

The Gluconeogenic Enzyme Phosphoenolpyruvate Carboxykinase in Arabidopsis Is Essential for Seedling Establishment

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Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the gluconeogenic production of sugars from storage oil in germinating oilseeds. Here, we present the results of analysis on PEPCK antisense Arabidopsis plants with a range of enzyme activities from 20% to 80% of wild-type levels. There is a direct correlation between enzyme activity and seedling establishment during early post-germinative growth, thus demonstrating the absolute requirement of PEPCK and gluconeogenesis in this process. Soluble sugar levels in the 35S-*PCK1* antisense seedlings are reduced and seedling establishment can be rescued with an exogenous supply of sucrose. We observed an increase in the respiration of acetyl coenzyme A units released from fatty acid β -oxidation and a corresponding decrease in the production of sugars with decreasing enzyme activity in 2-d-old antisense seedlings. The 35S-*PCK1* antisense lines have a more extreme phenotype when compared with Arabidopsis mutants disrupted in the glyoxylate cycle. We conclude that the 35S-*PCK1* antisense seedlings are compromised in the ability to use both storage lipid and storage protein through gluconeogenesis to produce soluble sugars.

In oilseed plants, ATP-dependent phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.49) is proposed to have a fundamental role in the provision of Suc during germination and early post-germinative seedling growth in oilseed species. Fatty acids from seed storage lipids are catabolized to acetyl CoA to produce sugars for seedling growth and development by β -oxidation, the glyoxylate cycle, and gluconeogenesis (Kornberg and Beevers, 1957; Calvin and Beevers, 1961; Beevers, 1980).

There is strong evidence that PEPCK is a key enzyme in the regulation of flux through the gluconeogenic pathway in early post-germinative seedling growth. First, studies in marrow (*Cucurbita pepo*) have demonstrated that the maximum catalytic activity of PEPCK is higher than and changes simultaneously with the flux through the gluconeogenic pathway during post-germinative growth (Leegood and ap Rees, 1978a). Second, PEPCK and Fru 1,6-bisphosphatase (FBPase) are the only enzymes in the conversion of oxaloacetate to Glc 6-phosphate during gluconeogenesis that are substantially displaced from equilibrium in vivo (Leegood and ap Rees, 1978b). Third, studies using the PEPCK inhibitor 3-mercaptopycolinic acid demonstrated that PEPCK has a regulatory role in gluconeogenesis (Leegood and ap Rees, 1978a), with a high flux control coefficient of between 0.7 to 1.0 reported in gluconeogenic

marrow cotyledons (Trevanion et al., 1995). More recently, however, Runquist and Kruger (1999) calculated the flux control coefficient for the glyoxylate cycle enzyme isocitrate lyase (*ICL*) in germinating castor bean (*Ricinus communis*) seedlings to be 0.66 ± 0.09 , suggesting that the flux control coefficient for PEPCK is unlikely to be as high as previously reported. The flux through the gluconeogenic pathway is also regulated by FBPase (Leegood and ap Rees, 1978b), but the relative contributions of PEPCK and FBPase in the regulation of gluconeogenesis have yet to be determined.

Besides a role in gluconeogenesis, PEPCK fulfils additional functions in plants. The photosynthetic carbon dioxide-concentrating mechanisms found in plants with Crassulacean acid metabolism and both PEPCK-type and some NADP-malic enzyme-type C_4 plants contain PEPCK activity that plays a role in decarboxylating C_4 acids (Leegood et al., 1996; Walker and Leegood, 1996; Winkler et al., 1999). A broad range of C_3 plant tissues also contain PEPCK activity, including cauliflower (*Brassica oleracea*) florets (Mazelis and Vennesland, 1957), turnip taproot (Cooper and Benedict, 1968), grape (*Vitis vinifera*; Ruffner and Kliever, 1975), apple (*Malus domestica*), kiwifruit (*Actinidia deliciosa*), and aubergine (*Solanum melongena*; Blanke et al., 1988). Within plants, PEPCK has been located in developing seeds (Walker et al., 1999), phloem and trichome tissues, oil and resin ducts (Leegood and Walker, 1999), and ripening tomato (*Lycopersicon esculentum*) fruit (Bahrami et al., 2001), but the role of PEPCK in these tissues remains speculative. There is support for the involvement of

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PEPCK in pH stability and nitrogen and amino acid metabolism (Walker et al., 1999, 2001; Lea et al., 2001) and more recently, evidence that PEPCK plays a role in decarboxylating C_4 acids via a partial C_4 cycle in the vascular system of C_3 plants (Hibberd and Quick, 2002).

Two *PCK* genes have been identified in the Arabidopsis genome, *PCK1* on chromosome 4 (Munich Information Center for Protein Sequences [MIPS; <http://mips.gsf.de>]; MIPS code At4g37870) and *PCK2* on chromosome 5 (MIPS code At5g65690). Both are predicted to encode cytosolic enzymes, with *PCK1* the predominant gene expressed during early post-germinative growth (Rylott et al., 2001).

The *PCK1* and *PCK2* genes exhibit 80% identity at the cDNA level. The *PCK1* gene shares 91% identity with a 1,509-bp cold-inducible canola (*Brassica napus*) PEPCK cDNA (GenBank accession no. U21745; Sáez-Vásquez et al., 1995) and 81% identity with a 2,408-bp PEPCK cDNA isolated from senescing cucumber (*Cucumis sativus*) cotyledons (Kim and Smith, 1994; GenBank accession no. L31899). The predicted open reading frames of *PCK1* and *PCK2* encode for proteins of 671 and 628 amino acids, respectively, with 71% identity. During purification, PEPCK has been shown to undergo a rapid proteolytic cleavage, with the native protein for cucumber of 74 kD cleaved to 64 kD (Walker et al., 1995). The predicted molecular masses of *PCK1* and *PCK2* are 73.4 and 68.7 kD, respectively.

The coordinate induction of β -oxidation and glyoxylate cycle genes during storage lipid mobilization has been well documented. The acyl-CoA oxidase (*ACX3*; Eastmond et al., 2000; Froman et al., 2000) and keto-acyl-CoA thiolase (*PED1/KAT2*; Hayashi et al., 1998; Germain et al., 2001) genes of β -oxidation are both induced transcriptionally during early post-germinative seedling growth. Transcription also plays a major role in regulating the abundance of the glyoxylate cycle enzymes. Genes encoding the glyoxylate cycle enzymes *ICL* and malate synthase (*MS*) are controlled at the level of transcription (Comai et al., 1989; Graham et al., 1990; Sarah et al., 1996) and *ICL* and *MS* are both coordinately expressed during early post-germinative growth and senescence (Gut and Matile, 1988; Comai et al., 1989). However, relatively little is known about the regulation of plant PEPCKs. In cucumber, *PCK1* expression parallels *MS* and *ICL* (Kim and Smith, 1994), and more recently, coordinate expression of genes of the β -oxidation pathway, glyoxylate cycle, and gluconeogenesis have all been demonstrated during germination in Arabidopsis (Rylott et al., 2001).

Two allelic mutants in the β -oxidation keto-acyl-thiolase gene, *ped1* (Hayashi et al., 1998) and *kat2* (Germain et al., 2001) and the acyl-CoA oxidase gene, *acx3* (Eastmond et al., 2000) have recently been characterized. Germinating seedlings of the *ped1* and *kat2*

mutants are unable to catabolize storage lipid into acetyl CoA, and in the absence of an exogenous supply of Suc, do not establish. This failure to establish is characterized by an arrest in seedling growth, with failure of the cotyledons to expand or roots to elongate. However, *icl* seedlings are able to use storage lipid and establish without an exogenous supply of Suc under optimal growth conditions. Arabidopsis seeds contain approximately 25% storage protein (Baud et al., 2002), and it is likely that this contributes gluconeogenic substrate for establishment of *icl* seedlings.

Following the observations and conclusions drawn from both β -oxidation and glyoxylate cycle mutants, we hypothesize that gluconeogenesis would be essential for oilseed germination and seedling establishment. In this study, we have used the *PCK1* gene, which is the predominant *PCK* gene expressed during early seedling growth, to produce 35S-*PCK1* antisense Arabidopsis plants with a range of PEPCK activities to functionally characterize the role of PEPCK.

RESULTS

To determine the level of expression of the *PCK* genes in Arabidopsis, reverse transcriptase (RT)-PCR was used. Figure 1A shows that *PCK1* is the predominant *PCK* gene expressed in 2-d-old seedlings. The *PCK1* gene is also highly expressed in mature leaf, root, and flower tissue whereas *PCK2* is expressed principally in root and flower.

One hundred and ninety-six 35S-*PCK1* antisense transformants were identified after selection on hygromycin. Of these, 60% showed a significant reduction in the level of seedling establishment in the T_2 generation (data not shown). After initial characterization, five homozygous lines with T-DNA insertions at one or two loci, based on segregation analysis in the T_2 and T_3 generations, and representing a range of PEPCK activities were selected for further analysis (lines 16, 64, 122, 192, and 195). Analysis using RT-PCR on cDNA from 2-d-old seedlings indicated that transcript levels of *PCK1* were decreased in all five lines, with line 16 showing the lowest steady-state levels of *PCK1* mRNA (Fig. 1B). Transcript levels of *PCK2* in 2-d-old seedlings were much lower than *PCK1* (Fig. 1A), and *PCK2* levels were unaltered in the 35S-*PCK1* antisense lines (Fig. 1C). Primers were made to the putative cytosolic FBPase (MIPS code At1g43670) based on similarity with a confirmed cytosolic FBPase from canola reported by Strand et al. (2000). Analysis using RT-PCR showed that the transcript levels of this FBPase were unaltered in the 35S-*PCK1* antisense lines (data not shown).

Figure 2A illustrates the peak in PEPCK activity observed in 2-d-old, untransformed, wild-type Arabidopsis seedlings. This corresponds with the peak in

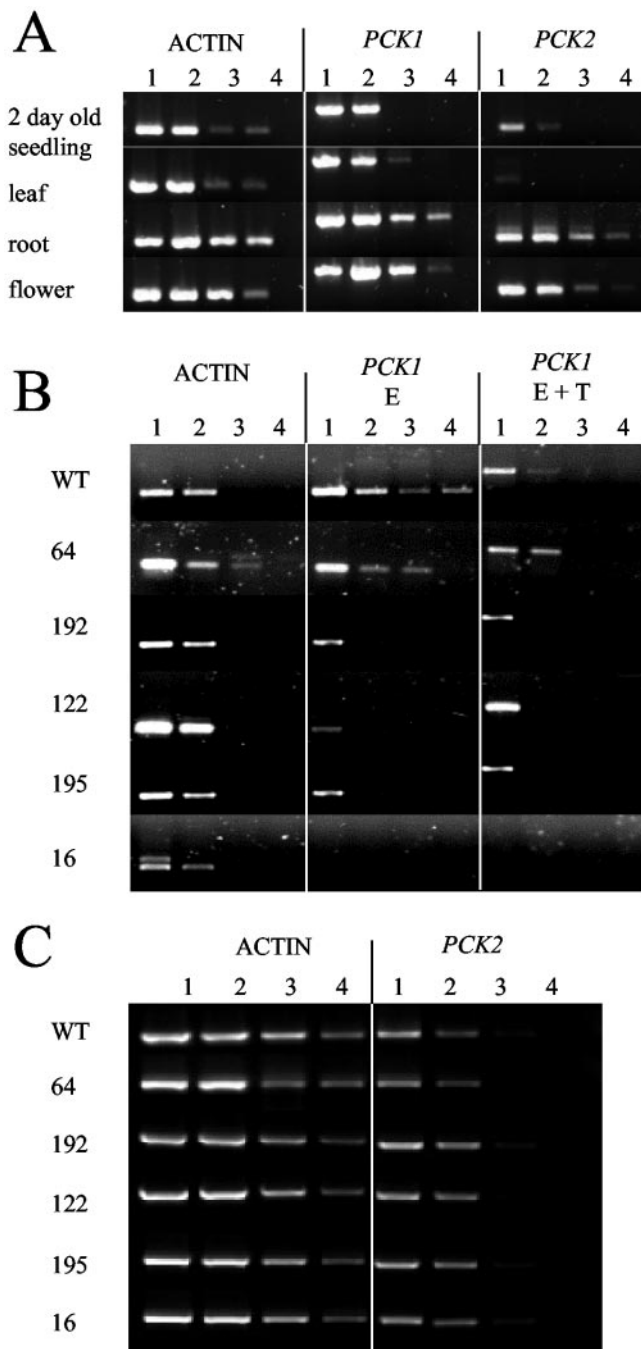


Figure 1. A, RT-PCR on cDNA from 2-d-old seedlings, leaf, root, and flower of untransformed wild-type Col0. Actin cDNA was amplified using primers *ACT2S* and *ACT2A*, *PCK1* using *PCK1-1001* and *PCK1-1709*, and *PCK2* using *PCK2-1032* and *PCK2-1536*. B, RT-PCR on cDNA from 2-d-old seedlings from untransformed wild-type Col0 and five 35S-*PCK1* antisense lines: 64, 192, 122, 195, and 16. Endogenous (E) *PCK1* cDNA only, was amplified using *PCK1-72* and *PCK1-573*. Endogenous (E) and transgene (T) *PCK1* cDNA was amplified using primers *PCK1-1001* and *PCK1-1709*. C, *PCK2* cDNA from 2-d-old seedlings was amplified using primers *PCK2-1032* and *PCK2-1536*. PCR was carried out on undiluted (lanes 1), one-tenth (lanes 2), one-hundredth (lanes 3), and one-thousandth (lanes 4) dilutions of cDNA.

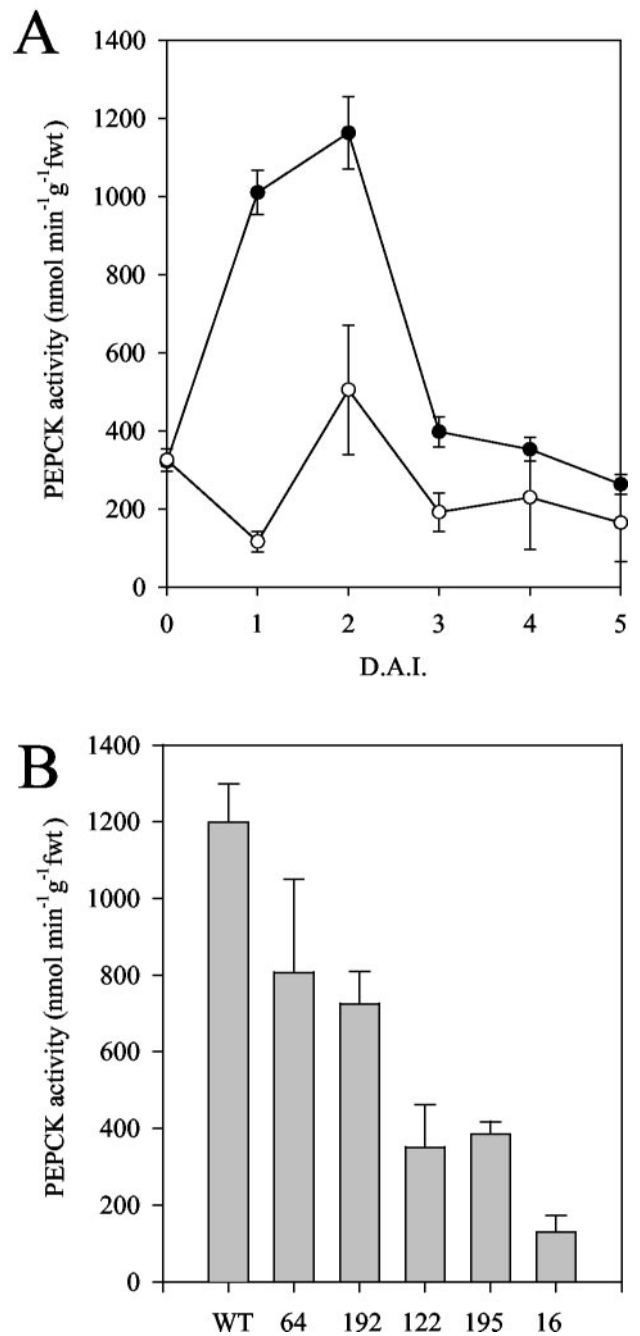


Figure 2. A, PEPCK activity in 0- to 5-d-old untransformed wild-type Col0 (●) and 35S-*PCK1* antisense line 122 (○) seedlings. B, PEPCK activity in 2-d-old untransformed wild-type Col0 and 35S-*PCK1* antisense seedlings. Seedlings were grown on one-half strength Murashige and Skoog medium supplemented with 20 mM Suc. Values are the mean \pm SE of three measurements made on three separate batches.

activity in β -oxidation and glyoxylate cycle enzymes (Germain et al., 2001; Rylott et al., 2001) and the rapid decline in storage lipid levels seen in *Arabidopsis* seedlings grown under these conditions (Germain et al., 2001). This peak was significantly reduced in the 35S-*PCK1* antisense line 122. The decreased PEPCK

activity also mirrored the pattern of transcript expression seen in the antisense lines; line 16 exhibited the lowest level of activity, <11% of untransformed, wild-type Arabidopsis in 2-d-old seedlings (Fig. 2B). These results demonstrate that the *PCK1* gene is responsible for the peak in activity seen in 2-d-old seedlings.

In the absence of an exogenous supply of Suc, the ability of the 35S-*PCK1* antisense seedlings to establish was compromised. This was characterized by an arrest in seedling growth, with failure of the cotyledons to expand or roots to elongate. No true leaves were produced in these seedlings (Fig. 3). Seedlings of the 35S-*PCK1* antisense lines that had failed to establish could be rescued by transferring to plates containing 20 mM Suc; these seedlings then grew and established normally.

Seedling establishment, without exogenous Suc, was further compromised by a reduction in light intensity (Fig. 4A) and in the dark, hypocotyl lengths in 5-d-old 35S-*PCK1* antisense seedlings were also reduced, by up to 68% in line 16 (Fig. 4B). To test the relationship between PEPCK activity and seedling establishment, measurements were made on 17 additional homozygous antisense lines to provide a more complete range of PEPCK activities. There was a statistically significant correlation between PEPCK activity and seedling establishment ($r = 0.750$, $P < 0.01$; Fig. 5). To investigate whether a decreased level of endogenous Suc in the seedlings was linked to the reduction in seedling establishment, we measured the soluble sugar levels (Glc, Fru, and Suc) in seedlings grown without an exogenous supply of Suc. As seedling growth is compromised in the antisense lines, we measured the levels of Suc in 2-d-old seedlings, when the wild-type and antisense lines are at similar developmental stages. We found a significant reduction in the levels of soluble sugars in the four lines with the lowest PEPCK enzyme activities (Fig. 6). To examine whether storage lipid catabolism was compromised in these 35S-*PCK1* antisense seedlings,

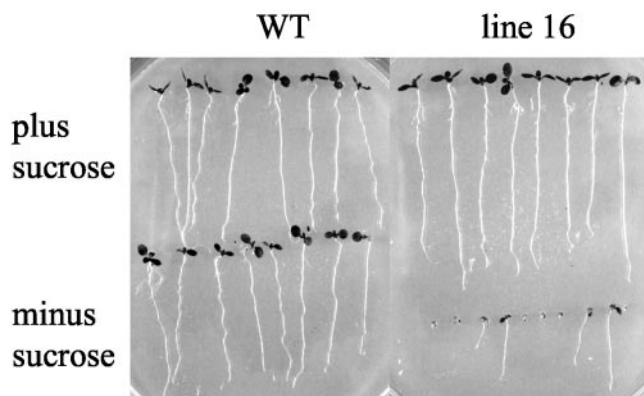


Figure 3. Untransformed wild-type Col0 and 35S-*PCK1* antisense seedlings grown on one-half-strength Murashige and Skoog media with or without 20 mM Suc for 10 d at $160 \mu\text{mol m}^{-2} \text{s}^{-1}$.

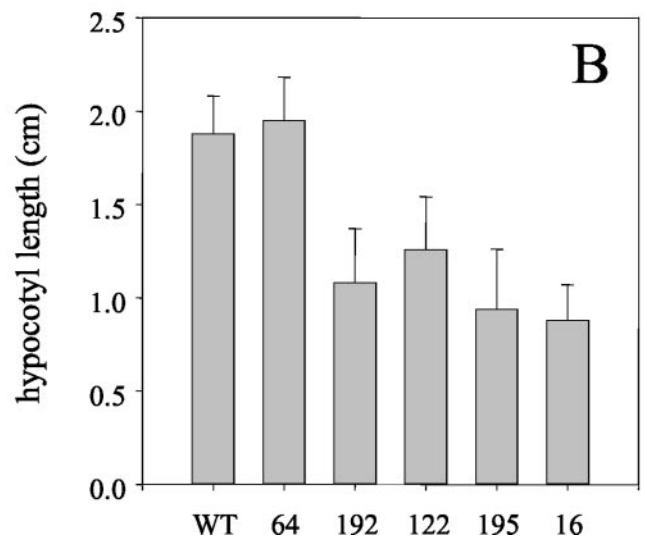
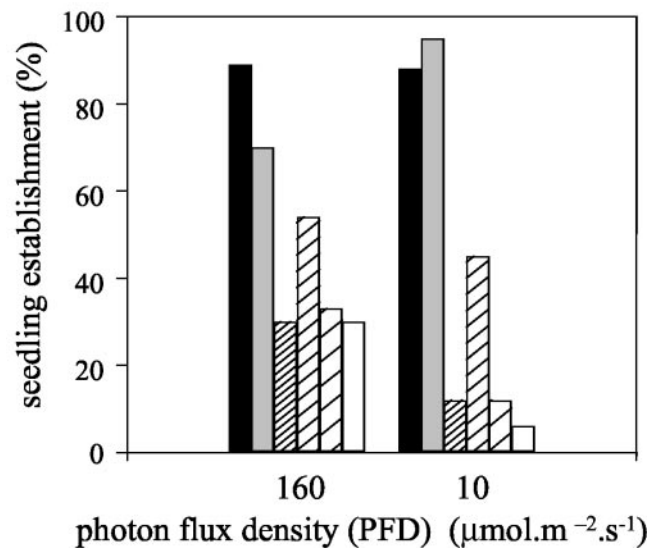


Figure 4. A, Seedling establishment in high ($160 \mu\text{mol m}^{-2} \text{s}^{-1}$) and low ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) photon flux densities. Black box, untransformed wild-type Col0; gray box, line 64; narrow hatched box, line 122; medium hatched box, line 192; wide hatched box, line 195; white box, line 16. Suc was not included in the medium; values are the mean \pm SE of measurements on three separate batches of 30 seedlings. B, Hypocotyl length of 35S-*PCK1* antisense and wild-type etiolated seedlings. Seedlings were grown in the dark on medium without Suc for 5 d. Values are the mean \pm SE of measurements on three separate batches of 30 seedlings.

total fatty acid levels were analyzed using GC-based fatty acid analysis (Browse et al., 1986). All five of the 35S-*PCK1* antisense lines revealed no significant alterations in the rate of storage lipid catabolism from that of untransformed Arabidopsis seedlings. Figure 7A shows that the levels of eicosenoic acid (20:1), which is specific to storage triacylglycerol (Lemieux et al., 1990), in seedlings from line 16 decreased at the same rate as wild-type seedlings both with and with-

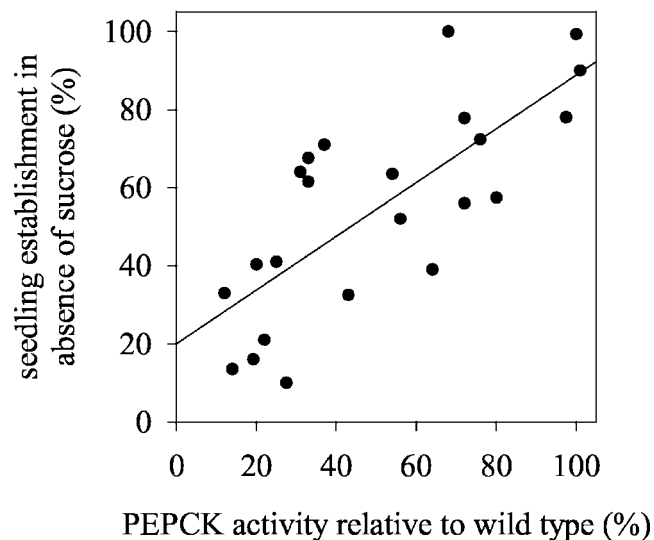


Figure 5. The relationship between seedling establishment and PEPCK activity in 2-d-old seedlings from independent 35S-*PCK1* antisense lines grown on one-half-strength Murashige and Skoog medium without Suc.

out Suc. The observed delay in storage lipid breakdown in the presence of Suc is in agreement with previous reports (Eastmond et al., 2000; Martin et al., 2002). In germinating castor bean seedlings, storage protein is converted to Suc via gluconeogenesis (Stewart and Beever, 1967). To investigate whether storage protein breakdown was reduced in the anti-sense lines, protein extracts from germinating seed-

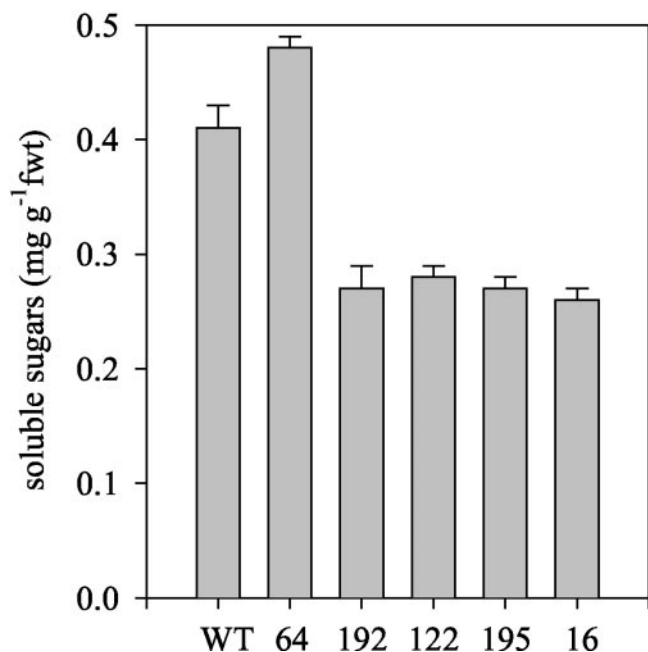


Figure 6. Soluble sugar levels (Glc, Fru, and Suc) in 2-d-old untransformed wild-type Col0 and 35S-*PCK1* antisense seedlings; mean \pm SE of measurements made on five replicates. All values are the mean \pm SE of measurements made on three separate batches of seedlings.

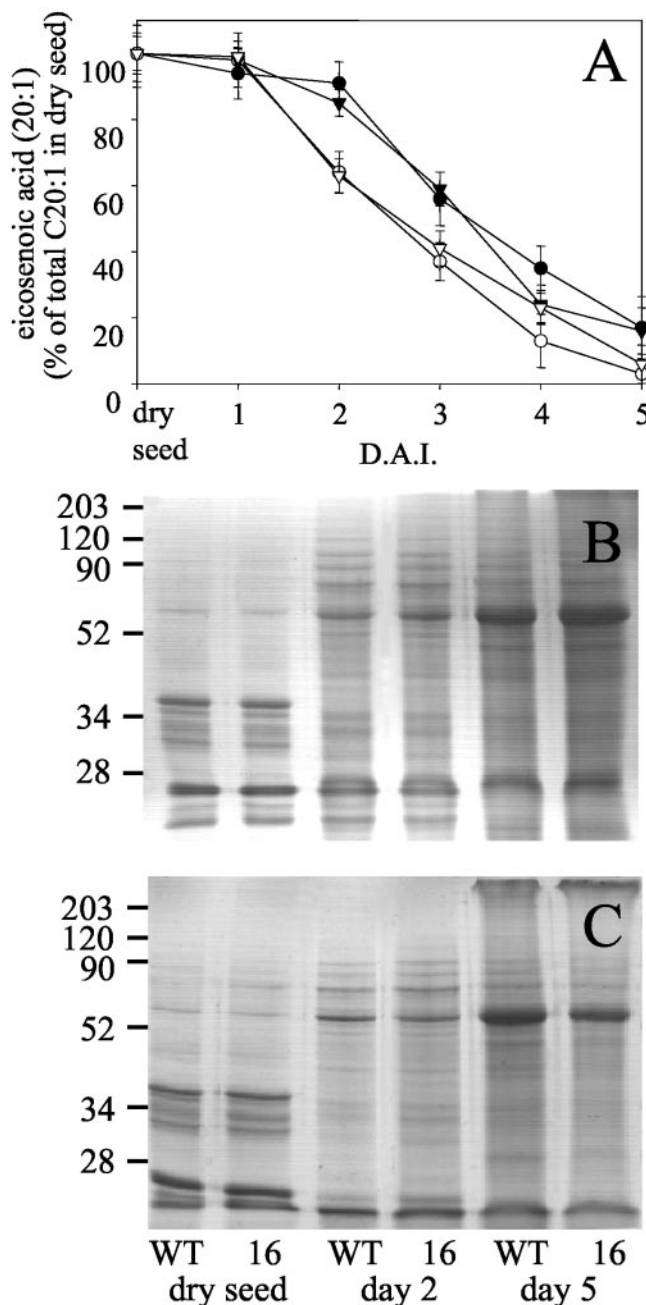


Figure 7. A, Levels of eicosenoic acid (20:1), which is specific to storage triacylglycerol in wild-type and 35S-*PCK1* antisense line 16 seedlings 0 to 5 d after imbibition (D.A.I.). Wild type (●) and line 16 (▼) grown on one-half-strength Murashige and Skoog media with 20 mM Suc, wild type (○) and line 16 (▽) grown without Suc. SDS-PAGE gel of protein extracts from seedlings of wild-type and 35S-*PCK1* antisense line 16 grown on one-half-strength Murashige and Skoog medium containing 20 mM Suc (B) and on one-half-strength Murashige and Skoog medium without Suc (C). Protein extract equivalent to eight seedlings was loaded onto each lane. Molecular weight markers are in kilodaltons.

lings from wild-type and 35S-*PCK1* antisense line 16 were visualized on SDS-PAGE gels (Fig. 7, B and C). The legumin-type globulins (also referred to as 12S

globulin or cruciferin in Arabidopsis; Sjødahl et al., 1991) are present between approximately 45 and 16 kD. Dry seeds of both wild type and line 16 have comparable levels of storage proteins. The storage proteins are catabolized at similar rates in wild-type and line 16 seedlings grown on media with (Fig. 7B) or without 20 mM Suc (Fig. 7C).

Because lipid and protein catabolism appeared unaltered, but soluble sugar levels were reduced, presumably as a consequence of compromised gluconeogenesis, we investigated whether more of the storage carbon was being respired in the 35S-*PCK1* antisense lines than in untransformed Arabidopsis by feeding [14 C]acetate to 2-d-old seedlings. To test the relationship between PEPCK activity and 14 C incorporation into carbon dioxide and sugars, measurements were made on the same additional set of 17 lines used in Figure 5 (Fig. 8). There was a negative correlation between PEPCK activity and the incorporation of 14 C from acetate into carbon dioxide and a positive correlation for the accumulation of 14 C from acetate into sugars. Although the r values for these correlations were not significant ($r = -0.420$ and $r = 0.370$ respectively), similar trends were seen in two separate experiments.

The reduction of PEPCK activity seen for 2-d-old seedlings in Figure 2B was mirrored in the rosette leaves, with line 16 showing the greatest reduction (Fig. 9). However, the level of PEPCK activity in rosette leaves was approximately 20-fold lower than in 2-d-old seedlings. Once seedlings had become established and transplanted to soil, there was no observable difference in the growth habit throughout the remainder of the life cycle.

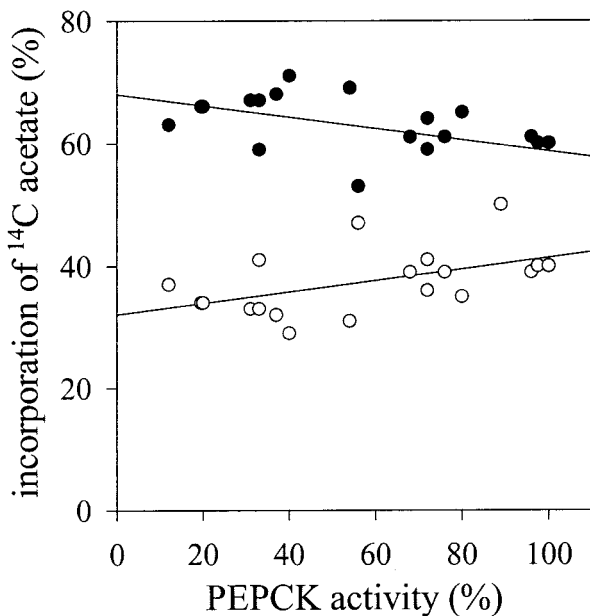


Figure 8. Incorporation of [14 C]acetate into sugars (○) and carbon dioxide (●) in wild-type Col0 and 35S-*PCK1* antisense 2-d-old seedlings.

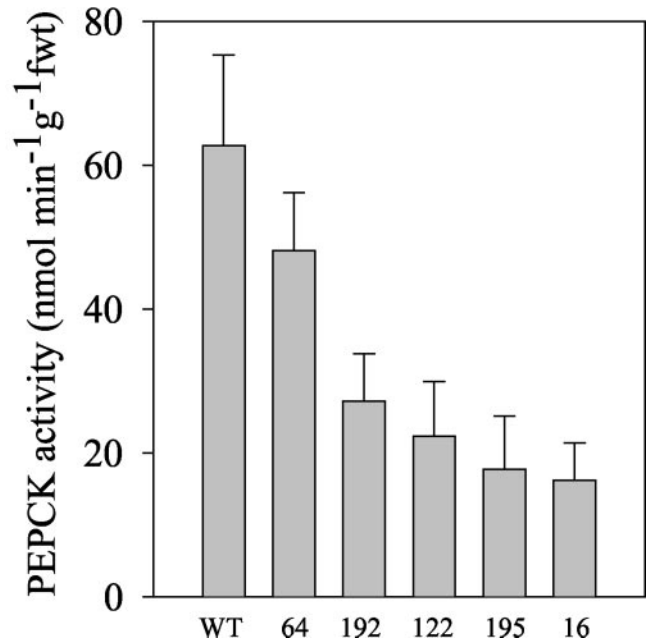


Figure 9. PEPCK activity in rosette leaves of untransformed wild-type Col0 and 35S-*PCK1* antisense lines. Values are the mean \pm SE of measurements made on three separate batches of tissue.

DISCUSSION

The direct correlation between seedling establishment and PEPCK activity (Fig. 5) in the different antisense lines demonstrates the essential role of *PCK1* in this process. However the [14 C]acetate feeding experiments presented in Figure 8 imply that PEPCK does not exert a high level of control over gluconeogenic flux. Previous studies have suggested higher levels of flux control for PEPCK. Leegood and ap Rees (1978a) noted a 3-fold increase in incorporation of [2- 14 C]acetate into carbon dioxide in the presence of the PEPCK inhibitor 3-mercaptopicolinic acid. Trevanion et al. (1995), also using 3-mercaptopicolinic acid reported the flux control coefficient of PEPCK as 0.7 to 1.0. However, our results and those of Leegood and ap Rees (1978a) and Trevanion et al. (1995) were all obtained by feeding exogenous [14 C]acetate, which gives only an indirect measurement of the fate of β -oxidation-derived acetyl CoA units. More recently, the flux control coefficient for another enzymatic step in the conversion of acetyl CoA to Suc, namely ICL, was reported to be 0.66 ± 0.09 (Runquist and Kruger, 1999) thus questioning a major role for PEPCK in this process. Irrespective of the data obtained through the [14 C]acetate feeding experiments, the fact remains that decreasing PEPCK activity has a profound effect on the ability of seedlings to establish and therefore must play a significant role in regulating flux through the pathway.

The linear curve fitted to the data in Figure 5 suggests that in the total absence of PEPCK activity, 20% of the seedlings would still establish. To deter-

mine whether this is the case would require the removal of PCK2 activity in addition to PCK1 because the *PCK2* gene is also expressed in young seedlings albeit at lower levels (Fig. 1A).

In the 35S-*PCK1* antisense lines, storage lipid is still catabolized during early post-germinative growth, even without a supply of exogenous sugars. Our results suggest that there is an increase in the respiration of acetyl CoA units released from fatty acid β -oxidation and a corresponding decrease in the production of sugars in the 35S-*PCK1* antisense lines with decreasing PEPCK activity (Fig. 8). These results indicate that when the capacity for the conversion of storage lipid to Suc is reduced, there is an increased flux of carbon derived from fatty acid β -oxidation into respiration. A similar conclusion was reported for an Arabidopsis mutant of the glyoxylate cycle gene, *ICL* (Eastmond et al., 2000). In 2-d-old *icl* seedlings, the glyoxylate cycle is blocked; consequently, there is no net flux of carbon from lipid into gluconeogenesis, and lipid is instead respired. However, seedling establishment is not compromised in the *icl* mutant when grown under optimal conditions, demonstrating that the glyoxylate cycle is not essential for seedling establishment under these conditions.

In addition to lipid reserves, Arabidopsis seeds contain reserves of storage protein (Baud et al., 2002). Stewart and Beevers (1967) demonstrated that amino acids from storage protein in the endosperm of castor bean are converted to Suc by gluconeogenesis and translocated to the germinating seedling. In the *icl* mutant, gluconeogenic substrate can still be provided by the breakdown of this storage protein, which is presumably in sufficient quantities for seedling establishment under optimal conditions. Under the suboptimal conditions of reduced light, or photoperiod, the frequency of seedling establishment in the *icl* mutant decreases as these additional reserves are depleted before photosynthetic competency is attained (Eastmond et al., 2000; Eastmond and Graham, 2001). Decreasing the amount of light available for photosynthesis also further reduces the percentage of seedling establishment in the 35S-*PCK1* antisense lines. In these lines, the combination of suboptimal growth conditions and reduction in available Suc from the restricted gluconeogenic pathway further compromise seedling establishment. The decrease in soluble sugars in the 35S-*PCK1* antisense seedlings at d 2 is already 30% (Fig. 6), which is similar to that reported for the *icl1* mutant (Pritchard et al., 2002). We conclude that the levels of soluble sugars are close to the threshold required for seedling establishment at this time, and even a relatively small drop can have a profound effect.

Unlike the *icl* and 35S-*PCK1* antisense lines, the β -oxidation mutants *ped1* and *kat2* are unable to catabolize lipid reserves, even in the presence of an exogenous supply of Suc. Furthermore, without a supply of exogenous Suc, post-germinative growth

does not occur in *ped1* and *kat2*, even under optimal growth conditions (Hayashi et al., 1998; Germain et al., 2001). Thus in the *icl* mutant, the catabolism of storage protein, in combination with respiration of storage lipid, provides enough fuel to accomplish seedling establishment under optimal growth conditions. However, the *ped1* and *kat2* mutants demonstrate that the catabolism of protein reserves alone is insufficient to attain seedling establishment. Moreover, unlike the *icl* mutant, the β -oxidation cycle is essential under all environmental conditions. In the 35S-*PCK1* antisense lines, although the phenotype is less acute than the *ped1* and *kat2* mutants, it is still more severe than the *icl* mutant. Even under optimal growth conditions, the frequency of seedling establishment is reduced compared with untransformed, wild-type seedlings. The 35S-*PCK1* antisense seedlings are compromised in the ability to use both storage lipid and storage protein through gluconeogenesis to produce soluble sugars. This demonstrates that whereas a disrupted glyoxylate cycle can be compensated for by optimal growth and early onset of photosynthetic capacity in young seedlings, gluconeogenesis is essential irrespective of growth conditions.

No alterations in mature plant phenotype in the 35S-*PCK1* antisense lines were observed. Metabolite profiling analysis targeted at the detection of phenylpropanoids and amino acids also did not reveal any alterations in the antisense lines. In the rosette leaves, PEPCK activity is 20-fold less than in seedlings, consistent with leaves as photosynthetic organs, being capable of producing an autotrophic supply of Suc. Furthermore, the distribution of PEPCK activity in leaves is localized to particular cell types such as trichomes (Leegood and Walker, 1999). It is possible, therefore that alterations, particularly of metabolites, in specific cell types within whole organs in the 35S-*PCK1* antisense lines may have been masked by an unaltered phenotype in the rest of the organ. Despite the large reduction in PEPCK activity (80% of wild type in line 16) achieved in the antisense lines, this may not be a sufficient reduction to produce an altered phenotype in the mature plant. In addition, *PCK2* gene expression levels are unaltered in the 35S-*PCK1* lines, and *PCK2* may play a more dominant role in mature plant organs. Thus additional studies are required to further dissect the roles of PEPCK activity in whole plants.

CONCLUSIONS

In conclusion, we have used an antisense approach to functionally characterize the role of *PCK1* during early post-germinative growth. In combination with the *icl* mutant studies, we have demonstrated that alternative storage reserves to lipid, most probably storage protein, can contribute substrate for gluconeogenesis, and thus gluconeogenesis is essential for seedling establishment in the oilseed Arabidopsis.

MATERIALS AND METHODS

35S-*PCK1* Antisense Construct

The cDNA expressed sequence tag H36251 was identified in the GenBank DNA sequence database as a PEPCK (*PCK1*), and the clone was subsequently obtained from the Arabidopsis Biological Resource Center (DNA Stock Center, Ohio State University, Columbus). The cDNA clone was provided as an *Escherichia coli* bacterial colony containing the cloning vector λ -Ziplox (Invitrogen, Carlsbad, CA) into which the cDNA clone was directionally inserted 5' to 3'. The sequence was confirmed by partial sequencing of the 3' and 5' ends. The 1,239-bp PEPCK fragment was excised from λ -Ziplox by restriction digest of the plasmid with *Xba*I and *Sma*I and ligated into pJO530 (a pBIN19 derivative; Bevan, 1984), which had been previously linearized by restriction digestion with *Xba*I and *Sma*I. Plant transformation using the binary vector system was conducted using *Agrobacterium tumefaciens* strain G3V101 containing a vir⁺ Ti-plasmid lacking the T-DNA region. Restriction digest and PCR analysis confirmed the insertion of the PEPCK cDNA fragment in pJO530 in the antisense orientation.

Plant Material and Growth Conditions

Arabidopsis ecotype Columbia (Col0) plants were transformed by vacuum infiltration (Bechtold and Pelletier, 1998). T₁ seed was grown on hygromycin plates, and resistant seedlings were selected to soil. T₂ and T₃ progeny were screened for homozygous lines by segregation on hygromycin. Seeds were germinated in continuous light on 0.8% (w/v) agar plates containing one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962; plus 20 mM Suc where indicated) at 20°C after 3 d imbibition at 4°C in the dark. For experiments with etiolated seedlings, plates were transferred back to the dark at 20°C after 30-min exposure to white light.

RT-PCR Analysis

The cDNA was synthesized from 5 μ g of DNase-treated RNA using a Prostar First-strand RT-PCR kit (Stratagene, La Jolla, CA). PCR was performed using serial dilutions of cDNA, the reactions were heated to 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, and then a single 72°C for 10 min. As a marker for constitutive expression actin (*ACT2*; GenBank accession no. U41998), cDNA was amplified with the primers ACT2A (5'-CTT ACA ATT TCC CGC TCT GC-3') and ACT2S (5'-GTT GGG ATG AAC CAG AAG GA-3'). Endogenous and transgene *PCK1* cDNA was amplified using primers *PCK1*-1001 (5'-AGG GTC TTT TCA GTG TGA TGC-3') and *PCK1*-1709 (5'-CCA TAA CTG CCA CCA GAC CA-3'). Only endogenous *PCK1* cDNA was amplified using *PCK1*-72 (5'-GAA GAT AAC GAC CGG AGC AG-3') and *PCK1*-573 (5'-GGG AGC ACG ACC AGT CTT AG-3'). *PCK2* cDNA was amplified using *PCK2*-1032 (5'-CTG CAT TTT CTC AGC CAA CA-3') and *PCK2*-1536 (5'-CCG GTA TTT GAT CCG AGA TG-3').

Enzyme and Sugar Assays

PEPCK assays were performed on plant tissue extracts according to the method of Cooper et al. (1968). Soluble sugars (Glc, Fru, and Suc) were measured according to the method of Stitt et al. (1989).

Metabolism of [¹⁴C]Acetate

The [¹⁴C]acetate feeding experiments were as described by Eastmond et al. (2000).

Amounts of label incorporated into ethanol-soluble (acidic, neutral, and basic) components, ethanol-insoluble material, and carbon dioxide were determined as described by Canvin and Beevers (1961). The neutral component contained the soluble sugar fraction.

Lipid Analysis

Fatty acids were extracted from dry seeds and 0- to 5 d-old-seedlings grown on one-half-strength Murashige and Skoog media with and without

20 mM Suc. Fatty acids were measured using the method of Browse et al. (1986).

Storage Protein Extraction and SDS-PAGE Analysis

Seedlings were homogenized in 50 mM HEPES, pH 7, and 5 mM dithiothreitol and 0.5% (w/v) SDS was added, and the samples were heated to 100°C for 1 h and centrifuged for 5 min at 8,000g. The supernatant was mixed with loading buffer (125 mM Tris, pH 6.8, 25% [v/v] glycerol, 2.5% [w/v] SDS, 0.05% [w/v] bromophenol blue, and 2.5% [v/v] β -mercaptoethanol) and the samples loaded onto a 12% (w/v) acrylamide gels. SDS-PAGE was as described by Laemmli (1970).

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