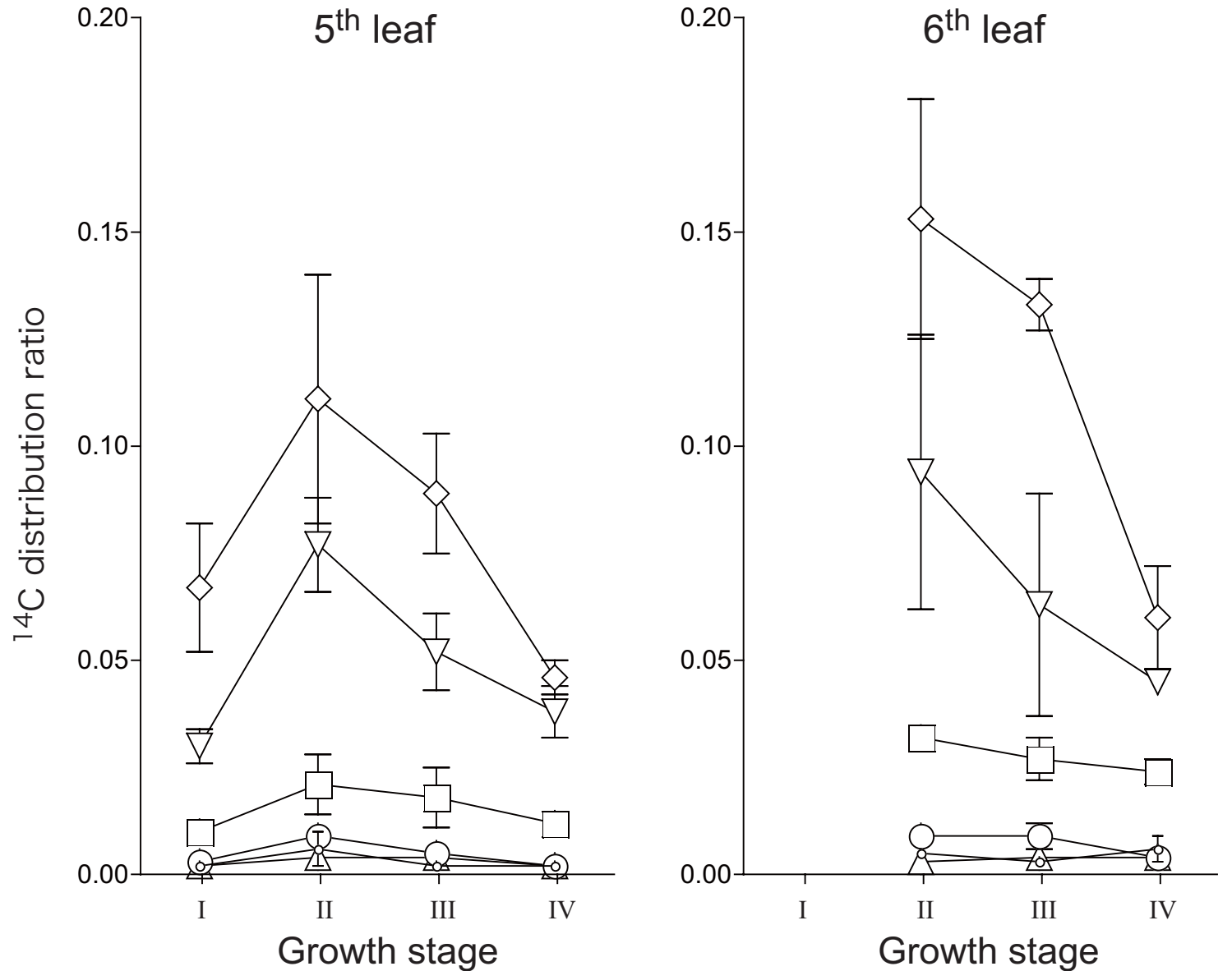


Fig. 3. Distribution of primary photosynthates of the phosphate ester fraction in the 5<sup>th</sup> (left figure) and 6<sup>th</sup> leaf (right figure) at different growth stages.

I, II, II, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

□ ; fructose 1,6-bisphosphate, △ ; UDP glucose, ▽ ; hexose 6-phosphate, ◇ ; 3 phospho glyceric acid, ○ ; phosphoenolpyruvate, ◊ ; glucose 3-phosphate.

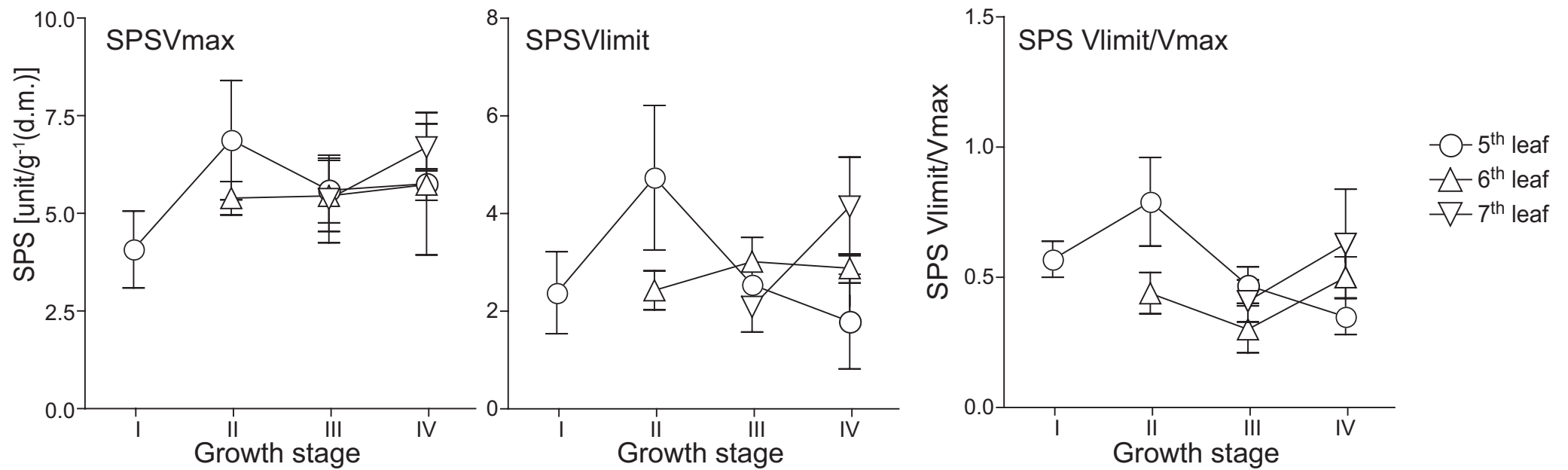


Fig. 4. Sucrose phosphate synthase (SPS) activity and the ratio of Vlimit and Vmax at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> eaf, respectively, were fully expanded. Data shown are means with SE (n=3).

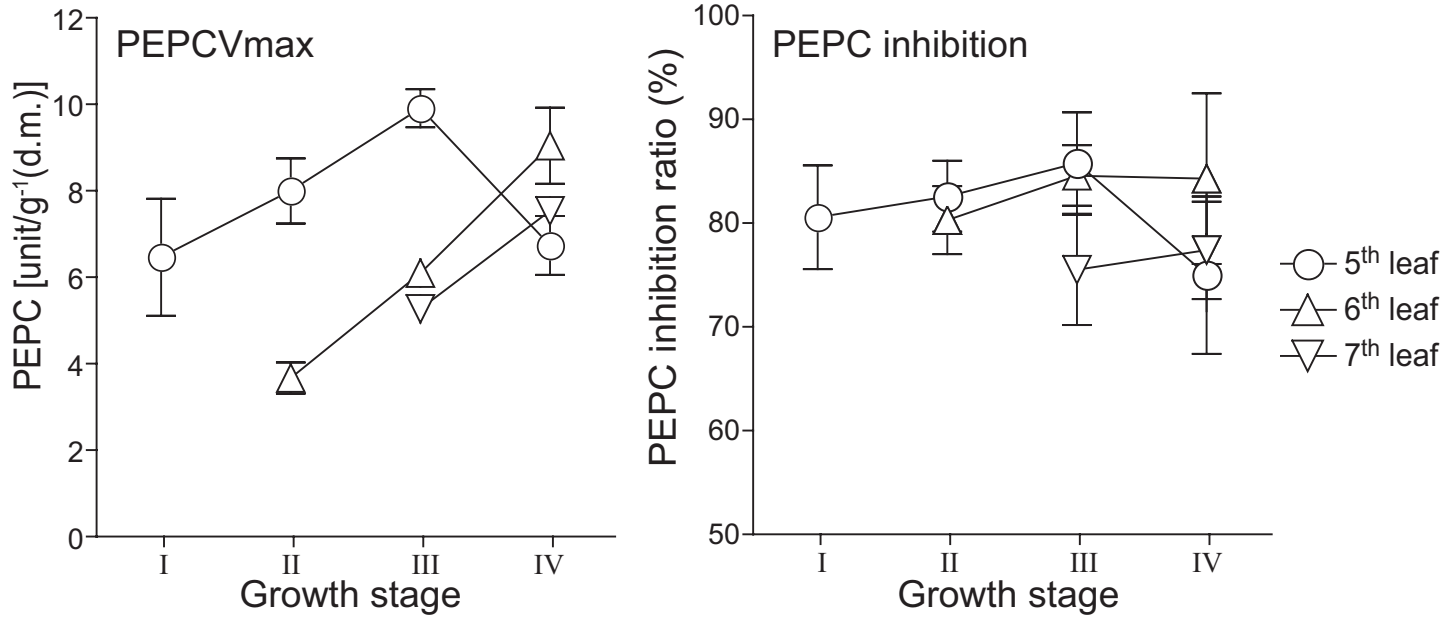


Fig. 5. Phosphoenolpyruvate carboxylase (PEPC) activity and the ratio of  $V_{\text{limit}}$  and  $V_{\text{max}}$  at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

PEPC  $V_{\text{max}}$  differed significantly between the 5<sup>th</sup> and 6<sup>th</sup> leaf. Differences between leaves were analysed by a General Linear Model followed by Bonferoni's post hoc test with  $P < 0.05$ .

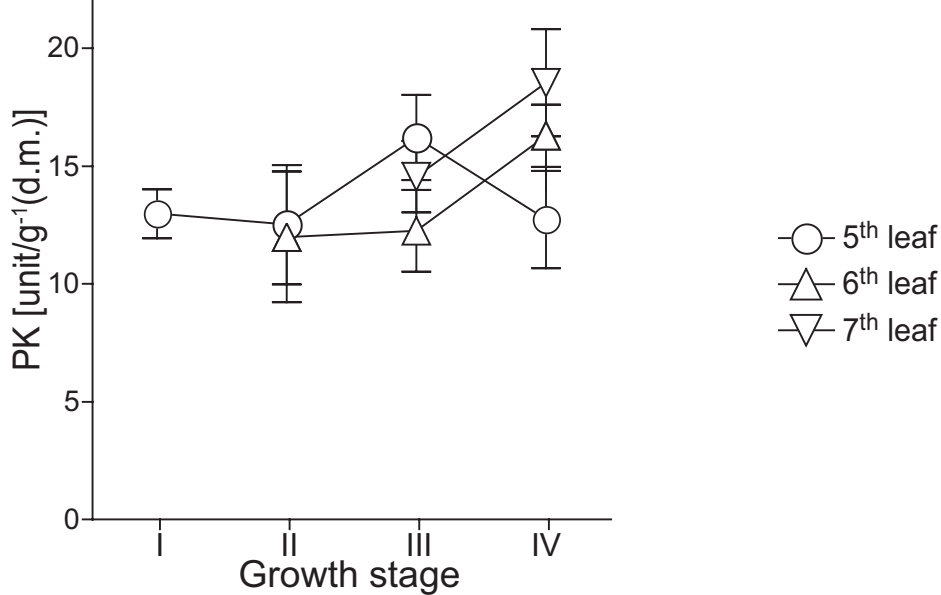


Fig. 6. Phosphoenolpyruvate kinase (PK Vmax) activity at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

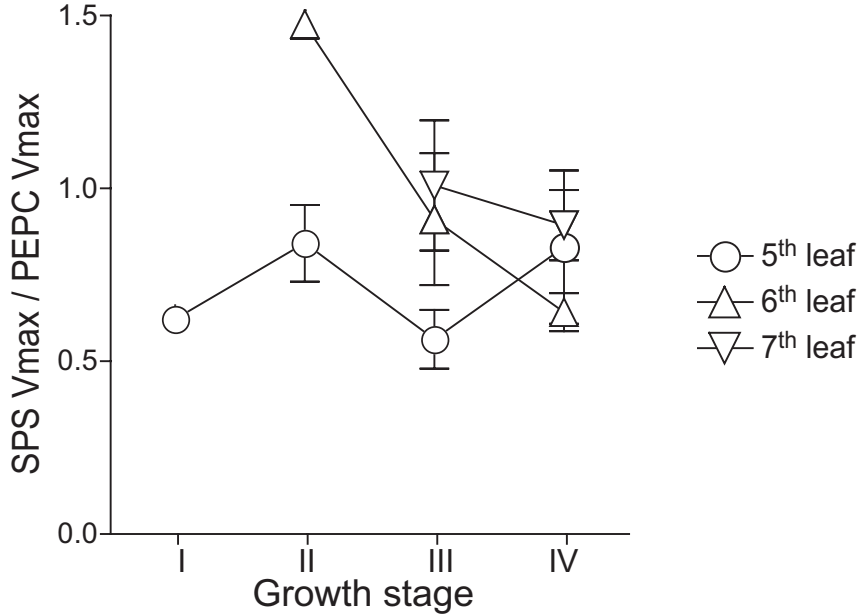


Fig. 7. Ratio of SPS Vmax and PEPC Vmax at different growth stages.

I, II, III, and IV indicate growth stages at which the 5<sup>th</sup>, 6<sup>th</sup>, 7<sup>th</sup> and 8<sup>th</sup> leaf, respectively, were fully expanded. Data shown are means with SE (n=3).

There was a significant difference between the 5<sup>th</sup> and 6<sup>th</sup> leaf, as revealed by General Linear Model analysis followed by Bonferoni' s post hoc test with  $P < 0.05$ .