

Nine 3-ketoacyl-CoA thiolases (KATs) and acetoacetyl-CoA thiolases (ACATs) encoded by five genes in *Arabidopsis thaliana* are targeted either to peroxisomes or cytosol but not to mitochondria

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Abstract The sub-cellular location of enzymes of fatty acid β -oxidation in plants is controversial. In the current debate the role and location of particular thiolases in fatty acid degradation, fatty acid synthesis and isoleucine degradation are important. The aim of this research was to determine the sub-cellular location and hence provide information about possible functions of all the putative 3-ketoacyl-CoA thiolases (KAT) and acetoacetyl-CoA thiolases (ACAT) in *Arabidopsis*. *Arabidopsis* has three genes predicted to encode KATs, one of which encodes two polypeptides that differ at the N-terminal end. Expression in *Arabidopsis* cells of cDNAs encoding each of these KATs fused to green fluorescent protein (GFP) at their C-termini showed that three are targeted to peroxisomes while the fourth is apparently cytosolic. The four KATs are also predicted to have mitochondrial targeting sequences, but purified mitochondria were unable to import any of the proteins in vitro. *Arabidopsis* also has two genes encoding a total of five different putative ACATs. One isoform is targeted to peroxisomes as a fusion with GFP, while the others display no targeting in vivo as GFP fusions, or import into isolated mitochondria. Analysis of gene co-expression

clusters in *Arabidopsis* suggests a role for peroxisomal KAT2 in β -oxidation, while KAT5 co-expresses with genes of the flavonoid biosynthesis pathway and cytosolic ACAT2 clearly co-expresses with genes of the cytosolic mevalonate biosynthesis pathway. We conclude that KATs and ACATs are present in the cytosol and peroxisome, but are not found in mitochondria. The implications for fatty acid β -oxidation and for isoleucine degradation in mitochondria are discussed.

Keywords Thiolase · Mitochondria · Peroxisomes · β -oxidation · Sub-cellular localization

Abbreviations

| | |
|-------------|--|
| AOX | Alternative oxidase |
| ACAT | Acetoacetyl-CoA thiolase |
| KAT | 3-Ketoacyl-CoA thiolase |
| Rubisco SSU | Small subunit of Ribulose 1,5-bisphosphate carboxylase/oxygenase |

Introduction

It is widely accepted that the complete β -oxidation of medium- and long-chain fatty acids in plants takes place in the peroxisomes (Hooks 2002), as it does in yeast (van Roermund et al. 2003). However, some biochemical evidence suggests that plant mitochondria can also carry out such β -oxidation of fatty acids (Masterson and Wood 2001). It has also become clear recently that plant mitochondria catalyse at least the initial steps in the degradation of branched-chain α -keto acids, derived from leucine, isoleucine and

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valine, through a branched chain α -keto acid dehydrogenase complex similar to the pyruvate and 2-oxoglutarate dehydrogenase complexes of the TCA cycle (Fujiki et al. 2000; Graham and Eastmond 2002; Taylor et al. 2004). In the case of the leucine carbon skeleton, the later steps of degradation are carried out entirely within the mitochondrion (Graham and Eastmond 2002; Taylor et al. 2004). In contrast, final degradation of valine derivatives requires both mitochondrial and peroxisomal steps (Lange et al. 2004). Meanwhile the complete oxidation of the products from the isoleucine carbon skeleton includes a β -oxidation step that requires a 3-ketoacyl-CoA thiolase (KAT) for the removal of an acetyl-CoA from 2-methylaceto-acetyl CoA to form propionyl-CoA. However, it is unclear if this thiolase catalysed β -oxidation of 2-methylaceto-acetyl CoA occurs in the mitochondrion in plants, as it does in mammals (Fukao et al. 2001), or whether it occurs in the peroxisome in plants akin to the final steps of valine metabolism (Lange et al. 2004).

Comparisons to *Saccharomyces cerevisiae* are not informative as yeast degrades branched chain amino acids not via the branched chain dehydrogenase complex route in mitochondria, but via the Ehrlich pathway involving pyruvate decarboxylase to form the corresponding aldehydes and an aldehyde dehydrogenase to form the corresponding alcohol in the cytosol (Derrick and Large 1993). Thus yeast does not need a thiolase for isoleucine degradation.

Two distinct forms of 3-ketoacyl-CoA thiolase are known. Type 1 3-ketoacyl-CoA thiolase (KAT; EC 2.3.1.16) is typically involved in the degradative process of fatty acid β -oxidation. The Type II enzyme is an acetoacetyl-CoA thiolase (ACAT; EC 2.3.1.9), typically involved in the mevalonate pathway where it functions in the biosynthetic direction. However, ACATs are not exclusively involved in mevalonate synthesis. In mammals that undertake both fatty acid β -oxidation and isoleucine catabolism in mitochondria, the former is performed by a KAT while the later is performed by an ACAT (Pereto et al. 2005).

In Arabidopsis, thiolase has been reported to be associated with both peroxisomes and mitochondria in sucrose density gradients (Footitt et al. 2002). Kruff et al (2001) and Heazlewood et al (2004) have both claimed the thiolase KAT2 encoded by At2g33150 to be present in purified mitochondria in large-scale proteome analyses. This thiolase has previously been proposed to be a component of isoleucine catabolism in mitochondria (Taylor et al. 2004). However, the question is a type I enzyme, while in mammals it is the mitochondrial type II ACATs that have been implicated in isoleucine catabolism (Pereto et al.

2005). The KAT2 thiolase encoded by At2g33150 has a predicted type 2 peroxisomal targeting sequence (PTS2) conforming to the consensus R-(X)₆-H/Q-A/L/F with a downstream cysteine residue required for proteolytic cleavage (Baker and Sparkes 2005), and is imported into peroxisomes in vitro (Johnson and Olsen 2003). At2g33150 is well known to be essential for peroxisomal β -oxidation (Germain et al. 2001). However, the protein encoded by At2g33150 is also predicted to be targeted to mitochondria by three different targeting prediction programs (Heazlewood et al. 2004). Furthermore, changing a single amino acid in the peroxisomal targeting signal of the KAT precursor in mammals, a glutamic acid residue to any non-acidic residue, resulted in targeting to both mitochondria and peroxisomes (Tsukamoto et al. 1994). This raises the possibility that the thiolase encoded by At2g33150 is targeted to peroxisomes and mitochondria, representing a dual targeted protein.

This study was carried out to define the subcellular localization of the products from the putative KATs and ACATs in Arabidopsis. This was achieved by identifying all possible thiolase genes in Arabidopsis and comparing their sequences to known-location type I and type II thiolases in yeast, mammals and fungi. We then used transcript sequence data to produce all the possible cDNAs for these gene products. These cDNAs were used with in vivo and in vitro organelle targeting assays to define subcellular localization of type I and II thiolases in Arabidopsis and gene expression profiling data was compared to define likely functional links.

Materials and methods

Identification of genes and cDNAs encoding thiolase

The predicted protein sequence encoded by At2g33150 previously shown to be a thiolase was used to define other thiolase encoding genes in Arabidopsis (Germain et al. 2001), and the sequences from other species as previously published (Pereto et al. 2005). A similarity tree was made using ClustalW multiple sequence alignment and neighbour joining (Thompson et al. 1994, 1997). The gene structures were obtained from The Arabidopsis Information Resource annotation version 6 (TAIR6) and three individual cDNAs were amplified for all possible cDNAs. The cDNAs produced from the genes were defined using the Arabidopsis genome tiling array (Mockler et al. 2005; Yamada et al. 2003). Targeting predictions of the

encoded proteins were carried out using a variety of prediction programs: TargetP (Emanuelsson et al. 2000), Mitoprot (Claros and Vincens 1996), Subloc (Hua and Sun 2001), Ipsort (Bannai et al. 2002), Predotar (Small et al. 2004), Mitpred (Kumar et al. 2006) and PeroxP (Emanuelsson et al. 2003). Percentage identity and similarity was calculated using MatGAP v2.02 (Campanella et al. 2003).

Subcellular targeting of predicted thiolase proteins

The coding sequences of the predicted thiolase proteins were cloned in frame with the coding region of GFP in pGEM 3Zf(+) containing the 35S CaMV promoter (Chew et al. 2003). The alternative oxidase (AOX) coding region fused to GFP (Lee and Whelan 2004), and the red fluorescent protein (RFP) fused to a type 1 peroxisomal SRL targeting signal from pumpkin (Pracharoenwattana et al. 2005), were used as mitochondrial and peroxisomal controls respectively. The constructs were used to transform *Arabidopsis* suspension culture cells by biolistic transformation as previously outlined (Thirkettle-Watts et al. 2003). Fluorescence patterns were obtained 48 h after transformation by visualization under an Olympus BX61 fluorescence microscope and imaged using the Cell^R imaging software. In vitro protein import assays into mitochondria isolated from *Arabidopsis* suspension cell cultures were carried out as described in Lister et al. (2004). In vitro mitochondrial uptake assays were performed by adding precursor protein to 100 µg of isolated mitochondria in 200 µl in the presence of respiratory substrate (succinate 5 mM) and ATP (1 mM) and ADP (200 µM) in import buffer (0.3 M sucrose, 50 mM KCl, 10 mM MOPS pH 7.2, 5 mM KH₂PO₄, 1% (w/v) BSA, 1 mM MgCl₂, 1 mM methionine and 5 mM DTT). Reactions were incubated at 24°C for 20 min then divided into two equal aliquots and placed on ice. To one aliquot Proteinase K was added to a final concentration of 40 µg/ml and incubated for 15 min on ice, followed by the addition of PMSF to 2 mM to terminate protease digestion. The mitochondria were pelleted, washed twice in ice-cold import buffer. The final pellet was resuspended in SDS-PAGE sample buffer and proteins separated in 12% (w/v) polyacrylamide gels, then dried. Radiolabelled proteins were visualized by exposing to a BAS TR2040 imaging plate for 24 h and reading on a BAS 2500 Bio imaging analyser (Fuji, Tokyo). Outer membrane ruptured mitochondria (Mit-OM) were prepared after the import assay to test for the intra-organelle location of imported protein. Rupture of the outer membrane allowed access of added protease to

intermembrane space components or inner membrane proteins exposed to the intermembrane space. Mit-OM were prepared by resuspending 100 µg of mitochondrial protein in 10 ml SEH buffer (250 mM sucrose, 1 mM EDTA, 10 mM Hepes pH 7.4) and then adding 155 µl of 20 mM Hepes pH 7.4 and incubating on ice for 20 min. To restore osmolarity 25 µl of 2 M sucrose and 10 µl of 3 M KCl was added and mixed, re-pelleted and washed in import buffer. Valinomycin was added to a final concentration of 1 µM where indicated prior to the addition of the precursor protein to mitochondria and commencement of the import assay. AOX was used as a positive control and the small subunit of Ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco SSU) as a negative control. As some precursor proteins displayed protease insensitivity even in the presence of valinomycin the sensitivity of the precursor proteins alone to added protease was tested. Sensitivity of the in vitro synthesized radiolabelled proteins was tested by adding proteinase K to the synthesized protein alone in the absence of mitochondria to ensure that the added protease could digest the protein.

In silico expression analysis

Expression correlation for genes encoding KATs and ACATs was performed using the Expression Angler program on the Botany Array Resource (Toufighi et al. 2005). The Genevestigator *Arabidopsis* microarray database was used to analyse the response of the genes of interest in this study in a variety of tissue types (Zimmermann et al. 2004). The meta-analyser tool was the function utilized, ATH1 22k array wild type only arrays were chosen and the genes of interest were selected. The data was visualized using a linear scale from a total of 1860 array experiments. TMeV (TIGR Multiple Experiment Viewer) programme was used to cluster the genes and stresses, and Euclidean distance and complete linkage were chosen for the hierarchal clustering (Eisen et al. 1998; Saeed et al. 2003).

Results

The *Arabidopsis* thiolase gene families

Searches of the *Arabidopsis* genome identify five loci with sequence similarity to genes encoding known thiolase proteins (Germain et al. 2001). Comparison of predicted amino acid sequences shows that they fall into two classes. Three loci encode the Type I class of enzyme, KAT 1, 2 and 5, typically involved in

acetyl-CoA formation in fatty acid β -oxidation by removal of a 2-carbon chain from a 3-ketoacyl-CoA. The other two genes encode the type II class of enzyme, ACAT 1 and 2, typically involved in acetoacetyl-CoA formation from two molecules of acetyl-CoA (Fig. 1A, Supplementary Fig. 1A). The type I genes have previously been annotated as *KATI*, *KAT2* and *KAT5* (3-ketoacyl-CoA thiolase) (Germain et al. 2001), based on the chromosome on which they are found (At1g04710, At2g33150 and At5g48880, respectively). All three are closely related to known peroxisomal located type I thiolases in human, mouse and yeast and this cluster also contains representatives from the fungus *Neurospora crassa* and the monocot rice (*Oryza sativa*). The matrix and membrane-bound type I mitochondrial thiolases involved in fatty acid degradation in human, mouse and *Drosophila* cluster separately and do not contain members from the completed genome sequences of fungi, Arabidopsis or rice.

The two Arabidopsis type II thiolases (here referred to as *ACAT1* and *ACAT2*, At5g47720 and At5g48230, respectively) cluster with the known cytosolic type II thiolases from yeast and the cytosolic and mitochondrial type II thiolases from human, mouse and *Drosophila*. The monocot rice also has two type II thiolases that cluster in this set and *N. crassa* contains a single type II gene that clusters with the yeast cytosolic type II protein.

The sequence divergence of mitochondrial type I thiolases in mammals from the peroxisomal type I KATs in plants, fungi and animals makes the presence of *KAT2* in Arabidopsis mitochondria appear unlikely based on phylogenetic evidence if thiolase location is conserved. However, the sequence analysis does not define the location of the type II ACAT proteins in Arabidopsis and leaves open the possibility of a mitochondrial location of at least one of these proteins, especially given the presence of multiple type II thiolases in both plants and mammals.

Definition of the number of products from each Arabidopsis thiolase gene

Analysis of EST sequences and tiling array data shows that *KATI* and *KAT2* loci each encode single polypeptide sequences (Fig. 1B) (Mockler et al. 2005; Yamada et al. 2003). In contrast, *KAT5* encodes two proteins that differ at the N-terminus due to alternative RNA splicing that generates either 13 (*KAT5.1*) or 14 (*KAT5.2*) exons (Fig. 1B). The N-terminal sequences of proteins encoded by *KATI*, *KAT2* and *KAT5.2* include putative PTS2-type sequences, while the protein encoded by *KAT5.1* does not (Fig. 1B).

Fig. 1 Classification of thiolase (*KAT* and *ACAT*) genes and gene structure in Arabidopsis. **(A)** A phylogenetic tree was generated using the neighbour joining method, using ClustalW of thiolase proteins from a variety of organisms. *KAT* = 3-ketoacyl-CoA thiolases, *ACAT* = acetoacetyl-CoA thiolases. **(B)** Gene structure and predicted proteins encoded by *thiolase* genes. The differences in the proteins encoded by each locus are indicated in bold where evidence for more than one cDNA exists. The open white boxes indicate exons

Interestingly, proteins encoded by *KATI*, *KAT2*, *KAT5.1* and *KAT5.2* proteins are predicted to be targeted to mitochondria by at least two of three different programs (Table 1). Analysis of EST sequences and tiling array data shows that *ACAT1* and *ACAT2* loci also encode three and two proteins respectively (Fig. 1B). Differential RNA splicing results in the protein encoded by *ACAT1.1* lacking ten amino acids at the C-terminus relative to the protein encoded by *ACAT1.2*. The protein *ACAT1.3* has 20 different amino acid residues at the N-terminus relative to *ACAT1.1*. None of the proteins has predicted organelle-targeting information (Table 1). Differential RNA splicing also accounts for the N-terminal 6 amino acid residues of the protein encoded by *ACAT2.1* being replaced by 11 different amino acid residues in the case of the protein encoded by *ACAT2.2* (Fig. 1B). Neither protein has predicted organelle-targeting information (Table 1).

Targeting of type I and II thiolases in vivo

To localize thiolases in vivo, GFP and RFP fusions were employed. GFP and RFP have been used extensively to study protein targeting to mitochondria, peroxisomes and chloroplasts (Heazlewood et al. 2005). To demonstrate specific mitochondrial and peroxisomal targeting in vivo and our ability to distinguish the two, an AOX-GFP construct (Lee and Whelan 2004) and an RFP-PTS1 construct (Pracharoenwattana et al. 2005), were employed. The two gene constructs were co-delivered into Arabidopsis suspension culture cells using a biolistic gene gun (Thirkettle-Watts et al. 2003). After 48 h individual cells expressing both GFP and RFP fluorescence were imaged. The results show that GFP and RFP were targeted to discrete organelles consistent with specific targeting to mitochondria and peroxisomes respectively (Fig. 2).

To examine thiolase targeting we made translational fusions with GFP at the C-terminus since peroxisomal (PTS2) and mitochondrial targeting sequences are both N-terminal. Thiolase cDNAs encoding all nine proteins were linked to the GFP coding region and cloned downstream of the CaMV 35S promoter. They were

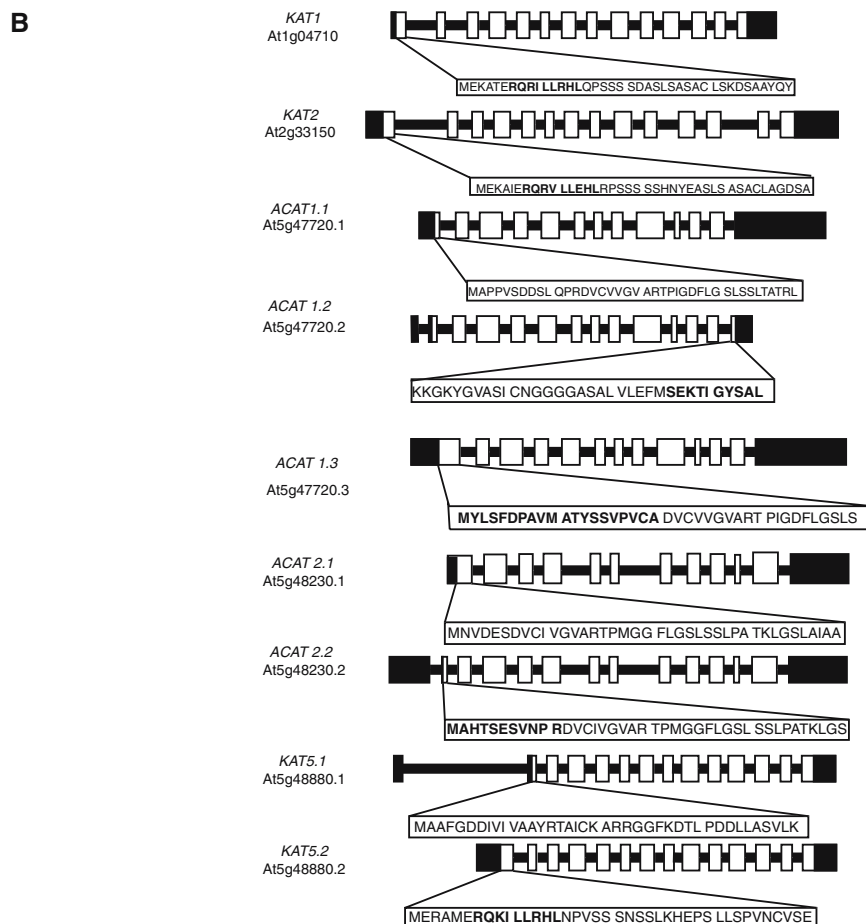
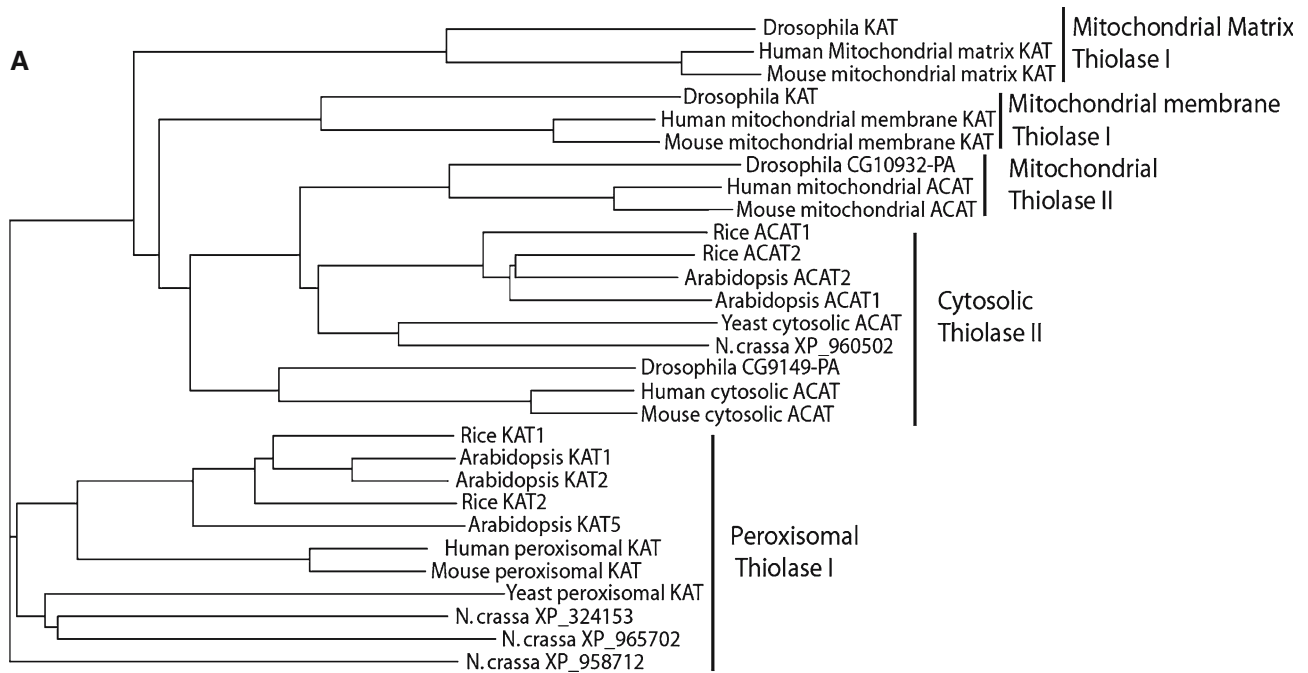


Table 1 Summary of the subcellular location of thiolase proteins

| Protein | Locus | Target prediction | Peroxisomal targeting | Proteomic | In vivo | In vitro | Location | Function |
|----------|-------------|-------------------|-----------------------|--|---------|----------|------------|------------------------|
| KAT 1 | At1g04710 | M | PTS2 | | P | NM | Peroxisome | |
| KAT 2 | At2g33150 | M | PTS2 | M ^{a,b} , C ^c , N ^d | P | NM | Peroxisome | β -oxidation |
| KAT 5.1 | At5g48880.1 | M | – | | NT | NM | Cytosol | Flavonoid biosynthesis |
| KAT 5.2 | At5g48880.2 | M | PTS2 | | P | NM | Peroxisome | Flavonoid biosynthesis |
| ACAT 1.1 | At5g47720.1 | None | – | | NT | NM | Cytosol | |
| ACAT 1.2 | At5g47720.2 | None | – | | NT | NM | Cytosol | |
| ACAT 1.3 | At5g47720.3 | None | – | | P | NM | Peroxisome | |
| ACAT 2.1 | At5g48320.1 | None | – | | NT | NM | Cytosol | Mevalonate pathway |
| ACAT 2.2 | At5g48230.2 | None | – | | NT | NM | Cytosol | Mevalonate pathway |

Targeting prediction = M (mitochondria) if two or more predictions indicate a mitochondrial location. Peroxisomal Targeting = the presence of a Type 1 or 2 peroxisomal targeting signal. Proteomic = evidence for location from independent proteomic studies, M = Mitochondria, C = chloroplast and N = nuclear.

^{a,b} Krufft et al (2001) and Heazlewood et al (2004), ^c Kleffmann et al (2004), ^d Pendle et al (2005). In vivo = targeting ability as by GFP tagging, P = peroxisomal and NT = no targeting. In vitro tested ability to target to mitochondria, NM = not taken up into isolated mitochondria. Final column indicates the location concluded and suggested role in metabolism

each delivered into Arabidopsis suspension culture cells together with the RFP–PTS1 construct. After 48 h individual cells expressing both GFP and RFP fluorescence were imaged, and the images merged. The results show that KAT1, KAT2 and KAT5.2 and ACAT 1.3 were targeted to peroxisomes, as indicated by coincidence of GFP and RFP images (Fig. 2). In contrast, ACAT1.1, ACAT1.2, ACAT2.1, ACAT2.2 and KAT5.1 show diffuse fluorescence throughout the cell indicating that no specific targeting to any organelle has occurred, suggesting a cytosolic localization. With peroxisomal targeting of KAT1, KAT2, KAT5.2 and ACAT 1.3, although it was apparent that the patterns of GFP and RFP were essentially identical, the higher intensity of the former means that when merged the green fluorescence was dominant in some cells.

Protein import into isolated mitochondria

None of the thiolases were apparently targeted to mitochondria in vivo. However, it is possible that uptake was prevented by the GFP fusion, or that mitochondria normally take up less thiolase than peroxisomes, such that GFP fluorescence from mitochondria did not reach an intensity to be detected. To test these possibilities we examined the ability of isolated mitochondria to take up all nine thiolases. Each protein was synthesized in a rabbit reticulocyte lysate translation system in the presence of radiolabelled methionine, and then tested for import into mitochondria isolated from Arabidopsis cell cultures. As a control the import and processing of AOX and Rubisco SSU were examined, the former as a positive control for import and the latter as a control to demonstrate

the specificity of import into isolated mitochondria (Chew et al. 2003; Chew and Whelan 2004). In this case the AOX precursor protein (36 kDa) was imported and cleaved to a mature protein (32 kDa) as previously demonstrated (Fig. 3, lanes 1 and 2) (Whelan et al. 1995). Addition of protease resulted in the 32-kDa mature form being resistant to protease digestion indicating uptake by mitochondria. This resistance was abolished by the addition of valinomycin that dissipates the inner membrane potential (Fig. 3, lanes 4 and 5) (Tanudji et al. 2001). The phosphate translocator from maize was used as an additional control, after uptake into mitochondria and rupture of the outer membrane protease digestion results in a small cleaved protected fragment of 33 kDa, indicating that the added protease has access to inside the outer membrane (Bathgate et al. 1989; Murcha et al. 2004, 2005; Winning et al. 1992). In contrast to the mitochondrial controls, Rubisco SSU was not protected from protease digestion indicating it was not imported into mitochondria (Fig. 3).

When the nine thiolase proteins were tested for mitochondrial uptake two distinct patterns were observed, KAT1, ACAT 1.1, ACAT 1.2, ACAT 1.3, KAT5.1 and KAT5.2 did not yield any protease protected products upon incubation with mitochondria and thus were deemed not to be imported (Fig. 3). KAT2, ACAT2.1 and ACAT2.2 yielded resistant products upon incubation with mitochondria. Although KAT2 was not proteolytically cleaved by mitochondria, a protease resistant product with a lower mol mass was obtained when mitochondria were treated with Proteinase K (Fig. 3, lanes 1–3). Notably this was also generated in the presence of valinomycin (Fig. 3, lanes 4–5). However, upon rupture of the outer

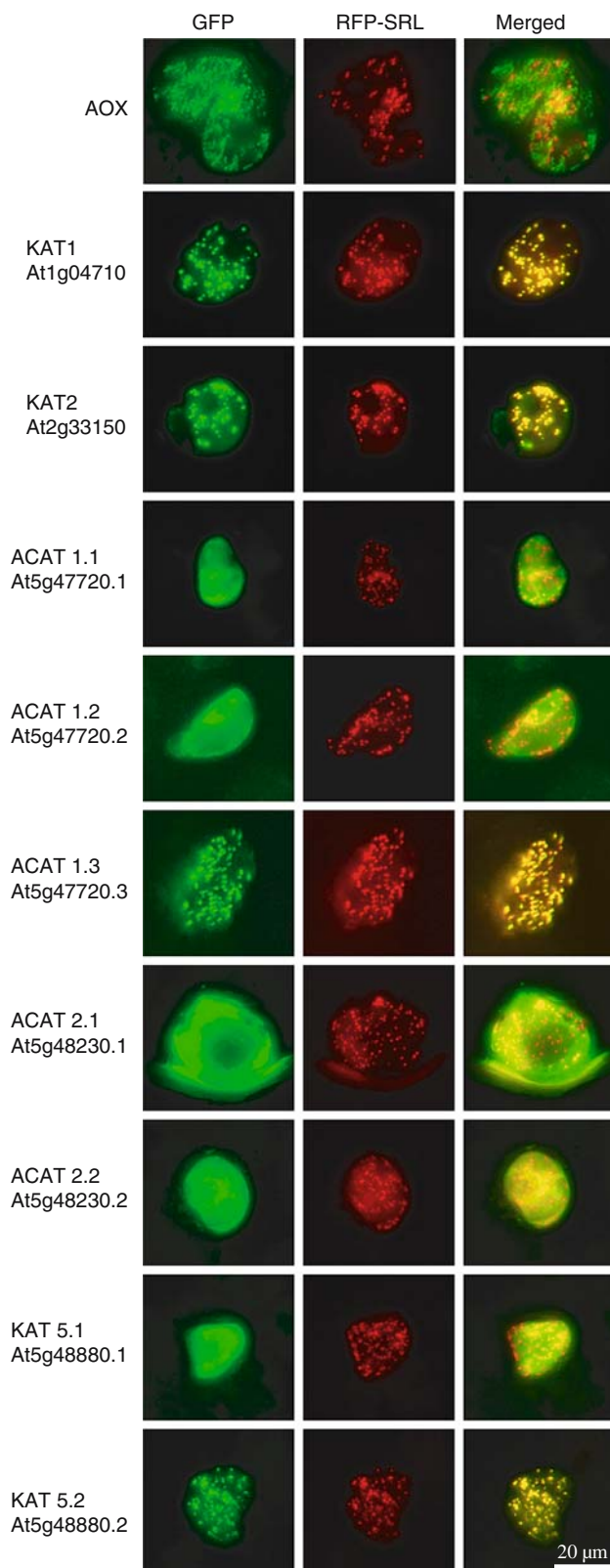


Fig. 2 In vivo targeting ability of thiolases in Arabidopsis. The cDNA coding sequences of thiolase from Arabidopsis were tagged with GFP to assess targeting ability. Each cell shown was transformed with both a GFP construct and with RFP with a type I PTS. Each panel shows the localization of GFP targeted either by the mitochondrial protein alternative oxidase (AOX) or by thiolases (GFP panel). A peroxisomal pattern obtained in the same cell with the RFP with a type I PTS is shown (RFP-SRL panel) together with the merged images (Merged panel)

membrane this product was absent (Fig. 3, lanes 6–9). In the case of ACAT2.1 and ACAT2.2 a similar pattern was observed except that the protected protein had the same molecular mass as the protein added to the import assay (Fig. 3, lanes 1–5). Again with rupture of the outer membrane no protease protection was observed (Fig. 3, lanes 6–9).

Although the protease protected fragments produced upon incubation of KAT2, ACAT2.1 and ACAT2.2 may suggest uptake by mitochondria, their presence when valinomycin was added to the import assay and their sensitivity when the outer mitochondrial membrane is ruptured suggests that they may represent protease resistant products in the presence of intact mitochondria. The protease susceptibility of KAT2, ACAT2.1 and ACAT2.2 was tested by the ability of added protease to digest the radiolabelled precursor protein. Incubation of KAT2.1, ACAT2.1 and ACAT2.2 with proteinase K alone indicated that they were resistant to protease digestion; in contrast AOX was completely digested (Fig. 4). Thus it was concluded that there was no uptake of any radiolabelled thiolase proteins into isolated mitochondria, in agreement with the GFP targeting (Fig. 2).

Co-expression analysis

The probable functions of these type I and type II thiolases in their determined subcellular locations was examined by analysis of co-expression of these genes in microarray data from Arabidopsis (Fig. 5). We used the Expression Angler co-expression correlation tool from the Botany Array Resource (Toufighi et al. 2005) to find the most co-expressed genes based on microarray hybridization data on Arabidopsis 22K genechips; the top 25 co-expressed genes are shown in each case (Fig. 5A, Supplementary Table 1). This analysis shows that *KAT2* co-expresses (Correlation >0.65–0.79) more highly with a range of peroxisomal fatty acid degradation components in the peroxisome than with any other nuclear genes in Arabidopsis. These included the fatty acid multifunction protein MFP2 (At3g06860), citrate synthase (At2g42790), acyl-CoA oxidases (At5g65110, At3g51840) and enoyl-CoA hydratase (At4g16210)

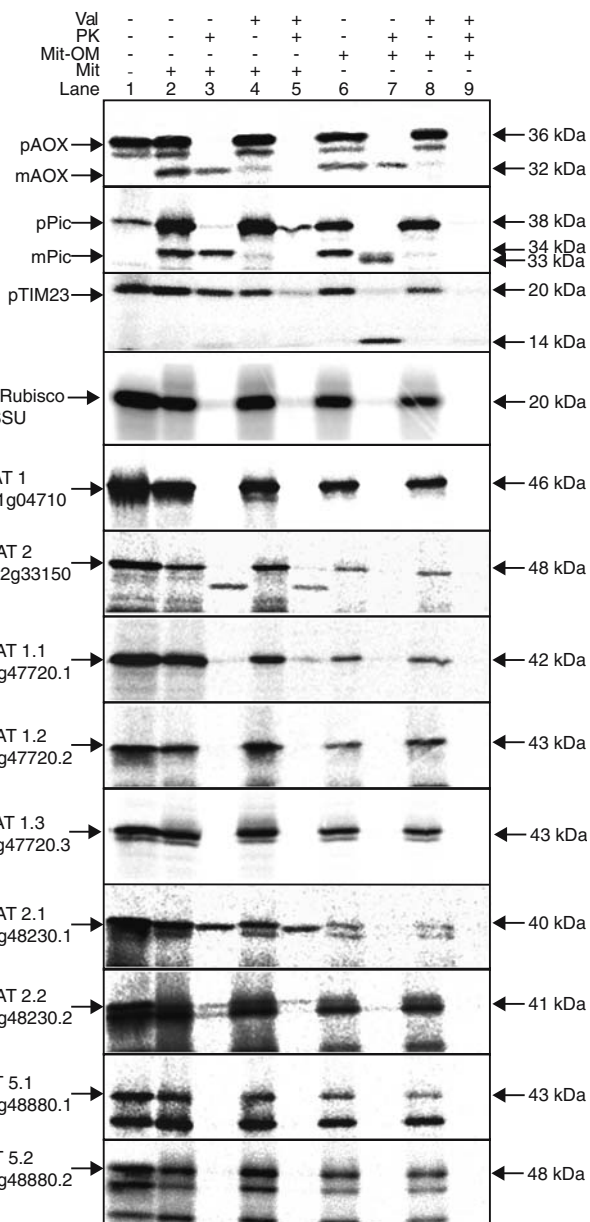


Fig. 3 In vitro import of radiolabelled thiolase proteins into mitochondria isolated from Arabidopsis. Lane 1, precursor protein alone. Lane 2, precursor protein incubated with mitochondria under conditions that support import into mitochondria. Lane 3, as lane 2 with proteinase K added after incubation of precursor with mitochondria. Lane 4 and 5, as lane 2 and 3 with valinomycin added to the import assay prior to the addition of precursor protein. Lanes 6–9 as 2–5 except that the mitochondrial outer membrane was ruptured after the incubation period with precursor protein but prior to addition of proteinase K. Apparent mol mass are indicated in kDa. Abbreviations: Mit = mitochondria, Mit-OM = mitochondria with outer membrane ruptured, PK = proteinase K, Val = valinomycin, AOX = alternative oxidase, Pic = phosphate carrier, Rubisco SSU = small subunit of ribulose-1, 5 bisphosphate carboxylase/oxygenase, p = precursor protein band, m = mature protein band

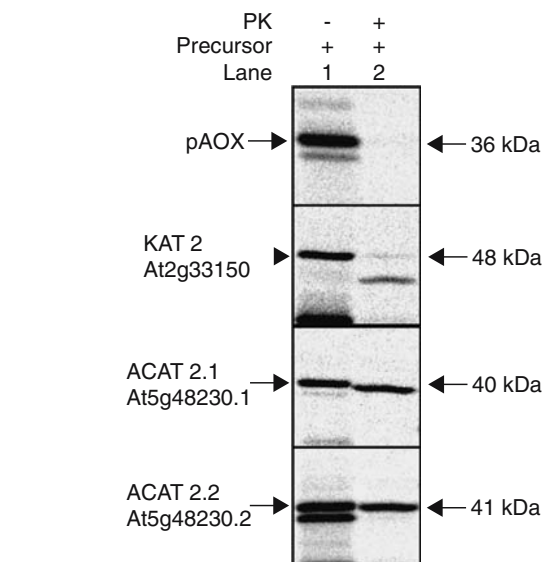


Fig. 4 Protease susceptibility of KAT 2, ACAT 2.1 and ACAT 2.2. The ability of proteinase K to digest thiolases was tested by incubating the protease with radiolabelled protein. Alternative oxidase was used as a control and apparent mol mass are indicated in kDa

(Fig. 5A). Curiously, Expression Angler showed *KAT5* is not co-expressed with β -oxidation enzymes, but instead with a series of flavonoid biosynthesis enzymes (Correlation >0.6–0.71), including flavanone 3-hydroxylase (At3g51240) 4-coumarate CoA ligase (At1g65060), chalcone synthase (At5g13930), chalcone isomerase (At5g05270). Notably this pathway requires short acyl-CoAs for biosynthesis.

The gene encoding a type II enzyme ACAT2 (At5g48230) was found by Expression Angler to be co-expressed with a range of genes, but notably, hydroxymethylglutaryl-CoA synthase (At4g11820) and mevalonate diphosphate decarboxylase (At2g38700, At3g54250) were highly co-expressed (Correlation >0.80) (Fig. 5, Supplementary Table 1). This is consistent with the role of type II genes in the cytosolic mevalonate pathway leading to isoprene-containing compounds such as sterols and terpenoids.

The isoleucine catabolism pathway involves the branched chain amino acid dehydrogenase complex (At5g09300, At3g13450, At3g06850), isovaleryl-CoA dehydrogenase (At3g45300), enoyl-CoA hydratase (At4g31810) in mitochondria, and then 3-hydroxy-2-methylbutyryl-CoA dehydrogenases and the fatty acid multifunction proteins (At4g29010, At3g06860, At3g15290), in addition to a thiolase, but these fore-mentioned genes do not appear to be co-expressed with any of the thiolase genes (data not shown).

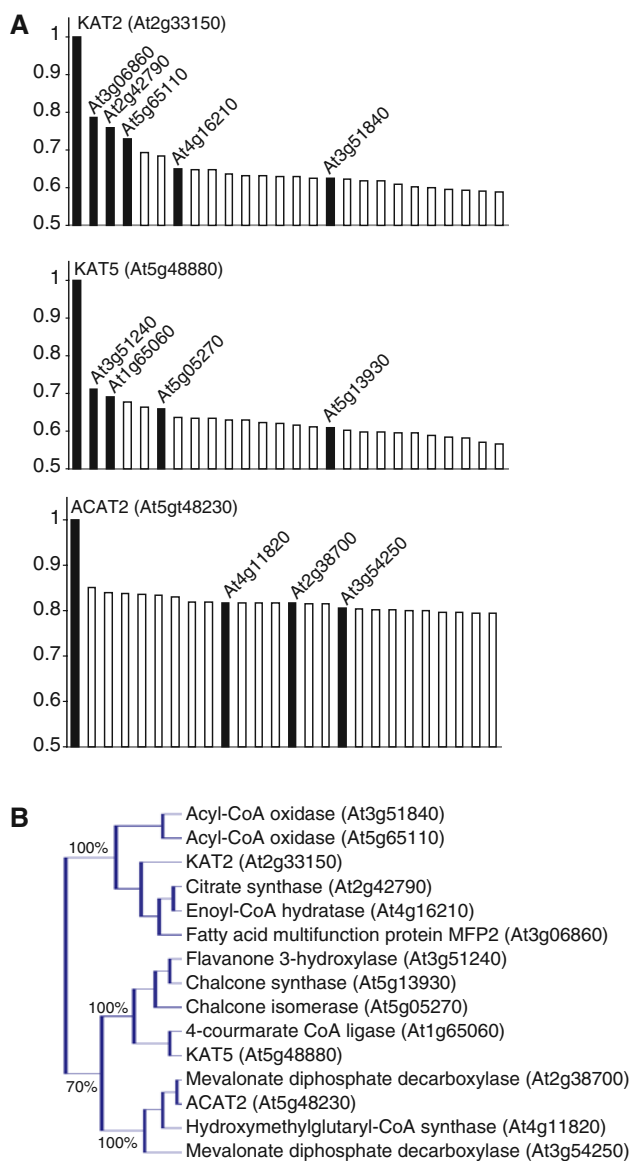


Fig. 5 In silico expression analysis. **(A)** The 25 genes co-expressed to the greatest extent with each thiolase gene were determined using the Expression Angler tool from the Botany Array Resource. The annotated genes indicated for *KAT2*, *KAT5* and *ACAT2* are those for which the proteins encoded by these genes could function in peroxisomal fatty acid degradation, flavonoid biosynthesis and the mevalonate pathway, respectively. **(B)** Clustering analysis of co-expressed genes with *KAT2*, *KAT5* and *ACAT2* to determine which (*KAT* or *ACAT*) branch with co-expressed genes as determined by Expression Angler. Each *KAT* or *ACAT* is located in a distinct group in agreement with analysis by Expression Angler, with good bootstrap values supporting the branch points

To confirm the co-expression groups in Fig. 5A, we used Genevestigator (Zimmermann et al. 2004) to cluster *KAT2*, *KAT5* and *ACAT2* and their cohort of highlighted co-expressed genes across a series of microarray data based on tissue specific expression (Fig. 5B). This bootstrapped cluster tree showed three

separate groupings of genes, confirming the Expression Angler analysis of distinct expression patterns of these three thiolases, correlating with distinct roles in metabolism. Note this type of expression analysis cannot distinguish differential roles for isoforms of proteins resulting from alternative splicing as the probe sets used to determine expression do not distinguish between splice forms.

Discussion

Table 1 summarizes the results of our knowledge on the subcellular localization of thiolase protein in Arabidopsis. Although some thiolase proteins contain a predicted mitochondrial targeting signal both in vitro and in vivo protein localization assays indicate that they are not imported into mitochondria. This conflicts with proteome analysis of isolated mitochondria suggesting a mitochondrial localization for *KAT2* (Heazlewood et al. 2004; Krufft et al. 2001). We propose that this is due to contamination by peroxisomal proteins and that *KAT2* is not an authentic mitochondrial protein. Heazlewood et al (2004) reported a low level of contamination of their mitochondrial samples with peroxisomes, consistent with the potential for some false positive identifications in this shotgun proteomic study. The apparent requirement of a thiolase for isoleucine catabolism in mitochondria (Taylor et al. 2004) is not a strong argument for the role of *KAT2* in mitochondria, as mitochondrial type I enzymes in animals are structurally distinct from Arabidopsis *KATs* (Fig. 1). The terminal step and isoleucine degradation might be best served by the type II rather than a type I enzyme, and we have now shown convincingly that type II thiolases are in the cytosol and/or peroxisome in Arabidopsis (Fig. 2).

Our data suggests that at least in Arabidopsis, thiolases involved in β -oxidation are not present in mitochondria, despite the fact that some biochemical evidence has suggested this may take place in mitochondria of pea (Masterson and Wood 2001). The results presented here however cannot be definitive for all plant species as it is possible that genes encoding thiolases in other plant species may have mitochondrial targeting ability due to the fact that at least some thiolase genes in Arabidopsis encode proteins that have predicted mitochondrial targeting ability. Thus relatively small changes are likely required to achieve mitochondrial targeting of plant thiolases, as has been observed with some peroxisomal thiolases from other organisms (Danpure et al. 2003; Tsukamoto et al. 1994).

The data on enzymes required for isoleucine degradation from 2-methyl-3-hydroxybutyryl-CoA through to propionyl-CoA increasingly suggests that this part of the biochemical pathway is a non-mitochondrial activity. Of the three 3-hydroxy-2-methylbutyryl-CoA dehydrogenases in *Arabidopsis* (At4g29010, At3g06860, At3g15290), At3g06860 has been located to peroxisomes by three separate reports using GFP tagging (Cutler et al. 2000; Koh et al. 2005; Tian et al. 2004) and At3g15290 has been located to chloroplasts by mass spectrometry (Kleffmann et al. 2004). The type II ACAT thiolases are all non-mitochondrial (Fig. 2), being present in either the cytosol or peroxisome from our own data. Transport of 2-methyl-3-hydroxybutyryl-CoA out of mitochondria has not been investigated, but the substrate specificity of an array of known mitochondrial carriers from the Mitochondrial Carrier Protein (MCP) family, the Preprotein and Amino acid Transporter (PRAT) family and ATP Binding Cassette (ABC) transporters remain to be studied in *Arabidopsis* (Pohlmeier et al. 1997; Rassow et al. 1999; Brugiere et al. 2004; Picault et al. 2004). The distribution of pathways of amino acid biosynthesis and metabolism between organelles and the cytosol is relatively common in plants, but in the case of isoleucine metabolism, although the metabolic enzymes involved are now relatively clear, the transport activities that facilitate this pathway between mitochondria, the cytosol and the peroxisome remain to be elucidated.

Co-expression analysis of transcript data can be a powerful tool to confirm other data or provide leads for further analysis. In this case, the co-expression results for KAT2 are consistent with all our experimental data. This gene encodes a peroxisomal thiolase and is co-expressed with other peroxisomal proteins involved in the same process, namely β -oxidation of fatty acids. For ACAT2 the subcellular location, enzyme class and co-expression also coincide to suggest a role in mevalonate biosynthesis leading to isoprenes. The KAT5 co-expression result was a surprise as this protein was suspected to be involved in β -oxidation based on its enzyme class. However, the different location of KAT5.1 and KAT5.2 (Table 1), the fact that KAT5 does not maintain β -oxidation in seedlings of the KAT knockout but can partially complement for the lack of KAT2 when driven by 35S expression (Germain et al 2001), and the co-expression link with flavonoid biosynthesis rather than β -oxidation genes (Fig. 5), suggests that while KAT5 encodes a thiolase, it has a distinct role to KAT2 in acyl-CoA metabolism in plants.

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