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Conservation of two lineages of peroxisomal (Type I) 3-ketoacyl-CoA thiolases in land plants, specialization of the genes in Brassicaceae, and characterization of their expression in *Arabidopsis thaliana*

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Abstract

***Arabidopsis thaliana* has three genes encoding type I 3-ketoacyl-CoA thiolases (*KAT1, KAT2,* and *KAT5*), one of which (*KAT5*) is alternatively transcribed to produce both peroxisomal and cytosolic proteins. To evaluate the potential importance of these four gene products, their evolutionary history in plants and their expression patterns in *Arabidopsis* were investigated. Land plants as a whole have gene lineages corresponding to *KAT2* and *KAT5*, implying conservation of distinct functions for these two genes. By contrast, analysis of synteny shows that *KAT1* arose by duplication of the *KAT2* locus. KAT1 is found in the Brassicaceae family, including in the genera *Arabidopsis, Capsella, Thellungiella* (=*Eutrema*) and *Brassica,* but not in the more distantly related Caricaceae (order Brassicales*)*, or other plants*.* Gene expression analysis using qRT-PCR and β-glucuronidase reporter genes showed strong expression of *KAT2* during germination and in many plant tissues throughout the life cycle, consistent with its observed dominant function in fatty acid β-oxidation. *KAT1* was expressed very weakly while *KAT5* was most strongly expressed during flower development and in seedlings after germination. Isoform-specific qRT-PCR analysis and promoter β-glucuronidase reporters revealed that the two splicing variants of *KAT5* have similar expression profiles. Alternative splicing of *KAT5* to produce cytosolic and peroxisomal proteins is specific to and ubiquitous in the Brassicaceae, and possibly had an earlier origin in the order Brassicales. This implies that an additional function for KAT5 arose between 43 and 115 mybp. We speculate that this *KAT5* mutation was recruited for a cytosolic function in secondary metabolism.**

Key words: 3-ketoacyl-CoA thiolase, β-oxidation, Brassicales, evolution, flowering, germination, peroxisome.

Introduction

Germination and seedling establishment in oilseed species such as *Arabidopsis thaliana* require peroxisomal β-oxidation to degrade the seed storage lipids that fuel this stage of development (Eastmond and Graham, 2001; Graham, 2008). As well as its role during seed germination, β-oxidation is a significant pathway for the synthesis of the hormones jasmonic acid (JA) and indole-3-acetic acid (IAA) in plants (Baker *et al.*, 2006), and is required for the turnover of fatty acids in plant cells during development and senescence (Yang and Ohlrogge, 2009). Three core enzymes catalyse peroxisomal β-oxidation: acyl-CoA oxidase (ACX), multifunctional protein (MFP, which can exhibit hydratase, dehydrogenase, epimerase and isomerase activities), and L-3-ketoacyl- CoA thiolase (KAT).

The *ACX* and *MFP* gene families, represented by six and two genes, respectively, have been extensively characterized in *Arabidopsis* (Graham, 2008; Arent *et al.*, 2010). By contrast, of

the three *KAT* genes, only the role of KAT2 has been investigated in detail (Hayashi *et al.*, 1998; Germain *et al.*, 2001; Footitt *et al.*, 2007a). Thiolases of two types are distinguished. Type 1 enzymes (KAT: EC 2.3.1.16) are typically peroxisomal and catalyse the thiolysis of acetyl-CoA units from the thiol end of the fatty acyl-CoA during fatty acid catabolism. Type 2 enzymes (acetyl-CoA acetyltransferase or ACAT; EC 2.3.1.9) are typically cytosolic and are involved in acetoacetyl CoA synthesis in the mevalonate biosynthesis pathway. The three *KAT* genes in *Arabidopsis* were designated as *KAT1* (At1g04710), *KAT2* (At2g33150), and *KAT5* (At5g48880) based on the chromosome on which they are located (Germain *et al.*, 2001). Subsequent analysis has determined that while both *KAT1* and *KAT2* encode single peroxisome targeted proteins, *KAT5* encodes the cytosolic KAT5.1 and the peroxisomal KAT5.2 isoforms. The $KAT5.2_{px}$ transcript differs from $KAT5.1_{cyt}$ in the 5' region, with an additional exon encoding a peroxisome targeting signal type 2 (PTS2), and alternate transcription and translation start sites (Carrie *et al.*, 2007).

Genes involved in lipid mobilization, including β-oxidation, the glyoxylate cycle, and gluconeogenesis, are expressed co-ordinately during early seedling growth in *Arabidopsis*, with transcript levels and enzyme activities peaking at 48 h after the commencement of germination (Rylott *et al.*, 2001). Of the three *Arabidopsis KAT* genes, expression of *KAT2* is dominant during this stage of the life cycle, with *KAT2* transcript levels far more abundant than *KAT1* and *KAT5* (Germain *et al.*, 2001; Kamada *et al.*, 2003). Genes of lipid mobilization decline in expression level in the late stages of seedling establishment, a process associated with peroxisome matrix remodelling as the function of the organelle changes from one primarily concerned with oil mobilisation to metabolism associated with photosynthesis (Rylott *et al.*, 2001; Kamada *et al.*, 2003; Pracharoenwattana and Smith, 2008; Lingard *et al.*, 2009). There is growing evidence for the functional importance of β-oxidation in reproductive tissue and seed development (Richmond and Bleecker, 1999; Rylott *et al.*, 2003, 2006; Chia *et al.*, 2005; Footitt *et al.*, 2007b; Schilmiller *et al.*, 2007). Expression of the *Arabidopsis KAT* genes has been detected in reproductive tissue (Kamada *et al.*, 2003), however, detailed analysis has not been reported, nor have $KAT5.1_{cyt}$ and $KAT5.2_{px}$ transcript expression patterns been distinguished. To characterize the function of members of the *KAT* gene family further, the phylogenetic relationships of the genes in sequenced plant genomes was investigated and a detailed analysis of their patterns of expression in *Arabidopsis thaliana* was conducted. Comparative genomics has highlighted the dynamic expansion, specialization, and contraction of plant genomes (Wang *et al.*, 2011; Rutter *et al.*, 2012) and evolution of the *KAT* gene family in the Brassicaceae provides an excellent and specific example of this.

Materials and methods

Bioinformatics

KAT protein sequences were retrieved from the collection of sequenced plant genomes at Phytozome v8.0 (http://www.phytozome.org) and as individually submitted sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov). *Thellungiella parvula* (*Eutrema parvulum*) KAT sequences were obtained from the Compare Genomes site (http://genomevolution.org/CoGe/index.pl). Sequence visualization and manipulations were done using the Geneious (v5.4.6) package (Drummond *et al.*, 2011). Multiple sequence alignment of full-length predicted protein sequences was made in Geneious using the MAFFT Alignment plug-in (Katoh *et al.*, 2002) and phylogenetic analysis of this alignment was done using the PHYML plugin (Guindon and Gascuel, 2003) with the WAG substitution model and 1000 bootstrap replicates. Synteny analysis was done using the CoGe and SynMap (http://genomevolution.org/CoGe/index.pl) comparative genome analysis tools (Lyons *et al.*, 2008).

GUS promoter reporter analysis

KAT gene promoter sequences, including the 5'-UTR and intergenic DNA upstream of the start codon, were amplified by PCR from wild-type Col-0 genomic DNA for Gateway cloning. Primer sequences are given in Supplementary Table S1 at *JXB* online. Amplified promoter fragments extended approximately 2 kb upstream from the start codons (2156 bp for the *KAT2* promoter, 2151 bp for $KAT5.1_{cyt}$, and 2125 bp for $KAT5.2_{px}$) or until the adjacent gene (*KAT1*, 1010 bp) as detailed in Figure 3A. Promoters were cloned into the GUS/GFP reporter plasmid pHGWFS7 (Karimi *et al.*, 2002). *Agrobacterium* strain CV3130-C58 harbouring the pHGWFS7-KAT promoter vectors was used to transform *Arabidopsis* Col-0 plants using the floral dip method of Clough and Bent (1998). Transformed plants were screened for homozygosity over three generations by selection on hygromycin. Plants were grown for GUS staining under continuous light conditions to control for the diurnal regulation of promoter activity. Seedlings were grown on half-strength MS media (without sucrose). Flowers and siliques were removed from 6-week-old soil-grown plants and stained for GUS expression (Weigel and Glazebrook, 2002).

Quantitative RT-PCR

Aerial tissue samples (about 100 mg) were taken from 5-week-old *Arabidopsis* Col-0 plants grown in soil under continuous light conditions, and root samples were taken from hydroponically grown plants. Germinating seed samples utilized approximately 20 mg of dry seed that had been spread on plates containing half-strength MS media then stratified for 48 h before being transferred to continuous light. Samples were ground into a fine powder with a mortar and pestle pre-cooled with liquid nitrogen. Germinating seed RNA was extracted using the RNAqueous Kit with Plant RNA Isolation Aid (Ambion) and included LiCl precipitation. RNA from other plant tissues was isolated using an Aurum (Bio-Rad) kit. RNA was treated with Turbo DNA-free DNase (Ambion) and 1 μg used as the template for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad). cDNAs were diluted 1/5 for quantitative PCR. Primer pairs for qRT-PCR that spanned introns were designed using Primer3. Due to the similarity of the $KAT5.1_{cyt}$ and $KAT5.2_{px}$ sequences, primers were designed in the respective 5' UTRs and therefore could not bound introns. Primer sequences are listed in Supplementary Table S1 at *JXB* online.

qRT-PCR was performed on a Roche LC480. The reaction volume was 5 μl and included 1× LightCycler 480 SYBR Green I Master (Roche), 0.5 μl diluted cDNA, and 0.1 μl of 20 μM primers. Cycle conditions were: 95 °C for 10 min; 45 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Melt curve analysis of real-time PCR products was performed to verify amplification of a single product. Crossing point values were calculated under high confidence. Four biological replicates per tissue sample were examined, with at least two technical replicates of each real-time PCR. The average crossing point value of two technical replicates was used to calculate expression relative to an internal reference gene adjusted by primer efficiencies. Two reference genes were tested: *ACT2* (At3g18780) and the clathrin adaptor complex subunit (*CACS*; At5g46630), identified by Czechowski *et al.* (2005) as a more suitable reference gene for transcript normalization. For analysis, *CACS* was used for normalization; the average relative expression and standard error for the four biological replicates are shown.

Results

KAT2 and KAT5 isoforms are conserved in higher plants

KAT protein sequences were obtained from Phytozome v8.0 to investigate the evolutionary history of the gene family. The Phytozome database includes genome sequences of 31 plant species that encompass major clades of plant (Viridiplantae) evolution, including green algae, mosses, spikemosses (lycophytes), monocot grasses, eudicots, and dicots. Interrogation of this database using *Arabidopsis* KAT2 (At3g33150) in a BLAST query yielded 82 protein sequences. This number was reduced to 71 hits (see Supplementary Table S2 at *JXB* online) after exclusion of proteins with obviously poor matches and partial alignments. KAT sequences from *Brassica napus* and *Thellungiella parvula* (which are not represented in Phytozome v8.0) were obtained from NCBI and CoGe, respectively, to yield a total of 76 KAT protein sequences from 33 species.

There was at least one KAT isoform encoded in each of the 33 genomes, with the majority (29/33) encoding two or more (see Supplementary Table S2 at *JXB* online). Notably, both green alga species (*Volvox carteri* and *Chlamydomonas reinhardtii*) only possessed a single gene. The exon–intron structure of the genes is highly conserved in land plants, with almost all of the *KAT* coding sequences from higher plants being assembled from 14 exons that span similar regions of the protein in each species (see Supplementary Table S2 at *JXB* online). In land plants, β-oxidation is a peroxisomal process and KAT proteins would thus be expected to localize to that organelle. Accordingly, 74/76 proteins possess a predicted peroxisome targeting signal 2 (PTS2) close to the N-terminus, the only exceptions being one of the two *Selaginella moellendorffii* isozymes (see Supplementary Table S2 at *JXB* online, Taxon #6) and *C. reinhardtii*, in which the KAT protein includes an incomplete PTS2 (RLAVLSRQF) (see Supplementary Table S2 at *JXB* online, Taxon #2). *C. reinhardtii* (and some other chlorophytes) have previously been noted to have peroxisomes that, although capable of PTS1 and PTS2-mediated import (Hayashi and Shinozaki, 2012), differ significantly from those in other plant lineages in lacking both catalase, which is found in their mitochondria, and glycolate oxidase, which is functionally replaced by a mitochondrial glycolate dehydrogenase (Kato *et al.*, 1997; Atteia *et al.*, 2009). In addition, a number of enzymes of β-oxidation have been identified in the *C. reinhardtii* mitochondrial proteome, including KAT, an acyl-CoA dehydrogenase (ACAD; EC 1.3.99.3), an enoyl-CoA hydratase (E-CoAH1; EC 4.2.1.17), but not acyl activating enzymes or acyl-CoA oxidases (Atteia *et al.*, 2009).

The amino acid multiple sequence alignment of plant KAT protein sequences was analysed to generate a maximum likelihood phylogenetic tree (Fig. 1). Although many other plant thiolases are available via Genbank, the analysis was restricted to those from sequenced genomes available at Phytozome and CoGe so as to obtain the best picture of KAT diversity within and between species. The only exception to this was the inclusion of the two KAT sequences available from *B. napus* for which the full genome sequence is not yet available that were included due to their special relevance to evolution of the family in the Brassicales. The

tree was rooted with the KATs from the green algae *V. carteri* and *C. reinhardtii*. KAT sequences of higher plants were divided into two relatively well-supported clades (Fig. 1). These clades were denoted KAT2 and KAT5 corresponding to the *Arabidopsis* KAT isozyme they contained. The taxonomic orders of the species are indicated on the tree for clarity. KAT proteins from the Poales formed two discrete clusters, one embedded in the KAT5 clade, and the second resolved basal to KATs of all angiosperms. We nominally label this later as KAT2 representatives of the Poales. Thus, of the 29 species of higher plants, 27 were represented by two KAT isoforms corresponding to KAT2 and KAT5. The two exceptions (*Medicago truncatula* and *Aquilegia coerulea*; see Supplementary Table S2 at *JXB* online) have only one annotated *KAT* gene. Whether the absence of another *KAT* gene from these genomes is genuine or due to incomplete sequencing or annotation remains to be determined. Interestingly, both moss genomes also encode two KAT proteins, but unlike those of higher plants these were not phylogenetically separated on the tree into KAT2 or KAT5 isoforms.

KAT1 *is a duplication of* KAT2 *unique to the Brassicaceae lineage*

The Brassicales appear to have experienced further duplication of *KAT* genes. The KAT2 cluster (Fig. 1) also contains six species (*Arabidopsis thaliana*, *A. lyrata*, *Capsella rubella*, *Thellungiella halophila*, *T. parvula*, and *Brassica rapa*) with a third *KAT* gene (*KAT1*) in addition to the *KAT2* and *KAT5* paralogues (see Supplementary Table S2 at *JXB* online). *AtKAT1* does not appear to have orthologues other than in the Brassicaceae, suggesting that it is a duplication of *KAT2* peculiar to that lineage. Indeed, there is significant synteny between the regions of *A. thaliana* chromosomes 1 and 2 surrounding the *KAT1* and *KAT2* genes. A fragment of about 2.2 Mbp on chromosome 2 shows nearly complete correspondence of genes with span of 1.3 Mbp on chromosome 1 (see Supplementary Fig. S1A at *JXB* online). It is likely that the duplication between chromosomes 1 and 2 was followed by a localized segmental inversion on one of the chromosomes, because about 170 kb is inverted with respect to the rest of the duplicated region (see Supplementary Fig. S1B at *JXB* online). In contrast, there is no synteny between the *AtKAT2* or *AtKAT1* genomic regions and that of *AtKAT5* (not shown).

These syntenic chromosome arrangements are also found in *A. lyrata*, *T. parvula*, and *C. rubella* around genes corresponding to *KAT1* and *KAT2* (not shown). *BrKAT1* (*B. rapa*) is likely to have had the same origin at *AtKAT1*, but the situation is less clear because the *Brassica* lineage underwent whole genome triplication after the split from the *Arabidopsis* lineage (Mun *et al.*, 2009). There are three clear orthologues of *AtKAT2* in the *B. rapa* genome (Fig. 1; see Supplementary Table S2 at *JXB* online). Genomic regions around these *B. rapa* KAT2 genes display significant synteny with each other and with the *AtKAT2* genomic region (see Supplementary Table S3 at *JXB* online). In addition, there are three other regions displaying synteny with these, only one of which has retained a *KAT* gene. This locus, accession Bra030586 (see Supplementary Table S2 and S3 at

Fig. 1. Phylogenetic analysis of the plant 3-ketoacyl-CoA thiolase (KAT) family proteins. Multiple sequence alignment of full-length KAT protein sequences predicted from sequenced plant genomes was made using MAFFT (Katoh *et al*., 2002) and phylogeny estimated using PHYML (Guindon and Gascuel, 2003). Numbers at the nodes are per cent bootstrap agreement from 1000 replicates. Major

Fig. 2. Alignment of dicotyledonous plant thiolase proteins. Consensus sequences were derived from four groups of sequences comprising dicotyledonous KAT2 or KAT5 and Brassicales KAT2 or KAT5 sequences. AtKAT2 and AtKAT5 are included as samples of specific sequences. Typical plant KAT proteins are about 460 amino acids; the first approximately 200 residues are shown. Highly conserved residues (present in >90% of sequences) in each of the four consensus sequences are shown in capital letters, while moderately conserved residues (present in 50–90% of sequences) in lower case. Non-conserved positions are depicted by a period (.) and gaps introduced into the alignment by a dash (-). Symbols used to indicate residues with strongly similar properties on the Gonnet PAM 250 matrix are: # (N, D, Q or E); % (F or Y);! (I or V). Residues conserved between the different consensus sequences are highlighted with black shading and grey shading indicates consensus sequence residues that are not identical but have similar properties. The red highlighting indicates residues that clearly distinguish KAT5. The blue box shows the PTS2 near the N-terminus of the proteins and the yellow highlighting the alternative initial Met that is conserved in all Brassicales KAT5 proteins.

JXB online) groups with *AtKAT1* in the phylogenetic analysis (Fig. 1). These latter three regions correspond to approximately the same genes duplicated between *KAT2* and *KAT1* in *A. thaliana* (see Supplementary Fig. S1B at *JXB* online). This evidence suggests that the duplication occurred before the *Brassica–Arabidopsis* split (and also before whole genome triplication in *Brassica*). It is also interesting to note that there are six regions in the *B. rapa* genome that display synteny with the *AtKAT5* region (see Supplementary Table S3 at *JXB* online). All correspond to approximately the same span of the *A. thaliana* genome (~620 Mbp), but in *B. rapa* only one of the six regions has retained a *KAT5* gene. There are no such regions of synteny between *CpKAT2* and other genomic regions in *Carica papaya*, a more distantly related species in the order Brassicales. The separation of Brassicaceae, which have maintained a *KAT1* orthologue, from Caricaceae, which have not, can be dated to between 43 and 115 mybp (see inset, Fig. 1); these ages thus provide the upper and lower bounds for the timing of the segmental duplication.

Dual targeting of KAT5 is conserved in Brassicales

AtKAT5 is alternately transcribed to produce two species of mRNA, *KAT5.1_cyt* and *KAT5.2_px* (Fig. 3A). These possess alternative start (Met) codons that direct the encoded proteins to the peroxisome and the cytosol, respectively (Carrie *et al.*, 2007). Further

investigation of the plant *KAT* gene family revealed that all *KAT5* orthologues from the Brassicales (represented by two families in Phytozome; Brassicaceae and Caricaceae), but not other plant orders, have potential alternative start codons in the same relative positions (Fig. 2). Thus, *A. thaliana*, *A. lyrata, Capsella rubella, T. halophila, T. parvula, B. rapa*, and *B. napus* (Brassicaceae) and *Carica papaya* (Caricaceae) *KAT5* orthologues putatively encode KAT5.1 and KAT5.2 isoforms. Examination of PASA assembled ESTs (via Phytozome v8.0) provided, where data were available, evidence for alternate transcription of *KAT5* for each of these species (see Supplementary Table S2 at *JXB* online).

Examination of alignments of plant thiolase proteins highlights differences between KAT2 and KAT5 and between KAT5s of Brassicales and other dicotyledonous species (Fig. 2). Groups of Brassicales KAT sequences that were identified as KAT2 or KAT5 in Fig. 1 were aligned separately using Multalin (http://multalin.toulouse.inra.fr/multalin/multalin.html). Similarly, other dicotyledonous plant KAT2 or KAT5 sequences were aligned (i.e. excluding those from Brassicales) and the four resulting consensus sequences compared (Fig. 2). After the conserved PTS2 (residues 7–15 in Fig. 2), all taxa have a linker that shows little conservation (residues 25–30). The sequence following this (residues 31–51) is highly conserved in all KAT2 proteins and less so in KAT5. Moreover, Brassicales KAT5s differ substantially from the KAT2s and the KAT5s of other dicots in

clades are named after similarity to *Arabidopsis thaliana* KAT2 (At2g33150) and KAT5 (At5g48880) isozymes and taxonomic order of the species is annotated to the right. The length of the branch connecting the ancestral (Volvocales) KATs has been shortened by 50%. Inset: The evolutionary history and timing of Brassicales species represented in the main tree, derived from Beilstein *et al.* (2010), Dassanayake *et al.* (2011), and Mun *et al.* (2009).

this region and it includes the alternate Met described above and a four amino acid deletion relative to all other KAT proteins. Interestingly, although *C. papaya* (a basal Brassicales taxon) possesses the alternate Met in its KAT5 protein, it does not have the four amino acid deletion and the sequence surrounding the second Met is more similar to the typical KAT5 (and KAT2) consensus (not shown). Unfortunately, evidence for or against alternative transcription of *CpKAT5* is not available because the EST collection is small and does not include the 5' ends of the transcripts. A second region that distinguishes between KAT2 and KAT5 isoforms is found in residues 173–192. Here, KAT2 sequences are highly conserved and there is little conservation in the KAT5 sequences (Fig. 2).

Based on protein sequence similarity and EST data, we suggest that alternative transcription and protein localization of KAT5 isoforms is unique to the order Brassicales and originated with a mutation to make a second Met in a lineage ancestral to *C. papaya* (i.e. at least 115 mybp; Fig. 1) followed by further mutation and deletions between 115 and 43 mybp to yield the typical Brassicales KAT5 sequence.

Patterns of KAT *gene expression by* promoter::GUS *reporters*

Eleven *KAT1-GUS*, nine *KAT2-GUS*, ten *KAT5.1-GUS*, and ten *KAT5.2-GUS* independent transformants were obtained and screened for GUS activity. Lines giving GUS staining patterns representative of the group were selected for more detailed analysis. The promoter-reporter construct *KAT5.2-GUS* includes the *KAT5* promoter up to the first start codon joined to the *GUS* reporter gene; *KAT5.1-GUS* includes the 5' UTR and first exon of *KAT5.2* and thus includes the full putative promoter that might be expected to drive independent transcription of *KAT5.1$_{cyt}$* (Fig. 3A).

GUS expression in 2–6-d-old seedlings indicated low levels of expression for *KAT1-GUS* and *KAT5.2-GUS*, with higher levels for *KAT2-GUS* and *KAT5.1-GUS* (Fig. 3B). In *KAT1-GUS* lines, activity was nearly absent but some GUS staining was observed in the root tip (indicated by the arrow in Fig. 3B). *KAT2-GUS* was expressed strongly in the cotyledons and hypocotyls, as well as in the root tip, with this activity diminishing significantly by day four. *KAT5.1-GUS* expression was observed in the cotyledons, but appeared to be stronger near the apical meristem and in the root at day two. Expression in apical parts and the root tip diminished significantly during the five days analysed, but was maintained in the rest of the root. *KAT5.1-GUS* activity was also observed in the new leaves forming at the meristem. *KAT5.2-GUS* had lower expression levels with some staining of the petioles and also just behind the root tip during the first three days of germination. Seedlings grown for 16 d on MS medium were also stained for GUS (see Supplementary Fig. S2 at *JXB* online). Evidence for promoter activity was not seen in *KAT1-GUS* lines. *KAT2-GUS* and *KAT5.1-GUS* were expressed in the roots, petioles, and new leaves of the rosette and *KAT5.2-GUS* promoter activity was mostly limited to petioles (see Supplementary Fig. S2at *JXB* online). Collectively, these results suggest that there are genomic elements in the region between the two *KAT5* start codons that contribute significantly to transcription of the gene

and that potentially drive expression of the isoforms to different levels.

KAT2 *and* KAT5 *show differential expression patterns in reproductive tissue*

In flowers, as in seedlings, *KAT1-GUS* promoter expression was essentially absent at all stages (Fig. 4). *KAT2-GUS* expression was absent during early flower development, and was first observed at early stages in young anthers and petals, corresponding to stage 12 of flower development. As the flowers matured, strong staining was observed in the anther filament and petals as well as the tip of the gynoecium and developing ovules. *KAT5.1-GUS* reported activity from the earliest stages of flower development, primarily in anthers, but was not present beyond stage 12 of flower development. Staining in *KAT5.2-GUS* lines was seen during the middle stages (~9–12) of flower development and was largely absent at the later stages. Promoter analysis in silique tissue indicated activity in the developing seeds for *KAT2-GUS*, *KAT5.1-GUS*, and *KAT5.2-GUS*, but not for the *KAT1-GUS* lines (Fig. 4).

Publicly available *Arabidopsis* thiolase gene expression data retrieved from the BAR eFP browser (http://bar.utoronto.ca/) (Winter *et al.*, 2007) broadly agrees with the experimental data from promoter analysis (see Supplementary Fig. S3 at *JXB* online). *KAT1* transcript levels were uniformly low. *KAT2* transcript abundance vastly exceeded *KAT1* and *KAT5*, with expression highest in germinating seeds (and also in dry seed and senescent leaves; see Supplementary Fig. S3A, B at *JXB* online). In flowers and developing seed, *KAT2* expression was initially relatively low, and increased in maternal tissues from about stage 12 of flower development. KAT2 expression increases appreciably in the style once developing seeds have passed the torpedo stage (see Supplementary Fig. S3C, D, E at *JXB* online). As seen in the GUS reporter lines, *KAT5* transcript abundance follows a complementary distribution to *KAT2*, with expression highest during early stages of flower and seed development (see Supplementary Fig. S3C, D, F at *JXB* online). In terms of the total abundance of transcript, *KAT5* is substantially lower in most tissues (see Supplementary Fig. S3A, B at *JXB* online). However, *KAT5* expression level matches *KAT2* in early flower and seed development, after which *KAT2* transcript abundance increases several-fold while *KAT5* decreases substantially (see Supplementary Fig. S3D at *JXB* online).

As data derived from microarray analysis does not allow differentiation between *KAT5.1$_{cyt}$* and *KAT5.2$_{px}$* transcripts, qRT-PCR was used to detail the microarray expression data further (Fig. 5). Firstly, in germinating seeds (Fig. 5A), the *KAT2* expression level was high and spiked 12 h after stratification but decreased again by the time seeds began to germinate at 48 h. *KAT1* expression was much lower, but followed a similar pattern to *KAT2*. *KAT5* expression was examined by use of primers located towards the 3' end of the transcript, which assessed total transcript level (*KAT5$_{total}$*), and by two alternative primer pairs at the 5' end that were able to discriminate *KAT5.1$_{cyt}$* and *KAT5.2$_{px}$*. *KAT5$_{total}$* levels, initially very low, began to rise significantly by 48 h when seeds were

Fig. 3. Activity of *KAT:GUS* promoter reporters during seedling establishment. (A) Thiolase gene structure and promoter regions used for reporter analysis. UTRs are indicated by white boxes, coding sequences by black boxes, introns by thick black lines, and upstream promoter regions by thin lines. The red line shows the region of promoter cloned upstream of the start codon. In the case of *KAT1*, the gene adjacent to the promoter is indicated by the shaded box. The common coding regions and the alternative start codons between *KAT5.1cyt* and *KAT5.2px* are indicated and emphasize the differences between the 5' ends of the alternative transcripts. (B) GUS expression in Col-0 seedlings. Seeds of thiolase promoter-GUS reporter lines were stratified then seedlings grown in continuous light on half-strength MS media. Seedlings were stained for GUS activity from 2–6 d after transfer to the light. The length of the scale bar is 0.5 mm.

germinating. *KAT5.2px* followed a similar pattern to *KAT5total* and *KAT5.1cyt* remained barely detectable during the first 48 h of germination (Fig. 5A). In tissues of mature plants, *KAT1* expression was low and relatively uniform (Fig. 5B). *KAT2* and *KAT5total* were expressed at comparable levels in most tissues, although *KAT5total* expression exhibited significantly higher peaks in flowers and siliques. *KAT5.2px* transcript was expressed at low levels in most plant tissues, with substantially higher expression in flowers and siliques, and *KAT5.1cyt* was expressed exclusively in the flowers and siliques (Fig. 5B). Together with the GUS reporter and microarray data, these observations suggest a role for both *KAT2* and *KAT5* in fertility, in both flowers and siliques, with a role for *KAT5* primarily during early flower and embryo development and *KAT2* during seed filling and maturation.

Discussion

Evolution of KAT *genes*

Ancient Viridiplantae taxa such as green algae have a single *KAT* gene, whereas almost all sequenced land plant genomes (mosses and higher plants) possess at least two *KAT* genes. Phylogenetic analysis of predicted KAT protein sequences suggests that duplication of *KAT* genes may have been associated with the adoption of a land habitat and that two *KAT* genes have been selectively maintained ever since. In angiosperms the encoded proteins cluster into two groups that may be defined by their similarity to *A. thaliana* KAT2 and KAT5. Where a genome encodes three or more KAT proteins, the extra isozymes cluster with the respective KAT2 or KAT5 from that species suggesting that they are

Fig. 4. *KAT:GUS* promoter reporter activity in flowers and siliques. Flowers and siliques were removed from soil-grown *KAT:GUS* reporter plants at various stages of flower and silique development, and stained for GUS activity. The length of the scale bar is 0.5 mm.

Fig. 5. Quantitative RT-PCR analysis of *KAT* transcript abundance in *Arabidopsis thaliana*. (A) *KAT* transcript abundance during germination. (B) Relative expression of KAT transcripts in different plant organs. The abundance of *KAT1*, *KAT2*, *KAT5$_{total}$*, *KAT5.1$_{cyt}$*. and *KAT5.2$_{px}$* transcripts was measured and normalized to reference gene (*CACS*) transcript levels. Error bars represent standard error from the mean (*n*=4).

more recent lineage-specific duplications (Fig. 1). For example, in the KAT2 clade, *Populus trichocarpa*, *Linum usitatissimum*, and *Glycine max* show duplicates clustered together, and there are three closely related *Brassica rapa* KAT2 proteins. KAT1 in the Brassicaceae appears to be an older duplication in that it was found in six species from four Brassicaceae genera (*Arabidopsis*, *Capsella*, *Thellungiella*, and *Brassica*) and there is extensive synteny between genomic sequences surrounding *KAT1* and *KAT2* in these species (see Supplementary Fig. S1 and Table S3 at *JXB* online). Despite its relatively high rate of divergence (branch lengths between KAT1s in Fig. 1 are long compared with those between KAT2s in the Brassicales), and very low levels of expression (Figs 3–5; see Supplementary Figs S2 and S3 at *JXB* online), *KAT1* has persisted in the Brassicaceae for at least 43 million years suggesting a selective advantage in maintaining this gene.

Roles for thiolases during the plant life cycle

In *A. thaliana*, both *KAT2* and *KAT5* are expressed in distinct (although not mutually exclusive) patterns. *KAT2* is dominant during seed germination and its expression is co-ordinated with that of other β-oxidation genes, including *MFP2*, *PXA1*, *ACX1*, *LACS6*, and *LACS7* (Fulda *et al.*, 2004), during the first 2–3 d of seedling establishment when the bulk of storage lipid breakdown occurs. Indeed, the *kat2-1* mutant can germinate, but does not establish unless supplied with sugar in the medium (Germain *et al.*, 2001). *KAT2* expression is high throughout the life cycle with other peaks of expression during later stages of flower and seed development, and in senescence. By comparison, *KAT5* expression is relatively minor during germination and strongest in young flowers and early in seed development.

Strong expression of *KAT2* and *KAT5* in inflorescences and siliques (Figs 4, 45; see Supplementary Fig. S3 at *JXB* online) and compromised reproductive success in mutants of core β-oxidation genes including *kat2*, *cts*, *acx1 acx5*, and *lacs6 lacs7* (Footitt *et al.*, 2007*a*,*b*; Schilmiller *et al.*, 2007) suggests a role for β-oxidation in reproduction. β-oxidation potentially contributes to reproductive success via its roles in fatty-acid turnover and/or synthesis of the hormones JA and IAA. Auxin can be synthesized via tryptophan-dependent and -independent pathways, and can also be derived from stored forms; either IAA conjugates (amino acid, sugar or peptide), or from indole-3-butyric acid (IBA) via one cycle of β-oxidation (Baker *et al.*, 2006). Similarly, JA biosynthesis involves the precursor, 3-oxo-2-(2'-[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), undergoing three cycles of β-oxidation to produce JA (Baker *et al.*, 2006).

Many mutants disrupted in JA synthesis or signalling (including *acx1 acx5*) display male sterility, usually in the form of defective pollen development, that can be rescued by exogenously supplied JA (Schilmiller *et al.*, 2007). *kat2* mutants, however, are not sterile, presumably due to compensation of KAT function by KAT5 (Afitlhile *et al.*, 2005). KAT2 appears to be the dominant thiolase in IBA metabolism: *kat2* mutant seedlings have an IBA-resistant phenotype while *kat1* and *kat5* knockouts do not (Wiszniewski *et al.*, 2009). It is likely that β-oxidation of IBA to IAA contributes significantly to levels of free IAA in seedlings (Strader *et al.*, 2011). In flowers, IAA generated in

the stamens controls flower development by promoting anther growth and suppressing petal development (Aloni *et al.*, 2006). Anther elongation in *cts* and *kat2-1* mutants is inhibited, but it can be rescued by exogenous supply of 1-naphthaleneacetic acid (NAA; a synthetic auxin) or IAA (but not by JA) (Footitt *et al.*, 2007*b*). High *KAT2* expression in anther filaments and *KAT5* expression in anthers (Fig. 4) is consistent with an essential role for β-oxidation-mediated hormone metabolism in flower development.

During fertilization, pollen tube growth is extremely rapid and lipids may provide some of the energy required to drive this growth. Lipid bodies and peroxisomes accumulate in developing and mature *Arabidopsis* (Kuang and Musgrave, 1996) and olive (Rodríguez-García *et al.*, 2003) pollen. They then diminish in number during pollen tube growth. *In vitro* germination tests of pollen from *kat2-1* and *cts* mutants revealed reduced germination frequency and shorter pollen tube length, but these could be rescued by exogenous sucrose supply, suggesting lipid degradation via β-oxidation may contribute to the growth of germinating pollen tubes *in vivo* (Footitt *et al.*, 2007*b*).

β-oxidation is also implicated during seed maturation. While fatty acid accumulation in developing embryos originates maternally as photosynthetically fixed carbon, β-oxidation of stored oil may provide respiratory substrates following the breaking of the trophic connection with maternal tissue (Baud *et al.*, 2002). In fact, a 10% decrease in seed oil content has been observed to occur in *B. napus* embryos late in their development (Eastmond and Rawsthorne, 2000) and β-oxidation, the glyoxylate cycle, and gluconeogenesis are all active in developing embryos (Eastmond and Graham, 2001; Chia *et al.*, 2005). A lower respiration rate in *kat2-1* mutant ovules compared with the wild type suggests that β-oxidation is important for carbon flow into sugars via gluconeogenesis and respiration (Footitt *et al.*, 2007*a*). This is corroborated by strong expression of *KAT2* and *KAT5* in silique tissue (Figs 4, 45; see Supplementary Fig. S3 at *JXB* online) implying a role for both thiolases in reproductive success.

A specific function for thiolases in Brassicales?

The apparent coincident origin and maintenance in the Brassicales (but not other plant orders) of a third *KAT* gene (*KAT1*; Fig. 1) and a *KAT5* orthologue that is alternatively transcribed to produce cytosol- and peroxisome-targeted proteins (Fig. 2) raises the question as to whether these events were related and facilitated a specific function for thiolases in Brassicales. The first product of the phenylpropanoid pathway, *trans*-cinnamic acid, represents a branch point to either flavonoids or benzenoid metabolism (Boatright *et al.*, 2004). Intriguingly, *KAT5* coexpresses with genes of flavonoid metabolism (Carrie *et al.*, 2007). Benzoic acid (BA) synthesis from *trans*-cinnamic acid can occur cytosolically or in peroxisomes. In the first case, hydration is followed by retro-aldol cleavage (to release an acetic acid molecule) and dehydrogenation of benzaldehyde to yield BA. BA can then be activated to form benzoyl-CoA. In peroxisomes, CoA-activated *trans*-cinnamic acid undergoes one cycle of β-oxidation: hydration followed by dehydrogenation and KAT-mediated thiolysis to yield acetyl-CoA and benzoyl-CoA. Both of these pathways have been shown in petunia to contribute to the plant BA pool

(Boatright *et al.*, 2004). Mutations that reduce thiolase activity in flowers also result in reduced BA or benzoyl-CoA accumulation. For example, silencing of the *Petunia hybrida PhKAT1* results in a significant decrease in BA and volatile benzenoid compound production in petunia petals (Moerkercke *et al.*, 2009). Arabidopsis *chy1* knockout mutants exhibit greatly reduced KAT activity (Lange *et al.*, 2004) and *chy1* mutant seeds are deficient in BA and BA-containing glucosinolates (benzoyloxy-glucosinolates) (Ibdah and Pichersky, 2009). As glucosinolate synthesis, including benzyl- and benzoyloxy-glucosinolates, is essentially a cytosolic process, a cytosolic KAT5 may have been co-opted in the Brassicales for benzenoid metabolism. We suggest that future research should address KAT function in secondary metabolism including benzoyloxyglucosinolate and flavonoid synthesis.

The complement, structure, and expression of three *KAT* genes in the Brassicaceae has survived genome expansion and contraction in the lineage and persisted through millions of years. In the same time span, plant metabolism has undergone some major changes including the evolution of C$_4$ photosynthesis. This implies that *KAT* gene specialization has a fundamentally important function.

Supplementary data

Supplementary data can be found at *JXB* online.

Acknowledgements

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