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Storage Reserve Mobilisation and Seedling Establishment in Arabidopsis

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1.1 INTRODUCTION

During seed development large quantities of carbon, nitrogen and minor nutrients are stored from the mother plant. These will fuel post-germinative seedling establishment until chloroplast and root development are complete and photosynthesis can begin. Around 70% of our food comes from seeds, and much of the rest comes from animals fed on seeds, so seed storage reserves are of central importance to human existence (Bewley and Black, 1994).

Carbon storage in the form of triacylglycerol (TAG) is a ubiquitous feature of seed plants, even in cereals that store the majority of their carbon as starch. During Arabidopsis seed development starch accumulates transiently but is eventually converted to TAG, which is stored in organelles known as liposomes, or oil bodies. Cells of the embryo and endosperm are packed full of oil bodies (Figure 1), which comprise up to 45% of the dry weight of the mature Arabidopsis seed (O'Neill et al., 2003). TAG accumulation depends on the action of genes that promote embryo identity and seed dormancy such as *LEAFY COTYLEDON1, FUSCA3* and *ABSCISIC ACID INSENSITIVE 3,* and requires the activity of the Apetala2 transcription factor *WRINKLED1*, which regulates carbon flow through glycolysis in the developing seed (Focks and Benning, 1998; Cernac and Benning, 2004). The close relationship of Arabidopsis to major and emerging oilseed crops makes the study of Arabidopsis fatty acid metabolism especially relevant, and the current state of the art knowledge gained from Arabidopsis underpins modern attempts to engineer plants to produce neutraceutical polyunsaturated fatty acids, to improve oil crops for biodiesel production, and for the provision of oil to replace dwindling and increasingly expensive petrochemical supplies (Thelen and Ohlrogge, 2002; see www.oilcrop.com).

This chapter will describe the pathways required for the breakdown and mobilisation of seed oil in Arabidopsis. This requires the hydrolysis of TAG by lipases and subsequent ß-oxidation of the resultant fatty acids in the peroxisome. This produces acetyl-CoA, which is converted to citrate and then either used for respiration, or through the glyoxylate cycle and gluconeogenesis is converted into soluble sugars to support metabolism and growth (Figure 2). The activity of these pathways is tightly coordinated with the control of seed dormancy and germination. However, in Arabidopsis it has been shown that, in the final stages of seed maturation, seed oil content actually falls, indicating that reserve mobilisation begins prior to germination (Baud et al., 2002). This correlates with the appearance of transcripts for key genes in ß-oxidation and the glyoxylate cycle (Schmid et al., 2005).

2.1 LIPOLYSIS

Lipolysis is the first step of lipid reserve mobilisation, yet at present it remains the most enigmatic. Various lipases with activity against TAG have been purified from plants, including oilseed rape, maize, and castor bean, but no genetic evidence has been presented that any are required for TAG hydrolysis in germinating seeds. The well known acid lipase from castor bean endosperm has recently been cloned (Eastmond, 2004b). This lipase is anchored to oil bodies by a long hydrophobic stretch of amino acids and is highly active against the TAG triolein *in vitro*. Yet the acid pH optimum and peak of expression during seed develop-

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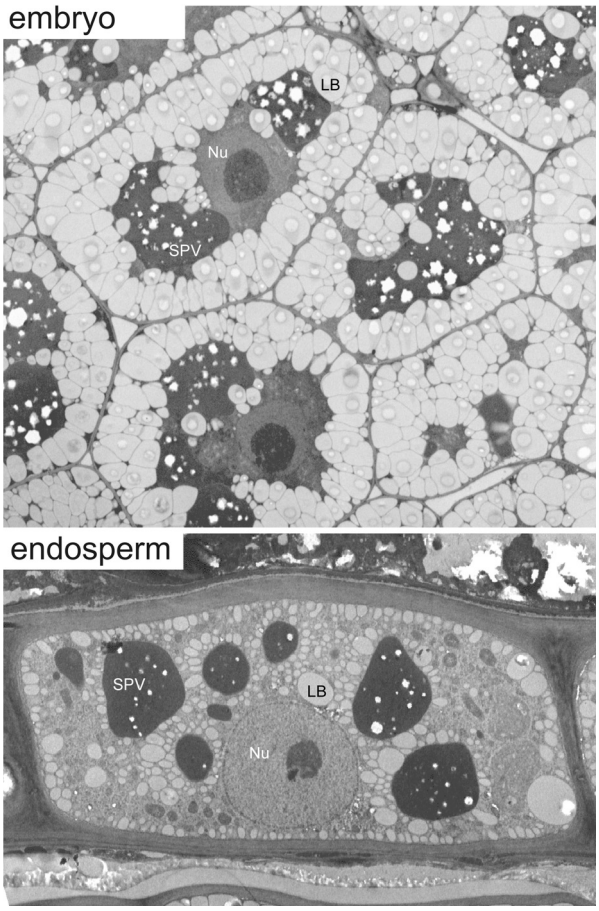


Figure 1. Transmission electron micrographs of imbibed Arabidopsis seeds showing embryo and endosperm cells packed with lipid or oil bodies. Abbreviations: LB, lipid bodies; Nu, nucleus; SPV, storage protein vacuole.

ment make this an unlikely candidate for the lipase required for post-germinative reserve mobilisation. Recently a candidate for Arabidopsis TAG lipase was identified by El-Kouhen et al., (2005). This protein shares weak homology with mammalian gastric lipases and was expressed during seed germination. However, the recombinant protein only showed high activity towards short chain TAGs not found in Arabidopsis seed storage reserves, and an insertion mutant still retained wild type TAG lipase activity and could break down TAG at the normal rate.

A more likely candidate for Arabidopsis TAG lipase is the gene encoded by the *sugar dependent 1* (*sdp1*) locus (Eastmond, 2006). The *sdp1* mutant cannot break down seed storage lipid and shows poor hypocotyl elongation in the dark that can be promoted to wild type levels by applying an alternative carbon source such as sucrose. As we shall see, this phenotype is diagnostic for an impairment in storage reserve mobilisation (Eastmond et al., 2000b; Cornah et al., 2004; Penfield et al., 2004). The gene corresponding to this mutation has been cloned and the gene product possesses a patatin-like serine esterase domain also found in mammalian adipose tissue lipase and yeast TAG lipase (Zimmerman et al., 2004). SDP1 is active against long chain TAGs *in vitro*, and GFP fusions show that it is associated with oil bodies during germination. Hence *SDP1* is the only candidate for plant TAG lipase for which convincing genetic evidence for function has been presented. SDP1 has only low activity against diacylglycerol and no activity against monoacylglycerol *in vitro* suggesting that further genes encoding these lipases are yet to be identified. The Arabidopsis genome contains one gene (At3g57140) closely related to SDP1, the corresponding protein showing 74% identity at the amino acid level. Expression of At3g57140 is detected in pollen and mature seeds according to Genevestigator. TAG oil bodies are also known to be associated with pollen and a novel group of oil-body associated proteins, termed oleosins have been shown to be present inside the pollen of Arabidopsis (Wu et al., 1997; Kim et al., 2002). It is thus tempting to speculate that this SDP1 homologue plays a role in hydrolyzing oil body TAGs in pollen.

2.2 FATTY ACID ENTRY INTO THE PEROXISOME

Fatty acids released from TAG by lipolysis must be transported into the peroxisome in order to proceed through ß-oxidation. The same goes for essential cofactors required such as Coenzyme-A (CoA). Hence transporters must exist that facilitate the transfer of these elements into the peroxisome during germination and early seedling establishment. One of these is encoded by the locus known variously as *PED3, PXA1* and *CTS* (Hayashi et al., 2002; Zolman et al., 2001a; Footitt et al., 2002). This locus encodes a plant homologue of the human adrenoleukodystrophy (ALD) gene, made famous by the film “Lorenzo's Oil.” Arabidopsis mutants in which this homologue is disrupted were shown to exhibit a block in fatty acid breakdown, suggesting that this gene might be important for the transport of carbon derived from TAG into peroxisomes (Zolman et al., 2001a; Hayashi et al., 2002; Footitt et al., 2002). *PED3/PXA/CTS* (hereafter referred to as *CTS*) is co-localized with both catalase and enzymes involved in peroxisomal ß-oxidation showing that CTS is a peroxisomal protein (Footitt et al., 2002). The *cts* mutants are also resistant to 2,4-dichloro-phenoxy-butyric acid (2,4-DB), demonstrating a general block in fatty acid ß-oxidation.

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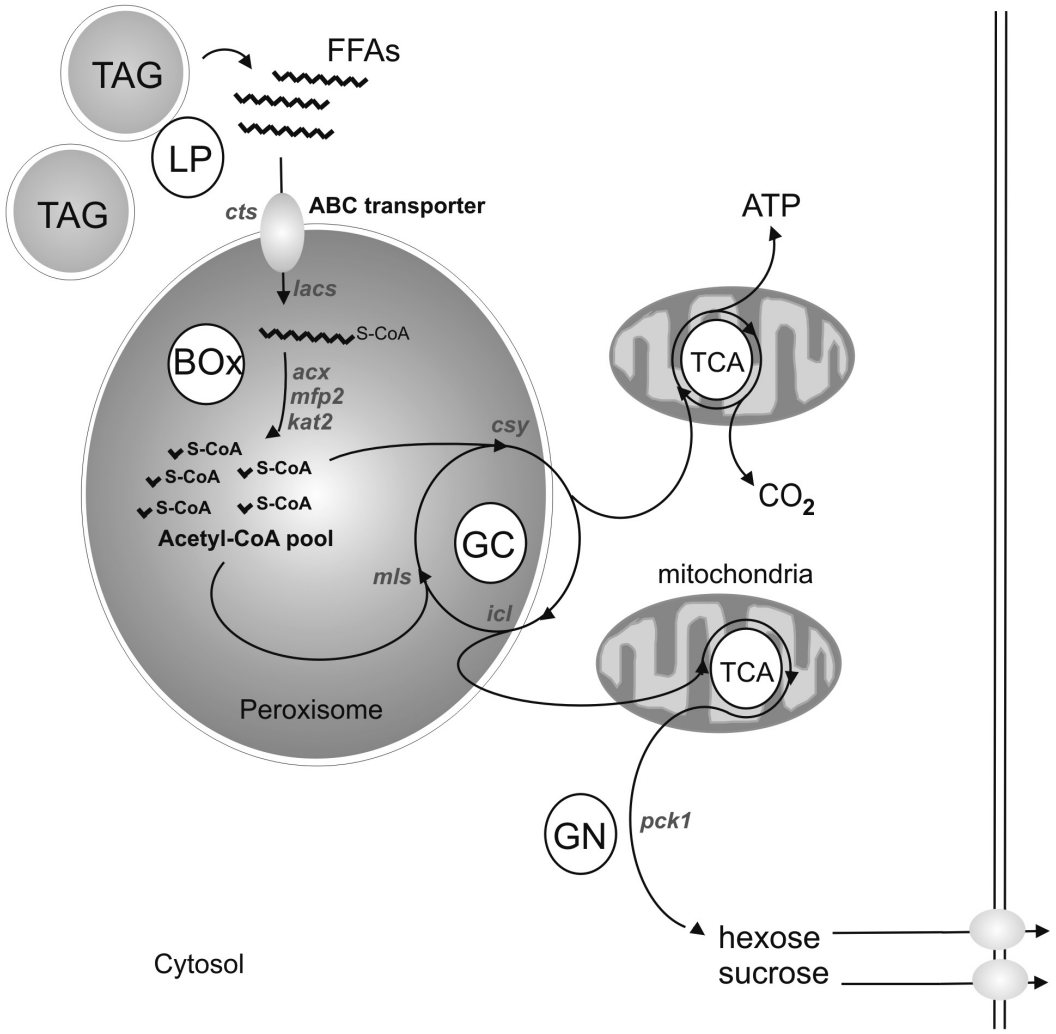


Figure 2. An overview of the major metabolic pathways required for lipid reserve mobilization in germinating Arabidopsis seeds.

The human and yeast homologues of ALD have been shown to transport acyl-CoA esters from the cytosol into the peroxisome (Mosser et al., 1993; Hettema et al., 1996; Verleur et al., 1997). Hence, simply by homology, CTS appears most likely to be an acyl-CoA transporter (but see section 2.3 for a detailed discussion on this issue). Supporting this hypothesis *cts* mutants accumulate high levels of long chain acyl-CoA esters, which also indicates either a defect in acyl-CoA transport or catabolism. These major metabolic defects cause the complete failure of post-germinative seedling establishment, a phenotype that is rescued by the addition of an exogenous carbon supply into the growth medium. Furthermore, *cts* mutants also display a germination defect (see section 2.7).

2.3 FATTY ACID ACTIVATION FOR ß-OXIDATION

Before the fatty acids derived from TAG can proceed through ß-oxidation they must be esterified to CoA. This reaction is catalysed by long chain acyl-CoA synthetases

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(LACS; *EC 6.2.1.3*; Shockey et al., 2002). The reaction occurs in two steps: firstly fatty acids are converted to the adenylate acyl-AMP using ATP; the carbonyl carbon of the adenylate then reacts with the thiol group of CoA yielding acyl-CoA and releasing AMP (Groot et al., 1976). Interestingly, the two LACS required for post-germinative fatty acid breakdown are localised in the peroxisome (Fulda et al., 2002). The important role of two LACS isoforms, LACS6 and LACS7 in fatty acid breakdown was demonstrated by Fulda et al., (2004). The *lacs6 lacs7* double mutant is defective in seedling establishment and cannot break down storage lipid. They are not 2,4-DB resistant, presumably because the short chain 2,4DB substrate is acted on by a third LACS isoform. However, *lacs6 lacs7* cannot break down fatty acids stored in TAG. The phenotype of these mutants strongly argues in favour of fatty acid activation inside the peroxisome.

So why would a peroxisomal CoA synthetase activity be required if acyl-CoA esters are being transported into the peroxisome? Clearly, a thorough characterisation of the substrate specificity of the CTS transporter is required to settle this issue. However, several alternative models can be envisaged (Figure 3). First, the substrate of CTS may be free fatty acids, rather than acyl-CoA esters. In this scenario the free fatty acids are then activated by LACS in the peroxisomal lumen. In a second model, the transport of acyl-CoA esters results in their cleavage and the peroxisomal LACS activity is required to reactivate them. Third, an alternative route of entry for free fatty acids into the peroxisome may operate in addition to the transport of acyl-CoA esters by CTS. These free fatty acids would then require activation by the LACS in the peroxisomal lumen. The final model is conceptually similar to the last, except that CTS is simply a CoA transporter, and again, the fatty acids enter the peroxisome by an alternative route. There is ample precedent for this: the yeast homologue of ALDP can transport free CoA (Hettema et al., 1996).

2.4 PEROXISOMAL ß-OXIDATION

All three families of ß-oxidation genes are up-regulated coordinately at the level of transcription during Arabidopsis seed germination and early postgerminative growth (Rylott et al., 2001). This correlates with the period of most rapid fatty acid degradation. Put simply, fatty acid ß-oxidation converts acyl-CoA esters into multiple mole-

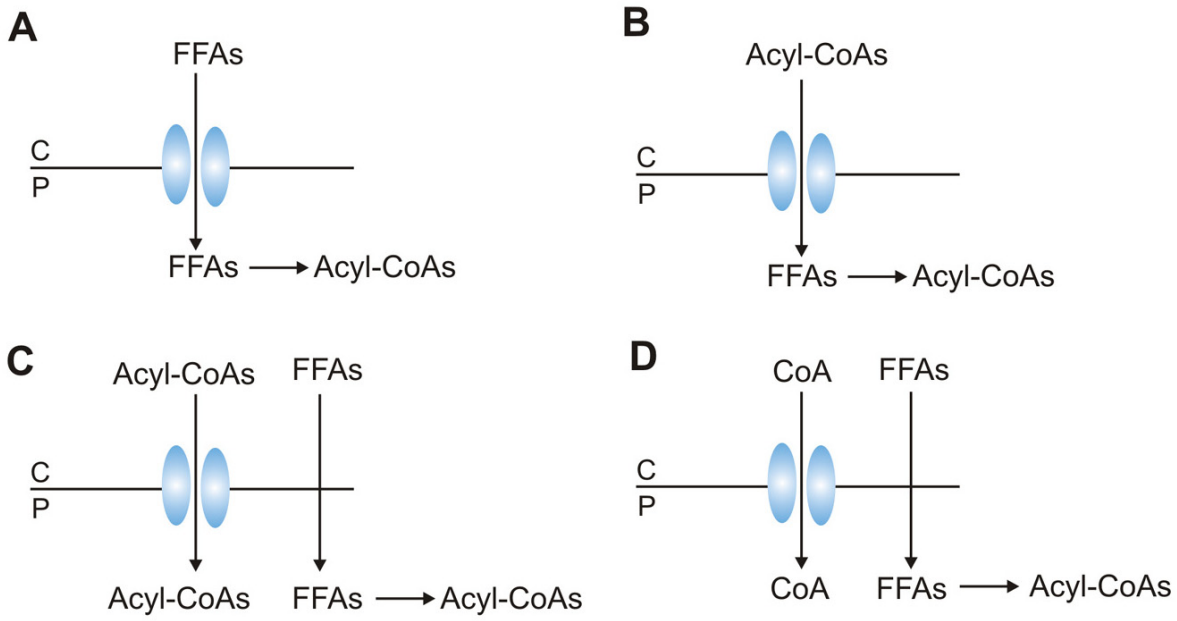


Figure 3. Alternative models to explain the requirement for both the CTS putative acyl-CoA transporter and peroxisomally localized acyl-CoA synthetases for lipid breakdown. A. CTS can transport free fatty acids. B. CTS transports CoA esters, but transport results in cleavage therefore necessitating reactivation. C. CTS transports acyl-CoAs, but free fatty acids enter through a second route. The CoA is then recycled by ß-oxidation and used to activate these fatty acids. D. CTS is simply a free CoA transporter and fatty acids enter through an alternative route. C, cytosol; P, peroxisome.

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cules of the two carbon unit acetyl-CoA (Figure 4). For instance one molecule of stearic acid (C18:0) derived stearoyl-CoA will be oxidized to 9 molecules of acetyl-CoA. The acetyl-CoA produced by ß-oxidation can be converted to carbohydrate via the glyoxylate cycle (see section 3.1), or can be used as a respiratory substrate to release energy for the developing seedling. Peroxisomal ß-oxidation has been the subject of both forward and reverse genetic studies in Arabidopsis, and the role of each step in the pathway has been characterized in detail. Forward genetic studies have taken advantage of the ability of ß-oxidation to metabolise two non-toxic compounds, indolebutyric acid (IBA) and 2,4-dichlorophenoxybutryric acid (2,4DB) into the toxic compounds indoleacetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4D). IBA is an endogenous plant auxin present at low levels in plants including Arabidopsis (Ludwig-Müller et al., 1993). Analysis of multiple mutants shows that ß-oxidation is essential both for Arabidopsis embryo development, seedling establishment, and storage TAG breakdown. We shall see that ß-oxidation also has a role in the control of seed dormancy, senescence and the biosynthesis of plant hormones.

2.4.1 Acyl-CoA Oxidases Catalyse the First Step in Fatty Acid ß-Oxidation

Acyl-CoA oxidases (ACXs) catalyse the first step of the peroxisomal ß-oxidation sequence where acyl-CoA is oxidized to Δ^2-*trans*-enoyl-CoA. The reaction requires FAD as a co-factor generating $FADH_2$ which is then oxidized by flavoprotein dehydrogenase producing hydrogen peroxide. This is a damaging oxidant, which is immediately converted to H_2O and O_2 by dedicated antioxidant systems (see section 2.5). Studies have shown that plants contain a family of *ACX* isoenzymes with distinct but slightly overlapping acyl-CoA chain length specificities (Hooks et al., 1999). Four Arabidopsis *ACX* isogenes have been functionally characterized by heterologous expression and these encode long chain (*ACX2*), medium long chain (*ACX1*), medium (*ACX3*) and short (*ACX4*) chain acyl-CoA oxidase activities (Hooks et al., 1999; De Bellis et al., 2000; Froman et al., 2000; Eastmond et al., 2000a; Hayashi et al., 1999). In addition two other *ACX* genes have been identified in the *Arabidopsis* genome (Graham and Eastmond, 2002). *ACX5* and *ACX6* are most closely related to *ACX1* and *ACX3,* respectively.

Mutants disrupted in all six *ACX* genes have been described (Eastmond et al., 2000a; Rylott et al., 2003a; Adham et al., 2005; Pinfield-Wells et al., 2005). Of these the *acx1-2, acx3-1* and *acx4-1* mutants show weak resistance of root growth on IBA, and *acx3-1* and *acx4-1* show resistance to 2,4DB, suggesting a defect in ß-oxidation (Eastmond et al., 2000a; Rylott et al., 2003a). The 2,4DB resistance of *acx3-1* and *acx4-1* is a reflection of their short chain length specificity. The *acx5-1* mutant has been shown to enhance the IBA resistant phenotype of *acx1-2* (Adham et al., 2005). Lipid breakdown and seedling establishment are unaffected in the single mutants, due to the overlapping substrate specificities of the gene products. The *acx3-1 acx4-1* double mutants abort during the first

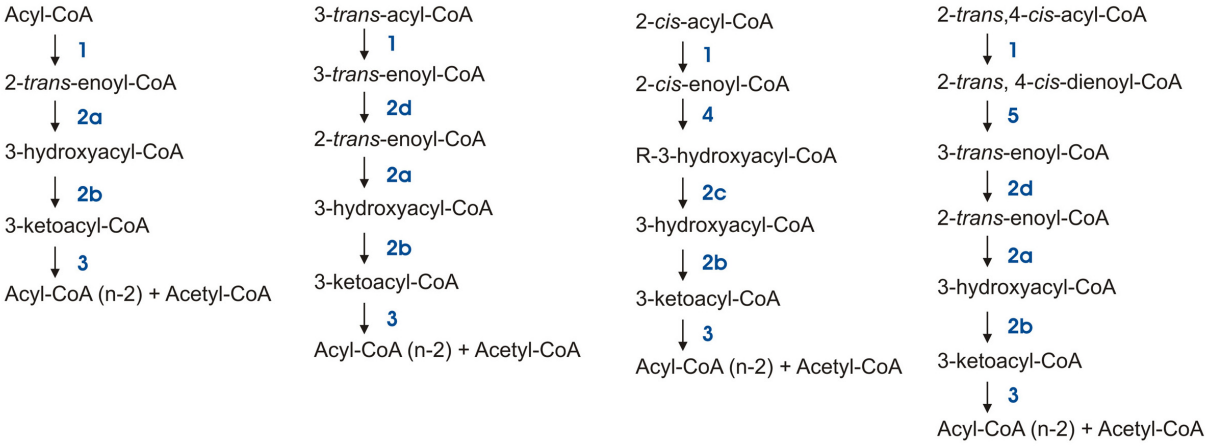


Figure 4. Pathways for the catabolism of saturated and unsaturated acyl-CoAs by ß-oxidation showing the activities required for each step. 1. acyl-CoA oxidase. 2 Multifunctional protein: a. 2-*trans*-enoyl-CoA hydratase. b. L-3-hydroxyacyl-CoA dehydrogenase. c. D-3-hydroxyacyl-CoA epimerase. d. Δ^3,Δ^2-enoyl-CoA isomerase. 3. 3-ketoacyl-CoA thiolase. 4. 2-enoyl-CoA hydratase. 5. 2,4-dienoyl-CoA reductase.

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phase of embryo development due to a complete block in short-chain acyl-CoA oxidase activity. It is proposed that the accumulation of short chain acyl-CoAs results in toxicity to the developing embryo, or that this activity is essential for embryo development (Rylott et al., 2003a). The *acx1 acx2* double mutant is unable to break down seed storage lipid and is compromised in seedling establishment, but this phenotype is not as severe as in the *cts* mutants. The *acx1 acx2* double mutant also accumulates acyl-CoA esters and is strongly resistant to IBA, indicating a block in ß-oxidation.

2.4.2 The Multifunctional Protein and the ß-oxidation of Unsaturated Fatty Acids

The Multifunctional protein (MFP) is an unusual protein in that it contains the domains for four distinct catalytic activities: 2-*trans* enoyl-CoA hydratase (*EC 4.2.1.17*), L-3-hydroxyacyl-CoA dehydrogenase (*EC 1.1.1.35*), D-3-hydroxyacyl-CoA epimerase (*EC 5.1.2.3*) and Δ^3,Δ^2-enoyl-CoA isomerase (*EC 5.3.3.8*; Behrends et al., 1988; Guehnemann-Schaefer and Kindl, 1995). MFP has a complex role in ß-oxidation, using different combinations of activities for the ß-oxidation of saturated and unsaturated fatty acids. Saturated fatty acids and those containing a double bond at the Δ^2 position in the *trans* configuration are acted on by the 2-*trans* enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities. These are often referred to as the 'core' activities of the MFP, catalyzing the hydration of 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA and the subsequent oxidation of 3-hydroxy acyl-CoA (Frevert and Kindl, 1980). The dehydrogenase step requires NAD^+ and generates NADH, so a system is needed within the peroxisome to regenerate NAD^+ (discussed in section 2.5).

Core ß-oxidation functions through a 2-*trans*-enoyl-CoA intermediate. Yet many Arabidopsis fatty acids have unsaturated double bonds either on an odd-numbered carbon or in the *cis*-configuration, resulting in the production of enoyl-CoA intermediates that cannot be directly catabolised via the core activities. If a fatty acid has a *cis* double bond at an odd numbered carbon the 3-*cis*-enoyl-CoA generated by core ß-oxidation must be converted to the *trans* configuration before the core activities of the MFP can get to work. This requires a third MFP activity, Δ^3,Δ^2-enoyl-CoA isomerase. However, Arabidopsis seeds contain large quantities of unsaturated fatty acids, particularly linolenic and linoleic acid, both of which contain double bonds in the *cis* configuration at even carbons. These require two additional activities of the multifunctional protein, D-3-hydroxyacyl-CoA epimerase and the Δ^3,Δ^2-enoyl-CoA isomerase activity, before the fatty acid can proceed through core ß-oxidation (Graham and Eastmond, 2002).

The two Arabidopsis isoforms of the multifunctional protein have been characterised genetically. The first is encoded by the *ABNORMAL INFLORESCENCE MERISTEM 1* (*AIM1*) locus (Richmond and Bleeker, 1999). Mutants in this gene display normal seedling establishment, but subsequently show highly stunted vegetative growth with the production of abnormal leaves and inflorescences. The *aim1* mutant is largely infertile. AIM1 is clearly the predominant MFP isoform active during post-germinative growth. In contrast, mutants in *MFP2* show a sugar dependent short hypocotyl phenotype in the dark, accumulate acyl-CoAs during seedling establishment, and break down TAG only slowly (Rylott et al., 2006). Although the *mfp2* mutant phenotype is weaker than other ß-oxidation mutants it is probable that the activity of AIM1 compensates somewhat for the loss of MFP2 during germination and seedling establishment. *aim1 mfp2* double mutants are embryo lethal (Rylott et al., 2006).

Recently, an Arabidopsis isoform of $\Delta^{(3,5)}$, $\Delta^{(2,4)}$-dienoyl-CoA isomerase has been identified (Goepfert et al., 2005). This enzyme is highly expressed in young seedlings and is therefore likely to have a role in the catabolism of polyunsaturated fatty acids during seed germination and seedling establishment, although this hypothesis requires genetic confirmation. Arabidopsis also contains various candidates for further enzymes that might play a role in the catabolism of unsaturated fatty acids, such as putative enoyl-CoA hydratases (Graham and Eastmond, 2002). However, these remain functionally uncharacterized.

2.4.3 3-L-ketoacyl-CoA thiolase 2 is the Predominant Thiolase Isoform in Germinating Seeds

3-L-ketoacyl-CoA thiolase (KAT) catalyses the last step of fatty acid ß-oxidation involving thiolytic cleavage of 3-ketoacyl-CoA to acyl-CoA (C_{n-2}) and acetyl-CoA (C_2). The acyl-CoA (C_{n-2}) then re-enters the ß-oxidation pool again as many times as necessary. There are four putative thiolase genes in Arabidopsis, each of which contain putative peroxisomal targeting sequences (Hooks, 2002). Using a 2,4-DB screen, Hayashi et al. (1998) isolated a thiolase deficient mutant called *peroxisome defective 1* (*ped1*), The *ped1* mutant is deficient in the KAT2 isoform of thiolase and exhibits defective seedling establishment that can be rescued by sugar provision, and 2,4DB resistant root growth, indicating a block in ß-oxidation. Subsequently, analysis of a T-DNA insertion allele, *kat2-1*, showed increased accumulation of acyl-CoA esters compared to wild type, as has been observed in both *cts* and *acx1 acx2*

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mutants (Germain et al., 2001). This mutant is also unable to break down TAG. The KAT2 protein accounts for all the thiolase activity in germinating seeds.

2.4.4 Peroxisomal Citrate Synthase (pCYS) is Required for ß-oxidation

Although traditionally viewed as a glyoxylate cycle enzyme, pCYS has recently been shown to be required for ß-oxidation (Pracharoenwattana et al., 2005). There are three putative pCYS isoforms in Arabidopsis, but one, *CYS1*, is expressed only during seed development. Consequently the *csy2 csy3* double mutant has revealed the role of this enzyme during seed germination and vegetative growth. This mutant strongly resembles previously described ß-oxidation mutants. TAG breakdown is blocked and seedling establishment is compromised without an alternative carbon source. The mutant seedlings are also 2,4DB resistant. These phenotypes are not shared with other glyoxylate cycle mutants (Eastmond et al., 2000b; Cornah et al., 2004). The *csy2 csy3* double mutant also exhibits a severe vegetative phenotype and cannot survive through to the reproductive stage. In this way it resembles the phenotype of *aim1* plants (Richmond and Bleeker, 1999). Pracharoenwattana et al., (2005) also show that pCSY is also required for lipid respiration, indicating that soluble carbon is supplied from the peroxisome to the mitochondria. They propose that the block in ß-oxidation is due to peroxisomes being unable to export acetyl CoA, which accumulates in the absence of CYS activity. Previously, the existence of an acyl-carnitine-like shuttle was postulated, based on the phenotype of a putative acyl-carnitine carrier protein mutant, which produced seeds that could germinate and break down storage lipid but could not progress to autotrophic growth (Lawand et al., 2002). This developmental block could be overcome by provision of exogenous sugar or transfer of seedlings to the dark. The function of the gene affected by the *bou de soufflé* mutation, a lesion in a putative acyl-carnitine carrier protein that requires sugars for seedling establishment in the light (Lawand et al., 2002), is yet to be determined.

2.5 THE PEROXISOMAL SYSTEM FOR THE RECYCLING OF NAD^+ AND THE REMOVAL OF REACTIVE OXYGEN SPECIES

It is generally accepted that in mature peroxisomes no exchange of NAD^+ or NADH occurs with the cytosol or any other organelles (van Roermund et al., 1995). The action of the multifunctional protein requires NAD^+ and generates NADH, so a system must exist within the peroxisome for the oxidation of NADH to allow ß-oxidation to continue. Mettler and Beevers (1980) proposed that NAD^+ is regenerated within the peroxisome through the action of peroxisomal malate dehydrogenase (Figure 5). In this proposal peroxisomal malate dehydrogenase operates in the reverse direction from that expected for the glyoxylate cycle, generating NAD^+ and malate. The latter is exported to the cytosol or mitochondria for conversion to oxaloacetate, which is then transported back to the peroxisome to continue the glyoxylate cycle. The oxaloacetate is believed to be transported back and forth in the form of aspartate. This mechanism is strongly supported by genetic evidence in yeast, as mutants in peroxisomal malate dehydrogenase are blocked in ß-oxidation (van Roermund et al., 1999). Recent work shows that Arabidopsis mutants deficient in peroxisomal malate dehydrogenase also are deficient in ß-oxidation (see Baker et al., 2006), confirming Mettler and Beevers original model.

Although the role of the malate shuttle in the regeneration of NAD^+ is well established there are alternative proposed mechanisms which may operate in parallel. ß-oxidation also produces large amounts of potentially damaging hydrogen peroxide, which requires reduction. This can be achieved through the action of catalase, which is abundant in peroxisomes, and in Arabidopsis has been shown to be required for photorespiration in high light, but no evidence of an effect on fatty acid catabolism during seedling establishment was observed, suggesting that catalase could be less important than previously expected during storage reserve mobilisation (Vandenabeele et al., 2004). The importance of the peroxisome anti-oxidant system is neatly demonstrated by the tocopherol deficient *vitamin E deficient 2* (*vte2*) mutant of Arabidopsis (Sattler et al., 2004). The *vte2* mutant seedlings show a syndrome characteristic of a block in ß-oxidation, such as poor seedling establishment and a failure to break down stored lipid. The fatty acids in *vte2* mutants show evidence of peroxidation and other oxidative covalent modifications which prevent their passage through the ß-oxidation pathway.

A peroxisome membrane associated ascorbate peroxidase (APX) with a high affinity for H_2O_2 is also proposed to be part of the peroxide scavenging system (Lisenbee et al., 2003; Lisenbee et al., 2005). APX reduces ascorbate to monodihydroascorbate which can be reconverted to ascorbate by a second enzyme, monodihydroascorbate reductase, using NADH as a cofactor. Hence this system could also have the potential benefit of recycling NAD^+ for ß-oxidation, in conjuction with the malate shuttle. The importance of this system in germinating seeds has not been tested genetically, however.

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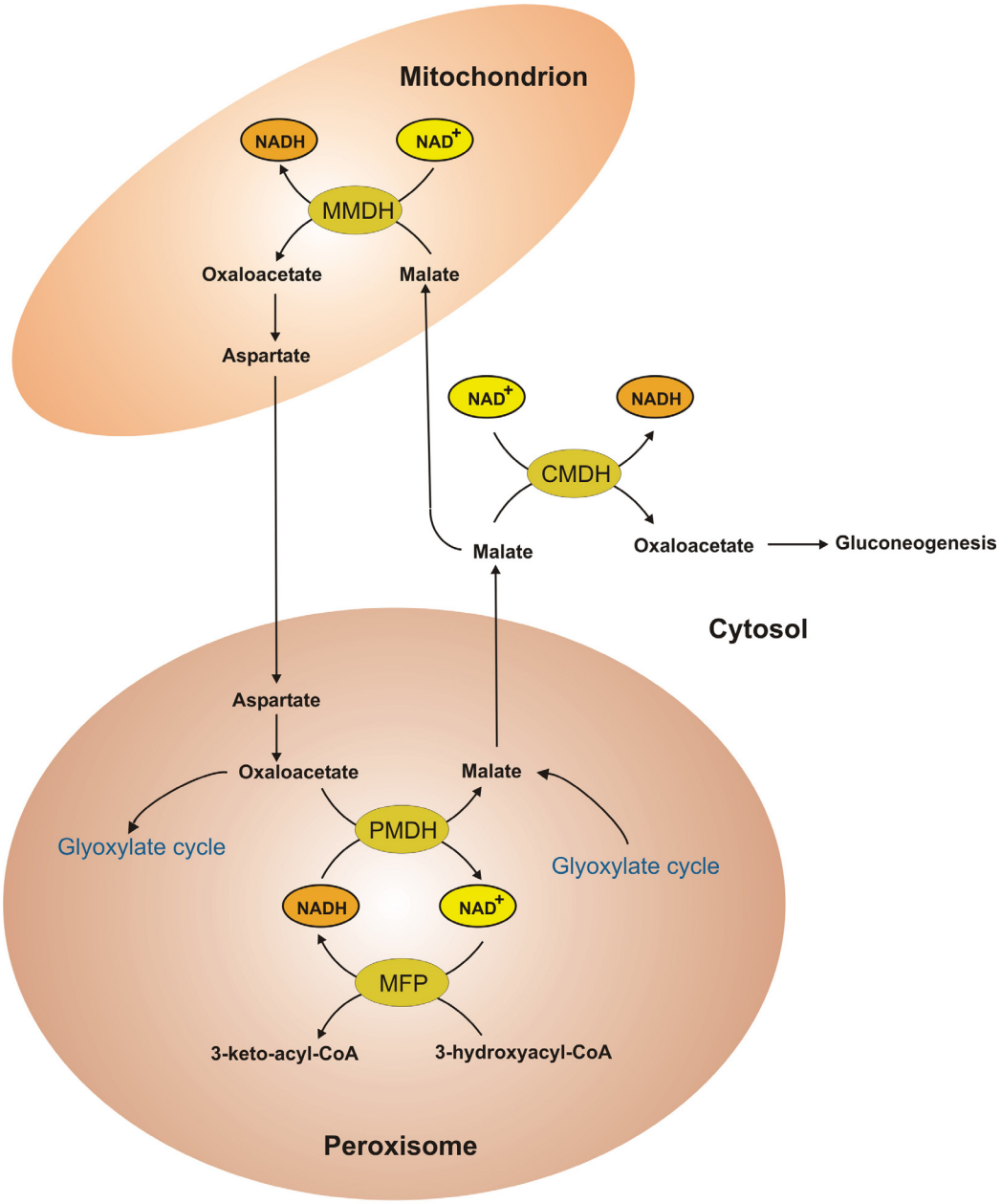


Figure 5. The malate aspartate shuttle proposed by Mettler and Beevers' for the recycling of NAD^+ by peroxisomal malate dehydrogenase. Reducing equivalents in the form of malate are exported from peroxisomes and converted to oxaloacetate (OAA) in either the cytosol or mitochondria. OAA in turn is converted to aspartate which re-enters peroxisomes where it is converted back to OAA and continues through the glyoxylate cycle. PMDH, peroxisomal malate dehydrogenase; CMDH, cytosolic malate dehydrogenase; MMDH, mitochondrial malate dehydrogenase.

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2.6 PEROXISOME MORPHOLOGY IS ALTERED IN RESERVE MOBILISATION MUTANTS

Blocking storage reserve mobilisation is known to modify peroxisome size in young seedlings. The *kat2-1* mutant, the *acx1 acx2* double mutant, and *mfp2* mutants all show peroxisomes that appear up to four times larger than the corresponding wild type and *kat2-1* peroxisomes also contain inclusion bodies of unknown composition Germain et al., 2001; Hayashi et al., 2001; Pinfield-Wells et al., 2005; Rylott et al., 2006). The same enlargement phenotype has been described for the *lacs6 lacs7* double mutants (Fulda et al., 2004), but interestingly *cts* peroxisomes are normal (Footitt et al., 2002). All of these mutants are defective in TAG breakdown and accumulate acyl-CoA esters, yet it is likely that *cts* mutants accumulate acyl-CoAs outside of the peroxisome. As the actual amounts of accumulated acyl-CoAs in these mutants are very low it is unlikely that it is the mere mechanical pressure or surfactant effects inside the peroxisomes that cause the enlargement to occur. This has led to the suggestion that acyl-CoA esters might have a positive role in the regulation of peroxisome size control in Arabidopsis (Graham et al., 2002b).

2.7 ß-OXIDATION AND THE REGULATION OF SEED DORMANCY

The *ped1/kat2* and *pxa1/ped3* mutants were originally described as having a postgerminative arrest phenotype that is rescued by an exogenous carbon source (Hayashi et al., 1998; Germain et al., 2001). This contrasts with that of the *cts* mutants, most of which fail to even germinate (Russell et al., 2000). This phenotype is not affected by the inclusion of sugars in the germination medium, suggesting that failure to germinate is not a result of carbon limitation to the embryo (Footitt et al., 2002). The germination of *cts* mutants is not rescued by seed after-ripening or cold stratification, but is moderately improved by the inclusion of nitrate in the media. Yet crosses to non-dormant mutants, such as *leafy-cotyledon 1* (*lec1*) and *abscisic acid insensitive 3* (*abi3*), rescue the non-germinating *cts* mutant phenotype. Hence it is hypothesised that CTS is required for the progression from dormant to non-dormant seed. If the *cts* seed coat is breached by nicking then the increased dormancy of *cts* mutant seeds is also overcome, showing that CTS regulates the exit from coat-imposed dormancy. Careful re-evaluation of the *kat2* phenotype has shown that *kat2* seeds exhibit a similar germination phenotype to *cts*, albeit slightly less severe (Pinfield-Wells et al., 2005). Furthermore, *acx1 acx2* double mutants lacking long chain acyl-CoA oxidase activity and the peroxisomal citrate synthase double mutants *csy2 csy3* also share the *cts* "forever dormant" phenotype, clearly implicating the entire ß-oxidation pathway in the regulation of seed dormancy and germination (Pinfield-Wells et al., 2005; Pracharoenwattana et al., 2005). However the *lacs6 lacs7* double mutants germinate normally, suggesting that the catabolism of long chain acyl-CoA esters is not required for dormancy exit (H. Pinfield-Wells and I. Graham, unpublished). Furthermore, mutants deficient in the glyoxylate cycle and gluconeogenesis germinate normally, so it appears that peroxisomal ß-oxidation is specifically implicated in dormancy regulation, but that the substrate is not the long chain fatty acids stored in TAG. The germination of ß-oxidation mutants is not rescued by applied gibberellins, suggesting that ß-oxidation may be required for the GA response in seeds.

2.8 ß-OXIDATION IS REQUIRED FOR JASMONATE SYNTHESIS

The biosynthesis of the oxylipin hormone jasmonic acid requires the action of peroxisomal ß-oxidation. Jasmonic acid synthesis begins with the peroxidation of linolenic acid in the chloroplast and the subsequent production of 12-oxo-phytodienoic acid (OPDA) which can be stored in the chloroplast membranes. This is transported to the peroxisomes where reduction and two rounds of ß-oxidation results in the production of jasmonate. The CTS transporter is required for the transport of jasmonate precursors into the peroxisome, although it is unclear whether free OPDA or the CoA ester is the substrate (Theodoulou et al., 2005). Of the Arabidopsis acyl-CoA oxidases, *ACX1* is transcriptionally induced by jasmonate and this induction requires the jasmonate response gene *COROTONINE INSENSITIVE 1* (*COI1*; Cruz Castillo et al., 2004). Induction of *ACX1* by wounding also requires *COI1*, suggesting that this is a mechanism through which jasmonate can feedback up-regulate its own biosynthesis. The *KAT2* gene is also transcriptionally induced by wounding, but not by jasmonate, suggesting that the up-regulation of KAT2 is a primary response to wounding. One further isoform of KAT, KAT5, appears to be systemically up-regulated by wounding (Cruz Castillo et al., 2004). Genetic evidence supports

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the major role of *ACX1* and *KAT2* in jasmonate biosynthesis. The *acx1* mutant accumulates only low levels of jasmonate after wounding, while antisense inhibition of KAT2 results in an 80% reduction in jasmonate levels in wounded tissue (Pinfield-Wells et al., 2005; Cruz Castillo et al., 2004). Recently, acyl-CoA synthetases have also been identified that have the ability to activate precursors of jasmonate for entry into ß-oxidation (Schneider et al., 2005).

2.9 ß-OXIDATION AND BRANCHED CHAIN AMINO ACID CATABOLISM

Leucine, isoleucine and valine are branched chain amino acids (BCAAs) requiring ß-oxidation for their catabolism. The sub-cellular location of BCAA catabolism has been a topic of debate, with evidence for the involvement of both mitochondria and peroxisomes (reviewed in Graham and Eastmond, 2002). BCAAs are first oxidatively transaminated to form alpha-keto acids that then undergo oxidative decarboxylation and esterification to produce acyl-CoA esters. There is now good evidence from studies in Arabidopsis of the mitochondrial localization of the first steps in BCAA catabolism, namely branched chain aminotransamination (Schuster and Binder, 2005), oxidative decarboxylation of the resulting alpha-keto acids (Taylor et al., 2004) and acyl-CoA dehydrogenation (Daschner et al., 2001). The acyl CoA dehydrogenase enzyme is equivalent to the peroxisomal acyl CoA oxidase enzyme, performing the first oxidative reaction on acyl-CoAs. However, rather than transferring electrons to molecular oxygen to form hydrogen peroxide, the dehydrogenase enzymes feed electrons in the electron transfer chain via an electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF-QO). A recent report of an Arabidopsis mutant disrupted in the mitochondrial ETF-QO showed that it accumulates intermediates in leucine catabolism, thus confirming its role in BCAA catabolism (Ishizaki et al., 2005). Remarkably, this mutant also accumulated phytanoyl-CoA — an intermediate in chlorophyll degradation, which indicates that at least the first steps in catabolism of phytanoyl CoA occur in the mitochondria. Further breakdown of this 20-carbon straight chain compound is expected to involve peroxisomal ß-oxidation, but little is known about transport between the organelles.

Biochemical studies from the Gerhardt laboratory on peroxisomes and mitochondria from mung bean hypocotyls previously demonstrated that the production of BCAA associated acyl-CoA esters occurred almost exclusively in peroxisomes (Gerbling and Gerhardt 1988; Gerbling and Gerhardt 1989). How does this fit with the mitochondrial location of the first steps in BCAA catabolism seen in Arabidopsis? It is possible that once branch chain acyl CoAs are produced they are transported to the peroxisome for subsequent degradation. Characterization of a 2,4 DB resistant Arabidopsis mutant, *chy1/dbr5*, disrupted in the peroxisomal ß-hydroxyisobutryl-CoA hydrolase enzyme of valine catabolism, does in fact indicate that the core ß-oxidation reactions in BCAA catabolism take place in peroxisomes (Zolman et al., 2001b; Lange et al., 2004). Disruption of valine catabolism in *chy1/dbr5* apparently results in accumulation of the toxic intermediate, methylacrylyl-CoA, which inhibits the 3-ketoacyl-CoA thiolase enzyme and thus indirectly blocks peroxisomal ß-oxidation (Zolman et al., 2001b; Lange et al., 2004). Details of the extent of peroxisomal involvement in BCAA catabolism and the associated transport of metabolites between the mitochondria and peroxisome remain to be resolved, but it is tempting to speculate that the CTS transporter or related proteins could play an important role, particularly since this transporter is active on IBA and 2,4 DB.

2.10 ß-OXIDATION, MEMBRANE LIPID TURNOVER, AND SENESCENCE

Peroxisomal ß-oxidation serves two important roles during leaf senescence; the synthesis of jasmonate and the catabolism of membrane fatty acids. The carbon from fatty acids is believed to be converted into soluble form and subsequently exported for use in other areas of the plant. A microarray analysis of dark induced senescence showed that multiple genes required for lipid catabolism are coordinately induced (Lin and Wu, 2004). However, unlike the situation in cucumber, where carbon starvation also induces the expression of the glyoxylate cycle genes *ICL* and *MLS* (Graham et al., 1992), no induction of the glyoxylate cycle is evident in Arabidopsis. Instead, high expression of the plastidial enzyme *PYRUVATE ORTHOPHOSPHATE DIKINASE* (*PPDK*) was noted, and it was postulated that this enzyme might be involved in an alternative carbon salvage pathway. Interestingly, PPDK is also highly expressed in germinating seeds, further supporting the putative gluconeogenenic role of this enzyme in Arabidopsis. Alternatively, the acetyl-CoA produced by fatty acid ß-oxidation could simply be respired. More recently it has been shown that the very high induction of ß-oxidation gene expression seen in dark induced senescence is only partially evident during normal leaf senescence (Buchanan-Wollaston et al., 2005), although this may simply reflect the rate at which the two processes occur. Recent work clearly shows that phytanoyl-CoA

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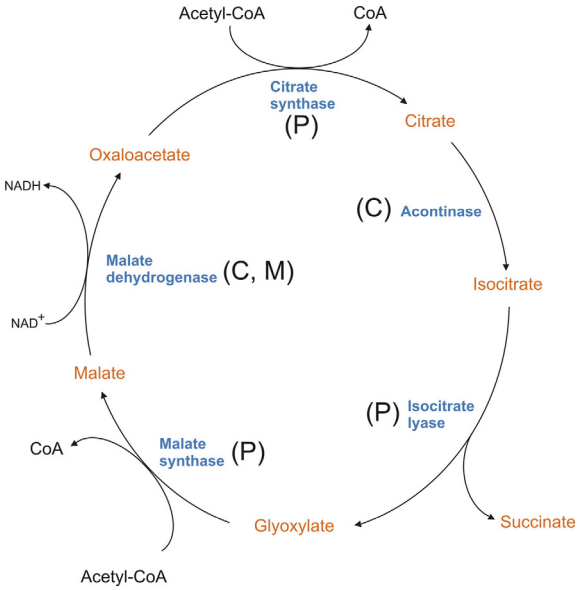


Figure 6. Schematic representation of the glyoxylate cycle, a modified TCA cycle for the assimilation of two carbon molecules. The glyoxylate cycle requires enzymes in the peroxisome (P) and cytosol (C), and possibly also the mitochondrion (M).

accumulates during dark induced senescence in Arabidopsis (Ishizaki et al., 2005). Phytanoyl-CoA is a breakdown product of chlorophyll, and the appearance of the CoA ester suggests that ß-oxidation is important in its catabolism. The precise role of fatty acid catabolism during senescence will require elucidation using genetic studies.

3.1 THE GLYOXYLATE CYCLE AND GLUCONEOGENESIS

The role of the glyoxylate cycle is to convert two carbon acetyl-CoA units produced by ß-oxidation into four carbon carbohydrates (**Figure 6).** These can then be converted into hexose by gluconeogenesis, and subsequently used for cell wall biosynthesis, or converted into sucrose for transport throughout the seedling. The existence of the glyoxylate cycle, which is absent in animals, was first demonstrated in plants in the castor bean endosperm shortly after germination (Kornberg and Beevers, 1957). The endosperm is the major storage tissue in castor beans, so full conversion of stored TAG to sucrose and transport to the embryo is obviously necessary for utilization. So what then in the case of Arabidopsis where the embryo is the predominant storage tissue, and the endosperm but a single cell layer separating the embryo from the seed coat in the mature seed? Surprisingly, we have shown that Arabidopsis too stores up to 10% of its TAG in the endosperm (Penfield et al., 2004). Furthermore, although the endosperm is not essential for seedling establishment, if it is removed hypocotyl elongation in the dark is severely reduced. As we shall see, this phenotype is characteristic of mutants affected in the glyoxylate cycle or gluconeogenesis, and is restored to wild type by the provision of sucrose (Penfield et al., 2004; Eastmond et al., 2000b). The utilisation of Arabidopsis endospermic reserves depends, as expected, on gluconeogenesis. These results suggest that the germinating embryo in the dark partitions its resources, as reserve mobilization in the cotyledons (where the majority of TAG is stored) cannot compensate for the loss of 10% of the seed's lipid from the endosperm. The glyoxylate cycle is partitioned between the peroxisome and the cytosol in plants, including Arabidopsis (Courtois-Verniquet and Douce, 1993; Hayashi et al., 1995).

3.1.2 Abolishing Isocitrate Lyase Activity Compromises Gluconeogenesis but not Lipid Respiration

Arabidopsis contains one gene for *ISOCITRATE LYASE* (*ICL*) and two insertion mutants have been described (Eastmond et al., 2000b). *icl* mutants cannot convert acetate into sugars, but can use acetate as a respiratory substrate. Although the seedling establishment of *icl* mutant seedlings is not compromised under optimal conditions, they do show defective hypocotyl elongation in the dark unless an exogenous carbon source is supplied. This is probably due in part to the failure of reserve mobilization from the endosperm. However, *icl* mutants do establish slowly, and as such would be expected to be at a severe disadvantage when growing in competition with other wild type seedlings. Hence, the glyoxylate cycle, viewed in the traditional sense, is not essential for Arabidopsis seed germination or early seedling growth.

3.1.3 *malate synthase* Mutants are Capable of a Reduced Rate of Gluconeogenesis

Alongside ICL, MALATE SYNTHASE (MLS) is the only other enzyme unique to the glyoxylate cycle. Arabidopsis

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contains only one gene for MLS, and mutants in this gene resemble *icl* mutants in that they display a short hypocotyl phenotype in the dark that can be restored to wild type by the provision of sugars (Cornah et al., 2004). Interestingly, acetate feeding experiments clearly demonstrate that *mls* mutants are capable of some gluconeogenesis, even though the traditional proposed route of carbon flux is blocked. This is reflected in the observation that the hypocotyl phenotype of *mls* mutants is marginally less severe than that of *icl*. Cornah et al., (2004) suggest that in the absence of MLS the glyoxylate produced by ICL can enter gluconeogenesis by hijacking enzyme activities from the photorespiratory pathway, and perhaps also PPDK.

3.1.4 Phospho*enol*pyruvate carboxykinase 1 (PCK1) is Required for Gluconeogenesis

Arabidopsis contains two isoforms of PCK. However, mutant and antisense experiments have shown that only PCK1 is important during seedling establishment (Rylott et al., 2003b; Penfield et al., 2004). *pck1* mutants are deficient in PCK activity and show a short hypocotyl in the dark phenotype that is restored to wild type by the inclusion of sugars in the medium. Otherwise PCK1 mutants show no obvious vegetative phenotype. However, PCK may also be required for carbon concentration in the chloroplasts of the Arabidopsis vascular bundle, as has been described in C4 plants (Hibberd and Quick, 2002).

3.2 GLYCEROL UTILISATION FROM TAG

TAG contains a molar ratio of 3:1 fatty acids to glycerol, and hence glycerol comprises a small but significant carbon reserve in the seed. As lipases release fatty acids from the glycerol backbone a large quantity of free glycerol is released. High concentrations of glycerol are known to inhibit plant growth: glycerol is converted to glycerol-3-phosphate, a competitive inhibitor of the glycolytic enzyme glucose-6-phosphate isomerase (Aubert et al., 1994). Two alternative pathways have been proposed for glycerol utilisation in plants, one of which is strongly supported by both genetic and biochemical evidence. In a screen for Arabidopsis mutants resistant to the inhibitory effects of glycerol on root growth Eastmond (2004a) identified an Arabidopsis gene encoding glycerol kinase. The *glycerol insensitive 1* (*gli1*) mutant lacked glycerol kinase activity and accumulated high levels of glycerol in young seedlings. Interestingly, this led to increased resistance to abiotic stresses such as dehydration or salt exposure. Hypocotyl elongation in the dark was similar to wild type in the *gli1* mutant, but the *gli1* mutant enhanced the hypocotyl growth deficiency of *icl* mutants, suggesting that gluconeogenesis from glycerol drives hypocotyl elongation in the absence of the glyoxylate cycle in *icl* mutants. The glycerol-3-phosphate produced by glycerol kinase is believed to be converted to the glycolytic intermediate dihydroxyacetone phosphate by the enzyme glycerol-3-phosphate dehydrogenase, and can then proceed through gluconeogenesis or be used as a respiratory substrate.

4.1 THE REGULATION OF STORAGE RESERVE MOBILISATION: BALANCING CARBON AND NITRATE

Various treatments are known to block the mobilisation of storage reserves, and all are effective only if applied soon after germination. For instance, Arabidopsis seeds on MS media supplemented by 6% glucose germinate but fail to green or develop true leaves (Zhou et al., 1998), and lipid mobilisation is blocked (To et al., 2002). This effect is also dependent on the nitrogen status of the seedlings, as reducing the nitrate in the media reduces the amount of glucose required for the developmental arrest phenotype (Martin et al., 2002). A similar effect can be seen in seedlings germinating in the presence of both ABA and glucose. While ABA blocks germination this effect is relieved by the inclusion of carbohydrate in the media (Garciarrubio et al., 1997; Finkelstein and Lynch, 2000). The germinated seedlings do not green and the development of true leaves is blocked, however. Hence available carbohydrate and ABA both block lipid breakdown while nitrate antagonises these effects.

All these effects are only seen if seedlings are exposed to these conditions immediately after germination, and after two days of growth this window is passed (Lopez-Molina et al., 2001). It has been suggested that during this time ABA and high sugar levels can force the seedling to revert to a “storage mode” that existed during seed development (Rook et al., 2003). This transition from an embryonic storage mode to source tissues breaking down TAG and exporting sugars might involve large-scale changes in chromatin structure, as one factor, PICKLE (PKL), known to be responsible for the repression of embryonic characters in germinating seeds shares homology with mammalian chromatin remodelling factors (Ogas et al., 1997 Ogas et al., 1999). The *pkl* mutant exhibits an incompletely penetrant phenotype in the primary root characterised by oil body retention and swelling. It is convincingly argued that the *pkl* mutant is compromised in the transition from a seed developmental mode to a post-germinative mobilisation mode. The *pkl* mutant phenotype is enhanced by gibberellin (GA) deficiency, suggesting that GA has a role in

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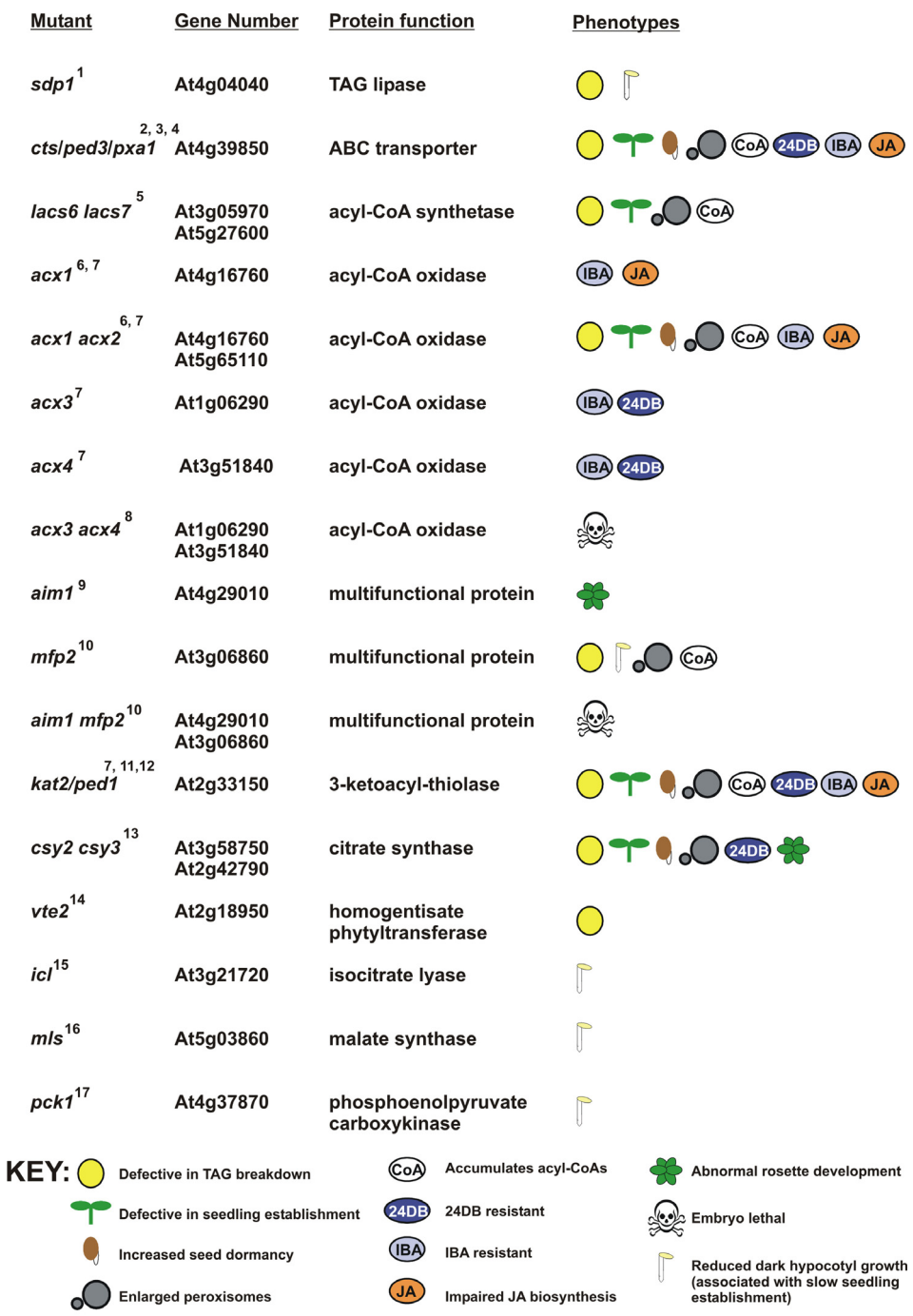


Figure 7. Key to phenotypes of mutants affecting Arabidopsis storage reserve mobilization and required pathways. Superscript numbers indicate references for the described phenotypes. 1. Eastmond, 2006; 2. Footitt et al. 2002; 3. Hayashi et al 2002; 3. Zolman et al., 2001a; Fulda et al., 2004; 6. Pinfield-Wells et al., 2005; 7. Adham et al., 2005; 8. Rylott et al. 2003a; 9. Richmond and Bleeker, 1999; 10. Rylott et al., 2006; 11. Hayashi et al., 1998; 12. Germain et al., 2001; 13. Pracharoenwattana et al., 2005; 14. Sattler et al., 2004; Eastmond et al., 2000; Cornah et al., 2004; Penfield et al., 2004.

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this switch. Such a mechanism would serve to couple the onset of reserve mobilisation with seed germination.

Even though ABA inhibits lipid breakdown it cannot completely block fatty acid catabolism, or the expression of genes encoding the key enzymes of fatty acid ß-oxidation or the glyoxylate cycle (Pritchard et al., 2002). ABA treated seeds accumulate sucrose, but ABA treated *icl* and *kat2* mutants do not, showing that the accumulated sucrose is derived from lipid breakdown. It has recently been shown that lipid catabolism in the presence of ABA occurs in the endosperm, while the embryo itself remains largely quiescent (Penfield et al., 2004). Our recent work shows that *ABSCISIC ACID INSENSITIVE 4* (*ABI4*) is the key regulator of lipid catabolism in response to ABA. ABI4 is required for the ABA repression of storage lipid breakdown in the embryo and is not expressed in the endosperm, where ABA does not regulate either lipid catabolism or gluconeogenesis (Penfield et al., 2006). Stress during germination, such as low water availability, also induces an embryo specific block on fatty acid catabolism, suggesting that under these conditions the seed can enter a second period of quiescence fuelled by reserves stored in the endosperm. The key adaptive advantage of this system is that reserve mobilisation is accompanied by breakdown of soluble carbohydrates such as sucrose that are central to desiccation tolerance (Footitt et al., 2002). Hence, by solely catabolising endospermic reserves, the embryo itself retains desiccation tolerance and therefore the option to delay the point at which it commits fully to the seedling establishment program.

CONCLUSIONS

Lipid reserve mobilisation is one of the most extensively genetically characterised biochemical processes in plants (Figure 7). These analyses have revealed novel roles for these pathways, such as the unexpected role of ß-oxidation in seed dormancy (Footitt et al., 2002; Pinfield-Wells et al., 2005; Pracharoenwattana et al., 2005). However, there is still much to learn about the functions of ß-oxidation during the vegetative phase, where mutants such as *aim1* and *csy2 csy3* have strong phenotypes. This contrasts with the lack of vegetative phenotypes in *cts* mutants, suggesting the existence of further unknown entry routes for ß-oxidation substrates into the peroxisome. The recent discovery that ß-oxidation is required for chlorophyll breakdown (Ishizaki et al., 2005) suggests there are further unknown substrates for this pathway in plants.

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