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Developmental regulation of photosynthate distribution in leaves of rice

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Abstract

mRNA expression patterns of genes for metabolic key enzymes sucrose phosphate synthase (SPS), phospho*enol*pyruvate carboxylase (PEPC), pyruvate kinase, ribulose 1,5-bisphosphate carboxylase/oxygenase, glutamine synthetase 1, and glutamine synthetase 2 were investigated in leaves of rice plants grown at two nitrogen (N) supplies ($N_{0.5}$, $N_{3.0}$). The relative gene expression patterns were similar in all leaves except for 9th leaf, in which mRNA levels were generally depressed. Though increased N supply prolonged the expression period of each mRNA, it did not affect the relative expression intensity of any mRNA in a given leaf. SPS V_{max}, SPS limiting and PEPC activities, and carbon flow were examined. The ratio between PEPC activity and SPS V_{max} was higher in leaves developed at the vegetative growth stage (vegetative leaves: 5th and 7th leaves) than in leaves developed after the ear primordia formation stage (reproductive leaves: 9th and flag leaves). PEPC activity and SPS V_{max} decreased with declining leaf N content. After using $^{14}CO_2$ the ^{14}C photosynthate distribution in the amino acid fraction was higher in vegetative than in reproductive leaves when compared for the same leaf N status. Thus, at high PEPC/SPS activities ratio, more ^{14}C photosynthate was distributed to the amino acid pool, whereas at higher SPS activity more ^{14}C was channelled into the saccharide fraction. Thus, leaf ontogeny was an important factor controlling photosynthate distribution to the N- or C-pool, respectively, regardless of the leaf N status.

Additional key words: glutamine synthetases; leaf development; mRNA expression; *Oryza*; phospho*enol*pyruvate carboxylase; pyruvate kinase; ribulose-1,5-bisphosphate carboxylase/oxygenase; sucrose phosphate synthase.

Introduction

The distribution of photosynthetically fixed carbon between amino acids and saccharides is regulated by the relative activities of sucrose phosphate synthase (SPS) and phospho*enol*pyruvate carboxylase (PEPC; Champigny and Foyer 1992, Foyer *et al*. 1994, Huber *et al*. 1994). In C_3-plants, PEPC acts to replenish TCA cycle intermediates which serve as source material for the synthesis of amino acids (Stitt 1999). On the other hand, C distribution to sucrose increases with rising SPS content (Worrel *et al*. 1991, Signora *et al*. 1998, Murchie *et al*. 1999). Galtier *et al*. (1993) demonstrated a positive relationship between SPS activity and the sucrose/starch ratio by changing the content of SPS in tomato transformed with a maize SPS gene, resulting in increased starch degradation in the chloroplasts. Similar effects were reported by Galtier *et al.* (1995) and Micallef *et al*. (1995). Thus, once dihydroxyacetone phosphate (DHAP) is released from the chloroplast, the rate of sucrose synthesis is regulated by SPS activity. On the other hand, DHAP released from chloroplasts can also be catabolized by pyruvate kinase (PK) or PEPC to fuel the synthesis of amino acids *via* the intermediates of the TCA cycle. When the C_4-PEPC gene from *Flaveria trinervia* was introduced into potato plants, the C flow was redirected from sugar and starch synthesis to malate and amino acid synthesis (Rademacher *et al*. 2002). *Vice versa*, increased expression levels of SPS resulted in a decrease of amino acid synthesis (Laporte *et al*. 2001).

Previously we demonstrated that the ratio of SPS activity to PEPC activity in leaves of rice dramatically increased around the panicle primordia formation stage (Nakamura *et al.* 1997). The photosynthate distribution to

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Abbreviations: DHAP – dihydroxyacetone phosphate; EDTA – ethylenediamine-tetraacetic acid; F_6P – fructose-6-phosphate, G_6P – glucose-6-phosphate; GS1 – glutamine synthetase 1; GS2 – glutamine synthetase 2; MOPS – morpholinopropanesulphonic acid; PEP – phospho*enol*pyruvate; PEPC – phospho*enol*pyruvate carboxylase, PK – pyruvate kinase; PMSF – phenylmethanesulfonyl fluoride; PVP – polyvinylpyrrolidone; RuBPCO – ribulose-1,5-bisphoshate carboxylase/oxygenase; SPS – sucrose phosphate synthase; UDPG – UDP-glukose.
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amino acids was large at the vegetative growth stage, but decreased after the panicle primordia formation stage regardless of nitrogen availability (Nakamura *et al*. 1997). One might expect that changes in C flow will also affect other enzymes, especially those involved in C and N metabolism in leaves.

In this study, we evaluated the interactive role(s) of C- and N-metabolism-related enzymes. We studied mRNA expression levels of SPS, PEPC, PK, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), and glutamine synthetases (GS1, GS2) at different phases of leaf development. PK regulates C flow from DHAP to the TCA cycle, and RuBPCO is the key enzyme for photosynthesis. Both GS1 and GS2 couple the ammonium ion to 2-keto glutarate. The cytosolic GS1 produces the substrate for further transamination reactions, and GS2 catalyzes the glycine to serine conversion in the chloroplasts (Buchanan-Wollaston and Ainsworth 1997). We determined mRNA expression patterns under low and high N, because N application promotes the expression of RuBPCO (Plumley and Schmidt 1989), GS (Sakakibara *et al*. 1992), and PEPC (Suzuki *et al*. 1994).

In rice, amino acid (and protein) synthesis is more active during the vegetative growth stage, while saccharide synthesis is stimulated after the initiation of the reproductive stage. We did not detect a correlation of this physiological switch with expression patterns of C- and N-metabolism-related enzymes. Therefore, we determined changes of PEPC/SPS activity and the distribution of fixed ^{14}C to amino acids and the sucrose pool during rice plant development, in order to characterize the enzymatic basis of the physiological transition.

Materials and methods

Experiment 1

Plants: Rice plants (*Oryza sativa* L. cv. Michikogane) were sown on April 18 in a greenhouse and were transplanted in batches of six to 5 000 cm^3 pots when they had reached the 5-leaf stage (June 12) and the soil was put under submerged condition with tap water. To each pot, 2 g P_2O_5 and 1 g K_2O were applied as superphosphate and potassium sulphate, respectively. Ammonium sulphate was added at 0.5 g N ($N_{0.5}$ treatment, standard concentration) or 3 g N ($N_{3.0}$ treatment, excess concentration) per pot. All experiments were carried out with 4 replications.

Sampling: For the analysis of mRNA expression, samples (upper two thirds of the leaf, not including the growth zone) were taken on May 31, June 19, June 28 (vegetative growth stage), July 8, July 19 (panicle formation stage), July 29, August 6 (flowering stage), August 17, August 26 (milk-ripe stage), September 4, September 11, September 20, and September 29. N content was determined for all samples, except for the small sized ones taken on May 31 and June 19. Samples from the vegetative, panicle formation, flowering, and milk-ripe stages were also used for analyses of enzyme activity and mineral content. Leaf samples were taken from the 3^{rd}, 5^{th}, 7^{th}, 9^{th}, and flag leaf (11^{th} leaf). For the determination of mRNA expression, samples were collected between 08:30 and 09:00, immediately frozen in liquid N, and stored at –80 °C until analysis. For the analysis of enzyme activity and mineral content, samples were taken between 09:00 and 10:00 and weighed. Half of the samples were frozen in liquid N and stored at –80 °C until analysis; the other half was dried at 80 °C for 48 h for mineral content analysis.

N content was determined by the semi-micro Kjeldahl method.

RNA extraction and Northern analysis: Probes for PEPC and RuBPCO determination were supplied by Dr. Makoto Matsuoka. Probes for PK, SPS, GS1, and GS2 were amplified using appropriate pairs of primers (5'-AAATTGTCCCCCAAAGATGGACTA and 5'-TCA GCTGTACAGAAGCAGTTGTGC for PK, 5'-ATCGAC GGCGAGCGGGGG and 5'-GCAGGTAGTCCTGGT CGGGG for SPS, 5'-ACCGCCGACATCAACACC and 5'-TGGAAGCCCAGCAAGAAC for GS1, and 5'-GGG AGACTGGAATGGAG and 5'-TCGAGGGAAGGACG CAG for GS2) and rice cDNA as template.

Total RNA was isolated by the SDS/phenol method (Palmiter 1974). 10 µg of total RNA was denatured in 1× MOPS, pH 7.0, 16 % (v/v) formaldehyde, and 50 % formamide (v/v), and was separated on a 1 % agarose gel that contained 1× MOPS, pH 7.0 and 5 % formaldehyde (v/v). After electrophoresis, the purity of the RNA was checked by the expression of clear ribosomal RNA bands (data not shown), then RNA was transferred to a *Hybond-N^+* membrane (*Amersham Biosciences*, Piscataway, NJ, USA), and fixed by UV radiation (*CL-1000*, *UVP*, Upland, CA, USA). The membranes were then hybridized in 5× SSPE, pH 7.4, 5× Denhardt's solution, 0.5 % (m/v) SDS, and 20 µg cm^{-3} denatured salmon-sperm DNA at 65 °C for 18 h with probes that had been labelled with ^{32}P using the random-priming method (*BcaBEST^{TM} Labeling Kit*, *Takara Bio*, Shiga, Japan). The membranes were washed twice in 2 × SSPE, pH 7.4 and 0.1 % (m /v) SDS at room temperature, and then in 1× SSPE, pH 7.4 and 0.1 % (m /v) SDS at 65 °C for 15 min; finally they were rinsed with 0.1 × SSPE and 0.1 % (m /v) SDS.

After incubation with the SPS, PEPC, and PK probes, membranes were washed with boiling 0.5 % (m/v) SDS, and rinsed with distilled water after cooling. These membranes then were used for re-probing with the GS1, GS2, and RuBPCO probes. Membranes were exposed to an Imaging Plate (*Fuji Bas-IIIs*, *Fuji Film*, Tokyo, Japan) for 30 h that was then examined with an image analysis

system (*Bas1000*, *Fuji Film*).

Protein extraction and activity analyses: 1-g sample was ground in liquid N and proteins were extracted at 4 °C by 6 cm^3 of 50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.1 % (v/v) *Triton X-100*, 10 % (v/v) glycerine, 1 % (m/v) PVP, and 0.1 % (m/v) PMSF. The extract was centrifuged for 15 min at 13 500×*g* at 4 °C. An aliquot of the supernatant was put on a 2.5 cm^3 column, which had been prepared with 2.3 cm^3 *Sephadex G-25* (*Amersham Biosciences*) and equilibrated with the extraction buffer, excluding *Triton X-100*.

PEPC activity was determined by the coupled reaction with malate dehydrogenase (Huber and Huber 1996). 20 mm^3 of the crude enzyme solution and 480 mm^3 of reaction buffer (50 mM MOPS-NaOH, pH 7.5, 15 mM $NaHCO_3$, 5 mM PEP, 15 mM $MgCl_2$, 0.25 mM NADH, and 12 units of malate dehydrogenase) were separately preheated at 30 °C, mixed for 10 s, and 5 min were allowed for the reaction. A reaction buffer without NADH was used as a blank. The decrease rate of NADH was determined using a spectrophotometer (*UV-1600*, *Shimadzu*, Tokyo, Japan). One unit of activity was defined as the amount of protein which turns over 1 µmol of NADH in 1 min.

In the case of SPS, we determined both V_{max} and limiting activity (Quy *et al*. 1991). V_{max} was measured under optimum conditions, while the limiting activity was measured at reduced concentration of activators [glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P)] and in the presence of a deactivator (phosphate). 13 mm^3 of crude enzyme solution were added to 40 mm^3 of reaction buffer (for the measurement of V_{max}: 50 mM MOPS-NaOH pH 7.5, 15 mM $MgCl_2$, 10 mM UDPG, 10 mM F_6P, 40 mM G_6P, and 2.5 mM DTT; for the determination of limiting activity: 50 mM MOPS-NaOH pH 7.5, 15 mM $MgCl_2$, 20 mM UDPG, 3 mM F_6P, 12 mM G_6P, 2.5 mM DTT, and 10 mM phosphate). The reaction buffer and crude enzyme solution were separately preheated at 37 °C, mixed, and the reaction was allowed to proceed for 15 min. 70 mm^3 of 30 % (m/v) KOH were added to stop the reaction, and the solution was heated (100 °C for 10 min) to precipitate proteins. For a blank, 30 % KOH solution was added before the reaction. The released amount of sucrose phosphate was determined by adding 1 cm^3 of 0.14 % (m/v) anthrone in 75 % H_2SO_4 and keeping the solution at 40 °C for 20 min, then storing it on ice until measurement of the absorbance at 620 nm (*UV-1600*, *Shimadzu*, Tokyo, Japan). One unit of activity was defined as the amount of protein which produces 1 µmol of sucrose phosphate in 1 min.

Experiment 2

Plants: Rice seeds were sown on April 20 in a greenhouse and were transplanted to 5 000 cm^3 pots in batches of four on June 1. To each pot, 1 g N, 2 g P_2O_5, and 1 g K_2O were added as ammonium sulphate, superphosphate, and potassium sulphate, respectively. All experiments were carried out with 4 replications. Samples were taken on June 29 (vegetative growth stage), July 11 (panicle formation stage), July 20 (flowering stage), and August 11 (milk-ripe stage).

$^{14}CO_2$ assimilation and ^{14}C analysis: Each leaf was covered with clear polyethylene bag filled with natural air. $^{14}CO_2$ was liberated underneath the bag by mixing 5 cm^3 of 0.18 mM $NaHCO_3$ with 1.85 MBq $NaH^{14}CO_3$ and 5 cm^3 of 4.9 M $HClO_4$, and the plants were allowed to assimilate for 5 min under natural irradiance (between 800 and 1 200 µmol m^{-2} s^{-1}). The CO_2 concentration after this period was not lower than 200 µmol mol^{-1} in any of the experiments. All leaves were collected between 0 and 30 min after exposure to $^{14}CO_2$, and were frozen in liquid N for storage.

Total ^{14}C was measured as described by Shinano *et al*. (1996), and the distribution of ^{14}C among various classes of chemical compounds (sugars, free amino acids, organic acids including sugar phosphates, protein amino acids, polysaccharides) was determined as detailed by Shinano *et al*. (1994).

Mineral analysis and enzyme activities were determined as described for Exp. 1.

Results

Experiment 1

N content of all leaves declined over time, but the rate of decrease was greater in the $N_{0.5}$ than $N_{3.0}$ treatment (Fig. 1).

Expression of individual mRNAs: Using same amount of total RNA as a reference, relative amounts of specific mRNAs were compared in leaves of any given position (Fig. 2). As the content of total RNA from the last samplings was too small for further analysis (data not shown), the values for individual mRNAs were assumed to be 0 at these dates (Fig. 2). This occurred earlier in the $N_{0.5}$ treatment, since leaf senescence commenced earlier in $N_{0.5}$ than $N_{3.0}$ treatment.

SPS: In all leaves, relative SPS mRNA levels increased with time, and decreased after full expansion of the leaf (Fig. 2). At 34 d after transplanting (DAT), the relative levels were higher in the 5th leaf than in the 7th and 9th leaves.

PEPC: The relative amounts of PEPC mRNA reached a maximum just before senescence. Contents were higher in the 3rd and 5th leaves than in the 7th and 9th ones.

PK: The expression pattern was similar to that of PEPC. Relative expression levels were similar in all but the 9th leaf.

RuBPCO: The relative expression level of RuBPCO mRNA generally declined gradually over the experimental period.

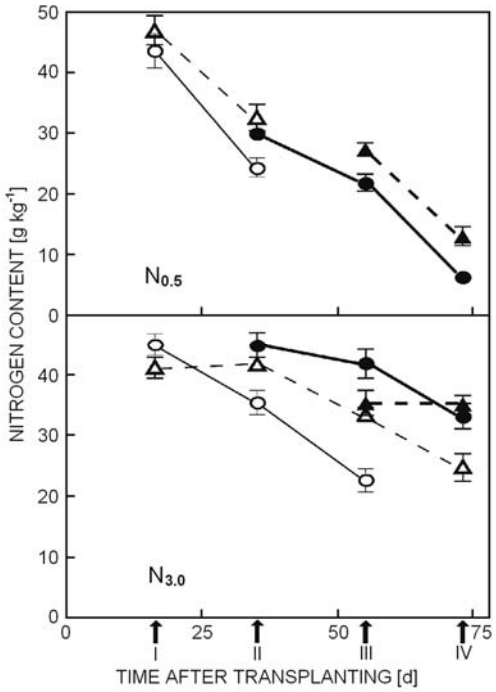

Fig. 1. Development of the contents of N and total RNA in various leaves (○ 5th leaf; Δ 7th leaf; ● 9th leaf; ▲ flag leaf) of rice plants under standard ($N_{0.5}$) and excess ($N_{3.0}$) nitrogen treatments. *Error bars* indicate S.E. I: vegetative growth stage, II: panicle formation stage, III: milk-ripe stage, IV: maturing stage.

GS1: The expression level of GS1 was low initially, but then increased. This was especially evident in the 5th, 7th, and flag leaves (Fig. 2).

GS2: The pattern of GS2 expression was similar to that of SPS (Fig. 2), but its peak occurred one week earlier.

SPS and PEPC activities: In plants of the $N_{0.5}$ treatment, PEPC activity decreased with time, but it was rather constant in the $N_{3.0}$ treatment (Fig. 3). The maximum PEPC activity measured in leaves of any given position tended to be higher in leaves that developed earlier (leaves 5 and 7) than in younger ones (leaf 9 and flag leaf).

In the $N_{0.5}$ treatment, SPS V_{max} decreased over the period of observation in all leaves (Fig. 3). The decline in SPS limiting activity (which represents the actual activity in the tissue) was less pronounced. In the $N_{3.0}$ treatment, a decrease in V_{max} was observed in all leaves except for leaf 9, although the rate of decrease was small and activity was maintained high even at the last sampling date. No clear tendency became evident in the time-course of SPS limiting activity in this treatment.

The activities of SPS and PEPC were positively correlated in leaves that developed during the vegetative stage (vegetative leaves: 5th and 7th leaf) as well as in leaves developed during the reproductive stage (reproductive leaves: 9th and flag leaf, Fig. 4; results from both N treatments were pooled for this Fig.). The ratios of PEPC activity to SPS V_{max} or SPS limiting activity, respectively, were generally higher in vegetative than reproductive leaves (Fig. 4). There was positive correlation between N content and each of the enzyme activities tested in vegetative and reproductive leaves, except for SPS limiting activity in vegetative leaves (Fig. 5).

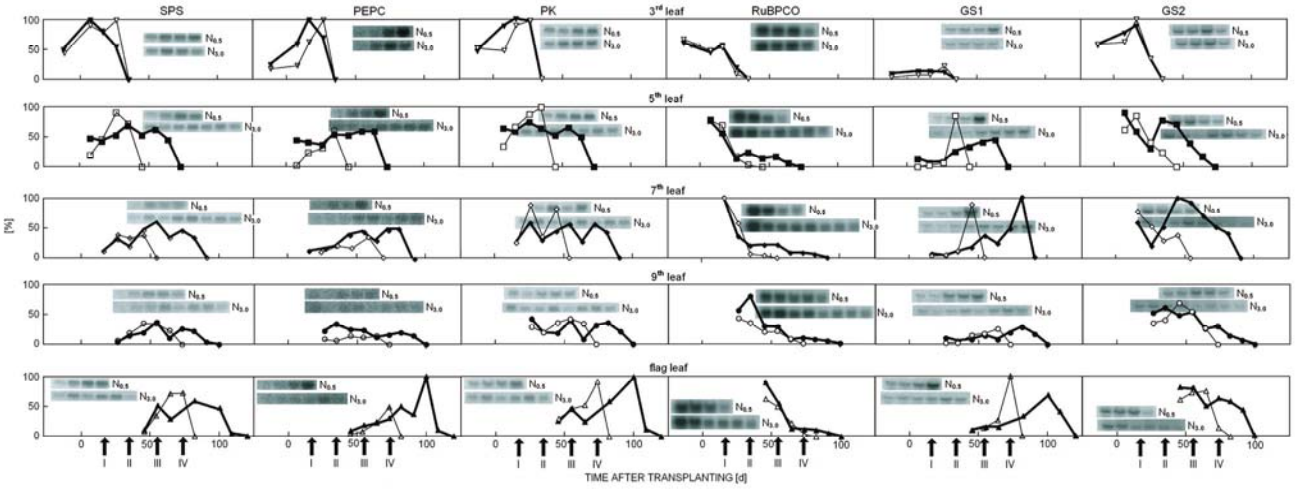

Fig. 2. Relative mRNA levels of SPS, PEPC, PK, RuBPCO, GS1, and GS2 in different leaves at different developmental stages. mRNA levels are expressed as values relative to the strongest expression for each enzyme in each N treatment. *Thin line*: $N_{0.5}$ treatment; *bold line*: $N_{3.0}$ treatment. I: vegetative growth stage, II: panicle formation stage, III: milk-ripe stage, IV: maturing stage.

Experiment 2

Distribution of ^{14}C: Before the panicle primordia formation stage (approximately 45 d after transplanting), the fraction of assimilated ^{14}C incorporated into free amino acids and protein amino acids (designated as the N-pool) at the end of the 5 min period of ^{14}C assimilation was higher than in later stages of all leaves examined (Fig. 6). The relative ^{14}C content of sugars and polysaccharides (the C-pool) increased accordingly.

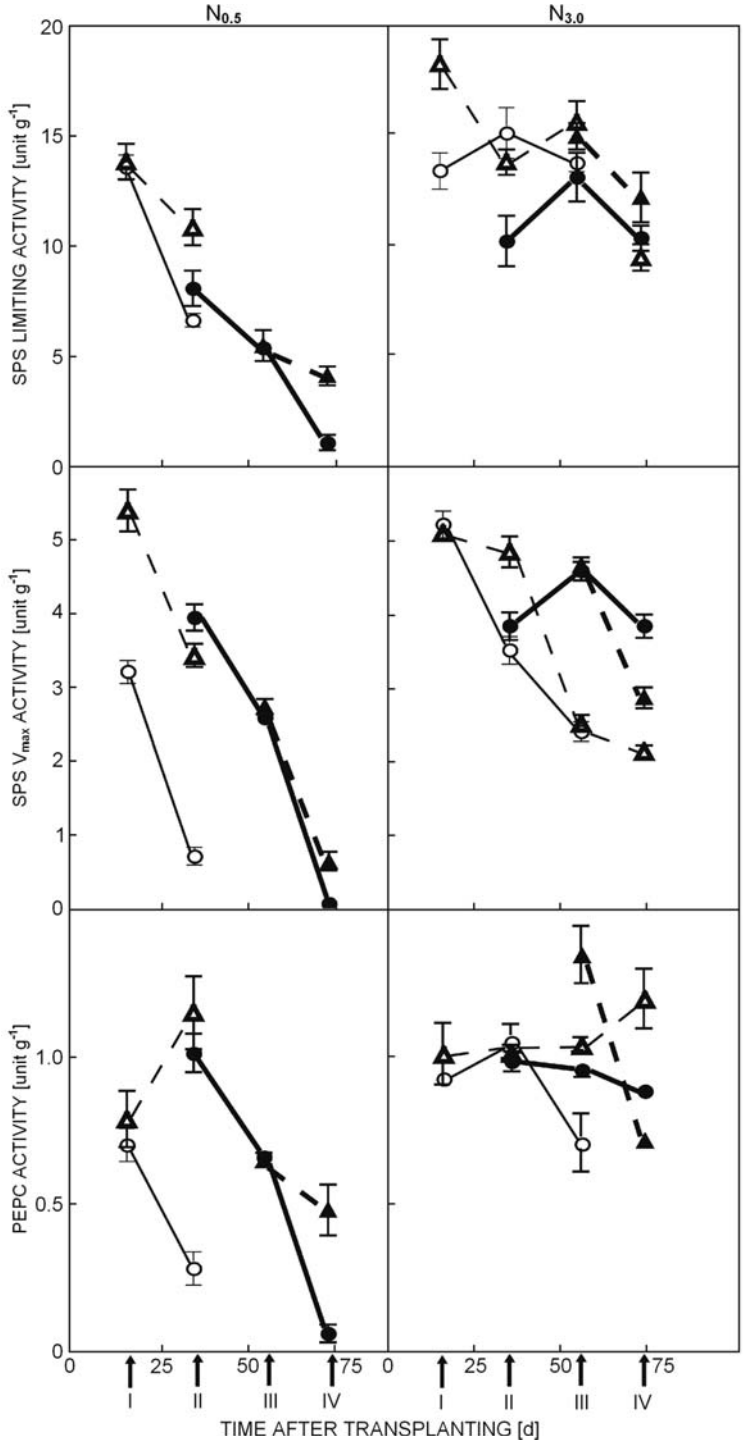

Fig. 3. Development of the activity of PEPC, SPS V_{max}, and SPS limiting activity in various leaves (○ 5th leaf; Δ 7th leaf; ● 9th leaf; ▲ flag leaf) of rice plants under standard ($N_{0.5}$) and excess ($N_{3.0}$) treatments. *Error bars* indicate standard error. I: vegetative growth stage, II: panicle formation stage, III: milk-ripe stage, IV: maturing stage.

Though the correlation between N content and ^{14}C distribution ratio to the N pool was positive both in vegetative (only leaf 6 was examined) and reproductive (leaves 8, 9, 10, and 11) leaves, the distribution ratio to N pool was higher in the vegetative leaf at any given value of N content (Fig. 7).

Discussion

To study changes of relative expression levels of mRNA during development, we selected 6 genes related to the acquisition of C (RuBPCO), the distribution of C to sucrose (SPS), the distribution of C to the TCA cycle (PEPC, PK), the distribution of C to the photorespiratory N cycle (GS2), and N translocation (GS1). The highest relative expression was observed in RuBPCO followed by GS2, SPS and PK, PEPC, and GS1. SPS facilitates C removal from chloroplasts, helping to maintain a high photosynthetic rate. PK is crucial for glycolytic C flux to pyruvate (Copeland and Turner 1987, Plaxton *et al*. 1993, Plaxton 1996). The relative expression levels of each mRNA were similar in leaves of different ages, except for the PEPC mRNA, which accumulated more strongly in leaves at late growth stages. Since ammonium and glutamine activate PEPC mRNA expression (Sugiharto *et al*. 1992), nitrogenous compounds derived from protein degradation during leaf senescence must be expected to stimulate PEPC mRNA expression.

GS1 is localised in the cytosol while GS2 acts in chloroplasts. Both enzymes catalyze the ATP-dependent condensation of NH_3 with glutamate to produce glutamine. The time-courses of relative mRNA levels of these enzymes differed in the rice leaves we studied (Fig. 2). GS1 protein content increased during leaf senescence, while the content of GS2 declined in parallel with the decrease in contents of chlorophyll, RuBPCO, and soluble proteins (Kawakami and Watanabe 1988, Kamachi *et al*. 1991). These patterns illustrate the roles of GS1 in the synthesis of translocable nitrogenous compounds (glutamine) in the senescent leaf, and of GS2 in capturing ammonium molecules which are released during photorespiration (Kozaki and Takeba 1996). Though mRNA levels do not necessarily correspond to contents of the corresponding proteins, the similarity of the time-courses of relative mRNA levels of RuBPCO and GS2 indicates the involvement of GS2 in photosynthesis-related processes Fig. 2).

In contrast to the later developing leaves, patterns of relative mRNA levels did not respond to N application in leaf 3 (Fig. 2). Apparently, the temporal patterns of gene expression in leaf 3 are already determined during early leaf development in the seed, before the seedling comes into contact with exogenous N sources. In younger leaves, mRNAs were accumulated at higher absolute levels and over a longer period in the high N treatment. However, mRNA concentrations expressed as relative levels were similar in the $N_{0.5}$ and $N_{3.0}$ treatments.

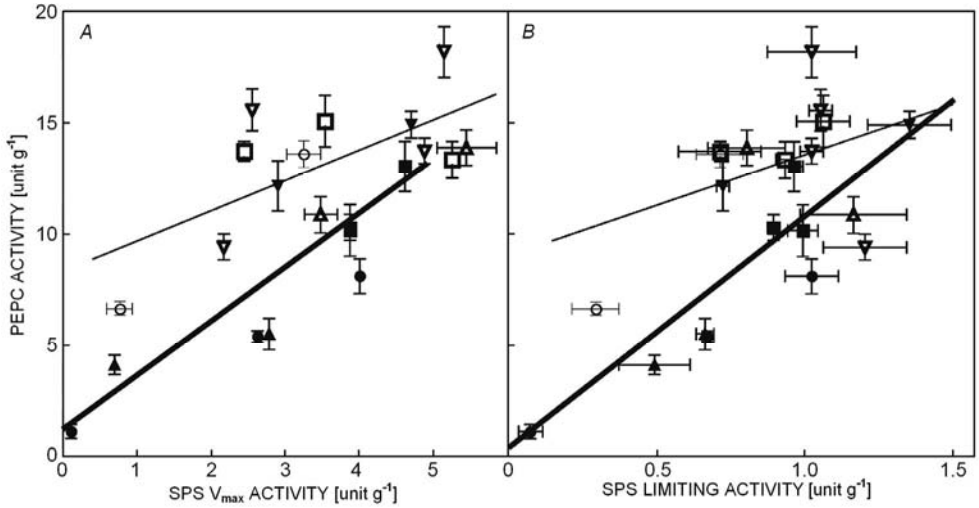

Fig. 4. Correlation of the activities of PEPC and SPS: *A*: Relationship between SPS V_{max} and PEPC activity. *B*: Relationship between SPS limiting activity and PEPC activity. *Thin lines* represent leaves developed during the vegetative growth stage (5^{th} and 7^{th} leaf); *bold lines* leaves developed during the reproductive growth stage (9^{th} and flag leaf). $N_{0.5}$: ○ 5^{th} leaf; Δ 7^{th} leaf; ● 9^{th} leaf; ▲ flag leaf; $N_{3.0}$: □ 5^{th} leaf; 7^{th} leaf; ■ 9^{th} leaf; ▼ flag leaf.
PEPC activity = 1.359 SPS V_{max} + 8.323 (r^2 = 0.369 a, n = 44) (5th and 7^{th} leaves, *narrow line*)
PEPC activity = 2.423 SPS V_{max} + 1.230 (r^2 = 0.687*, n = 40) (9^{th} and flag leaves, *thick line*)
PEPC activity = 4.529 SPS limiting activity + 9.027 (r^2 = 0.154, n = 44) (5^{th} and 7^{th} leaves, *narrow line*)
PEPC activity = 10.44 SPS limiting activity + 0.3492 (r^2 = 0.669*, n = 40) (9^{th} and flag leaves, *thick line*)
Asteroids denote significance of *t*-ratio; $^*p$<0.001.

In the 9th leaf, relative mRNA levels tended to be lower than in other leaves, regardless of the N treatment (Fig. 1). This might be related to the switch from the vegetative to the reproductive stage. In contrast to the older leaves, the primordium of leaf 9 is not formed at the early vegetative growth stage, but at the ear primordia formation stage. During the vegetative stage, N metabolism is highly active to support the generation of new organs, while at the reproductive stage C metabolism takes over to enable the accumulation of starch in the developing seeds. Therefore, we expected the expression patterns of genes related to C and N metabolism, respectively, to change in opposite senses in the 9th leaf. This was not the case; rather mRNA levels were generally depressed. Similarly, we expected PEPC mRNA to accumulate to higher levels at the vegetative growth stage, and SPS mRNA to increase at the reproductive stage. However, our expectation was not met.

In whole rice plants, the C/N ratio increases exponentially with growth (Osaki *et al*. 1992). Our results indicated that initial photosynthate distribution (within 5 min of carbon fixation) to the N-pool was high before the panicle primordia formation stage, but decreased thereafter (Fig. 6). Moreover, the balance of PEPC and SPS activities changed around the panicle primordia formation stage (Fig. 4, Nakamura *et al*. 1997). These findings were in accord with the proposal that the alternative allocation of DHAP-derived C to the synthesis of amino acids or sucrose is regulated by the relative strengths of PEPC and SPS (Champigny and Foyer 1992).

As shown in Fig. 4, the ratio of the activities of PEPC and SPS was higher in the 5th and 7th leaves (which developed during the vegetative growth stage) than in the 9th and flag leaf (whose primordia were formed after panicle primordia formation and which grow during the reproductive growth stage). The relationship between N content and SPS V_{max} as well as PEPC activity was significantly different between leaves growing in the vegetative or the reproductive stage (Fig. 5). Thus, not only leaf position (growth stage) but also the N content of the leaf regulates the activity of both enzymes. Moreover, N content and PEPC were strongly correlated (Fig. 5*C*), indicating that the relative activity of PEPC decreased as leaf N content declined during leaf development.

Our results (Fig. 5*C*) are in agreement with the fact that PEPC activity increased with N content in C_3 plants including wheat, barley, tomato (Koga and Ikeda 1997), ryegrass (Sagi *et al*. 1998), and Norway spruce (Wallenda *et al*. 1996). Moreover, PEPC activity is positively correlated with the N assimilation rate (Vanlerberghe *et al*. 1990). This makes sense as PEPC supplies C to the TCA cycle from which the synthesis of amino acids starts. SPS is a key enzyme regulating glucose synthesis (Stitt *et al*. 1987, Huber and Huber 1992). Both SPS and PEPC activities are tightly regulated by metabolites such as G_6P and phosphate in the case of SPS (Stitt *et al*. 1988), and malate, aspartic acid, and glutamic acid in the

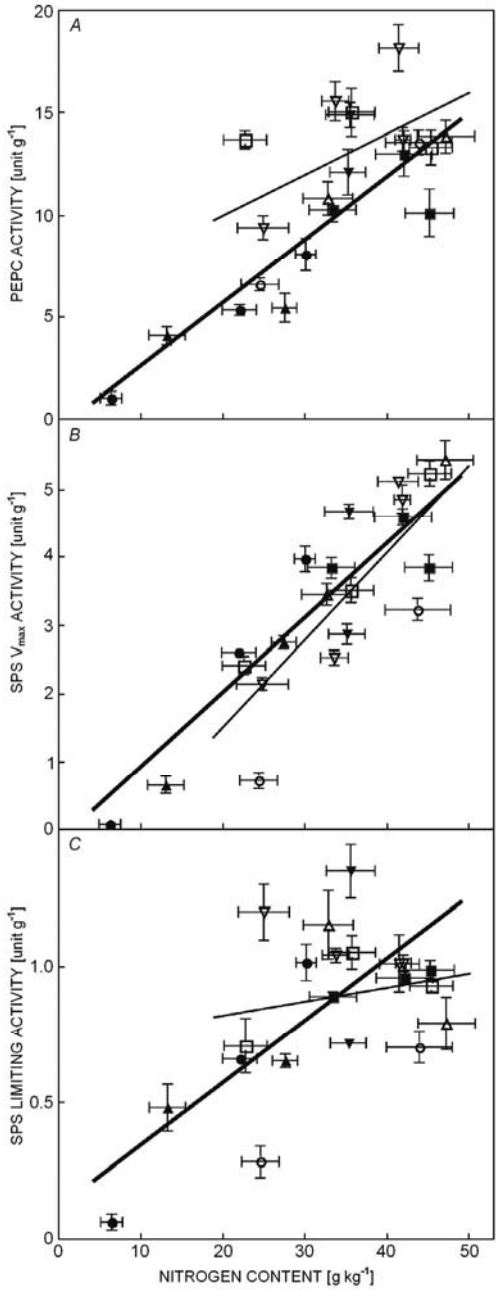

Fig. 5. Relationships between nitrogen content and (A) SPS V_{max}, (*B*) SPS limiting activity, or (*C*) PEPC activity. For symbols see Fig. 4.
PEPC activity = 0.200 N content + 5.98 (r^2 = 0.306*, n = 44) (5th and 7th leaves, *narrow line*)
PEPC activity = 0.309 N content – 0.467 (r^2 = 0.713*, n = 40) (9th and flag leaves, *thick line*)
SPS V_{max} = 0.128 N content – 1.06 (r^2 = 0.630*, n = 44) (5th and 7th leaves, *narrow line*)
SPS V_{max} = 0.110 N content – 0.178 (r^2 = 0.766*, n = 40) (9th and flag leaves, *thick line*)
SPS limiting activity = 0.00515 N content + 0.718 (r^2 = 0.027, n = 44) (5th and 7th leaves, *narrow line*)
SPS limiting activity = 0.0229 N content + 0.117 (r^2 = (0.636*, n = 40) (9th and flag leaves, *thick line*)
Asteroids denote significance of *t*-ratio. *p<0.001.

case of PEPC (Huppe and Turpin 1994) as well as by reversible phosphorylation by PEPC kinase and protein phosphatase (Vidal and Chollet 1997). Phosphorylation and dephosphorylation (Foyer *et al.* 1995, MacKintosh 1998) regulate activities of cytoplasmic SPS and PEPC while photosynthesis provides precursors as a signal to promote the activation (dephosphorylation) of SPS and activation (phosphorylation) of PEPC in the cytosol. High nitrate application promotes the activation of PEPC but not SPS (Foyer *et al.* 1995). Feeding maize with ammonium or nitrate increases PEPC protein content and mRNA *via* cytokinin-dependent transcription of the PEPC gene (Sugiharto and Sugiyama 1992, Suzuki *et al.* 1994). The regulation of PEPC protein by N nutrition proceeded by phosphorylation when nitrate was fed to N-deficient wheat leaf (Duff and Chollet 1995). These overall effects tend to activate sucrose synthesis by SPS and amino acid synthesis by PEPC. Thus the observed difference in the expression patterns of mRNA of SPS and PEPC was different from that of the enzymatic activity pattern, suggesting to be regulated by the biochemical function of enzyme.

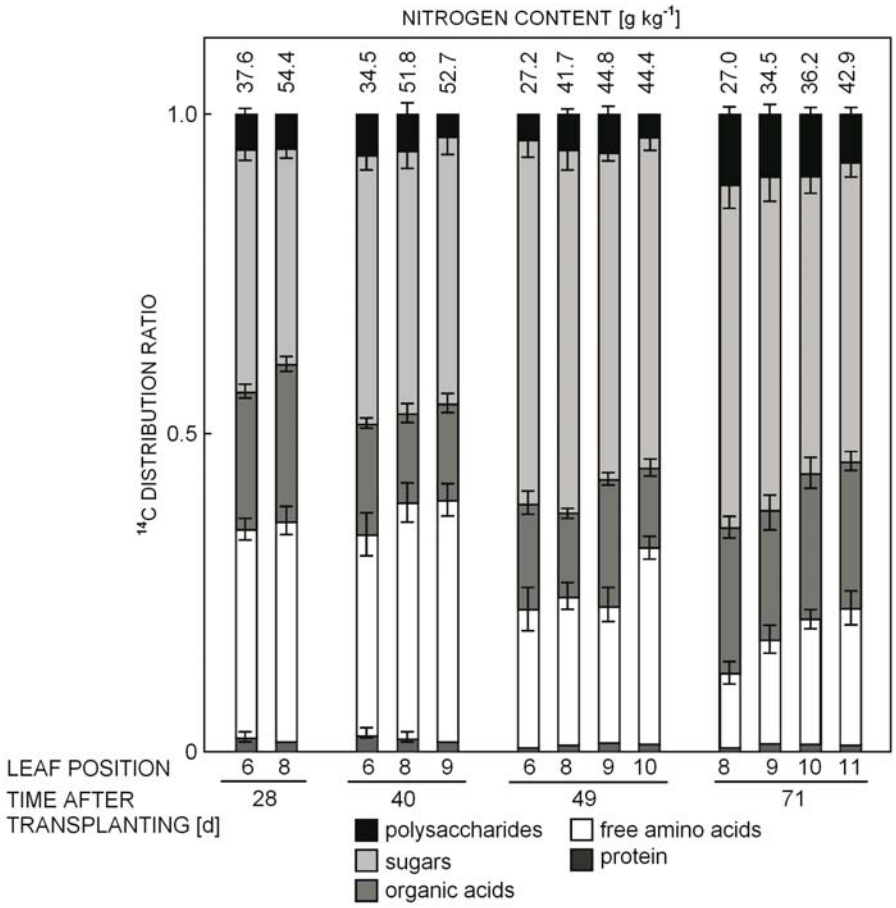

Fig. 6. ^{14}C distribution to various classes of compounds at the end of a 5-min period of exposure to ^{14}C assimilation in individual leaves at various developmental stages. *Values on top of the bars* indicate total nitrogen content. *Error bars* indicate standard error.

The ratio of the SPS limiting activity and the PEPC activity represent the relative intensity of C flow from DHAP to either sucrose or amino acids. In individual leaves, the ^{14}C distribution to the N-pool decreased with time, especially after the panicle primordia formation stage (Fig. 6). The ^{14}C distribution to the N- and C-pools is regulated primarily by the N status in leaves and leaf ontogeny (Fig. 8). We conclude that the photosynthates are distributed to the C- and N-pools before translocation from the leaves. However, ^{14}C distribution to the N-pool was not always strictly dependent on N content, but was also influenced by leaf position, that is, the leaf developmental stage (Fig. 7). The regulation by PEPC of C/N partitioning in nodules was also reported (Taybi *et al.* 2000, Bakrim *et al.* 2001), but the role of PEPC and SPS on the regulation of C/N partitioning along with the development of a leaf is not known.

Along with leaf development, as soon as the leaf reaches a specific stage of development, the sink to source transfer occurs in the leaf (Fellows and Geiger 1974, Turgeon 1989) while carbon and nitrogen do not equally behave in a similar manner. Osaki *et al.* (1988)

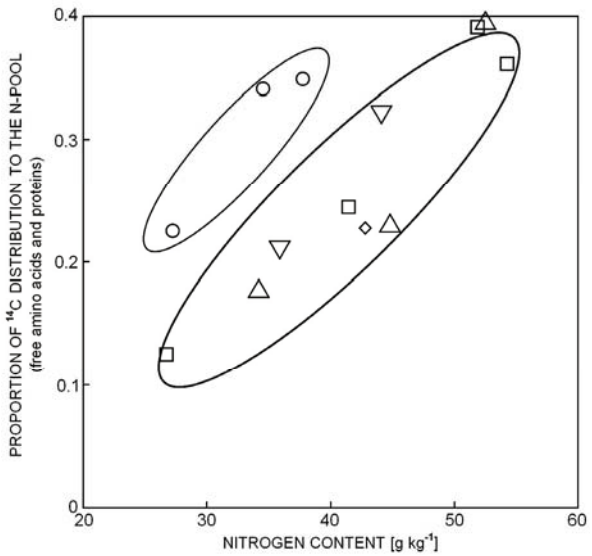

Fig. 7. Relationship between N content and the proportion of ^{14}C distribution to the N-pool (free amino acids and proteins) in leaves at the end of 5 min exposure to $^{14}CO_2$. The relationship between the parameters is significantly different in the 6th leaf from that in other leaves. ○ 6th leaf; □ 8th leaf; Δ 9th leaf; ◊ 10th leaf; 11th leaf.

demonstrated that nitrogen accumulation was almost ceased in rice, and the major part of N in reproductive organs was derived from nitrogen absorbed and once incorporated into proteins in leaves. Nevertheless, Tanaka and Osaki (1983) showed that major part of carbon in reproductive organ of rice was derived from the photosynthates that were acquired at about the same time. The physiological change in leaf may be regulated by hormones such as cytokinin, but there is no direct evidence that cytokinin regulates the C/N partitioning in plant.

We suggest that more sophisticated regulation of SPS and PEPC by content of metabolites and/or phosphorylation/dephosphorylation of each enzyme is regulated by mRNA expression. But to our knowledge the integrated information about the over-all regulation of SPS and PEPC has not been reported yet. Though our report did not demonstrate the amounts of each protein, we speculate about the existence of protein content regulation, because (*1*) the expression patterns of mRNA and enzyme activity were different (compare Figs. 2 and 3), and (*2*) SPS limiting activity to SPS V_{max} activity was not stable (Fig. 3).

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