Arabidopsis uses two gluconeogenic gateways for organic acids to fuel seedling establishment

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Gluconeogenesis is a fundamental metabolic process that allows organisms to make sugars from non-carbohydrate stores such as lipids and protein. In eukaryotes only one gluconeogenic route has been described from organic acid intermediates and this relies on the enzyme phosphoenolpyruvate carboxykinase (PCK). Here we show that two routes exist in Arabidopsis, and that the second uses pyruvate, orthophosphate dikinase (PPDK). Gluconeogenesis is critical to fuel the transition from seed to seedling. Arabidopsis pck1 and ppdk mutants are compromised in seed-storage reserve mobilization and seedling establishment. Radiolabelling studies show that PCK predominantly allows sugars to be made from dicarboxylic acids, which are products of lipid breakdown. However, PPDK also allows sugars to be made from pyruvate, which is a major product of protein breakdown. We propose that both routes have been evolutionarily conserved in plants because, while PCK expends less energy, PPDK is twice as efficient at recovering carbon from pyruvate.
Glucogenesis allows seedling establishment, a critical phase in the life cycle of plants that is fuelled by the breakdown of seed reserves. The efficiency of reserve remobilization has an impact on the success of plants in natural communities, but also on crops because seedling vigour is a key determinant of yield. Starch, lipids and proteins can all be used, although lipid reserves are considered the most widely distributed in nature. In the case of lipids, the first step in remobilization involves the hydrolysis of triacylglycerols, stored in oil bodies, to release free fatty acids and glycerol. Both products serve as gluconeogenic substrates but fatty acids account for ~95% of the carbon. The fatty acids enter the peroxisome and are activated to fatty acyl-CoA by the peroxisomal fatty acyl-CoA synthetases (FACS) (EC 6.2.1.3) and activated fatty acyl-CoA is then transported into the cytosol. The acetyl-CoA produced by this process is then converted to organic acids via the glyoxylate cycle.

In plants it is widely accepted that phosphoenolpyruvate carboxykinase (PCK, EC 4.1.1.49) then plays a pivotal role in gluconeogenesis by catalysing the conversion of the C4 dicarboxylic acid oxaloacetic acid to phosphoenolpyruvate (PEP) (EC 4.1.1.39) and PEP synthetase (EC 2.7.9.2) (ref. 9). This mechanism for PEP synthesis is generally believed to be the only physiological route by which eukaryotes bypass the practically irreversible glycolytic reaction catalysed by pyruvate kinase (EC 2.7.1.40). However, in certain bacteria an alternate pathway has also been described that allows C4 dicarboxylic acids to be converted via pyruvate to PEP for gluconeogenesis. For example, *Escherichia coli* can use the combined activities of malic enzyme (ME, EC 1.1.1.39) and PEP synthetase (EC 2.7.9.2) (ref. 10), while *Acetobacter xylinum* and *Sinorhizobium meliloti* can use ME and pyruvate, orthophosphate dikinase (PPDK, EC 2.7.9.1) (ref. 11). PPDK is best known for its role in C4 photosynthesis in plants (ref. 12). However, PPDK genes appear to be present in all species, regardless of their photosynthetic mechanism (ref. 13). The possibility that pyruvate phosphorylation contributes to plant gluconeogenesis was investigated in the 1960s through the application of C4-labelled pyruvate and alanine to the endosperm of germinated castor beans (*Ricinus communis*). The efficiency of sugar labelling from pyruvate was low and varied with the position of the label, consistent with pyruvate being decarboxylated rather than being converted directly to PEP (ref. 17). A further study showed that the efficiency of sugar labelling from alanine was much greater, despite the fact that alanine is directly deaminated to pyruvate (ref. 18). This discrepancy was attributed to a difference in experimental design for alanine feeding that promoted dark re-fixation of C4CO2 (ref. 18). Therefore, the data from these studies suggested that direct phosphorylation of pyruvate (for example, by PPDK) does not contribute to gluconeogenesis in castor endosperm.

In the model oilseed *Arabidopsis thaliana* a series of more recent studies have shown that both PPDK (ref. 16) and PCK (ref. 7) are expressed following seed germination, and that disruption of *PCK* function has a surprisingly modest effect on early seedling growth (ref. 7,8). These data raise the possibility that, in spite of the existing evidence from studies on castor beans (ref. 17,18), PPDK might provide an alternate route for organic acids to support gluconeogenesis in *Arabidopsis*. We therefore decided to test this hypothesis using a molecular genetic approach.

**Results**

**PCK1 and cytosolic PPDK are both required for seedling establishment.** To allow us to investigate the function of PPDK in *Arabidopsis*, we obtained *ppdk* and *pck1* (ref. 8) T-DNA insertion mutants and constructed a *ppdk-pck1* double mutant. Immunoblotting confirmed that 2-day-old *ppdk* and *pck1* seedlings lack their respective PPDK and PCK proteins and are therefore null mutants (Fig. 1c). Disruption of seed reserve mobilization is known to inhibit early seedling growth and establishment in *Arabidopsis* (ref. 1). Although PCK is considered to be essential for gluconeogenesis, *pck1* seedling growth is only modestly affected, either in the light or in darkness (ref. 7,8) (Fig. 2a). Seedling development in *ppdk* appears to be normal but *ppdk-pck1* shows a severe reduction in growth rate, measured by cotyledon

![Figure 1](image-url)
expansion in the light or hypocotyl extension in the dark (Fig. 2a). Early seedling growth can therefore be supported by PPDK, in the absence of PCK. The provision of mineral nutrition (Murashige and Skoog basal salts) makes no difference to hypocotyl extension in the dark, but exogenous sugar can completely rescue ppdk-pck1 growth (Supplementary Fig. 2a), which is consistent with the genes functioning in gluconeogenesis1. The ppdk-pck1 mutant could also be partially complemented using a genomic clone encoding cytosolic PPDK, while chloroplastic PPDK had no effect (Supplementary Fig. 2b).

High activities of PPDK and PCK have been reported in cells around veins of Arabidopsis20. A strategy that combined use of hairpin RNA interference constructs for each gene with a vein-specific enhancer trap21 suggested that PPDK and PCK in cells around veins are not required for seedling growth (Supplementary Fig. 3).

Although ppdk seedlings appear to grow normally under optimal laboratory conditions (Fig. 2a) such conditions are uncommon in natural environments. We therefore assessed the absolute fitness of ppdk plants by measuring the frequency of seedling establishment (equivalent to survival) when seeds are sown under a range of suboptimal light environments that are more prevalent in nature22. Using this assay, fewer ppdk seedlings survived (P<0.05, least significant difference (LSD) test, n = 3) than wild type (Fig. 2b–d). This included when access to light was delayed for a period after the completion of germination (Fig. 2b), as would be the case beneath the soil, or when light levels were low, or days were short (Fig. 2c,d). Survival of pck1 seedlings was also reduced under suboptimal conditions and this was accentuated in ppdk-pck1 (Fig. 2). These data suggest that both proteins are required to maximize the frequency of seedling establishment under suboptimal growth conditions. Even under optimal conditions pck1 and ppdk-pck1 seedlings that do establish exhibit delayed rosette growth and flowering (Supplementary Fig. 4).

**PPDK and PCK are required for normal seedling growth and establishment.** Representative images of WT, ppdk, pck1 and DM seedlings grown in the dark (left) or light (right) for 5 days. Scale bar, 1 cm.

**Figure 2 |**

**PPDK and PCK are required for normal seedling growth and establishment.** (a) Representative images of WT, ppdk, pck1 and DM seedlings grown in the dark (left) or light (right) for 5 days. Scale bar, 1 cm. The effect of (b) increasing periods of darkness before being transferred to light, (c) reduced light intensity and (d) reduced day length on the frequency of establishment of WT, pck1, ppdk and DM seedlings (as defined by the proportion of seedlings that develop true foliage leaves within 4 weeks after germination). Data are shown as means ± s.e. from three batches of a hundred seeds and asterisks represent a statistical difference from WT (P<0.05, LSD test, n = 3). Light intensity was 160 μmol m⁻² s⁻¹ except in c.

**PPDK and PCK1 are required for sugar accumulation following seed germination.** Quantification of total fatty acid (a,c) and protein (b,d) content in seeds (a,b) and 5-day-old seedlings (c,d) of WT, ppdk, pck1 and ppdk-pck1 grown on MS basic media, plus or minus 30 mM glucose. (e) Soluble sugar content in 2-day-old WT pck1, ppdk and DM seedlings. (f) Alanine content of two day old seedlings of WT, ppdk, pck1 and ppdk-pck1 grown on Murashige and Skoog (MS) basic media. Data in e are the sum of sucrose, glucose and fructose. All values are the mean ± s.e. of measurements on four separate pools of seeds or seedlings and asterisks represent a statistical difference from WT (P<0.05, LSD test, n = 4).
reports showing that mutant seedlings with gluconeogenic defects can still use lipids as a respiratory carbon source, particularly when sugar is provided.\(^{17,22,23}\)

In wild-type Arabidopsis seedlings soluble sugars accumulate shortly after germination as a result of gluconeogenesis from seed-storage lipids and this is impaired in mutants that are deficient in the pathway.\(^{22}\) We found that sugar levels are reduced by \(~70\%\) in 2-day-old ppk1 seedlings, consistent with the role of PPCK in gluconeogenesis\(^\text{23}\) (Fig. 3c). However, the ppdk mutant also has \(~30\%\) less sugar, and ppdk-ppk1 has \(~90\%\) less sugar than wild type (\(<P<0.05,\) LSD test, \(n=4\)). The significant impact of ppk1 and ppdk on sugar seed storage, and the additive effect observed in ppdk-ppk1, suggest that PPCK and PPDK are both required for normal gluconeogenic flux.

In addition to lipids, proteins can also serve as a gluconeogenic substrate\(^\text{18}\), and in some seeds they are the predominant reserve\(^\text{24}\). The carbon backbones of alanine, cysteine, glycine, serine, threonine and tryptophan can all be metabolized to pyruvate, the substrate of PPDK, and these amino acids make up around one-third of seed-storage protein in Arabidopsis. Uniquely, alanine is directly and reversibly converted to pyruvate and analysis of alanine content showed that in ppdk and ppdk-ppk1 seedlings, it increased (\(<P<0.05,\) LSD test, \(n=4\)) approximately threefold compared with ppk1 or wild type (Fig. 3f). This suggests an important gluconeogenic function for PPDK in remobilizing carbon skeletons present in amino acids that are catabolized to pyruvate.

PCK1 and PPDK play distinct roles in mobilization of stores. To follow the metabolism of gluconeogenic substrates derived from lipids and protein, short-term radiolabelling experiments were performed on 2-day-old seedlings using [2-\(^{14}\)C]-acetate and [U-\(^{14}\)C]-alanine.\(^\text{18,23}\) Seedlings were labelled for 4 h and the redistribution of \(^{14}\)C into metabolic fractions was determined and expressed as a percentage of the total metabolized (Fig. 4 and Supplementary Table 1). When wild-type seedlings were fed either acetate or alanine, \(~20\%\) of the total \(^{14}\)C was detected in sugars (Fig. 4a), suggesting that both metabolites act as gluconeogenic substrates.\(^\text{18,23}\) Feeding \(^{14}\)C-acetate to pck1 seedlings resulted in a substantial reduction in the percentage of \(^{14}\)C found in sugars (\(<P<0.05,\) LSD test, \(n=4\)) but no change in the percentage of \(^{14}\)C in this fraction was observed in ppdk (Fig. 4a,b). On the other hand, when \(^{14}\)C-alanine was fed to ppdk seedlings, the percentage of \(^{14}\)C in sugars was significantly reduced (\(<P<0.05,\) LSD test, \(n=4\)), while no change was seen in ppk1 (Fig. 4c,d). In ppdk-ppk1 the percentage of label found in sugars following \(^{14}\)C-alanine feeding was significantly (\(<P<0.05,\) LSD test, \(n=4\)) lower than in the ppdk single mutant; however, the percentage of label in sugar from \(^{14}\)C-acetate feeding was similar to ppk1. These labelling patterns are consistent with a model (Fig. 4e) in which PPDK participates in gluconeogenesis by remobilizing amino acids such as alanine that give rise to pyruvate, while PPCK is primarily involved in remobilization of acetyl-CoA derived from lipids.

In ppdk, more than 10\% of \(^{14}\)C-alanine is still metabolized to sugars versus \(~20\%\) in wild type (Fig. 4c). The most likely explanation for this is that in the absence of PPDK, pyruvate is converted to acetetyl-CoA by pyruvate dehydrogenase (EC 4.1.1.1), providing carbon skeletons to the glyoxylate or tricarboxylic acid cycles (Fig. 4e). The observation that total protein breakdown is not significantly impaired in ppdk seedlings (Fig. 3d) is also consistent with a diversion of flux rather than a blockage. The glyoxylate cycle in plants spans multiple subcellular compartments\(^1\) and thus the model in Fig. 4e is a simplification. Nevertheless, the route involving pyruvate dehydrogenase leads to the net loss of at least half the carbon as CO\(_2\) while conversion of pyruvate to sugar via PPDK results in no loss (Fig. 4e). Indeed, the percentage of \(^{14}\)C released as CO\(_2\) when ppdk is fed alanine is substantially greater than in wild type (Fig. 4d). Interestingly, animals and yeast lack PPDK but, by using a single pathway that combines pyruvate carboxylase (PC, EC 6.4.1.1) with PPCK, also achieve no net loss of carbon as CO\(_2\) when pyruvate supports gluconeogenesis (Supplementary Fig. 5).

When lipids are used as a gluconeogenic substrate in Arabidopsis the carbon conversion efficiency would be the same whether PPCK or PPDK are employed because PPDK must be coupled with the decarboxylating activity of ME (Supplementary Fig. 4), but...
PPDK has a higher cost in ‘energy’ because ATP is hydrolyzed to AMP, not ADP, and therefore requires an additional ATP to regenerate ADP via adenylate kinase (Fig. 4e). The strong impact of pck1 on sugar labelling from 14C-acetate (Fig. 4a) does not support a substantial role for ME in mediating flux from lipids to PEP via PPDK. We therefore propose that plants use a PPDK-dependent gluconeogenic pathway because it allows the most efficient recovery of carbon skeletons from amino acids in seed-storage proteins that are metabolized via pyruvate, while a PCK-dependent pathway is also present because it is more ‘energy-efficient’ for substrates metabolized to C₄ dicarboxylic acids, such as lipids and other amino acids.

PCK1 and PPDK deficiency leads to sugar-starvation responses. Compromised gluconeogenesis during early seedling growth would eventually be expected to lead to broad alterations to primary metabolism, and to induce sugar-starvation responses. To address this we undertook global analysis of gene expression using the GeneChip Arabidopsis ATH1 Genome Array from Affymetrix. Analysis of transcript abundance in 2-day-old seedlings for genes known to be involved in mobilization of storage lipids revealed a similar degree of overlap (180 and 64 genes, respectively) with the 278 SSM genes. Finally, ppdk-pck1 also shares a total of 823 more than twofold up- or downregulated genes in common with icl and 376 genes in common with mls (Table 2). We conclude that when PPDK is lacking, gluconeogenesis is reduced, but that this is not sufficient to lead to a significant sugar-starvation response under optimal growth conditions. However, when both PCK and PPDK are inactive, the impact on gluconeogenesis is eventually sufficient to induce a wide-scale starvation response as has been observed in glyoxylate cycle mutants.

**Table 2 | Overlap between differentially expressed genes in ppdk, pck1 and ppdk-pck1 and genes that are known to respond to sugar-starvation.**

| Genotypes | Number of overlapping genes |
|-----------|-----------------------------|
| ppdk versus SSM | 4 (1%) |
| pck1 versus SSM | 41 (15%) |
| mls versus SSM | 64 (23%) |
| DM versus SSM | 108 (38%) |
| icl versus SSM | 180 (64%) |
| ppdk versus mls | 5 |
| ppdk versus icl | 9 |
| ppdk versus pck1 | 14 |
| ppdk versus DM | 18 |
| pck1 versus mls | 168 |
| pck1 versus icl | 328 |
| DM versus mls | 376 |
| DM versus icl | 823 |
| DM versus pck1 | 946 |

DM, double mutant; SSM, Sugar starvation marker; SAM, Statistical Analysis of Microarrays. The overlap between genes significantly (P < 0.05, SAM, n = 3) more than two-fold up- or downregulated in 2-day-old ppdk, pck1, ppdk-pck1, mls and icl seedlings versus wild type and a set of 278 sugar-starvation marker (SSM) genes. Three microarray experiments were performed for each genotype.
Discussion

Our combined data show that, Arabidopsis, in common with some bacteria, uses PPDK as a gluconeogenic enzyme. To our knowledge, this is the first time that two distinct routes for the entry of organic acids into gluconeogenesis have been described in a eukaryote. Uniquely, we also provide evidence that the two routes have specialized functions in lipid and protein mobilization, respectively. Our findings are also surprising because they appear to be at odds with previous radiolabelling studies using endosperm of germinated castor beans, which suggested that very little gluconeogenic flux occurs through direct phosphorylation of pyruvate17,18. RNA sequencing data have shown that PPDK is expressed in germinated castor beans29. However, there might still be variation in PPDK activity between the two species and/or tissues. Castor seeds are anatomically very different from Arabidopsis, storing the bulk of their reserves in their large endosperm. In contrast, Arabidopsis seeds possess a greatly reduced endosperm consisting of a single-cell layer (aleurone) with the majority of the reserves stored in the embryo28. Using a translational fusion between cytosolic PPDK and GUS19, we observed that expression of PPDK in the aleurone of germinated Arabidopsis seed is much less widespread and persistent than in the embryo (Fig. 1a and Supplementary Fig. 6). Therefore, variation in PPDK expression does exist between different storage tissues in the same seed.

It is also conceivable that PPDK does support gluconeogenesis in castor endosperm despite the inefficiency with which it metabolizes exogenous pyruvate to sugars37. There is now considerable evidence that metabolic channelling takes place within glycolysis in Arabidopsis27 and in castor endosperm analysis of changes in metabolic intermediates in response to anoxia has also suggested that gluconeogenesis and glycolysis occur in separate intracellular regions28. It is therefore possible that supplying pyruvate to endosperm slices17 results in this metabolite entering the mitochondrion and being respirated, whereas pyruvate that is produced endogenously from amino acids, such as alanine18, may be channeled into gluconeogenesis. In the future, it would be interesting to investigate whether the compartmentalization and enzymatic properties of alanine aminotransferases (EC 2.6.1.2) could account for the comparatively efficient metabolism of alanine to sugars18. Arabidopsis contains four alanine aminotransferase genes and at least two are expressed in seedlings29,30.

In theory, the use of PPDK as a gluconeogenic enzyme is advantageous because it allows the most efficient remobilization of carbon skeletons from substrate that must be metabolized via pyruvate, such as certain gluconeogenic amino acids. Indeed, this subset of amino acids makes up around one-third of Arabidopsis seed-storage protein. It is plausible that the greater efficiency with which pyruvate can be used for gluconeogenesis is also a reason why PPDK or PEP synthase are used for gluconeogenesis in some bacteria, while animals and yeast employ an alternative route combining PC and PCK (Supplementary Fig. 5). To our knowledge, there is no firm molecular evidence that PEP synthase or PC exist in plants. Arabidopsis does have six genes encoding Mf31,32 that could allow PPDK to make a contribution to gluconeogenic flux from lipids, as has been shown for acetate in bacteria13,14. However, 14C-acetate labelling studies (Fig. 4a) and mutant analysis (Supplementary Table 2) suggest that this is not a major metabolic route in Arabidopsis.

In order for Arabidopsis PPDK to function in gluconeogenesis it must catalyse the synthesis of PEP in vivo. A recent study has shown that at cytosolic pH the specific activity of recombinant Arabidopsis PPDK is approximately eightfold lower in the PEP-forming direction than it is in the pyruvate-forming direction23. However, the $K_m$ for pyruvate (17 μM) is ~17-fold lower than it is for PEP and, at physiological pyruvate concentrations (~0.1 mM), PEP formation may therefore be favoured33. Furthermore, although high cytosolic concentrations of pyrophosphate (PPI) should inhibit PEP formation, PPI has relatively little effect on Arabidopsis PPDK activity in vitro33. Arabidopsis has also been found to contain an H$^+$-pyrophosphatase that suppresses PPI accumulation during early post-germinative growth and promotes gluconeogenesis34.

Given our discovery that PPDK plays a role in gluconeogenesis in Arabidopsis, it will be important to understand how this enzyme is regulated during the phase of seed-storage reserve mobilization. Gibberellins (GA) and abscisic acid (ABA) are known to play a key antagonistic role in seed dormancy and the control of germination27 and there is also evidence that they regulate storage-reserve breakdown, although their role appears to vary with species and tissue1. In Arabidopsis, transcriptional induction of PCK1 is repressed by ABA in the embryo but not the endosperm (aleurone) tissue of the seed25. A preliminary analysis of the effect of ABA on expression of the cytosolic PPDK–GUS fusion in embryos of inbred seeds suggests that PPDK is not sensitive to transcriptional repression by ABA (Supplementary Fig. 6) and thus its regulation differs from PCK1 (ref. 8). However, PPDK may be subject to other levels of regulation. For example, it is known that the PPDK-regulatory protein (PDRP) uses phosphoryl-donor nucleotides to reversibly phosphorylate an active-site threonine residue in PPDK, thereby inactivating the enzyme and a cytosolic PDRP has been identified in Arabidopsis35, although its precise physiological role is not yet understood.

In conclusion, the progressively compromised seedling growth evident in Arabidopsis ppdk, pck1 and ppdk-pck1 and the reduction in carbon recovery from seed-storage reserves in both the double and single mutants clearly shows that the gluconeogenic function of both PCK and PPDK enzymes is of relevance to plant fitness. The superior carbon recovery that PPDK allows would be advantageous to both bacteria and plants when growth is dependent on substrates such as lactate and gluconeogenic amino acids that can give rise to pyruvate. PPDK genes appear to be present in all plant genomes that have so far been sequenced16 and there is also evidence that PPDK is expressed in the storage reserve tissues of germinated seeds from a taxonomically diverse range of species including castor bean26, poplar27 (Populus balsamifera), rice38 (Oryza sativa), barley39 (Hordeum vulgare) and maize40 (Zea mays; Supplementary Table 3). Although it is possible that Arabidopsis independently and convergently recruited PPDK for gluconeogenesis, it is therefore more likely that both bacterial and plant lineages have maintained PPDK for this purpose since their divergence around 1.3 billion years ago.

Methods

Plant material and growth conditions. The PPDK and PCK1 insertion mutants in Arabidopsis ecotype Cvi (ppdk: SALK_073368 and pck1–2: SALK_032133) were identified in the SALK T-DNA collection41 and obtained from the European Arabidopsis Stock Centre (eNASC, University of Nottingham, UK). To assess seedling phenotype, metabolite content, protein and transcript levels, and to perform radiolabelling experiments, seeds were sterilized and sown on plates containing 0.8% w/v agar. Where stated, Murashige and Skoog basal salts (pH 5.7), 30 mM glucose and 20 μM ABA were also included. The seeds were then stratified for 3 days at 4°C, subjected to a 30-min light pulse of 200 μmol m$^{-2}$ s$^{-1}$ and routinely maintained in the light or dark at 22°C for up to 5 days. Experiments to determine the frequency of seedling establishment under different light regimes were performed as described previously37. In soil, Arabidopsis seed was sown on 3:1 compost:vermiculite in controlled growth chambers with 16-h photoperiods at 20°C, 60% humidity and 150 μmol m$^{-2}$ s$^{-1}$ light intensity. To assess seed production, plants were grown to maturity. Seeds were removed from silique and then sieved through a 0.4-mm mesh. For hypocotyl extension assays and vegetative plant growth and development, seedlings were imaged using a digital camera and hypocotyl length determined using the ImageJ software. Green fluorescent protein and chlorophyll were imaged using a Confocal Laser Scanning Microscope as described previously46.
Plasmid construction through to plant transformation. To generate hairpin constructs for both PCK and PPDK, sense and antisense regions of each gene were obtained by PCR using the primers listed in Supplementary Table 4. They were then fused to the LIPI intron 4 and placed under control of the GAL4VP16 upstream activation sequence as described previously. Briefly, SacI and KpnI/BglII sites were added to each sequence and used to clone sense fragments of each gene upstream of the LIPI intron. BamHI and SacI sites were used to allow maintenance of each gene to be cloned downstream of the LIPI intron. The flIII and SacI sites were then used to excise these fragments from the cloning vector and then place them into a binary vector containing the GAL4VP16 upstream activation sequence. Using floral dipping Agrobacterium tumefaciens was used to insert each construct into Arabidopsis and transgenic lines were selected on hygromycin. Homozygous single lines for PPDK and PCK were crossed to obtain double knockdown lines.

Metabolite measurements and radiolabelling experiments. Total fatty acid content and composition was determined using gas chromatography of fatty acid methyl esters (FAMEs) following direct transmethylation of tissue samples. Pools of 20 seeds or seedlings were added to vials containing 10 μL of methanol and 100 μL of 14C labeled 0.2 M acetic acid and 0.1 M sodium acetate. The vials were sealed and kept at 65 °C for 1 h. The total washes were combined and acidified using a mixture of hexane and 250 μL of 14C water at 40 °C. The resulting hexane layer was washed with 200 μL of 0.2 M NaOH and 100 μL of 0.1 M HCl. The solvent was evaporated and the residue taken up in 10 μL of 20% (v/v) ethanol at 85 °C for 1 h. The samples were then heated in a vacuum oven at 85 °C for 1 h and the resulting solutions were processed using thin-layer chromatography. The amount of 14C in each fraction was quantified by scintillation counting. In control experiments, >90% of the 14C in the neutral fraction was found to co-migrate with sucrose, glucose and fructose standards following paper chromatography.

Immunoblots and gene expression analysis. Soluble proteins were extracted by homogenizing 2-day-old seedling material in 100 mM potassium phosphate buffer (pH 7.4). The homogenate was spun in a centrifuge, the supernatant removed and the protein content quantified using the Bradford Assay. For immunoblotting 10 μL of protein was loaded on a 10% SDS–PAGE gel and transferred to nitrocellulose. For protein content quantification using the Bradford Assay. For immunoblotting 10 μL of protein was loaded on a 10% SDS–PAGE gel and transferred to nitrocellulose. The homogenate was spun in a centrifuge, the supernatant removed and the protein content quantified using the Bradford Assay. For immunoblotting 10 μL of protein was loaded on a 10% SDS–PAGE gel and transferred to nitrocellulose.

Statistical analysis. Analysis of variance was used to assess the differences in growth and metabolism between genotypes or treatments. Following significant (P < 0.05) F-test results, the means of interest were compared using the appropriate LSD value at the 5% (P = 0.05) level of significance, on the corresponding degrees of freedom. The GenStat (2011, 14th edition; VSN International) statistical system was used for these analyses. The significance analysis of microarray procedure was used to define significantly up- and downregulated genes (P < 0.05) with at least a twofold increase in expression.

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Additional information
Accession codes. Microarray data are available at uNASC (http://Arabidopsis.info/) under the registration number: NASCARRAY534, and also the iPlant collaborative at http://data.iplantcollaborative.org/quickshare/d884b0174e5c7974/Exp534.zip.

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