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Chewing the fat:β-oxidation in signalling and development.

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Abstract.

Peroxisomal β-oxidation is involved not only in fatty acid catabolism and lipid housekeeping but also in metabolism of hormones and amino acids in plants. Recent research in model species has led to new insights into the roles of this pathway in signalling and development, in particular regarding the involvement of β-oxidation in jasmonic acid biosynthesis. Analysis of associated processes, such as the glyoxylate cycle and redox metabolism has also highlighted the importance of integration of β-oxidation with cytosolic and mitochondrial metabolism. Mutations that disrupt β-oxidation can have extremely pleiotropic effects, indicating important and varied roles for this pathway throughout the plant life cycle and making this an exciting topic for future research.

Introduction

β-oxidation:new insights into an old pathway.

Fatty acids and fatty acid derived molecules have multiple roles in cells. In both prokaryotic and eukaryotic cells, fatty acids act as storage reserves that can be metabolised to produce energy, they are the building blocks of membrane lipids and act as signalling molecules. The breakdown of fatty acids via oxidation at the β-carbon and subsequent removal of two carbon units was discovered by Franz Knoop in 1904, making this one of the first metabolic pathways to be described. In plants, fatty acids are stored as triacylglycerides (TAG) in seeds of many species, and their catabolism by β-oxidation within the peroxisome to acetyl-CoA [1] and the subsequent conversion of acetyl-CoA to succinate via the glyoxylate cycle [2] provides germinating seeds with both carbon skeletons and energy before the seedlings develop the capacity to photosynthesise. The β-oxidation pathway is also important in the turnover of membrane lipids and the breakdown of branched chain amino acids [3]. Recent progress with mutant characterisation and metabolite profiling has led to the identification of new genes and enzymes, elucidation of substrate specificities, definition of the pathway of carbon metabolism, and new insights into the roles of this pathway in signalling and development. This review highlights recent advances that are leading to new insights into the physiological functions of an old pathway.

Identification and functional characterisation of genes associated with peroxisomal β-oxidation.

The core set of enzymes responsible for the β-oxidation of fatty acids have been studied extensively and the corresponding genes identified from a variety of organisms. Building on this knowledge, homology based searches of the Arabidopsis genome identified a comprehensive set of candidate genes involved in the breakdown of straight and branched

chain fatty acids in this model oilseed plant [3]. A combination of approaches including biochemical characterisation of heterologously expressed proteins, forward and reverse genetics has led to the functional characterisation of many of these candidate genes and identified additional ones (Table 1).

Substrates for peroxisomal β-oxidation have to be transported into the organelle and activated by esterification with Coenzyme A before they enter the β-oxidation spiral. Three different forward genetic screens (for germination potential, 2,4 dichloro-phenoxy-butyric acid (2,4-DB) resistance and indole butyric acid (IBA) resistance (Fig 1 and Table 1) identified the same peroxisomal ABC (ATP-binding cassette) transporter that is required for the movement of substrates for β-oxidation into the peroxisome [4-6]. Mutations at this locus (*cts* , *ped3* and *pxa1*) are compromised in fatty acid breakdown, seed germination and seedling establishment [4]. Functional characterisation of peroxisomal long-chain acyl-CoA synthetases (LACS) involved in activation of fatty acids has recently been achieved using a reverse genetics approach. The double mutant *lacs6-1 lacs7-1* is compromised in fatty acid breakdown and seedling establishment[7].

The core pathway of peroxisomal β-oxidation involves the repeated cleavage of acetate units from the thiol end of the fatty acyl-CoA (Fig 1). Three proteins are involved in this process: acyl-CoA oxidase (ACX); multifunctional protein (MFP) which can exhibit hydratase, dehydrogenase, epimerase and isomerase activities; and L-3-ketoacyl-CoA thiolase (KAT). Six *ACX* genes are encoded in the Arabidopsis genome and of these substrate specificities have been determined for ACX1 (medium-long chain), ACX2 (long – very long chain), ACX3 (medium chain) and ACX4 (short chain) [8-12]. Mutations in all six of these genes have shown that they have physiological roles during embryo development, seed germination and

seedling establishment [8,12-14]. Of the two Arabidopsis *MFP* genes one has been genetically characterised as *AIM1* (abnormal inflorescence meristem) and the second, *MFP2,* is predominantly expressed during seed germination [15,16]. Finally, of the three *KAT* genes *KAT2* (also known as *PED1; pe*roxisome *d*efective) encodes the major isozyme involved in seed germination [17,18]. Degradation of unsaturated fatty acids requires additional auxillary enzymes to convert double bonds at odd numbered carbons or *cis* double bonds to the 2-*trans*-enoyl-CoA intermediate that is the substrate for the 2-*trans*-enoyl-CoA hydratase activity of the multifunctional protein [3,19].

Profiling of acyl-CoA esters in the various fatty acid breakdown mutants has shown that the *cts* transporter mutant and the *lacs6-1 lacs7-1* synthetase double mutant both accumulate long chain acyl-CoAs [4,7]. The simplest explanation for the peroxisomal localisation of LACS6 and LACS7 is that the CTS (PXA1; PED3) transporter delivers fatty acids into the peroxisome and these are then activated. The increased levels of acyl-CoA esters in *cts* and *lacs6-1 lacs7-1* are most likely due to the activity of an as yet uncharacterised cytosolic acyl-CoA synthetase. Alternatively, the transported species might be the CoA derivative, as is the case for the *S.cerevisiae* peroxisomal ABC transporters [20] Disruption of specific enzymatic steps in β-oxidation also results in accumulation of acyl-CoAs. For example the *acx1-1 acx2-1* double mutant accumulates long chain acyl-CoAs [14] and the *acx3* and *acx4* mutants accumulate medium and short chain acyl-CoAs respectively [8,12]. In the *kat2(ped1), mfp2* and *acx1-1 acx2-1* double mutants long chain acyl-CoA levels are expected to be elevated in peroxisomes, which are enlarged compared to wild type [14,17,18,21,22] In contrast, in *cts* [4] and *lacs6-1 lacs7-1* mutants [7] acyl-CoAs are expected to be extra-peroxisomal and peroxisome size is normal. This has led to the suggestion that acyl-CoAs are involved in regulating various cellular processes including peroxisome morphology in plants [23].

Downstream metabolic integration

The β-oxidation cycle generates H_2O_2, NADH, and acetyl-CoA as the major end products. Removing the toxic H_2O_2 and regenerating NAD and CoASH are required to allow continued operation of the cycle. Recently, appreciable progress towards understanding how these products are further metabolized has provided insight into how these steps are integrated with downstream metabolism, both inside and outside the peroxisome, and how they might contribute to signalling.

H_2O_2 is not only potentially damaging through oxidation reactions, but is also a potential signalling molecule involved in stress responses [24,25]. H_2O_2 is inactivated through the action of catalase which is abundant in peroxisomes (Fig 2). Under conditions of stress, not only can β-oxidation increase in activity, but superoxide, nitric oxide and peroxynitrite are also produced in the peroxisome and these are reported to inhibit catalase activity [24,26]. Although components of the ascorbate-glutathione cycle have been demonstrated in peroxisomes [27], their role and quantitative importance is not clear [24]. An alternative possibility is the diffusion of H_2O_2 from the peroxisome and participation in cytosolic metabolism such as oxidation of ascorbate to monodehydroascorbate (MDA) by peroxisome-associated ascorbate peroxidase (APX) or in cell signalling (Fig 2; [24,28,29]). The high affinity of peroxisomal APX for H_2O_2 suggests that it could act as an efficient scavenger at the cytosolic face of the peroxisomal membrane [30]. However, the relative importance of these different potential fates of H_2O_2 requires detailed analysis.

The re-oxidation of NADH can also be potentially achieved by different processes. One involves oxidation by MDA reductase (MDAR) at the peroxisomal membrane (Fig 2). This is

potentially linked to APX activity, and might occur principally under conditions of oxidative stress. MDAR can also transfer electrons from NADH to oxygen either directly, or via a *b*-type cytochrome [31] to generate superoxide which in turn can generate H_2O_2 [24,26]. A third mechanism, which is strongly supported by genetic evidence, involves oxidation by peroxisomal malate dehydrogenase (pMDH). Yeast mutants lacking pMDH are unable to carry out fatty acid β-oxidation [32], which suggests pMDH is the only mechanism for NADH reoxidation in yeast peroxisomes. Similarly in Arabidopsis, knockout mutants lacking pMDH do not metabolise TAG during the first three days of seedling growth and are resistant to 2,4-DB (I. Pracharoenwattana and S.M.Smith personal communication). Thus NADH reoxidation by MDAR does not appear to operate to a significant extent in young seedlings. As *pmdh* mutant seedlings become photosynthetic they begin to break down TAG, presumably because NADH can be reoxidised by the hydroxypyruvate reductase (HPR) step of the photorespiratory pathway. (I. Pracharoenwattana and S.M.Smith personal communication) (Fig 2).

In establishing Arabidopsis seedlings, the glyoxylate cycle plays a central role in the conversion of acetyl units into 4-carbon acids for conversion to sugars [33]. The two glyoxylate cycle enzymes that use acetyl-CoA are peroxisomal citrate synthase (CSY) and malate synthase (MLS), and carbon is exported as citrate and malate (Fig 2). However, even in the absence of MLS, acetyl-CoA can be used by the seedling for gluconeogenesis because the glyoxylate from isocitrate lyase (ICL) can be metabolized by an alternative pathway [34]. At other stages of development when the glyoxylate cycle is absent, or when it is non-functional through mutation of ICL, acetyl units from β-oxidation are respired [35]. In yeast, acetyl units are transferred to the mitochondrion for entry into the TCA cycle via a carnitine shuttle mechanism [36]. In Arabidopsis, a putative acylcarnitine carrier in the mitochondrial membrane

has been found to be necessary for lipid breakdown and seedling growth in the light [37]. However, the proposal that this protein might be responsible for transfer of acetylcarnitine from the peroxisome into the mitochondrion is not consistent with the finding that peroxisomal CSY is required for continued β-oxidation [38]. It is concluded that unlike yeast, peroxisomal acetyl units in Arabidopsis are exported for respiration only after conversion to citrate.

Integration of peroxisomal and mitochondrial β-oxidation

Breakdown of the branched chain amino acids (BCAAs), leucine, isoleucine and valine, involves transamination, oxidative decarboxylation, esterification and β-oxidation of the resulting acyl-CoA esters in a series of reactions that appear to be primarily located in plant mitochondria [39-41]. However, characterization of the 2,4-DB resistant Arabidopsis mutants *chy1* and *dbr5* that are disrupted in the peroxisomal β-hydroxyisobutryl-CoA hydrolase enzyme of valine catabolism, has shown that, in agreement with earlier biochemical studies, at least some of the core β-oxidation reactions in BCAA catabolism take place in plant peroxisomes [3,42,43] Disruption of valine catabolism in *chy1* or *dbr5* apparently results in accumulation of the toxic intermediate, methylacrylyl-CoA which inhibits the 3-ketoacyl-CoA thiolase enzyme and thus indirectly blocks peroxisomal β-oxidation [42,43]. Details of other steps in valine catabolism remain to be resolved and it is not yet clear what, if any, transport mechanism operates to transfer intermediates from mitochondria to peroxisomes. A recent report of an Arabidopsis mutant disrupted in the mitochondrial electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF-QO) involved in electron transfer from acyl-CoA dehydrogenases showed that it accumulates not only intermediates in leucine catabolism but also, most remarkably, phytanoyl-CoA – an intermediate in chlorophyll degradation [44].

Further β-oxidation of this 20-carbon compound is predicted to involve peroxisomal β-oxidation and the challenge now is to work out the details of this important process.

Role of β-oxidation in the production of plant hormones.

The functions of β-oxidation include not only catabolism of fatty acids, but also action on a range of other substrates that give rise to signalling molecules, including indole acetic acid (IAA), Jasmonic acid (JA) and salicylic acid (SA), [6,45], therefore the β-oxidation machinery must accommodate a range of substrates with more complex structures, including aromatic or cyclopentanone rings and double bonds [45]. The isolation and characterisation of mutants defective in the conversion of indole butyric acid (IBA) to IAA (Fig 1) [6,13,46] has revealed the importance of β-oxidation for this process. IBA is an endogenous auxin which promotes secondary root formation and has been suggested to act as a 'slow release' form of IAA (reviewed in [47]) Other mutants with β-oxidation deficiency are also resistant to the effects of IBA (Table 1 and references therein) resulting in a 'long root' phenotype in the presence of this compound.

A biosynthetic scheme for JA was originally proposed in the early 1980s [48,49]. In the canonical pathway (Fig 3), 12-oxo-phytodienoic acid (OPDA) is synthesised in the plastid and transferred to the peroxisome where it undergoes reduction to 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), and acyl chain shortening by three rounds of β-oxidation to yield JA (Fig 3). Until recently it was not clear which specific peroxisomal components participated in JA biosynthesis and whether these were shared with fatty acid catabolism (comprehensively reviewed in [50]), but analysis of mutant alleles isolated by both forward and reverse genetics has proved effective in addressing this issue in Arabidopsis and tomato.

Oxylipin profiling of the *cts* mutant revealed a dramatic reduction in both basal and wound induced JA in leaves, suggesting that CTS (PXA1; PED3) is involved in import of OPDA (or its CoA ester) into peroxisomes in parallel with a second, probably passive, transport pathway [51]. Following import, OPDA is reduced by the action of oxophytodienoate reductase 3 (OPR3), the only OPR isoform with the correct stereospecificity [52,53]. *opr3* mutant plants are JA deficient and exhibit male sterility, confirming the lack of redundancy for this step [54,55]. It is not yet clear which JA intermediates are activated by esterification to CoA, although recombinant OPR3 accepts free OPDA and *opr3* plants lack OPC:8, suggesting that OPC:8 and not OPDA is subject to activation and β-oxidation [50,52,56]. However, the recent discovery of two peroxisomal 4-coumarate:CoA ligase (4-CL)-like acyl activating enzymes with strong *in vitro* preferences for OPDA and OPC:6 suggests that CoA esterification can occur at different stages in the pathway [57]. The detection of free OPC:6 and OPC:4 in plant tissue [49], together with the identification of thioesterases and additional acyl activating enzymes with putative peroxisomal targeting sequences [42,45,58,59] imply that intermediates can enter and exit β-oxidation, which could play an important regulatory role.

Analysis of *ACX* antisense and knockout plants has implicated Arabidopsis ACX1 in wound-induced JA biosynthesis *in vivo* [14,60] and characterisation of a JA-deficient mutant of tomato provided conclusive evidence for the involvement of a specific ACX isoform (LeAPX1A) in both basal and wound-induced JA formation in tomato leaves [61]. Recombinant LeACX1A was shown to use OPC:8-CoA as a substrate, in preference to fatty acyl-CoA. Given that both LeACX1A and AtACX1 isoforms accept a range of chain lengths, it is plausible that these enzymes also handle OPC:6- and OPC:4-CoA in repeated cycles of β-oxidation leading to JA [11,14,61]. In contrast to ACX, the role of MFPs in the context of JA biosynthesis has not yet been

investigated. Two studies have demonstrated the involvement of the KAT2-PED1 thiolase in wound-induced JA biosynthesis [60,62]. In neither case was JA completely abrogated, suggesting compensation from another KAT isoform. [17]. Thus, a combination of genetic and biochemical approaches has helped identify the roles of both core β-oxidation enzymes and novel components in JA biosynthesis.

New understandings of the role of β-oxidation in development revealed through genetic analysis

Both forward and reverse genetic screens have revealed the importance of proteins associated with β-oxidation during development and in response to stress (Table 1). In many cases analysis of single mutants has uncovered only subtle effects, whereas combinations of mutations that remove redundant functions show much stronger phenotypes. During the early stages of embryogenesis the absolute requirement for short and/or medium chain acyl-CoA oxidase activity was revealed by the lethality of the *acx3 acx4* double mutant [12], and the phenotypes associated with mutations of *aim1* and the *ped1 ped3* double mutant also indicate a requirement for β-oxidation for flower development [5,16]. In all these cases it is unclear whether the necessity is for β-oxidation to provide an energy source, or a lipid-derived signal molecule.

Mutations of several loci encoding proteins associated with β-oxidation lead to a requirement for exogenous carbohydrate for seedling establishment following germination, presumably to compensate for the failure to derive energy and carbon skeletons from TAG [5,7,13,17] . Genetic evidence has demonstrated that, in addition, β-oxidation is essential for germination prior to seedling establishment. The *cts* alleles have previously been described as displaying a 'forever dormant' phenotype, including non-responsiveness of imbibed seeds to chilling or

gibberellin [63] treatment , and similar phenotypes have recently been reported for *kat2* single and *acx1 acx2* and *csy2 csy3* double mutants[14,38]. Evidence for an important role for β-oxidation also comes from combination of *cts* with other mutations that alter phase two of germination (imbibed seeds prior to radicle emergence) [4,63] and demonstrate the specificity of β-oxidation for progression through this stage .

There appear to be two facets to the involvement of β-oxidation in germination, reserve mobilisation and developmental signalling [63-65]. TAG mobilisation occurs in both the endosperm and the embryo of imbibed seeds. Abscisic acid (ABA) reduces germination potential but does not completely inhibit TAG breakdown in imbibed seeds, and it has been shown that within the endosperm mobilisation is insensitive to exogenous ABA [64]. This is a key observation, as seed ABA treatment has often been used to mimic dormancy. It is possible therefore that TAG breakdown occurs in the endosperm of imbibed dormant seeds. The function of β-oxidation in the activation of germination could potentially be the result of either promotion of germination-associated processes, or repression of dormancy. Determining the after-ripening status of mutant seeds should allow discrimination between increased dormancy and reduced germination potential of these mutants, an important pre-requisite to determining the function of β-oxidation.

It appears likely that the requirement for β-oxidation for seedling establishment and germination are different. Whereas exogenous sugar removes the block to the establishment of germinated mutants, it does not increase the germination potential of non-germinating mutants [4,14,38,63] . This has been interpreted as indicating that the block to germination is not the result of the requirement for β-oxidation to supply carbon and energy (as it is for establishment). Other potential functions might be to facilitate either the synthesis or

degradation of signalling molecules that control germination. Of the many loci influencing germination potential that have been cloned none would appear to fall into this category. The apparent wide substrate specificity of CTS-PXA1-PED3 allows for the transport of compounds associated with both metabolism and hormone biosynthesis into the peroxisome, and therefore it will be important to determine which compounds are associated with an increase in germination potential. Although JA synthesis occurs via peroxisomal β-oxidation, there is no genetic evidence that this hormone has a major influence on germination in Arabidopsis. Similarly β-oxidation of precursors is one route to the synthesis of active auxin, but mutants of auxin synthesis and/or action have not been reported to affect germination. It is likely that other, as yet unidentified compounds are modified through peroxisomal β-oxidation to provide a chemical signal(s) required for the transition from dormancy to germination, and potentially for other phases of plant growth and development.

Conclusions and perspectives.

Characterisation of mutants in β-oxidation and associated processes has greatly advanced our understanding of the physiological significance of these pathways and their integration within the plant. It has also resulted in tantalising observations that suggest the involvement of products or substrates of β-oxidation in the control of peroxisome size and in the control of germination potential. Mutants affected in the conversion of OPDA to JA, and in the production and metabolism of various ROS species will in the future provide important insights into the relative contributions of these different signal molecules to diverse biological processes.

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Figure legends:

Figure 1 A. Pathways of core β-oxidation of straight chain saturated fatty acids, indole butyric acid and 2,4 dichloro-phenoxyacetic acid.

Prior to β-oxidation, substrates are activated by esterification to Coenzyme A (CoA). This reaction is catalysed by acyl activating enzymes (step 1), also known as acyl CoA synthetases. Acyl CoA is then converted to a 2-*trans*-enoyl CoA by acyl CoA oxidase (step 2). In subsequent steps, fatty acids are degraded via the L-isomers of 3-hydroxyacyl-CoA. Thus for straight chain, saturated fatty acids, only the "core" activities of 2-*trans* enoyl-CoA hydratase (step 3a) and L-3-hydroxyacyl-CoA hydratase (step 3b) are required. These enzymes catalyse the hydration of 2-trans-enoyl-CoA to 3-hydroxyacyl-CoA and subsequent oxidation to 3-ketoyl-CoA, and are encoded by the so-called multifunctional protein. In the case of fatty acids with *cis*-double bond(s) at odd- and even-numbered carbons, auxiliary activities are required, which may be provided by the multifunctional protein, or alternatively are catalysed by separate enzymes such as $\Delta^{3,5}\Delta^{2,4}$-dienoyl CoA isomerase [3,19]. These activities are of importance in plants, since the majority of fatty acids are polyunsaturated and therefore unable to be catabolised by the "core" pathway. In the final reaction of β-oxidation (step 4), 3-ketoacyl-CoA thiolase catalyses the thiolytic cleavage of 3-ketoacyl CoA to yield acyl CoA and acetyl CoA. This cycle is repeated with the removal of two carbon units until the fatty acid is completely oxidised. Activation requires one molecule of ATP and CoASH for each fatty acid and one cycle of β-oxidation requires one molecule each of CoASH, FAD, O_2, H_2O and NAD^+. NAD^+ is regenerated by several different routes, see text and figure 2. The

regeneration of FAD results in the generation of H_2O_2 which is removed by matrix-located catalase (step 5), or possibly also by peroxisomal ascorbate peroxidase at the cytosolic face of the peroxisomal membrane (not shown).

A single cycle of β-oxidation is required for conversion of the auxin precursors 2,4-dichloro-phenoxy-butyric acid (2,4-DB) and indole-butyric-acid (IBA) to 2,4-dichloro-phenoxy-acetic acid (2,4-D) and indole-acetic-acid (IAA) respectively. Action of a thioesterase is required to release the free acids from their CoA esters, following "core" β-oxidation (not shown). A number of different β-oxidation mutants show resistance to these compounds (table 1) but this could be due to either a direct or indirect block on their metabolism[13].

Figure 1B. Many β-oxidation mutants are resistant to the effects of 2,4 dichloro-phenoxy-butyric acid (2,4-DB).

Because 2,4-DB is metabolised via β-oxidation to 2,4 D, many mutants that disrupt β-oxidation either directly or indirectly result in resistance to this compound. This is illustrated here by the *cts1* mutant. The top row of plants shows wild type Arabidopsis (ecotype Landsberg erecta) and the *cts1* mutant (which is in the Landsberg erecta background) grown on 0.5x MS agar plus sucrose. Note that the *cts1* plants are smaller as has been reported for the *pxa1* mutant[6] which is defective in the same gene encoding a peroxisomal ABC transporter[4-6]. Wild type plants show a short root phenotype on 2,4-DB due to the metabolism of this compound to the auxin 2,4-D whilst the *cts1* mutant plants have a similar root length to the control (middle row). Both wild type and *cts1* mutant remain sensitive to the effects of 2,4-D as shown in the bottom row of plants. Plants were removed from the plates and arranged for photography.

Figure 2. Metabolism of products of β-oxidation.

Enzymes of β-oxidation are shown in green, those of reactive oxygen metabolism in blue, and those of other aspects of primary metabolism are shown in pink. Dashed lines show transfer of metabolites across the peroxisomal membrane. Only those steps of the glyoxylate cycle and photorespiration that are peroxisomal are shown.

Uptake of fatty acids (or possibly, fatty acyl-CoA) into the peroxisome is mediated by the ABC transporter, COMATOSE (CTS). Free fatty acids are activated by long chain acyl CoA synthetases (LACS) 6 and 7, which are associated with the lumenal side of the peroxisomal membrane. In each cycle of β-oxidation, one molecule of H_2O_2 is produced for each acyl-CoA molecule converted to 2-*trans*-enoyl-CoA by acyl CoA oxidase (ACX). NADH is produced by the dehydrogenase reaction catalysed by multifunctional protein. H_2O_2 is removed primarily by the abundant antioxidative matrix enzyme, catalase (CAT), but can also be scavenged at the cytosolic face of the peroxisome by membrane-anchored ascorbate peroxidase (APX), acting in concert with monodehydroascorbate reductase (MDAR) [28,30]. In Arabidopsis, MDAR is tightly associated with the membrane (Fig 2) but is also present in the cytosol and the peroxisomal matrix (not shown). The involvement of peroxisomal MDAR in H_2O_2 metabolism might require movement of ascorbate (ASC) and monodehydroascorbate (MDA) across the peroxisomal membrane, since the active site of peroxisomal MDAR is in the matrix, whereas that of APX faces the cytosol [29]. Thus MDAR regenerates cytosolic ascorbate and peroxisomal NAD^+ at the expense of NADH. An alternative fate of peroxisomal MDA is spontaneous disproportionation to dehydroascorbate followed by removal via dehydroascorbate reductase and glutathione reductase which have been detected in the matrix of plant peroxisomes (not shown; [24]). Cytosolic MDA might also be converted to ascorbate by cytosolic MDAR (not shown).

Abbreviations: ACX, acyl CoA oxidase; Hydratase, 2-trans-enoyl-CoA hydratase; HACoA DH, Hydroxyacyl-CoA dehydrogenase; KAT, 3-ketoacyl-CoA thiolase; MDH, malate

dehydrogenase; HPR, hydroxypyruvate reductase; SGAT, serine-glyoxylate aminotransferase; ICL, isocitrate lyase; MLS, malate synthase; CSY, citrate synthase; OAA, oxalaoacetate; Ser, serine; Hydroxy-pyr, hydroxypyruvate.

Figure 3 Overview of Jasmonic Acid (JA) biosynthesis.

JA synthesis is initiated in the chloroplast via the octadecanoid pathway starting from α-linolenic acid (18:3) or alternatively, in certain species, via the hexadecanoid pathway starting from hexadecatrienoic acid (16:3) [50,66]. These polyunsaturated fatty acids are thought to be released from membrane lipids by the action of phospholipases [50,67] and are converted to (9*S*, 13*S*)-12-oxo-phytodienoic acid [cis-(+)-OPDA] or dinor-OPDA (dnOPDA), respectively, by the sequential action of 13-lipoxygenase (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA, which exists in both free and membrane-esterified forms (MDGD-O [68]) is released from the plastid by an as yet unknown mechanism and transferred to the peroxisome. The ABC transporter, Comatose (CTS), is required for import of OPDA (or a CoA ester) into peroxisomes but a parallel pathway for passive transport of free OPDA probably also exists [51]. It is probable that dnOPDA is also imported in a similar fashion. Within the peroxisome, oxophytodienoate reductase (OPR3) catalyses reduction of the cyclopentenone ring to form the respective cyclopentanone, 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) [52-55]. The octanoic side chain of OPC:8 is then shortened by three cycles of β-oxidation to yield JA [48,49,69]. Note that the enantiomeric specificity of AOC yields (+)-7-iso-JA (shown). However, (+)-7-iso-JA readily isomerises to (3*R*,7*R*)-JA, which is the predominant form in plant tissues (not shown; [50]). Esterification to CoA is a pre-requisite for entry into the β-oxidation spiral but it is not yet clear which intermediates are activated. Peroxisomes contain two 4-coumarate CoA-ligase (4CL)-like acyl activating enzymes with strong *in vitro* preferences for OPDA and OPC:6, but further

peroxisomal activating enzymes await characterisation [45]. The first committed step of cyclopentanone β-oxidation is catalysed by acyl CoA oxidase 1 (ACX1) [14,61]. In β-oxidation of straight chain fatty acids, multifunctional proteins (MFP) catalyse the subsequent two steps but isoforms involved in JA biosynthesis have not yet been characterised. The final step is catalysed by the KAT2 thiolase in Arabidopsis, which forms acyl-CoA and OPC:n-2 [60,62]. JA is then presumably released from its CoA ester by a yet uncharacterised thioesterase. Cofactors have been omitted for clarity but note that regeneration of the FAD cofactor for ACX produces three molecules of H_2O_2 for each JA molecule synthesised.

Abbreviations: 13-HPOT: 13(*S*)-hydroperoxy-9(*Z*),11(*E*), 15(*Z*)-octadecatrienoic acid); allene oxide: 12,13(*S*)-epoxy-9-*Z*,11,15(*Z*)-octadecatrienoic acid).

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Table 1: Enzymes associated with beta-oxidation in Arabidopsis
G germination, E seedling establishment

Gene name and AGI code	Genetic screen	Genetic function	Protein function	Substrate specificity	Expression pattern	Sub-cellular Location	Reference
CTS *PED3* *PXA1* At4g39850	Forward: 1. G 2. 2,4-DB/ IBA resistant root elongation	G, E Root growth Developmental timing JA synthesis	ABC transporter	broad?	In all tissues tested, highest in E	Peroxisome membrane	4-6,51
LACS6 At3g05970	Reverse	E for *lacs6/7* double mutant.	Acyl CoA synthetase	Most active on 20:1 but significant activity with all LCFA tested	In all tissues tested, highest in E and developing seed,	Peroxisome (PTS2)	7,70
LACS7 At5g27600	Reverse	E for *lacs6/7* double mutant	Acyl CoA synthetase			Peroxisome (PTS1 and PTS2)	7,70
4CL-like At4g05160			Acyl CoA synthetase	OPC6:0	Induced by MeJA	Peroxisome (PTS1)	57
4CL-like At5g63380			Acyl CoA synthetase	OPDA		Peroxisome (PTS1)	57
ACX1 At4g16760	Forward; IBA resistant root elongation Reverse.Antisense	Wound induced JA synthesis G & E with ACX2	Acyl CoA oxidase	Medium-long chain saturated acyl CoA	In all tissues tested, highest in E and developing seed,	Peroxisome (PTS1)	11,13,14,60,71
ACX2 At5g65110	Reverse	G & E with ACX2	Acyl CoA oxidase	Long chain unsaturated acyl CoA	In all tissues tested. highest in E and developing seed,	Peroxisome (PTS2)	11,13,14,71
ACX3 IBR4 At1g06290	Forward: IBA resistant root elongation Reverse	Early embryo development with *ACX4*	Acyl CoA oxidase	Medium chain acyl CoA	In all tissues tested. highest in E and developing seed,	Peroxisome (PTS2)	8,9,12,13
ACX4 At3g51840	Reverse	Early embryo development with *ACX3*	Acyl CoA oxidase	Short chain acyl CoA	In all tissues tested. highest in E and developing seed,	Peroxisome (PTS1)	12,13
AIM1 At4g29010	Forward; Floral structure	Inflorescence structure, fertility	Multifunctional protein		Strong in adult tissues, especially siliques and flowers; weak in seedlings.	Peroxisome (PTS1)	16
MFP2 At3g06860	Reverse	E. Early embryo development with *AIM1*	Multifunctional protein	Broad	In all tissues tested. highest in E and developing seed.	Peroxisome (PTS1)	15,16,22
PED1	Forward; 2,4-DB	G & E	3-keto acyl	Probably broad	Highest in E.	Peroxisome	

KAT2 At2g33150	resistant root elongation Reverse.Antisense	JA synthesis	thiolase		Major thiolase activity in seedlings.	(PTS2)	14,17,18,60,62
KAT5 At5g48880			3-keto acyl thiolase		Highest in E	Peroxisome (PTS2)	17
KAT1 At1g04710			3-ketoacyl thiolase		Fairly constitutive	Peroxisome (PTS2)	17
ACH2 At1g01710			Acyl-CoA thioesterase	Saturated long chain acyl CoAs	Highest in mature leaves and flowers	Predicted PTS1	58
CSY1 At3g58740			Citrate synthase		Siliques	Predicted PTS2	38
CSY2 At3g58750	Reverse	G & E in conjunction with *CSY3*	Citrate synthase	Oxalo-acetate, acetyl CoA, Citrate	All tissues. Highest in flowers and E	Peroxisome (PTS2)	38
CSY3 At2g42790	Reverse	G & E in conjunction with *CSY2*	Citrate synthase	Oxalaoacetate, acetyl CoA, Citrate	All tissues. Highest in flowers and E	Peroxisome (PTS2)	38
DCI1 At5g43280			$\Delta^{35}\Delta^{24}$di enoyl-CoA isomerase	3,5-dienoyl CoAs	in all tissues tested Highest in E and developing seed,	Peroxisome (PTS1)	19
CHY1 At5g65940	Forward; IBA resistant root elongation	E	3-hydroxy-isobutyryl hydrolase	3-hydroxy-2 methyl propanoyl CoA		Peroxisome (PTS1)	42,43

Figure 1A

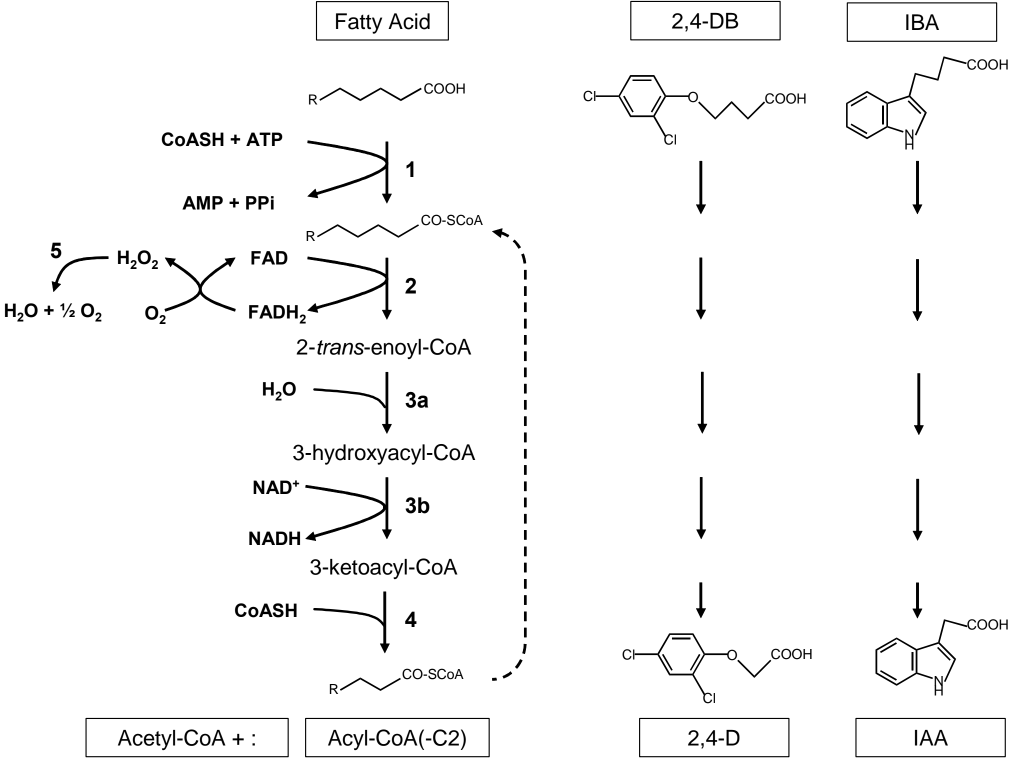

Figure 1B.

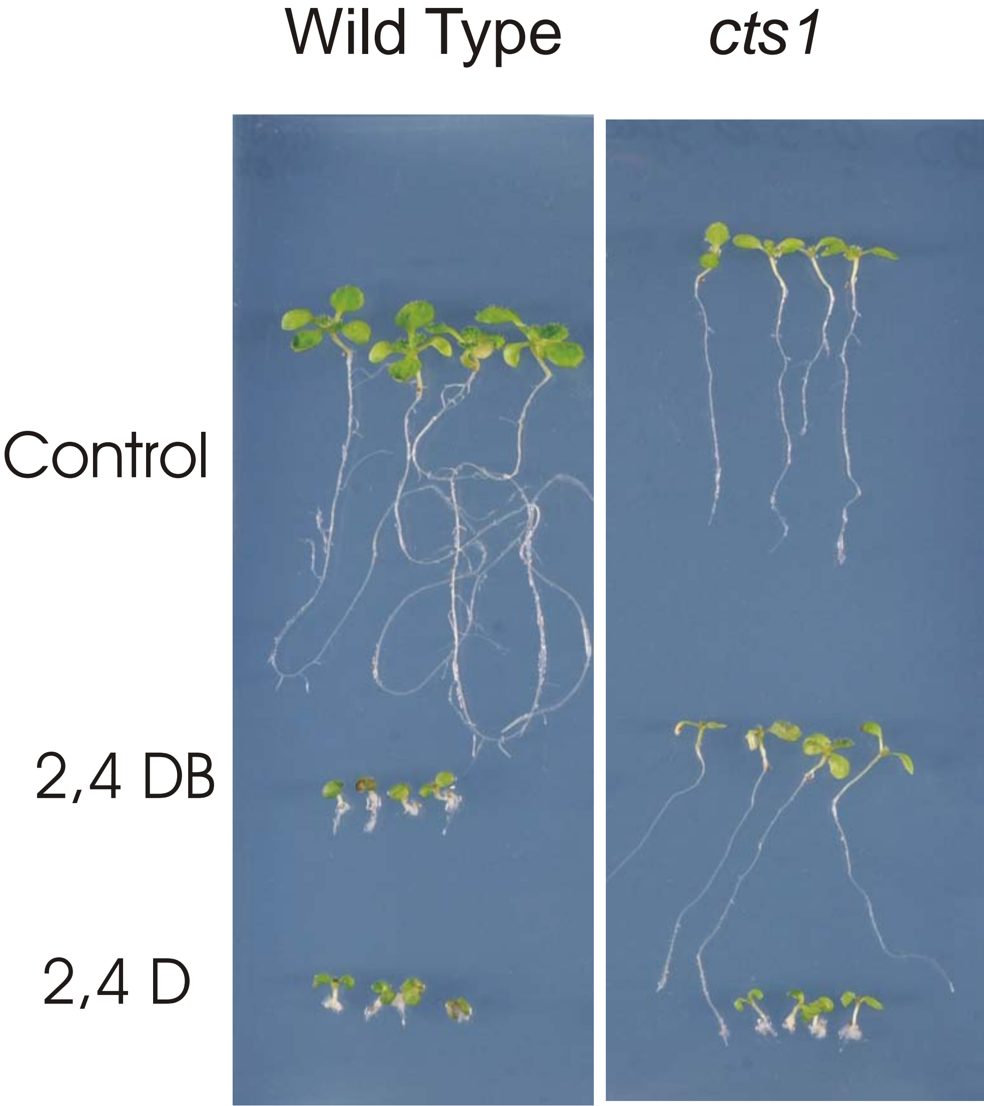

Figure 2

Free FA (*or* Acyl-CoA)
ATP
ADP + Pi
CTS
CoASH + ATP
LACS 6 & 7
AMP + PPi
Acyl-CoA
Peroxisome
Cytosol
O_2
H_2O
ACX
CAT
H_2O_2
2-*trans*-enoyl-CoA
Hydratase
Hydroxyacyl-CoA
NAD+
HACoA DH
NADH
MDH
Malate
OAA
HPR
Glycerate
Hydroxy-Pyr
SGAT
Ser
3-ketoacyl-CoA
Malate
MLS
CoASH
Glyoxylate
ICL
Isocitrate
Succinate
KAT
Acetyl-CoA
CSY
Citrate
OAA
Acyl-CoA
Signalling?
H_2O_2
H_2O
APX
ASC
ASC
MDA
MDA
MDAR
Malate
NAD+
MDH
OAA
NADH
Photorespiration
Glyoxylate cycle and Gluconeogenesis
TCA cycle

Figure 3