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Photosynthesis and Carbon Partitioning in Transgenic Tobacco Plants Deficient in Leaf Cytosolic Pyruvate Kinase[1]

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Whole-plant diurnal C exchange analysis provided a noninvasive estimation of daily net C gain in transgenic tobacco (*Nicotiana tabacum* L.) plants deficient in leaf cytosolic pyruvate kinase (PK_c-). PK_c- plants cultivated under a low light intensity (100 μmol m^{-2} s^{-1}) were previously shown to exhibit markedly reduced root growth, as well as delayed shoot and flower development when compared with plants having wild-type levels of PK_c (PK_c+). PK_c- and PK_c+ source leaves showed a similar net C gain, photosynthesis over a range of light intensities, and a capacity to export newly fixed $^{14}CO_2$ during photosynthesis. However, during growth under low light the nighttime, export of previously fixed $^{14}CO_2$ by fully expanded PK_c- leaves was 40% lower, whereas concurrent respiratory $^{14}CO_2$ evolution was 40% higher than that of PK_c+ leaves. This provides a rationale for the reduced root growth of the PK_c- plants grown at low irradiance. Leaf photosynthetic and export characteristics in PK_c- and PK_c+ plants raised in a greenhouse during winter months resembled those of plants grown in chambers at low irradiance. The data suggest that PK_c in source leaves has a critical role in regulating nighttime respiration particularly when the available pool of photoassimilates for export and leaf respiratory processes are low.

PK catalyzes the synthesis of pyruvate and ATP from PEP and ADP and is believed to be a major control point of plant and nonplant glycolysis (Plaxton, 1996). The enzyme has been demonstrated to be significantly displaced from equilibrium in vivo and has pronounced regulatory properties in vitro. Plant PK exists as cytosolic and plastidic isozymes (PK_c and PK_p, respectively), which differ substantially in their molecular and kinetic/regulatory properties. PK_c plays an important role in generating the precursor pyruvate for various biosynthetic pathways and mitochondrial respiration. The biosynthetic role of cytosolic glycolysis is central in actively growing autotrophic tissue (Plaxton, 1996), in which a significant proportion of the C that enters the glycolytic pathway is incorporated into numerous compounds such as amino acids, nucleic acids, fatty acids, and secondary metabolites. The exact contribution that these enzymatic steps provide in source leaves and in developing sink tissues remains unclear.

PK_c deficiency in nonplant species causes serious detrimental effects. However, our earlier studies revealed that transgenic tobacco plants (*Nicotiana tabacum* L.) deficient in leaf PK_c (PK_c-) grew from seed to seed, demonstrating the remarkable flexibility of plant PEP metabolism (Gottlob-McHugh et al., 1992; Knowles et al., 1998). Plant cells can use a variety of alternative metabolic routes to directly or indirectly circumvent the reaction catalyzed by PK_c. These could include the action of PEP phosphatase or the combined action of PEP carboxylase, malate dehydrogenase, and NAD-malic enzyme (Plaxton, 1996). It is also possible that the elevated levels of PEP observed in the PK_c- leaves (Gottlob-McHugh et al., 1992) results in an increased flux of glycolytic C from the cytosol to the chloroplast where the PEP may be metabolized by PK_p. Elimination of leaf PK_c can alter C metabolism and growth when total C supply is limited by growing the plants at reduced PPFD. Knowles et al. (1998) showed that, when grown at low PPFD, the PK_c- tobacco exhibited a delayed shoot and flower development, as well as a striking reduction in root growth. Since the lack of PK_c and the resulting altered glycolytic activity appeared to be confined to the leaves, we decided to further investigate the role of the leaves as sources of reduced C.

We recently described a procedure for evaluating immediate export rates during photosynthesis so that we could test and differentiate between the effect of environmental challenges, such as leaf warming, on the ability of the sources leaves to fix CO_2 and to export the reduced C products (Jiao and Grodzinski, 1996; Leonardos et al., 1996). These protocols were modified to study the effect of short- and long-term CO_2 enrichment on photosynthesis and export rates in source leaves of a number of C_3 and C_4 species (Jiao and Grodzinski, 1996, 1998; Grodzinski et al., 1998). The aim of the present study was to use these procedures to assess whole-plant gas exchange, photosynthesis, respiration, and export in intact, attached source leaves of two independent homozygous PK_c- tobacco lines. We

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Abbreviations: GM, Geiger-Müller; NCER, net carbon exchange rate; PK, pyruvate kinase; PK_c and PK_p, cytosolic PK and plastid PK, respectively; PK_c- and PK_c+, tobacco plants that are and are not deficient in leaf PK_c, respectively.

report that, when both PK_c- lines were cultivated under low irradiance, nighttime export of recently fixed CO_2 was reduced, whereas concurrent respiration of ^{14}C assimilates was enhanced. These findings provide a rationale for the reduced root development of the PK_c- plants.

MATERIALS AND METHODS

Plant Materials

Two transgenic tobacco (*Nicotiana tabacum* L.) lines that specifically lacked PK_c in their leaves were obtained because of the *trans*-inactivation phenomenon known as cosuppression (Gottlob-McHugh et al., 1992). Selfing of each parent line resulted in two PK_c- progeny lines, 14-1 and 15-7, in which the cosuppression was relatively stable, and a PK_c+ line, 18-7, which contained wild-type levels of PK_c (Knowles et al., 1998). The seeds obtained from the selfing of 14-1 and 15-7 (designated PK_c-1 and PK_c-2, respectively) plus the wild type and 18-7 (PK_c+1 and PK_c+2, respectively) were used in the present study. Seeds were germinated and grown in PROMIX-BX (Les Tourbières Premier LTÉE, Rivière du Loup, QC, Canada) in 20-cm pots in growth chambers at the University of Guelph. A 16-h photoperiod was maintained at 22°C ± 1°C during which the PPFD (400–700 nm) was 500 μmol m^{-2} s^{-1} (moderate light) or 100 μmol m^{-2} s^{-1} (low light). During the 8-h dark period the temperature was 18°C ± 1°C. Plants were fertilized biweekly with a nutrient solution, as described previously (Knowles et al., 1998).

In other experiments plants were grown during winter months (i.e. between November and March) in a greenhouse at the University of Guelph (latitude approximately 43.5°N). Solar-generated PPFD varied from 50 μmol m^{-2} s^{-1} on overcast days to more than 1000 μmol m^{-2} s^{-1} on sunny days. Artificial lighting was supplied by 1000-W Sylvania metal halide lamps throughout the 16-h photoperiod and maintained a minimum daytime PPFD of about 130 μmol m^{-2} s^{-1} at the plant level. Temperatures in the experimental greenhouse compartments were typically 24°C ± 1°C/18°C ± 1°C, day (16 h)/night (8 h).

PK Assay and Immunoblot Analyses

All measurements were made on the most recently fully expanded leaves, which were harvested, frozen in liquid N_2, and stored at −80°C until used. Enzyme extracts were prepared from leaves, as described previously (Gottlob-McHugh et al., 1992). PK activity was assayed spectrophotometrically at 25°C, as described by Plaxton (1989), and was corrected for PEP phosphatase activity by omitting ADP from the reaction mixture. One unit of PK activity is defined as the amount of enzyme resulting in the utilization of 1 μmol PEP min^{-1}. Activity values represent the means of quadruplicate determinations conducted with three separate extracts and were reproducible to within ±10% SE. Protein concentration was determined by the modified Bradford assay (Bollag and Edelstein, 1991) using bovine γ-globulin as the standard. Extracts were electrophoresed on 7.5% (w/v) SDS-polyacrylamide minigels and electroblotted onto a PVDF membrane. Immunoblotting was performed using affinity-purified anti-castor endosperm PK_c-IgG, and antigenic polypeptides were detected using an alkaline-phosphatase-conjugated secondary antibody, as described previously (Plaxton, 1989). Immunological specificities were confirmed by conducting immunoblots in which rabbit preimmune serum was substituted for the anti-PK_c-IgG.

Whole-Plant NCER and Daily C Gain

The growth rate of the plants was measured noninvasively by determining the whole-plant NCER using whole-plant gas-exchange chambers, as described previously (Dutton et al., 1988). Measurements were made at 24°C ± 1°C/18°C ± 1°C, day (16 h)/night (8 h), and ambient CO_2 (35 Pa) and O_2 (21 KPa). Plants grown under the moderate lighting regime were measured at 500 μmol m^{-2} s^{-1} PPFD at the level of the top leaf, whereas the plants grown under low light were measured at 100 μmol m^{-2} s^{-1} PPFD. One plant per chamber was used for the measurements. Because four chambers were run concurrently using a central IR gas-analysis system, CO_2 exchange measurements were made at 6- to 8-min intervals during a typical 50-h test (Leonardos et al., 1994). Six replications were conducted and based on the average diurnal gas exchange measurements; whole-plant daily C gain was calculated for each line under both PPFD levels.

Leaf Net CO_2 Exchange Rates

Plants were illuminated with Sylvania metal halide lamps (1000 W), which could provide a maximum of about 1800 μmol m^{-2} s^{-1} PPFD at the leaf level. The light response curves for photosynthesis were derived by using a series of neutral screens to reduce PPFD. The leaf gas-exchange rates of the most recently expanded leaves of 8- to 9-week-old plants were measured using an open-flow system described previously (Jiao and Grodzinski, 1996). Both leaf and air temperature in the plant chamber were maintained at 24°C ± 1°C. The inlet gas contained 35 Pa CO_2 and 21 KPa O_2. Chlorophyll content was determined as described by Wintermans and de Mot (1965).

Export and Storage of ^{14}C Assimilates during Photosynthesis

Export of newly fixed ^{14}C assimilates during steady-state photosynthesis was estimated as described previously (Jiao and Grodzinski, 1996). Plants were illuminated with Sylvania metal halide lamps (1000 W), which provided about 1200 μmol m^{-2} s^{-1} PPFD at the leaf level. A GM detector was mounted in the lower half of the leaf cuvette to continuously monitor radioactivity (i.e. ^{14}C accumulation) in the source leaves. To establish the time required to reach an equilibrium between the $^{14}CO_2$ of known specific activity and the ^{14}C-labeled Suc pool, leaves were first fed with $^{14}CO_2$ from 30 to 120 min and the labeled products were analyzed. The fed leaf was extracted with boiling 80% ethanol:water (v/v), and the major ^{14}C-labeled assimilates

were analyzed as described elsewhere (Jiao and Grodzinski, 1996). During feeding periods net $^{14}CO_2$ assimilation and ^{14}C accumulation rates of attached intact leaves were monitored continuously in a noninvasive manner using an IR gas and a GM detector, respectively. The measurement of the export rate of the newly fixed $^{14}CO_2$ during steady-state photosynthesis was calculated as the difference between the rate of $^{14}CO_2$ assimilation and the retention of ^{14}C assimilates 90 to 120 min after $^{14}CO_2$ feeding began. As reported below, isotopic equilibrium between the specific activity of the ^{14}C-Suc in the leaf and that of the $^{14}CO_2$ in the gas stream was established during this period. The CO_2 and O_2 levels were 35 Pa and 21 KPa, respectively. Whole-plant and leaf temperatures were 24°C ± 1°C and the RH was approximately 70%.

Leaf Dark Respiration and Nighttime Export of ^{14}C-Labeled Assimilates

The respiration and export of ^{14}C assimilates during the dark period that followed the $^{14}CO_2$ feeding were determined by trapping the $^{14}CO_2$ released and continuing to monitor the level of ^{14}C in the source leaf with the GM detectors mounted in the leaf cuvettes (Jiao and Grodzinski, 1998). After the $^{14}CO_2$ was supplied for 2 h and the rates of photosynthesis and concurrent export were determined, the lamps were extinguished and the leaves were supplied with a gas stream lacking $^{14}CO_2$. The CO_2 and O_2 levels were 35 Pa and 21 KPa during the 14-h chase period in the dark. The export of ^{14}C in the dark was corrected for loss of $^{14}CO_2$ due to respiration, which was determined by trapping the gas in 20% (w/v) KOH and measuring radioactivity by liquid-scintillation counting (Leonardos et al., 1996).

RESULTS

PK Activity and Immunoblot Analysis

PK activity assays and immunoblotting using anti-castor endosperm PK_c-IgG were used to assess the relative abundance of PK_c in extracts prepared from leaves of the same tobacco plants used in the physiological studies described below. The PK activities of fully expanded PK_c+ and PK_c- leaves harvested from plants grown under moderate or low light are reported in Figure 1. Extractable PK activity of the PK_c- leaves was reduced by 70% to 85% relative to the PK_c+ controls. Two to three separate extracts of the PK_c+ and PK_c- leaves were analyzed by immunoblotting, and representative results are shown in Figure 1. Immunoblots of PK_c+ leaf extracts revealed an intense immunoreactive polypeptide at 57 kD, which corresponds to subunits of tobacco leaf PK_c (Knowles et al., 1998). By contrast, antigenic staining of PK_c on immunoblots of PK_c- leaf extracts was either very faint (PK_c-1) or undetectable.

Plant Growth, Leaf Area, and Chlorophyll Content

As reported previously (Knowles et al., 1998), the PK_c- lines had a slower rate of development than did the two

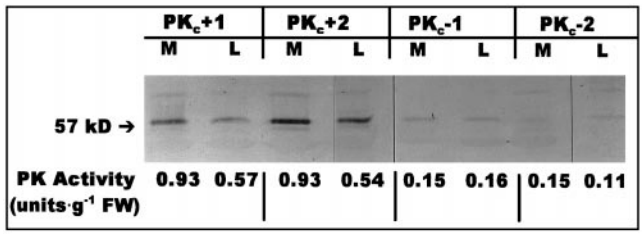

Figure 1. Immunological detection and activities of PK_c in extracts prepared from leaves of PK_c+ and PK_c- tobacco plants grown under moderate ("M" lanes) or low ("L" lanes) light intensities. Crude extracts were electrophoresed on 7.5% (w/v) SDS-polyacrylamide minigels (15 μg of protein per lane) and transferred to a PVDF membrane. Immunoblotting was performed using affinity-purified anti-castor endosperm PK_c-IgG (Plaxton, 1989). PK activities represent the means of quadruplicate determinations conducted with three separate extracts and were reproducible to within ±10% SE. FW, Fresh weight.

PK_c+ lines. Therefore, plants that were at an identical developmental stage were compared. The data reported here are from the stage at which the first flower bud was visible. Total leaf area per plant of the four lines grown under 500 μmol m^{-2} s^{-1} PPFD (moderate light) were similar (Table I). However, when plants were raised under 100 μmol m^{-2} s^{-1} PPFD (low light), the total leaf area of the PK_c-1 was less than that of the PK_c+ controls.

When leaf chlorophyll content of the PK_c- and PK_c+ lines were compared at a single light condition, the leaves of the PK_c- lines had more chlorophyll than did the leaves of the PK_c+ plants (Table I). Under moderate light leaves of each line had a greater chlorophyll content than leaves of that same line had when grown under low light (Table I).

Whole-Plant NCER and Daily C Gain

Figure 2 shows whole-plant NCER and daily net C gain of each tobacco line grown and measured at 500 or 100 μmol m^{-2} s^{-1} PPFD. Within each panel it is clear that photosynthesis at moderate light was greater than that at low light. However, whole-plant photosynthesis of the four lines was similar when compared at the same PPFD level (Fig. 2, A–D). The photosynthesis rates for PK_c-1 and PK_c-2 plants grown and measured at the moderate light intensity were 5.8 and 6.1 μmol C fixed m^{-2} s^{-1}, respectively. Whole-plant dark respiration rates ranged from 1.2 μmol C released m^{-2} s^{-1} in PK_c+1 to 1.6 μmol C released m^{-2} s^{-1} in PK_c-2. At the lower PPFD level, plant photosynthesis rates were one-third that of those at the moderate PPFD level, averaging 1.9 and 2.1 μmol C fixed m^{-2} s^{-1} for the PK_c-2 (Fig. 2D) and PK_c-1 lines, respectively (Fig. 2C). Dark respiration rates of plants grown under low light were also less than those of plants grown under moderate light. For example, in plants acclimatized to 100 μmol m^{-2} s^{-1} PPFD, the average rates of dark respiration during the 8-h night period were approximately 0.56 and 0.66 μmol C released m^{-2} s^{-1} for the PK_c+1 (Fig. 2A) and PK_c-2 lines (Fig. 2D), respectively.

At 500 μmol m^{-2} s^{-1} PPFD the daily C gain of all four tobacco lines was approximately 300 μmol C gained m^{-2},

Table I. *Total leaf area and chlorophyll content at flowering stage of tobacco plants cultivated under moderate or low light in growth chambers*

Each value represents the mean ± SE obtained with at least six leaves on six different plants. Significant differences among PK_c+ and PK_c- lines by Student's *t* test ($P < 0.05$) are indicated by superscript letters (a, b, c).

Plant Type	Leaf Area		Chlorophyll	
	Moderate light	Low light	Moderate light	Low light
	cm^2		$\mu g\ cm^{-2}$	
PK_c+1	2343 ± 101^a	2178 ± 88^a	39.3 ± 2.3^a	30.6 ± 1.0^c
PK_c+2	2252 ± 131^a	2175 ± 96^a	36.4 ± 1.2^a	27.1 ± 0.6^c
PK_c-1	2213 ± 86^a	1829 ± 62^b	43.8 ± 0.9^b	36.1 ± 1.9^a
PK_c-2	2208 ± 94^a	2083 ± 45^a	43.4 ± 0.6^b	35.3 ± 0.6^a

which was approximately 3-fold that measured at 100 μmol m^{-2} s^{-1} PPFD (Fig. 2, E–H). The data confirm that at the whole-plant level there was little difference in daily C gain per leaf area among the four tobacco lines.

Leaf Photosynthesis at Varying Irradiances

When intact attached source leaves were tested individually, net CO_2 fixation rates of PK_c- plants were similar to those of the PK_c+ plants grown at the same light level (Fig. 3). All plants grown at the moderate PPFD exhibited higher rates of leaf photosynthesis (Fig. 3A) than did plants grown at low PPFD (Fig. 3B). The PPFD required to saturate leaf photosynthesis was greater in plants acclimatized to the higher PPFD. In all of the plants grown under moderate and low light, the PPFD required to saturate photosynthesis was approximately 900 and 700 μmol m^{-2} s^{-1}, respectively (Fig. 3).

Establishing Conditions for Estimating Export Rates during Photosynthesis

The ^{14}C-labeling patterns in intact attached source leaves from PK_c- and PK_c+ plants are compared in Figure 4. Leaves of each line that had been grown at low or moderate PPFD were tested at similar stages of plant development at a PPFD that saturates photosynthesis. For ease of reporting the only results shown here are for leaves of the PK_c+1 line (Fig. 4, A, C, and E) and the PK_c-1 line (Fig. 4, B, D, and F). During the 120-min feed period total $^{14}CO_2$ fixation increased linearly (Fig. 4, A and B). The net photosynthetic rates were lower in plants grown at 100 μmol m^{-2} s^{-1} PPFD (refer to Figs. 3 and 5), but net CO_2 fixation rates were also constant during the 2-h feed. As illustrated in Figure 4, A and B, the rates of total ^{14}C retention in the source leaves were higher during the 1st h of the feed and reflects the fact that the label was being randomized among

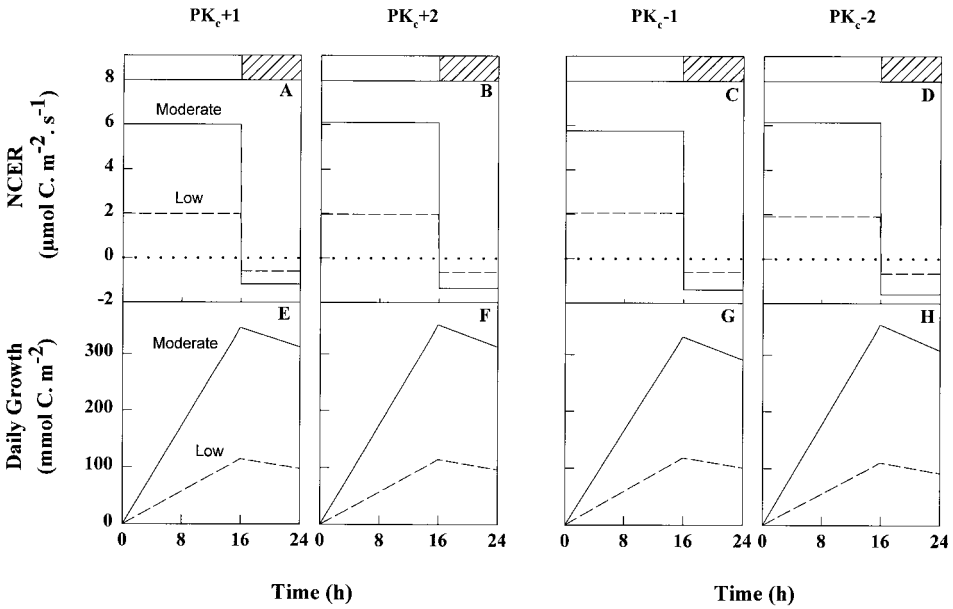

Figure 2. Whole-plant NCERs and net C gain during a 16-h photoperiod and an 8-h dark period in tobacco: PK_c+1 (A and E); PK_c+2 (B and F); PK_c-1 (C and G); and PK_c-2 (D and H). NCER was measured when the first flower bud appeared. Plants were both grown and measured at a PPFD of 500 (solid lines) or 100 (dashed lines) μmol m^{-2} s^{-1}. The CO_2 and O_2 levels were 35 Pa and 21 KPa, respectively. Day and night temperatures during the analysis were 22°C and 18°C, respectively. The data are the means of at least four replications.

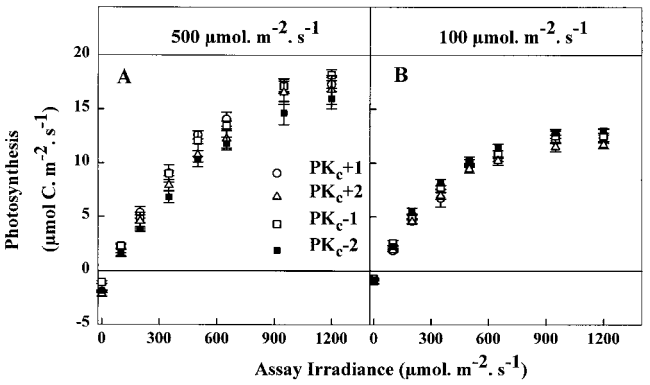

Figure 3. Leaf photosynthetic light-response curves of PK_c+ and PK_c- tobacco plants. Net photosynthesis was measured when the first flower bud appeared on plants grown in growth chambers under 500 (A) or 100 (B) μmol m^{-2} s^{-1} PPFD. Photosynthesis was measured at 25°C ± 1°C. The CO_2 and O_2 levels were 35 Pa and 21 KPa, respectively. Each value represents the mean ± SE obtained with at least six leaves on six different plants.

different intermediate pools before the export pools reached isotopic equilibrium with $^{14}CO_2$ in the gas stream.

A major labeled product accumulating in all leaves during the 2-h feed was ^{14}C-Suc (Fig. 4, C and D). Photosynthesis was greater and more ^{14}C-Suc accumulated in leaves of plants that had been grown under moderate light (Fig. 4, C and D, solid line) than in those grown under low light (Fig. 4, C and D, dotted line). Specific activities of ^{14}C-Suc increased rapidly in the leaves of the PK_c+ plants during the 1st h of feed (Fig. 4E). The specific activities of ^{14}C-Suc of plants grown under low light increased similarly. However, the specific activity of ^{14}C-Suc was lower in the PK_c- leaves (Fig. 4F) in the PK_c+ leaves (Fig. 4E). The specific activity of ^{14}C-Suc in PK_c- leaves was slightly higher in the plants grown under low light. Nevertheless, taken together the data clearly show that in all cases the specific activity of the ^{14}C-Suc was unchanged after 90 min (Fig. 4, E and F). Export rates reported below (Fig. 5) were calculated from ^{14}C retention and photosynthesis rates obtained between 90 and 120 min of the feed when it was assumed that the ^{14}C-Suc was leaving the leaf as readily as it was being synthesized from newly fixed $^{14}CO_2$.

Estimates of Export Rates during Photosynthesis and ^{14}C Partitioning during the Feed

The photosynthesis rates of plants grown under moderate light ranged from 14.5 to 16 μmol C fixed m^{-2} s^{-1} (Figs. 3 and 5A). All lines grown under low light exhibited significantly lower leaf photosynthesis rates than plants grown under moderate light (Figs. 3 and 5B). The photosynthesis rates of plants grown in greenhouses during winter months were similar to those raised in growth chambers at the lower PPFD level (Fig. 5, B and C). Approximately 55% to 65% of the ^{14}C assimilated was exported immediately in plants cultivated in growth chambers under either 500 (Fig. 5D) or 100 μmol m^{-2} s^{-1} PPFD (Fig. 5E). Only 45% to 50% was exported immediately in the plants grown in the greenhouse (Fig. 5F). However, the immediate export rates of ^{14}C-labeled photosynthate from leaves of both PK_c- lines were statistically similar to those of the PK_c+ lines regardless of the growth conditions (Fig. 5, D–F).

When leaves grown at the same light level were compared, there was no difference in the partitioning of label into starch and sugars. There appeared to be more label accumulated in starch in the plants growing in the greenhouse (Fig. 5, C and F), which reflected the fact that the immediate export rates were slightly lower in these plants. The patterns of ^{14}C distribution among the ethanol-soluble

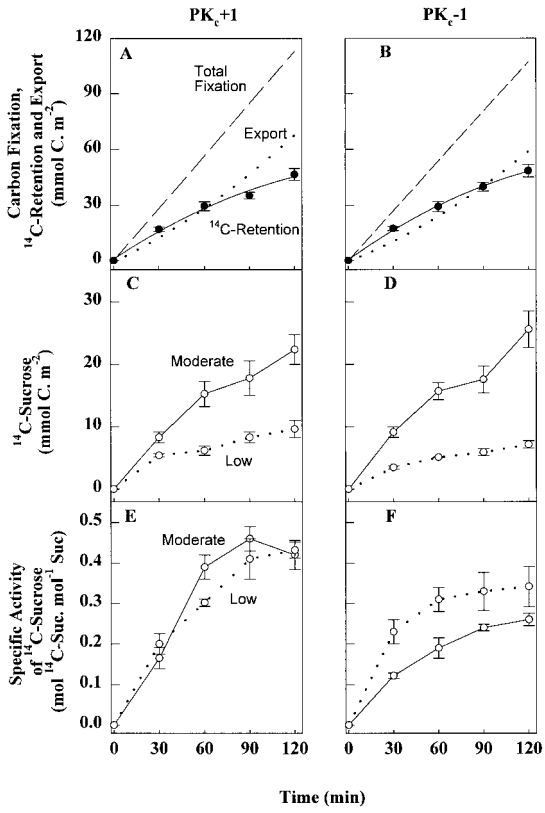

Figure 4. ^{14}C-Labeling patterns of intact, attached source leaves of PK_c+1 (A, C, and E) and PK_c-1 (B, D, and F) tobacco plants. A and B, Total $^{14}CO_2$ fixation, ^{14}C retention, and ^{14}C export of leaves of PK_c+1 (A) and PK_c-1 (B) plants grown at 500 μmol m^{-2} s^{-1} PPFD. Measurements were made during a 2-h $^{14}CO_2$ feed at 35 Pa CO_2, 25°C, and 1000 μmol m^{-2} s^{-1} PPFD. Total C fixation (dashed line) was calculated from IR gas analyzer data. The ^{14}C retention was monitored noninvasively with the GM detector (solid line) and invasively (●) following ethanol extraction. Export during steady-state $^{14}CO_2$ feeding (fine dotted line) was estimated as the difference between total fixation and ^{14}C retention in the leaf. C and D, Accumulation of ^{14}C-Suc in the source leaves of plants grown under low and moderate PPFD (i.e. 100 and 500 μmol m^{-2} s^{-1} PPFD), respectively, but analyzed at a saturating PPFD for photosynthesis. E and F, Time course for changes in specific activities of ^{14}C-Suc in the source leaves of plants grown under low and moderate PPFD levels, respectively. Each point represents the mean value (±SE) obtained with four leaves of four different plants.

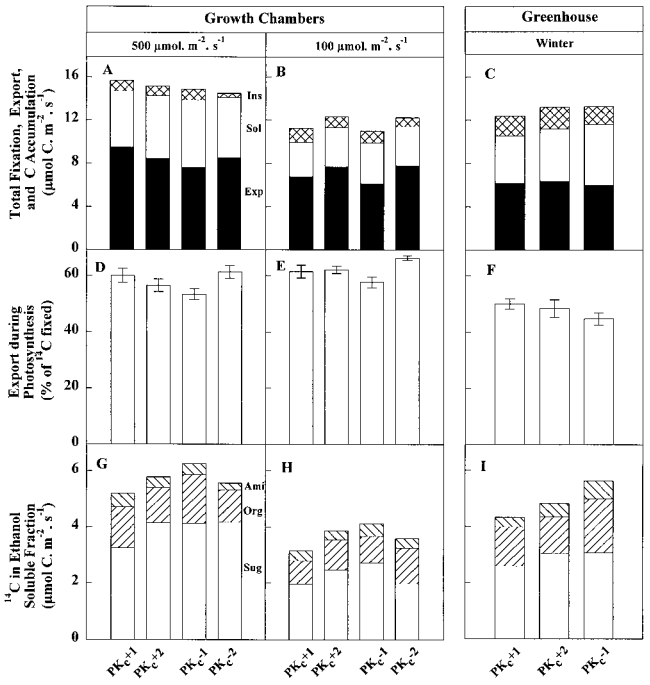

Figure 5. Leaf photosynthesis (entire bar in A–C), concurrent export rate (solid bar in A–C), and relative export flux during photosynthesis (D–F) of tobacco plants with wild-type levels of PK_c (PK_c+1 and PK_c+2) and two lines deficient in leaf PK_c (PK_c-1 and PK_c-2) grown under 500 (A) or 100 (B) μmol m^{-2} s^{-1} PPFD in growth chambers or in the greenhouse (C). Photosynthesis and concurrent export rates were measured when the first flower bud was visible. Photosynthesis and export were calculated from data obtained 90 to 120 min after $^{14}CO_2$ was first supplied at a leaf temperature of 25°C and at 1000 μmol m^{-2} s^{-1} PPFD, as described in the legend to Figure 4 and in "Materials and Methods." A to C, ^{14}C partitioning into the total ethanol-insoluble (Ins) fraction (i.e. starch), into the ethanol-soluble fraction (Sol), and into that exported (Exp) from the leaf at the end of a 120-min feed period. G to I, ^{14}C partitioning into the total sugar (Sug), organic acid (Org), and amino acid (Ami) fraction at the end of a 120-min feed period. The height of each bar represents the mean of measurements of at least four expanded leaflets of four different plants.

pools (i.e. sugars, organic acids, and amino acids) were similar in leaves of the PK_c- and PK_c+ lines (Fig. 5, G–I). For, example based on Student's *t* tests (not shown), there was no overall difference in the amount of ^{14}C accumulated in organic acids in leaves of PK_c- and PK_c+ plants at the end of the 2-h feed period. In all instances, ^{14}C sugars accounted for the largest pool (i.e. 60%–75% of the ethanol-soluble ^{14}C products). The major observations were that plants grown at 500 μmol m^{-2} s^{-1} PPFD had elevated photosynthesis rates and stored 35% to 40% more ^{14}C than did plants grown at 100 μmol m^{-2} s^{-1} PPFD (Fig. 5, G and H). Previously fixed $^{14}CO_2$ was primarily in the form of ^{14}C-Suc and ^{14}C-starch at the end of the feed periods (Figs. 4, C and D, and 5, A–C, G–I). The fate of the ^{14}C products that accumulated during the 2-h labeling period was investigated further.

Leaf Dark Respiration and Nighttime Export of Labeled Assimilates

Figure 6 shows examples of traces obtained from GM detectors, which monitored the accumulation of ^{14}C in leaves during the 2-h feeding period and the disappearance of label during the subsequent 14-h chase in the dark. Export and dark respiratory losses were variable during the chase. Although it was not determined whether the rapid decrease in radioactivity in the leaves at the beginning of the dark period was due to increased export or respiration, the overall pattern was similar in PK_c+ leaves (Fig. 6, A and B) and PK_c- (Fig. 6, C and D). During growth under moderate light, dark export of ^{14}C (Fig. 7A) and respiratory $^{14}CO_2$ evolution (Fig. 7D) were similar in leaves of all four lines. However, it is notable that during growth under low light the rates of ^{14}C-labeled photosynthate export in the dark were about 40% lower (Fig. 7B) and respiratory $^{14}CO_2$ evolution were 40% greater (Fig. 7E) in the PK_c- leaves as compared with the PK_c+ controls.

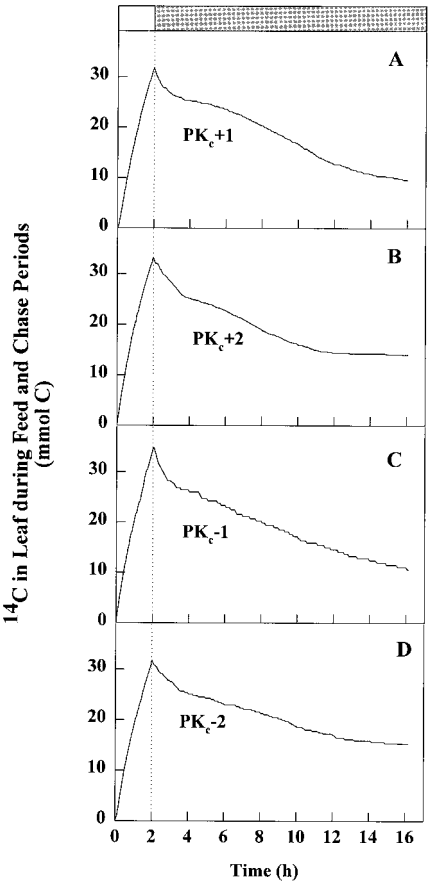

Figure 6. Effects of PK_c deficiency in source leaves of plants grown under low or moderate PPFD on the pattern of ^{14}C accumulation during the 2-h feed in the light and the subsequent disappearance of radioactivity during a 14-h chase period in the dark, as monitored noninvasively with a GM detector. During the dark chase period, CO_2 and leaf temperature were maintained at 35 Pa and 25°C, respectively. Each trace represents a typical analysis profile obtained from a single leaf. The experiment was repeated four times with the expanded leaves of four different plants.

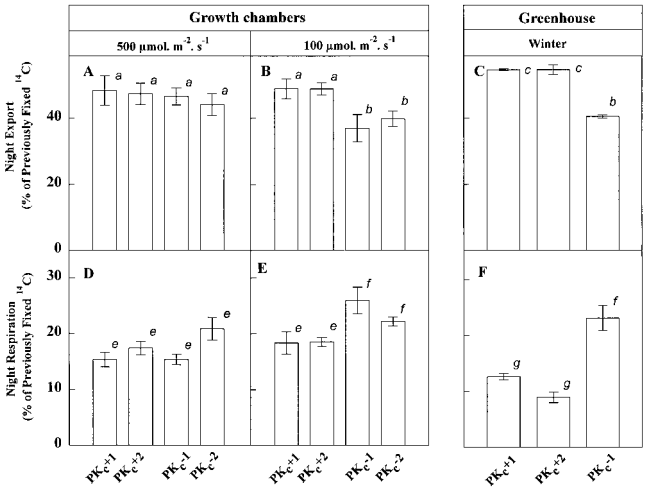

Figure 7. Nighttime export (A–C) and respiration (D–F) of previously fixed $^{14}CO_2$ in leaves of PK_c+ and PK_c- plants grown in growth chambers under moderate (A and D) or low (D and E) light or in a greenhouse during winter months (C and F). Dark export was corrected for respiration, which was determined by estimating the $^{14}CO_2$ released from the source leaves during the chase period. The height of each value represents the mean ± SE of measurements of four expanded leaves of four different plants. Significant differences among PK_c+ and PK_c- lines by Student's *t* test ($P < 0.05$) are indicated by superscript, italic letters (*a* to *g*) adjacent to the histobars.

Similarly, during winter months when natural PPFD levels are quite variable, leaves of PK_c- plants raised in the greenhouse also exported less ^{14}C (Fig. 7C) and released more $^{14}CO_2$ at night than did leaves of the PK_c+ controls (Fig. 7F).

DISCUSSION

Leaves of the tobacco plants used in the present investigation were either deficient in PK_c or they possessed wild-type levels of the enzyme (Fig. 1). PK activities of PK_c- leaves were only 15% to 30% of those of PK_c+ leaves. These reductions in PK activity are similar to those previously reported for leaves of the PK_c- plants (Gottlob-McHugh et al., 1992; Knowles et al., 1998). Furthermore, antigenic staining of PK_c was either absent or barely detectable on immunoblots of PK_c- leaf extracts (Fig. 1). Residual PK activity of PK_c- leaves has been attributed to PK_P (Gottlob-McHugh et al., 1992). The transgenic tobacco deficient in leaf PK_c exhibited a marked reduction in root growth, which became much more pronounced during growth under low light (Knowles et al., 1998). The objective of this study was to investigate the possibility that primary photoassimilate export from the source leaves was somehow modified by the deficiency of leaf PK_c.

The reduction in leaf PK_c (Fig. 1) did not affect whole-plant net C gain (Fig. 2), leaf photosynthesis (Figs. 3–5), ^{14}C partitioning in the leaf (Fig. 5G), or capacity of the leaf to export newly fixed CO_2 in the light or export stored ^{14}C assimilates during a subsequent nighttime chase when the plants were grown at 500 μmol m^{-2} s^{-1} (Fig. 7A). These results are consistent with those of Gottlob-McHugh et al. (1992), who demonstrated that leaves of the PK_c- transformants grown under 400 μmol m^{-2} s^{-1} PPFD exhibited normal rates of photosynthetic O_2 evolution (and respiratory O_2 consumption). There were no differences between plant groups in the capacity of their leaves to assimilate CO_2 over a range of PPFD levels, including the two PPFD levels at which the plants were grown. Similarly, the rates of export of newly fixed C during a $^{14}CO_2$ feeding were identical. These data indicate that in source leaves the PK_c is not an absolute prerequisite for (a) CO_2 fixation, (b) metabolism of ^{14}C photoassimilates, or (c) phloem loading and export during photosynthesis (i.e. in the light). The major difference between the PK_c- and the PK_c+ plants, which would account for the delayed development and reduced root growth of the PK_c- lines grown under low light, was the increased nighttime allocation of previously fixed C to respiration versus export (Fig. 7, B and E). The reduction in nighttime export could have a cumulative, negative influence on sink development.

At the time of appearance of the first flower bud, this organ functions as a strong sink for ^{14}C assimilates (B. Grodzinski and J. Jiao, unpublished data). At this stage, about 55% to 60% of the $^{14}CO_2$ being fixed was being exported immediately (Fig. 5, D and E). Also, about 40% to 45% of the ^{14}C photoassimilates remained in the leaf (Fig. 5, D and E). The ^{14}C retained in the leaves of all plants was available for further metabolism and export during dark periods. The plants acclimatized to 500 μmol m^{-2} s^{-1} PPFD fixed more $^{14}CO_2$ and therefore retained more ^{14}C at the end of the feed than did the plants cultivated under 100 μmol m^{-2} s^{-1} PPFD. Thus, there are two factors affecting mobilization and respiration rates during the dark chase period in the PK_c- lines, which must be considered in these experiments. The first is that these plants were clearly deficient in PK_c (Fig. 1). The second equally important consideration is that photosynthesis rates were lower and the pool sizes of ^{14}C intermediates were correspondingly lower in all plants acclimatized to 100 μmol m^{-2} s^{-1} versus plants acclimatized to 500 μmol m^{-2} s^{-1} PPFD. It appears that the PK_c step of cytosolic glycolysis can effectively be bypassed by alternative enzyme(s) if there are sufficient reserves of intermediates to drive other reaction sequences. In plants grown under low light there may be a higher, proportional respiratory cost associated with the translocation of ^{14}C intermediates at night. The enzyme (or enzymes) being used to bypass PK_c in the PK_c- less leaves may not regulate glycolytic flux in support of dark respiration as effectively as PK_c. Thus, the dark respiration of photoassimilates might be elevated, which could drain the pool of available Suc for phloem loading/translocation to sinks (hence, reducing export).

In cotton plants export in the dark was highly correlated with carbohydrate levels (i.e. starch) at the end of the photoperiod and with dark respiration rates (Hendrix and Grange, 1991). To some extent, $^{14}CO_2$ respiratory losses may reflect dark respiration rates in the leaves, which might be providing energy to sustain export in the dark. Nighttime export requires additional energy than in the light that can only be derived from stored photoassimilates (Giaquinta, 1972; Côté et al., 1992; Geiger and Servaites,

1994; Jiao and Grodzinski, 1998). Bouma et al. (1995) calculated that as much as one-third of the C stored in starch could be lost through dark respiration in support of the energy requirements for photoassimilate mobilization and export. However, in the PK_c- lines an increased respiration rate (i.e. $^{14}CO_2$ production) may not reflect an increased rate of ATP production via oxidative phosphorylation. There may in fact have been a reduction in ATP production via oxidative phosphorylation. In the absence of normal levels of PK_c some or all of the "enhanced" respiratory CO_2 loss might occur via the nonphosphorylating (cyanide resistant) pathway of mitochondrial electron transport (involving the alternate oxidase). PK_c regulation may be very important to the partitioning of electrons between phosphorylating and nonphosphorylating pathways of mitochondrial electron transport, since pyruvate is apparently the key activator of the alternative oxidase/CN-resistant pathway of mitochondrial respiration (Day et al., 1994). Thus, in PK_c- leaves a depletion of stored assimilates via a bypass pathway coupled with a reduced supply of energy via oxidative phosphorylation for loading and translocation processes may effectively jeopardize the opportunity for the PK_c- leaf tissue to mobilize carbohydrate reserves at night.

The PK_c- leaves had higher rates of $^{14}CO_2$ loss and exported less ^{14}C during the dark than did PK_c+ plants, but only during cultivation under low light when the total amount of ^{14}C assimilates produced was reduced. The plants were at a similar stage of development and the relative rates of export during photosynthesis (i.e. export expressed as a percentage of photosynthesis) of these leaves were similar (Fig. 5, D and E). A primary difference between the PK_c- plants grown at low versus moderate light was the amount of $^{14}CO_2$ initially fixed during the feed (Fig. 5, A, B, G, and H). Further studies are required to distinguish how PK_c and PK_c bypass enzymes are regulated when plants are cultivated under very low irradiances. Our previous study revealed that, to compensate for the physical deficiency of leaf PK_c, the PK_c- leaves did not up-regulate the activities the PK_c bypass enzymes PEP phosphatase or PEP carboxylase (Knowles et al., 1998). Thus, fine regulation of preexisting PK_c bypass enzymes may allow the PK_c- leaves to partially cope with PK_c deficiency. Further studies are required to assess whether this is the case and, if so, whether it might be achieved by the "constitutive" phosphorylation of the leaf PEP carboxylase into its phosphorylated, more active (i.e. less malate inhibited) state.

Leaves of PK_c+ and PK_c- plants raised in a greenhouse during winter months demonstrated photosynthesis (Figs. 5C), dark export, and respiration characteristics (Figs. 7, C and F) that were similar to those of plants grown in chambers under low light (Figs. 5B and 7, B and E, respectively). Growing the test plants in a greenhouse during our winter months represents a different light-limited growth condition. The plants cultivated in the greenhouse were subject to wide fluctuations in PPFD during the photoperiod. Most plants in nature are exposed to varying levels of PPFD during their development. Diurnal transitions are obvious, but cloud cover, sun flecking, and mutual shading are also commonplace events influencing leaf metabolism and function (Boardman, 1977). Our results suggest that the presence of PK_c and the operation of a "normal" glycolytic pathway are important in the production of energy needed for the mobilization of photoassimilates, when energy derived in the light from photosynthesis is not (directly) available, or when the levels of carbohydrates destined for export are too low to drive Suc synthesis, loading, and translocation processes required to meet sink demand.

In this study total nighttime export rates were not measured in all of the leaves in the canopy. Rather, the disappearance of label following a 2-h period of $^{14}CO_2$ labeling during which a steady rate of photosynthesis was maintained. In sugar beet, following longer labeling periods (e.g. 6 h), it was shown that during the first 2 to 3 h in the dark the source of C for export was from the Suc pool that accumulated during the light period and that the concentration of Suc was an important factor controlling the rate of translocation during the subsequent dark period (Geiger and Batey, 1967). Thereafter, starch was mobilized to form Suc for translocation. In tobacco ^{14}C-Suc accumulated during the feed period (Fig. 4, C and D) and likely served as a source of exported ^{14}C during the nighttime chase (Fig. 6). In the tobacco leaves acclimatized to growth at either the low or the moderate light, the fastest rate of disappearance of previously fixed ^{14}C occurred during the first hours of the dark period (Figs. 6 and 7). In this study nighttime export would be underestimated in all of the lines. A reduced export rate in the PK_c- lines during the dark chase period means that the actual reduction in translocation of photoassimilates among these plants was probably much greater than that reported here.

Taken together, our results underscore the problems of raising transgenic plants in different growth conditions and/or relying solely on data derived during growth under one light condition. To our knowledge, this is the first study in which the expression in leaves of a glycolytic enzyme was altered and photosynthate export rates were measured both in the light and in the dark. Selected modification of other reactions associated more directly with C transport and phloem-loading processes do alter translocation (Lerchl et al., 1995; Geigenberger et al., 1996; Hattenbach et al., 1997; Hausler et al., 1998). A major difference noted in the transgenic plants deficient in leaf PK_c was the reciprocal effect on C export and dark respiration in low-light-grown plants that were not assimilating CO_2 at a fast rate. This was not observed when the PK_c- plants were grown at a moderate light level. Further insight into the role of PK_c and glycolysis in maintaining homeostasis in source leaves may be provided by challenging transgenic plants with growth conditions in which nutrients, as well as light conditions, are varied.

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