Co-ordinate regulation of genes involved in storage lipid mobilization in *Arabidopsis thaliana*

E. L. Rylott*, M. A. Hooks† and I. A. Graham*[1]

*Centre for Novel Agricultural Products, Department of Biology, University of York, York YO10 5YW, U.K., and †University of Wales, Bangor, Gwynedd LL57 2UW, Wales, U.K.

Abstract

Molecular genetic approaches in the model plant *Arabidopsis thaliana* (*Col*0) are shedding new light on the role and control of the pathways associated with the mobilization of lipid reserves during oilseed germination and post-germinative growth. Numerous independent studies have reported on the expression of individual genes encoding enzymes from the three major pathways: β-oxidation, the glyoxylate cycle and gluconeogenesis. However, a single comprehensive study of representative genes and enzymes from the different pathways in a single plant species has not been done. Here we present results from *Arabidopsis* that demonstrate the co-ordinate regulation of gene expression and enzyme activities for the acyl-CoA oxidase- and 3-ketoacyl-CoA thiolase-mediated steps of β-oxidation, the isocitrate lyase and malate synthase steps of the glyoxylate cycle and the phosphoenolpyruvate carboxykinase step of gluconeogenesis. The mRNA abundance and enzyme activities increase to a peak at stage 2, 48 h after the onset of seed germination, and decline thereafter either to undetectable levels (for malate synthase and isocitrate lyase) or low basal levels (for the genes of β-oxidation and gluconeogenesis). The co-ordinate induction of all these genes at the onset of germination raises the possibility that a global regulatory mechanism operates to induce the expression of genes associated with the mobilization of storage reserves during the heterotrophic growth period.

Introduction

Lipid mobilization during post-germinative growth in oil seeds is a multistep process involving peroxisomal β-oxidation, the glyoxylate cycle and gluconeogenesis (Scheme 1). β-Oxidation involves three families of enzymes, acyl-CoA oxidases (ACXs), multifunctional protein (MFP, which exhibits 2-*trans*-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase and Δ^3,Δ^2-enoyl-CoA isomerase activities) and L-ketoacyl-CoA thiolase (thiolase). Activities or protein levels for these enzymes have been demonstrated in an extensive range of tissues and developmental stages [1–3]. An analysis of the almost complete genomic sequence of *Arabidopsis* reveals that there are at least six genes encoding ACX, four of which have been functionally characterized. *AtACX1* is a medium–long-chain ACX with a substrate optimum of $C_{14:0}$-CoA and *AtACX2* has a substrate optimum of $C_{18:1}$-CoA [4]. A short-chain ($C_{6:0}$) ACX, which we refer to as *ACX4* [5] and more recently a fourth ACX, *AtACX3*, with a substrate optimum of $C_{10:0}$-CoA to $C_{12:0}$-CoA have also been characterized [6,7].

Two genes encoding MFP have been characterized in *Arabidopsis*: *MFP2* is strongly expressed during germination [8], whereas *MFP1* is expressed predominantly in the silique and flower. A mutation in the *MFP1* gene, termed *aim*1, results in abnormal development of the inflorescence meristem [9].

Studies in cucumber [10] and pumpkin [11] have demonstrated a high level of thiolase gene expression during early post-germinative growth and in senescing cotyledons. Recently an *Arabidopsis* mutant, *ped1* (peroxisome deficient), which is disrupted in a 3-ketoacyl-CoA thiolase gene normally expressed strongly during germination, has been characterized. The *PED1* gene product is essential for post-germinative seedling establishment and this phenotype can be rescued by exogenous sucrose [12].

Isocitrate lyase (*ICL*) and malate synthase (*MS*) are key enzymes in the glyoxylate cycle, whereas phosphoenolpyruvate carboxykinase (*PEPck*) has a key role in gluconeogenesis [13]. Together, *ICL* and *MS* activities have been shown to increase co-ordinately with the pattern of lipid breakdown [14,15]. Both *ICL* and *MS* are also induced in senescing leaves and cotyledons [16,17] and in cell cultures and mesophyll protoplasts, in

Key words: β-oxidation, gluconeogenesis, glyoxylate cycle.

Abbreviations used: ACX, acyl-CoA oxidase; ICL, isocitrate lyase; MFP, multifunctional protein; MS, malate synthase; PEPck, phosphoenolpyruvate carboxykinase; *PED*, peroxisome deficient; RbcS, ribulose bisphosphate carboxylase.

[1]To whom correspondence should be addressed (e-mail iag1@cnap.york.ac.uk).

response to sugar deprivation [18]. Independent germination and sugar response elements in the *MS* [19] and *ICL* [20] promoters have been identified. Studies in cucumber have shown that *ICL* and *MS* are co-ordinately expressed with the *PEPck* gene during early post-germinative growth and senescence [21].

The objective of the present work was to establish the temporal expression of the key genes and enzymes of the major pathways of lipid mobilization in *Arabidopsis*. The effect of sucrose on germination and related gene expression has also been investigated because previous reports have implicated carbohydrates in the regulation of glyoxylate cycle gene expression [18–20].

Results

Stages of development

Arabidopsis seeds germinate and establish at different rates depending on the growth conditions. To standardize gene expression and enzyme activity results between experiments, it is important to define specific stages of development. In the present work, seedlings were grown at 20 °C under continuous white light at 70 $\mu E/m^2$. Stages of development were defined every 24 h of growth; these are shown in Figure 1(A).

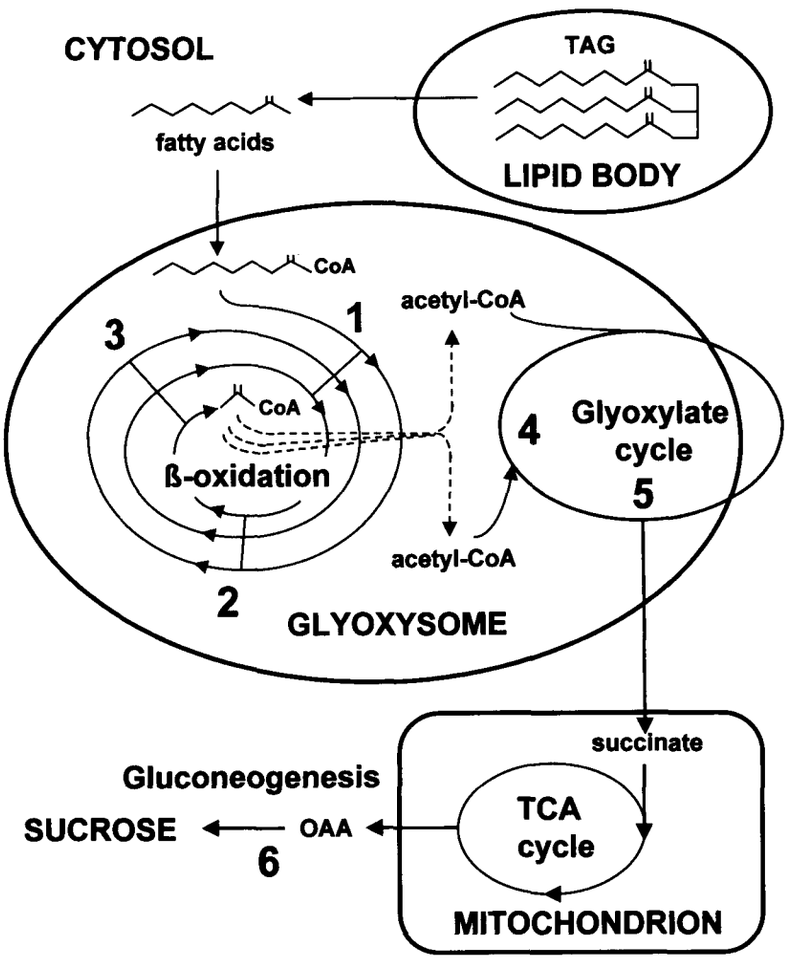
Effect of sucrose

The addition of low concentrations of sucrose (20 mM) to germinating seeds causes a slight delay

Scheme 1

Schematic representation of the pathways involved in storage lipid mobilization in oilseeds

Step 1, ACX; step 2, multifuctional protein; step 3, thiolase; step 4, MS; step 5, ICL; step 6, PEPck.

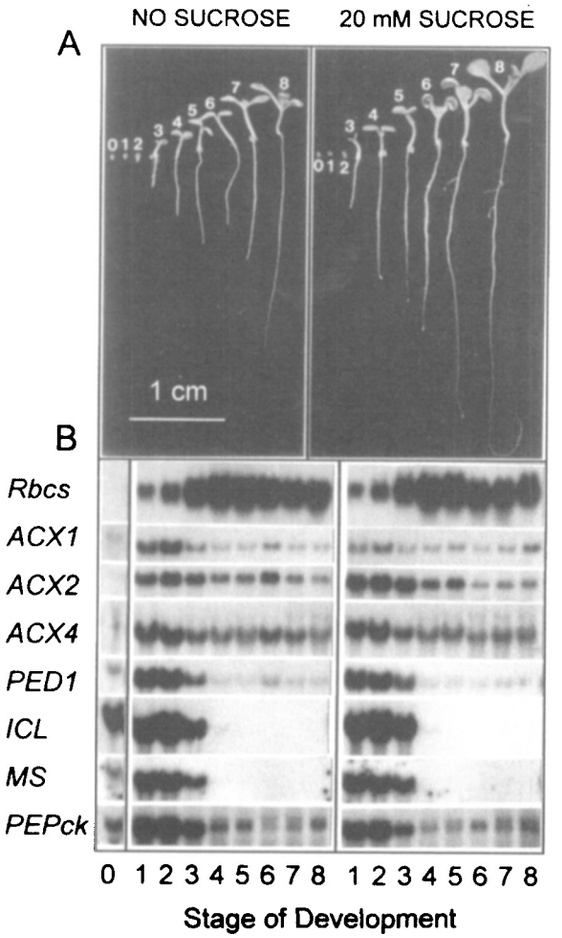


in radical emergence (stage 2), but thereafter results in more rapid seedling growth. The photosynthesis marker gene ribulose bisphosphate carboxylase (*RbcS*) was used to monitor the onset of photosynthetic competence of the seedlings from heterotrophic, storage reserve-dependent growth. The expression of *RbcS* and all the genes of lipid mobilization studied, showed an induction following imbibition (hydration) and were unaffected by the presence of 20 mM sucrose (Figure 1B).

Figure 1

Stages of seedling development (A) and Northern blot analysis of gene expression (B) from 0 to 8 days after imbibition

For (**B**), total RNA (10 μg) was loaded in each lane and blotted, and the filter was probed for *ACX1* (clone 35H7T7), *ACX2* (clone 5F12T7P), *ACX4* cDNA (clone 205E18T7), *PED1* (39H3T7), *MS* (GenBank accession no. T04260), *ICL* (Genbank accession no. Z18772), *PEPck* (GenBank accession no. H36251) and *RbcS* (GenBank accession no. T04228). The specific activities of the probes were similar and therefore the relative transcript abundances can be compared.



Expression during germination and post-germinative growth

In imbibed seeds (stage 0), *ICL*, *MS* and *PEPck* produced the highest levels of transcript, whereas levels of all the β-oxidation genes were low at this stage. mRNA levels of *ACX1*, *ACX2* and *ACX4*, *PED1*, *ICL*, *MS* and *PEPck* were all highly and co-ordinately induced following imbibition, peaking at stage 2. Significant levels of only the β-oxidation genes and *RbcS* were still detectable at stage 8.

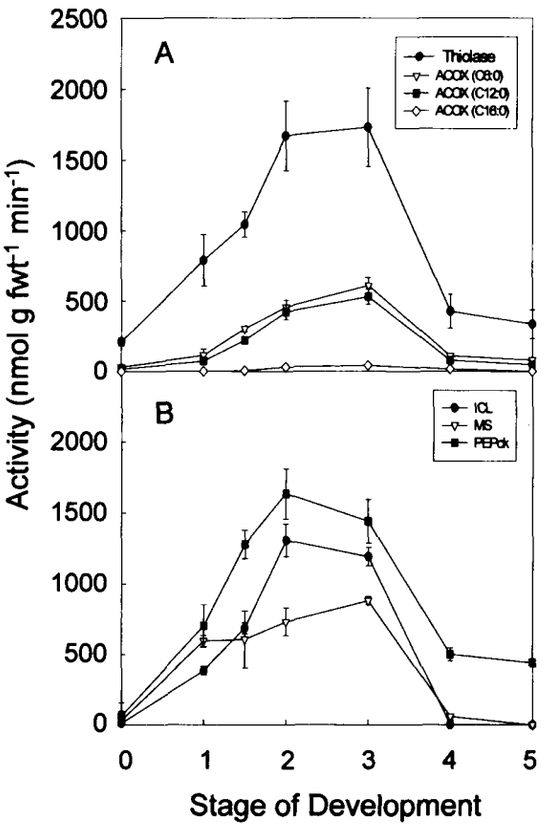
Enzyme activities in *Arabidopsis*

The activity of thiolase (determined with acetoacetyl-CoA as substrate) in imbibed seeds (stage 0) was significantly greater than the ACX $C_{6:0}$, ACX

Figure 2

Thiolase and ACOX activities (A) and ICL, MS and PEPck activities (B) during early post-germinative growth

Extracts were made as described in [4] and enzymes were assayed with the following methods: thiolase [27] with modifications as described in [28]; ACX [29] with $C_{6:0}$, $C_{12:0}$ and $C_{16:0}$ acyl-CoA substrates; ICL and MS [30]; PEPck [31]. Values are means±S.E.M. for measurements on three separate tissue extracts.



$C_{12:0}$, MS, ICL and PEPck activities. The ACX $C_{16:0}$ activity was not detectable in imbibed seed. Enzyme activity levels for all the genes involved in lipid mobilization rose rapidly, peaking at between stage 2 and stage 3. The thiolase activity was 42-fold the ACX $C_{16:0}$ activity and 3-fold that for ACX $C_{6:0}$ and ACX $C_{12:0}$ (Figure 2A). After stage 3, the levels of MS and ICL activity decreased abruptly to undetectable levels by stage 4 (Figure 2B). Decreased but significant levels of β-oxidation and *PEPck* gene transcripts persisted in photosynthetic seedlings up to stage 8, while the *RbcS* gene reached maximal levels by stage 4 and maintained these high levels up to stage 8. For all the genes involved in lipid mobilization, the pattern of activity mirrored that of gene expression with a 12–24 h time lag under the growth conditions used. Furthermore, the relative abundances of the mRNA transcripts matched with the relative levels of the enzyme activities. Of the β-oxidation genes, thiolase exhibited the highest level of both mRNA transcript and enzyme activity, whereas the *ACX1*, *ACX2* and *ACX4* genes all showed both lower mRNA transcript levels and enzyme activities.

Discussion

Comparing mRNA with enzyme levels

Although delayed by 12–24 h, the temporal expression pattern of the β-oxidation, glyoxylate cycle and gluconeogenic genes is reflected in the pattern of enzyme activity. The profile of mRNA expression seen here also correlates with the period of lipid mobilization during germination and post-germinative growth of *Arabidopsis* [6,22]. Studies in germinating cotton [15] and cucumber [14,23] show similar correlations between gene expression and enzyme activity for the glyoxylate cycle genes *ICL* and *MS* and concluded that the rapid increase in enzyme activity is regulated primarily at the level of transcription. The correlation between level of mRNA transcript and enzyme activity for all these lipid-mobilizing genes is further evidence that they are regulated at the level of transcription.

Existing evidence for transcriptional control

Reporter gene studies with cucumber *MS*-promoter–β-glucuronidase (GUS) fusions and *ICL*-promoter–GUS fusions in transgenic tobacco show temporal patterns of expression during seed germination and seedling establishment similar to the *ICL* and *MS* mRNA profiles in cucumber [24,25]. This work provides convincing evidence that the expression of these genes during oilseed germination is controlled at the level of transcription. More recently the isolation of an *ACX3*-promoter-trapped line that contained a fusion of the endogenous *ACX3* promoter to the GUS reporter gene has demonstrated that the expression of this gene during germination is also controlled at the level of transcription [6]. In addition to the reporter gene studies on *MS*, *ICL* and *ACX3*, the *MFP2* gene exhibits a strong correlation between mRNA expression and enzyme activity [8]. Combining these findings with the results presented here, it seems likely that all the lipid mobilization genes in the β-oxidation, glyoxylate and gluconeogenesis pathways are regulated at the level of transcription.

Role of the glyoxylate cycle

Both the mRNA levels and enzyme activities of the glyoxylate cycle genes *MS* and *ICL* are undetectable by stage 4, whereas the β-oxidation and gluconeogenesis genes are maintained at low levels after stage 4. This indicates that β-oxidation and gluconeogensis have additional roles in young seedlings, whereas involvement in storage lipid mobilization is the sole role of the glyoxylate cycle during this period of development.

Levels of thiolase and ACX activity

The thiolase activity reported here is consistent with previous studies for castor bean endosperm [26,27]. The ACX activities shown here are similar to those previously reported in *Arabidopsis* [4]. The low $C_{16:0}$ ACX activity relative to the medium-chain and short-chain ACX and thiolase activities suggest that $C_{16:0}$ ACX might be the limiting step in fatty acid β-oxidation during germination and post-germinative growth in *Arabidopsis*.

In conclusion, during storage lipid breakdown in *Arabidopsis* oilseeds, gene expression and enzyme activity are tightly co-ordinated both within and between the major pathways of β-oxidation, the glyoxylate cycle and gluconeogenesis. Existing evidence suggests that this regulation is mainly transcriptional and might occur by a global regulatory mechanism.

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Structure–function relationships of the liver and muscle isoforms of carnitine palmitoyltransferase I

V. A. Zammit[1], N. T. Price, F. Fraser and V. N. Jackson
Cell Biochemistry, Hannah Research Institute, Ayr KA6 5HL, Scotland, U.K.

Abstract

Elucidation of the membrane topology of carnitine palmitoyltransferase (CPT) I showed that the extreme N-terminus is involved in determining the sensitivity of the liver (L) isoform to malonyl-CoA and suggested that interaction between the two cytosolic segments of the CPT I molecule determines the kinetic characteristics of the enzyme. Work with chimaeric liver/muscle-isoform (L/M) proteins constructed from all six possible combinations of three domains [N-terminus plus transmembrane domain 1 (TM1), loop plus TM2 and C-domain] expressed in *Pichia pastoris* showed that the precise N–C and TM1–TM2 pairings determine the overall kinetic parameters of the protein. Discrete short sequences within the respective N-terminal regions have negative or positive effects on malonyl-CoA sensitivity (L-isoform) or the K_m for carnitine (M-isoform) in the full-length proteins, thus imparting to them their distinctive kinetic characteristics. Interactions within N-terminal domains also seem to be important in the targeting of the protein to microsomes in the *P. pastoris* expression system.

Key words: carnitine palmitoyltransferase, chimaeric proteins, malonyl-CoA, mitochondria, topology.

Abbreviations used: ACBP, acyl-CoA-binding protein; CPT, carnitine palmitoyltransferase; etomoxir, 2-[6-(4-chlorophenoxy) hexyl]oxirane carboxylic acid; L, liver; M, muscle; TM, transmembrane.

[1]To whom correspondence should be addressed (e-mail zammit@main.hri.sari.ac.uk).

Introduction

The study of the structure–function relationships of carnitine palmitoyltransferase (CPT) I received three important boosts in the past decade through (1) the cloning and sequencing of the cDNA coding for the liver (L)- and muscle (M)-isoforms [1,2] initially for the rat, and subsequently for other species [3], (2) the demonstration that the mature protein retained intact the N-terminus of