

# Altered Growth of Transgenic Tobacco Lacking Leaf Cytosolic Pyruvate Kinase<sup>1</sup>

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Previously, we reported that transformation of tobacco (*Nicotiana tabacum* L.) with a vector containing a potato cytosolic pyruvate kinase (PK<sub>c</sub>) cDNA generated two plant lines specifically lacking leaf PK<sub>c</sub> (PK<sub>c</sub>-) as a result of co-suppression. PK<sub>c</sub> deficiency in these primary transformants did not appear to alter plant development, although root growth was not examined. Here we report a striking reduction in root growth of homozygous progeny of both PK<sub>c</sub>- lines throughout development under moderate (600  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) or low (100  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) light intensities. When both PK<sub>c</sub>- lines were cultivated under low light, shoot and flower development were also delayed and leaf indentations were apparent. Leaf PK activity in the transformants was significantly decreased at all time points examined, whereas root activities were unaffected. Polypeptides corresponding to PK<sub>c</sub> were undetectable on immunoblots of PK<sub>c</sub>- leaf extracts, except in 6-week-old low-light-grown PK<sub>c</sub>- plants, in which leaf PK<sub>c</sub> expression appeared to be greatly reduced. The metabolic implications of the kinetic characteristics of partially purified PK<sub>c</sub> from wild-type tobacco leaves are discussed. Overall, the results suggest that leaf PK<sub>c</sub> deficiency leads to a perturbation in source-sink relationships.

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PK is a key regulatory enzyme of glycolysis that catalyzes the irreversible synthesis of pyruvate and ATP from PEP and ADP. Substantial evidence indicates that PK is a primary control site of plant glycolytic flux to pyruvate (Plaxton, 1996). Plant PK<sub>c</sub> and PK<sub>p</sub> differ in their molecular, immunological, and kinetic characteristics (Plaxton, 1989, 1996; Blakeley et al., 1990, 1991). Tissue-specific isoforms of PK<sub>c</sub> and PK<sub>p</sub> having unique physical and/or kinetic and regulatory properties have also been reported (Podestá and Plaxton, 1991, 1994b; McHugh et al., 1995; Hu and Plaxton, 1996; Plaxton, 1996). PK<sub>c</sub> plays a major role in generating the precursor pyruvate for various biosynthetic pathways and mitochondrial respiration, whereas PK<sub>p</sub> is thought to be important in supplying pyruvate and ATP for several plastidic biosynthetic pathways.

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Tobacco (*Nicotiana tabacum* L.) plants were transformed with a vector that was designed to examine the impact of overexpressing potato tuber PK<sub>c</sub> in plastids (Gottlob-McHugh et al., 1992). No transgenic plants were recovered that had increased expression of PK<sub>c</sub>. However, two primary transformants were unexpectedly produced that specifically lacked PK<sub>c</sub> in their leaves (PK<sub>c</sub>-). The reduction in tobacco PK<sub>c</sub> was likely attributable to the *trans*-inactivation phenomenon known as co-suppression or posttranscriptional homology-dependent gene silencing (Meyer and Saedler, 1996). Although PK<sub>c</sub> was immunologically undetectable in leaves, PK<sub>c</sub> protein levels similar to those of the wild type were observed in nonphotosynthetic tissues, including seeds and roots. Metabolite analyses indicated that the levels of PEP were substantially higher in the PK<sub>c</sub>- leaves than in wild-type leaves, consistent with a block in glycolysis at the step catalyzed by PK. Nevertheless, the transgenic tobacco plants lacking leaf PK<sub>c</sub> showed no alteration in shoot growth or leaf rates of photosynthetic O<sub>2</sub> evolution and respiratory O<sub>2</sub> consumption (Gottlob-McHugh et al., 1992). However, these plants were grown under optimal conditions and root growth was not examined.

In the present study, we report the analysis of homozygous progeny of these PK<sub>c</sub>- transformants grown under low or moderate light intensities. Our findings show that the absence of leaf PK<sub>c</sub> results in a striking decrease in root biomass and root:shoot ratios during growth under both light regimes, suggesting that an alteration in source:sink relationships was induced. Additionally, the altered leaf morphology and delayed development of PK<sub>c</sub>- tobacco grown under low light was noted. Finally, the characteristics of partially purified PK<sub>c</sub> from wild-type tobacco leaves lend support to other studies (Huppe and Turpin, 1994; Hu and Plaxton, 1996; Plaxton, 1996) suggesting a role for leaf PK<sub>c</sub> in regulating respiratory C flow for ATP production and in generating the C skeletons required for anabolic processes, including N assimilation.

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Abbreviations: PK, pyruvate kinase; PK<sub>c</sub> and PK<sub>p</sub>, cytosolic and plastidic pyruvate kinase isozymes, respectively; PK<sub>c</sub>- and PK<sub>c</sub>+, tobacco plants that are and are not deficient in leaf PK<sub>c</sub>, respectively; T-DNA, transfer DNA.

## MATERIALS AND METHODS

Tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) plants were raised in growth chambers with 16 h of light (100 or 600  $\mu\text{E m}^{-2} \text{s}^{-1}$ ; 25°C)/8 h of dark (20°C) at 70% RH in 8-inch pots containing potting mixture no. 1. Plants were provided with nutrient solution containing 257  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 57  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$ , 502  $\mu\text{M}$   $\text{K}_2\text{SO}_4$ , 243  $\mu\text{M}$   $\text{MgSO}_4$ , 246  $\mu\text{M}$   $\text{MgCl}_2$ , 748  $\mu\text{M}$   $\text{CaCl}_2$ , 10  $\mu\text{M}$   $\text{MnSO}_4$ , 1  $\mu\text{M}$   $\text{CuSO}_4$ , 1  $\mu\text{M}$   $\text{ZnSO}_4$ , 31  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 0.5  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 0.2  $\mu\text{M}$   $\text{CoSO}_4$ , 37.6  $\mu\text{M}$  Fe sequestrine, 3.8  $\mu\text{M}$  Fe, and 10 mM  $\text{KNO}_3^-$  applied biweekly. Plants were harvested for growth analysis at the beginning of the photoperiod. Roots were separated from the potting mixture by careful washing over a meshed sieve (2-mm mesh). All measurements were made on the most recently fully expanded leaves or primary roots from plants of the indicated ages. Tissue for enzyme and immunoblot analysis was immediately frozen in liquid  $\text{N}_2$  and stored until use.

### Southern-Blot Analysis

Total DNA from tobacco leaf tissue was isolated using a DNA isolation kit (GNOME, BIO 101, Vista, CA). After digestion with *EcoRI*, DNA (10  $\mu\text{g}$ ) was electrophoretically separated in 0.7% (w/v) agarose gels. DNA was transferred to Hybond-N nylon membrane (Amersham) and hybridized to a random-primer-labeled potato  $\text{PK}_c$ -cDNA probe according to protocols supplied by the manufacturer.

### Extraction and Measurement of Enzyme Activity

Enzymes were extracted from tobacco leaves and roots as described by Gottlob-McHugh et al. (1992); leaves were homogenized in three volumes and roots in four volumes of extraction buffer. PK activity was assayed spectrophotometrically as described by Plaxton (1989) and was corrected for PEP phosphatase activity by omitting ADP from the assays. Activities of ATP- and PPI-dependent phosphofructokinase and PEP carboxylase were determined as described by Podestá and Plaxton (1994a), whereas PEP phosphatase activity was measured as described by Duff et al. (1989). All enzyme assays were conducted at 30°C. Activity values are the means of triplicate determinations conducted on three to four separate extracts. One unit of enzyme activity is defined as the conversion of 1  $\mu\text{mol}$  of substrate per min. Apparent  $K_m$  values for PEP and ADP were calculated from the Michaelis-Menten equation fitted to a nonlinear, least-squares regression computer kinetics program (Brooks, 1992). All kinetic parameters are the means of three separate determinations and are reproducible to within  $\pm 10\%$  SE.

### Protein Determination

Protein concentration was determined by the dye-binding method of Bradford as described by Bollag and Edelstein (1991), with bovine  $\gamma$ -globulin as the standard.

### Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970) with a minigel apparatus (Bio-Rad) using 0.75-mm slab gels and a 7.5% (w/v) monomer concentration for the separating gel. Immunoblotting was performed using monospecific affinity-purified rabbit anti-castor endosperm  $\text{PK}_c$ -IgG, and antigenic polypeptides were detected using an alkaline-phosphatase-conjugated secondary antibody as described previously (Plaxton, 1989). Immunological specificities were confirmed by immunoblots in which rabbit preimmune serum was substituted for the anti- $\text{PK}_c$ -IgG.

### Partial Purification of Tobacco Leaf $\text{PK}_c$

Fully expanded wild-type tobacco leaves (90 g) were homogenized in 200 mL of 100 mM imidazole-HCl, pH 7.1, containing 5 mM  $\text{MgCl}_2$ , 20 mM NaF, 10 mM thiourea, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 20% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 3% (w/v) insoluble PVP. The filtered extract was centrifuged for 20 min at 46,000g. Butyl-Sepharose, DEAE-Fractogel (VWR Canlab, Mississauga, Ontario, Canada), and ADP-agarose chromatographies were conducted as described by Hu and Plaxton (1996). The final preparation was concentrated to about 2.0 mL using an ultrafilter (YM-30, Amicon, Oakville, Ontario, Canada), divided into 200- $\mu\text{L}$  aliquots, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$ .

## RESULTS

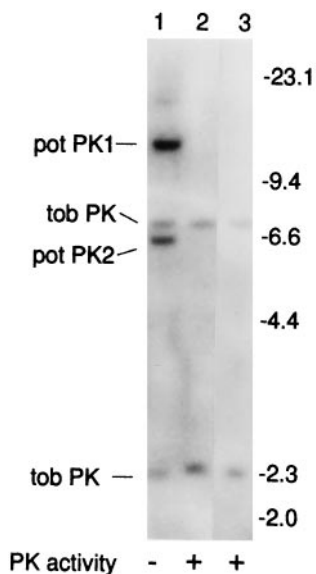
### Selection of Transgenic Tobacco Plants with Reduced $\text{PK}_c$ Activity

Two transgenic tobacco plant lines (1-121-35 and 7-121-35) that specifically lack  $\text{PK}_c$  in their leaves were obtained after transformation of tobacco with a vector designed to overexpress potato tuber  $\text{PK}_c$  in plastids. The absence of  $\text{PK}_c$  activity in the leaves was related to the co-suppression of the potato  $\text{PK}_c$  transgene and the endogenous tobacco  $\text{PK}_c$  (Gottlob-McHugh et al., 1992).

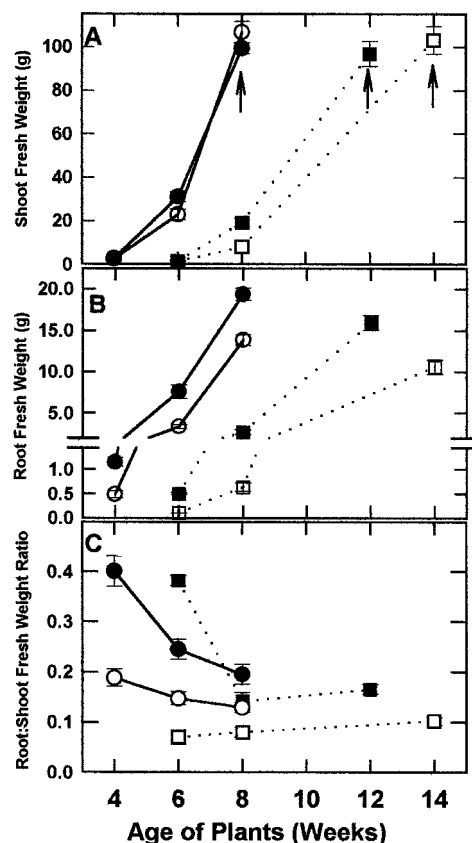
Plant 1-121-35 initially appeared to have two T-DNA copies integrated at different loci (Gottlob-McHugh et al., 1992). Upon further analysis of more than 50  $T_1$  progeny, this line was found to contain three segregating T-DNAs (results not shown). Analyses of leaf extracts using  $\text{PK}_c$  activity assays and immunoblotting with affinity-purified anti-castor endosperm  $\text{PK}_c$ -IgG revealed that about one-half of the progeny were  $\text{PK}_c^-$ , whereas the other half contained wild-type levels of  $\text{PK}_c$  activity and amount ( $\text{PK}_c^+$ ) (results not shown). Southern-blot analyses of these plants demonstrated that all of the progeny examined contained copies of the potato  $\text{PK}_c$  transgene. The reversion to  $\text{PK}_c^+$  generally appeared to be associated with a reduction in the number of potato  $\text{PK}_c$  transgenes (i.e. reduction from all three transgenes to two or one transgene[s]), but this correlation was imperfect. A few plants with all three transgenes became  $\text{PK}_c^+$  and several with fewer transgenes remained  $\text{PK}_c^-$ . The shoot and root growth of one

progeny line (14-1) derived from the selfing of line 1-121-35 was examined (see below). All seedlings from this line were kanamycin resistant and all progeny tested were PK<sub>c</sub><sup>-</sup>, suggesting that co-suppression in 14-1 was relatively stable.

The transgenic plant 7-121-35 contained two copies of the potato transgene. Selfing of 7-121-35 generated T<sub>1</sub> progeny that segregated 3:1 for kanamycin resistance. Southern-blot analyses of 30 of these progeny demonstrated that 22 contained both copies of the transgene (Fig. 1, lane 1), whereas 8 contained no copies of the transgene (Fig. 1, lane 2), indicating that the two copies of the transgene co-segregate. The same two bands (Fig. 1, pot PK1 and pot PK2) that were detected using the potato cDNA as a probe were also detected using nptII as a probe (results not shown). Of the 22 progeny that contained the transgene, all were found to be PK<sub>c</sub><sup>-</sup>, suggesting that the *trans*-inactivation event occurring in this line was more stable than in 1-121-35. The plants remained PK<sub>c</sub><sup>-</sup> whether they were homozygous or hemizygous for the transgene. All 8 of the progeny that did not contain the transgene were found to be PK<sub>c</sub><sup>+</sup>. The homozygous PK<sub>c</sub><sup>-</sup> progeny line 15-7 was selected for developmental studies of growth, PK activity, and PK<sub>c</sub> expression under both low and moderate light intensities (see below). The PK<sub>c</sub><sup>+</sup> line 18-7, which was generated from the selfing of 7-121-35, was used as a control (control 1). An additional PK<sub>c</sub><sup>+</sup> control (control 2) was wild-type tobacco (cv Petit Havana SR1).



**Figure 1.** Southern-blot analysis of T-DNA segregation in progeny of PK<sub>c</sub><sup>-</sup> plants. Each lane contains 10  $\mu$ g of *Eco*RI-digested tobacco DNA. The full-length potato cDNA (Blakeley et al., 1990) was used as the probe. Lane 1, Line 15-7 (PK<sub>c</sub><sup>-</sup>); lane 2, line 18-7 (control 1); lane 3, wild type (control 2). The positions of the hybridizing T-DNA fragments (pot PK1 and pot PK2), the cross-hybridizing endogenous tobacco PK fragments (tob PK), and the  $\lambda$ -*Hind*III size markers (in kb) are indicated.



**Figure 2.** Growth curves of PK<sub>c</sub><sup>-</sup> (line 15-7) and PK<sub>c</sub><sup>+</sup> (control 1; line 18-7) plants cultivated under moderate and low light intensities. A, Developmental profiles for shoot fresh weight; the arrows indicate the age at which maturation (i.e. onset of flowering) occurred. B, Developmental profiles for root fresh weight. C, Developmental profiles for root:shoot fresh weight ratios. ●, ■, PK<sub>c</sub><sup>+</sup> plants; ○, □, PK<sub>c</sub><sup>-</sup> plants; ●, ○, moderate light; ■, □, low light. All values represent the means  $\pm$  SE for 8 to 16 independent determinations.

### Growth and Development of Homozygous Progeny of Transgenic Plants

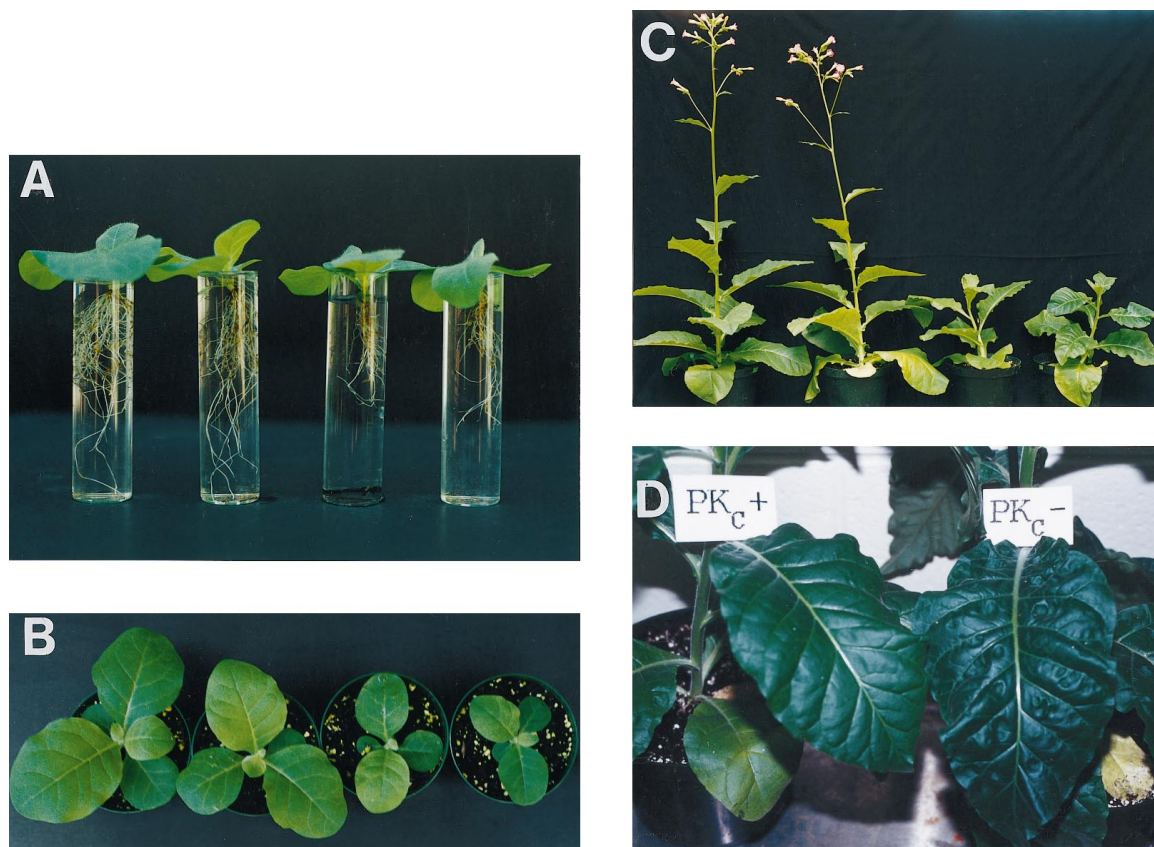
The effect of leaf PK<sub>c</sub> deficiency on tobacco growth was assessed throughout development under moderate and low light intensities (600 and 100  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>, respectively). The 4-, 6-, and 8-week-old plants grown under moderate light correspond to the four-leaf, eight-leaf, and mature (onset of flowering) phases, respectively. As previously noted (Gottlob-McHugh et al., 1992), the PK<sub>c</sub><sup>-</sup> plants grown under a moderate light intensity showed no alteration in shoot growth or shoot biomass relative to control plants (Fig. 2A). Both PK<sub>c</sub><sup>-</sup> and PK<sub>c</sub><sup>+</sup> plants bolted at the same time and the first flower opened at about 8 weeks. However, root growth was significantly impaired in the moderate-light-grown PK<sub>c</sub><sup>-</sup> (15-7) plants compared with PK<sub>c</sub><sup>+</sup> (control 1) plants at all three developmental stages (Fig. 2B). This resulted in a 32% reduction in the root:shoot ratio of mature plants (Fig. 2C). At maturity, the root:shoot ratios of the second PK<sub>c</sub><sup>-</sup> line (14-1) were similarly reduced by 27% after growth under moderate light ( $n = 6$ ).

Under low light, both root biomass and root:shoot ratios were again significantly decreased in the  $PK_c^-$  plants at all time points (Figs. 2, B and C, and 3A). In addition, however, differences were seen in the shoot growth and morphology of the  $PK_c^-$  plants relative to the  $PK_c^+$  controls (Figs. 2A and 3).  $PK_c^-$  shoots were smaller in 8-week-old plants, but after bolting were equivalent to those of the control plants at maturity (Figs. 2A and 3B). The reduced shoot biomass of the 8-week-old  $PK_c^-$  plants was apparently caused by a developmental impediment, because flowering was delayed by approximately 2 weeks (Figs. 2A and 3C). Although maturation of the  $PK_c^-$  low-light-grown plants was impeded, there were no observable differences in their flower morphology or ability to set seed. No differences in growth were observed between control 1 and control 2 or between the two  $PK_c^-$  lines (Fig. 3). Seed fresh weights, seed viability, and germination rates of the  $PK_c^-$  and  $PK_c^+$  plants were identical (results not shown). However, leaf morphology was altered such that pronounced indentations were apparent in fully expanded leaves of the mature  $PK_c^-$  plants grown under low light (Fig. 3D).

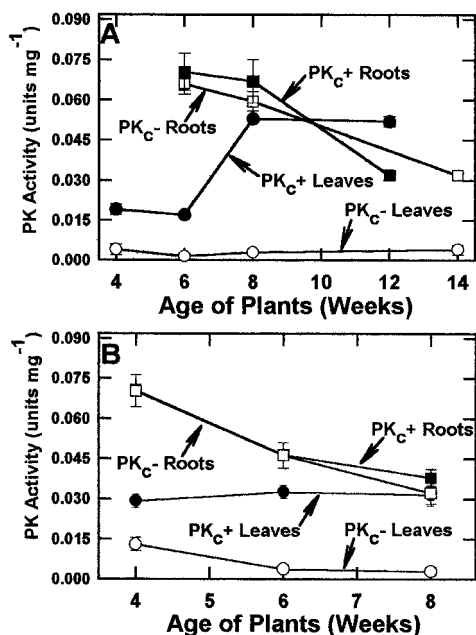
### Enzyme Activities and Immunoblotting

Developmental profiles for PK activity in leaves and roots of  $PK_c^+$  (control 1) and  $PK_c^-$  (15-7) tobacco plants grown under both light regimes are shown in Figure 4. PK specific activity in the most recently fully expanded leaves of control plants grown under low light increased from  $0.019 \pm 0.003$  units/mg at 4 weeks to  $0.052 \pm 0.004$  units/mg at 12 weeks (Fig. 4A). Root PK activity, however, decreased with age. The  $PK_c^-$  plants were similar to controls with respect to PK activity in roots but showed significantly reduced PK activity in fully expanded leaves at all developmental stages examined.

PK specific activities in fully expanded leaves of control 1 and  $PK_c^-$  (15-7) plants grown for 4 weeks under a moderate light intensity were  $0.029 \pm 0.003$  and  $0.014 \pm 0.002$  units/mg, respectively (Fig. 4B). Whereas leaf PK activities of moderate-light control 1 plants were relatively constant over the time course examined, the PK activity of  $PK_c^-$  plants decreased with age. At maturity, the activities measured in extracts prepared from leaves of control 1 and  $PK_c^-$  plants were lower than those of the respective flowering plants grown under low light (Fig. 4). PK activities in roots of the plants grown under moderate light were in the



**Figure 3.** Impact of the absence of leaf  $PK_c$  on the growth and development of tobacco cultivated under a low light intensity. Two  $PK_c^-$  lines, 15-7 and 14-1, are shown on the right, and the two  $PK_c^+$  lines, 18-7 (control 1) and wild type (control 2), are shown on the left in A through C. A and B, Reduced root and shoot growth, respectively, of 6-week-old  $PK_c^-$  plants. C, Delayed flowering of 12-week-old  $PK_c^-$  plants; D, Altered leaf morphology of flowering (14-week-old)  $PK_c^-$  (15-7) plants.



**Figure 4.** Developmental profiles for PK activity in leaves and roots of PK<sub>c</sub>- (line 15-7) and PK<sub>c</sub>+ (control 1; line 18-7) plants grown under low (A; 100 μE m<sup>-2</sup> s<sup>-1</sup>) or moderate (B; 600 μE m<sup>-2</sup> s<sup>-1</sup>) light intensities. All values represent the means ± SE of triplicate determinations conducted with four separate extracts.

same range and followed a similar pattern to those grown under low light.

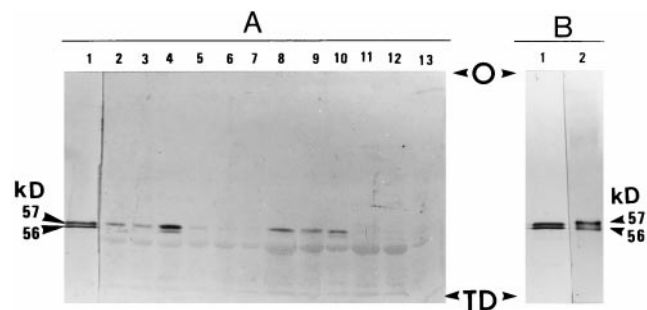
Immunoblotting using anti-castor endosperm PK<sub>c</sub>-IgG was employed to assess the relative abundance of PK<sub>c</sub> in extracts prepared from expanded leaves of PK<sub>c</sub>+ (control 1) and PK<sub>c</sub>- (15-7) plants cultivated under low and moderate light intensities. Two to three separate leaf extracts of the PK<sub>c</sub>- and PK<sub>c</sub>+ plants at each developmental stage were analyzed and representative results are shown in Figure 5A. With the exception of 6-week-old low-light-grown plants, the PK<sub>c</sub>- leaves appeared to lack PK<sub>c</sub> protein throughout development under both low and moderate light (Fig. 5A, lanes 5–8 and 11–13). The faint 57-kD immunoreactive band observed on the immunoblot of an extract from leaves of 6-week-old, low-light-grown PK<sub>c</sub>- plants (Fig. 5A, lane 5) was far less intense relative to that obtained with the respective PK<sub>c</sub>+ (control 1) extract (Fig. 5A, lane 2), and is consistent with the respective PK activities (Fig. 4A). In control 1 low-light-grown leaves, immunoreactive staining corresponding to PK<sub>c</sub> was less intense at 6 or 8 weeks than at maturity (Fig. 5A, lanes 2–4), consistent with the lower PK activities observed at the earlier developmental stages (Fig. 4). As with PK activity (Fig. 4B), the relative amount of immunoreactive PK<sub>c</sub> in leaf extracts derived from moderate-light-grown control 1 plants was relatively constant over the time course examined (Fig. 5A, lanes 8–10).

At maturity, extractable activities of PEP carboxylase, PEP phosphatase, and the PPI- or ATP-dependent phosphofructokinases were not significantly different in leaves or roots of control 1 versus PK<sub>c</sub>- (15-7) plants grown under

low light (not shown). There were no significant differences in soluble protein concentration between PK<sub>c</sub>- and PK<sub>c</sub>+ plants in either roots or leaves of plants grown under low or moderate light.

#### Characterization of Partially Purified PK<sub>c</sub> from Wild-Type Tobacco Leaves

Approximately 8 units of PK<sub>c</sub> having a final specific activity of 2.1 units/mg were isolated from wild-type tobacco leaves (overall yield, 10%; purification, 75-fold). The final preparation was devoid of PEP-phosphatase activity. The partially purified enzyme exhibited a broad pH-activity optimum of approximately 6.5 and a native molecular mass of about 220 kD, as estimated by gel filtration on a calibrated Superose 6 fast-protein liquid chromatography column. Immunoblotting using anti-castor endosperm PK<sub>c</sub>-IgG indicated that as in castor leaf PK<sub>c</sub> (Fig. 5B, lane 1), the tobacco leaf enzyme is composed of equal proportions of subunits having molecular masses of 57 and 56 kD (Fig. 5B, lane 2). Although at pH 7.6 the enzyme exhibited 70% of the V<sub>max</sub> at pH 6.5, its affinity for PEP and ADP was higher at pH 7.6 than at pH 6.5. Apparent K<sub>m</sub> values for PEP and Mg-ADP were 0.071 and 0.041 mM at pH 7.6, and 0.118 and 0.098 mM at pH 6.5, respectively. Mg-ATP, magnesium citrate, malate, 2-oxoglutarate, glutamate, and Pi inhibited the enzyme by about 40 to 60% when assayed at pH 6.5 or 7.6 with subsaturating concentrations of PEP and ADP (0.15 and 0.1 mM, respectively).



**Figure 5.** Immunological detection of castor and tobacco leaf PK<sub>c</sub>. Extracts were electrophoresed on 7.5% (w/v) SDS-polyacrylamide minigels and transferred to a PVDF membrane. Immunoblotting was performed using affinity-purified anti-castor endosperm PK<sub>c</sub>-IgG (Plaxton, 1989). A, Immunoblot analysis of extracts prepared from leaves of tobacco plants grown under low (lanes 2–7) and moderate (lanes 8–13) light intensities. Lane 1 contains 30 ng of homogeneous castor leaf PK<sub>c</sub> (Hu and Plaxton, 1996), whereas all other lanes contain 15 μg of protein from a tobacco leaf extract. Lanes 2, 3, and 4 represent 6-, 8-, and 12-week (mature) control 1 (line 18-7) leaf extracts, respectively; lanes 5, 6, and 7 represent 6-, 8-, and 14-week (mature) PK<sub>c</sub>- (line 15-7) leaf extracts, respectively; lanes 8, 9, and 10 represent 4-, 6-, and 8-week (mature) control 1 (line 18-7) leaf extracts, respectively; and lanes 11, 12, and 13 represent 4-, 6-, and 8-week (mature) PK<sub>c</sub>- (line 15-7) leaf extracts, respectively. B, Immunoblot of 30 ng of homogeneous castor leaf PK<sub>c</sub> (lane 1) (Hu and Plaxton, 1996) and 300 ng of partially purified PK<sub>c</sub> from wild-type tobacco leaves (lane 2). O, Origin; TD, tracking dye front.

## DISCUSSION

### Growth and Development of Transgenic Tobacco Lacking Leaf PK<sub>c</sub>

Our results are consistent with the previous observations (Gottlob-McHugh et al., 1992) that the transgenic tobacco plants resulting from transformation with sense constructs of the potato tuber PK<sub>c</sub> cDNA and placed under the control of the cauliflower mosaic virus 35S promoter had a specific elimination of PK<sub>c</sub> in their leaves. There was no significant difference in shoot or flower development of the homozygous progeny of the primary transformants lacking leaf PK<sub>c</sub> during growth under moderate light (Fig. 2A). This result agrees with those previously obtained with the primary transformants (Gottlob-McHugh et al., 1992). Of interest, however, was the significant reduction in root growth, and thus root:shoot ratios, that occurred when the PK<sub>c</sub>– plants were cultivated under moderate or low light (Figs. 2, B and C, and 3A).

Furthermore, shoot growth of the PK<sub>c</sub>– plants was significantly reduced by 8 weeks of growth under low light (Figs. 2A and 3B). This effect appears to be attributable to a developmental delay, because the shoot fresh weights of PK<sub>c</sub>– and control low-light-grown plants were equivalent when the two groups matured (i.e. when they began to flower) at about 12 and 14 weeks, respectively (Figs. 2A and 3C). The reason for the pronounced indentations in leaves of the PK<sub>c</sub>– plants grown under low light (Fig. 3D) is unknown. However, the normal seed development and germination of the PK<sub>c</sub>– plants illustrates that the absence of leaf PK<sub>c</sub> was not lethal under the conditions used.

### Enzyme Activities and Protein Expression

At 4 and 6 weeks of growth under a low light intensity, PK activity in leaves of homozygous PK<sub>c</sub>– progeny of the primary transformants was reduced by up to 75%, but was much less than the differences measured at maturity (Fig. 4A). Immunoblot analyses confirmed that with the exception of 6-week-old, low-light-grown plants (in which the amount of leaf PK<sub>c</sub> appeared to be greatly reduced), PK<sub>c</sub> was undetectable in leaf extracts of the PK<sub>c</sub>– plants (Fig. 5A). Taken together, the data of Figures 4A and 5A also indicate that the expression of leaf PK<sub>c</sub> in PK<sub>c</sub>+ plants is relatively lower during earlier phases of growth under low light than at the onset of flowering. In 4-week-old, moderate-light-grown plants, the difference between PK activities in PK<sub>c</sub>– and control leaves was only about 2-fold, whereas this difference was much greater (10-fold) in leaves of mature, 8-week-old plants (Fig. 4B).

The residual PK activities in PK<sub>c</sub>– leaves lacking PK<sub>c</sub> protein has been demonstrated to arise from the presence of chloroplastic PK (Gottlob-McHugh et al., 1992). The loss of PK<sub>c</sub>, an important site of cytosolic substrate-level phosphorylation of ADP to ATP, could be expected to be more deleterious during growth under the very low light irradiance of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ , when photosynthetic ATP production is severely compromised (photosynthesis in tobacco saturates at greater than 1200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). This provides a

rationale for the delayed development of the PK<sub>c</sub>– plants grown under low, but not moderate (i.e. 600  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), light intensities (Figs. 2A and 3B).

In contrast to leaves, the control and PK<sub>c</sub>– tobacco lines yielded similar developmental profiles for PK activity in roots (Fig. 4). This finding is consistent with the previous observation that the primary PK<sub>c</sub>– transformants and wild-type plants contained an equivalent amount of immunologically detectable PK<sub>c</sub> in their roots (Gottlob-McHugh et al., 1992). Therefore, the co-suppression of PK<sub>c</sub> appears to be specifically confined to leaf tissue. Enzymological studies have indicated that, similar to animal systems, higher plant PK<sub>c</sub> exists as a tissue-specific isozyme exhibiting substantial differences in several kinetic/regulatory properties that reflect the distinctive metabolic requirements of leaf versus seed tissues (Podestá and Plaxton 1991, 1994b; Hu and Plaxton, 1996; Plaxton, 1996). Further work is needed to assess the mechanism(s) whereby transformation of tobacco with a cDNA encoding potato tuber PK<sub>c</sub> results in the co-suppression or gene silencing of the putative leaf-specific isozyme of PK<sub>c</sub>.

### Tobacco Leaf PK<sub>c</sub> Is Feedback Inhibited by End Products of Respiration and N Assimilation

To further assess the potential function and regulation of tobacco leaf PK<sub>c</sub>, the enzyme was partially purified from wild-type leaves. The marked inhibition of tobacco leaf PK<sub>c</sub> by Mg-ATP, citric-acid-cycle intermediates, and glutamate is consistent with the suggestion (Hu and Plaxton, 1996; Plaxton, 1996) that PK<sub>c</sub> plays a key role in C<sub>3</sub> leaves in the regulation of the glycolytic flux for both mitochondrial respiration and the provision of C skeletons for anabolism. Any reduction in the levels of citric-acid-cycle intermediates by enhanced respiration and/or anabolic processes should relieve feedback inhibition of PK<sub>c</sub> and, consequently, stimulate the overall flux of cytosolic glycolysis to pyruvate. Because glutamate is the primary product of N assimilation by Gln synthetase/Gln 2-oxoglutarate aminotransferase, the inhibition of tobacco leaf PK<sub>c</sub> by glutamate should exert considerable feedback control on this enzyme. The elimination of PK<sub>c</sub> could therefore be expected to alter the leaf's ability to regulate glycolysis for respiration and to produce the C skeletons required for anabolic processes such as N assimilation.

### Plants Contain Metabolic Bypasses to PK<sub>c</sub>

The findings of the present and previous (Gottlob-McHugh et al., 1992) studies are compatible with the proposals (Black et al., 1987; Theodorou and Plaxton, 1995; Plaxton, 1996) that, because of their use of alternative enzymes and pathways, plants are able to maintain C flow through cytosolic glycolysis even in the absence of an "essential" enzyme such as PK<sub>c</sub>. Pathways unique to plants that can potentially circumvent the reaction catalyzed by PK<sub>c</sub> include PK<sub>p</sub>, PEP phosphatase, or the combined activities of PEP carboxylase, malate dehydrogenase, and NAD-malic enzyme (Duff et al., 1989; Plaxton, 1996). PEP phosphatase and PEP carboxylase have been suggested to be

important PK<sub>c</sub> bypasses during periods of nutritional Pi starvation, when PK<sub>c</sub> activity may be curtailed owing to ADP limitation (Theodorou and Plaxton, 1995; Plaxton, 1996). There was no significant up-regulation in the leaf or root activities of the putative PK<sub>c</sub> bypass enzymes PEP phosphatase and PEP carboxylase (or other key regulatory enzymes of cytosolic glycolysis such as ATP- and PPi-dependent phosphofructokinase) to compensate for the elimination of leaf PK<sub>c</sub>. Therefore, fine regulation of pre-existing enzymes may allow the PK<sub>c</sub>- leaves to compensate to some degree for the physical elimination of PK<sub>c</sub>. Further work is needed to determine if this is the case, and if so, whether it is possibly achieved by the constitutive posttranslational modification of leaf PEP carboxylase into its phosphorylated, more active state (Chollet et al., 1996).

### CONCLUDING REMARKS

To the best of our knowledge the present study is the first to show that specific "knockout" of an enzyme in one tissue (i.e., leaves) exerts a maximal detrimental effect on the development of another tissue (i.e. roots), in which the enzyme appears to be expressed normally. Preliminary in planta, pulse-chase <sup>14</sup>CO<sub>2</sub> radiolabeling studies have indicated that the stunted root growth of the low-light-grown transgenic tobacco plants deficient in leaf PK<sub>c</sub> may be caused by a 40% reduction in the nighttime export of recently assimilated <sup>14</sup>CO<sub>2</sub> from fully expanded source leaves (Jiao et al., 1997). This agrees with the fact that root growth is absolutely dependent on imported photosynthate, but not on imported amino acids, because inorganic N can be assimilated locally (Geigenberger et al., 1996). The reduced nighttime export of recently assimilated <sup>14</sup>CO<sub>2</sub> has been correlated with a doubling in the nighttime loss of respiratory <sup>14</sup>CO<sub>2</sub> by the PK<sub>c</sub>- leaves (Jiao et al., 1997). Presumably, the PK<sub>c</sub>-bypass enzyme that is being used by the PK<sub>c</sub>- leaves to compensate for the absence of PK<sub>c</sub> cannot regulate glycolytic flux in support of dark respiration to the same extent as PK<sub>c</sub>.

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