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An *Arabidopsis* mutant disrupted in valine catabolism is also compromised in peroxisomal fatty acid β-oxidation

Peter R. Lange[1], Peter J. Eastmond, Kathryn Madagan, Ian A. Graham*

Department of Biology, CNAP, Area 7, University of York, Heslington, York YO10 5YW, UK

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Abstract Characterisation of the *Arabidopsis dbr5* mutant, which was isolated on the basis of 2,4-dichlorophenoxybutyric acid (2,4-DB) resistance, revealed that it is disrupted in the *CHY1* gene. *CHY1* encodes a peroxisomal protein that is 43% identical to the mammalian β-hydroxyisobutryl-CoA hydrolase of valine catabolism. We show that 2,4-DB resistance and the associated sucrose dependent seedling growth are due to a large activity decrease of 3-ketoacyl-CoA thiolase, which is involved in peroxisomal fatty acid β-oxidation. ^{14}C-feeding studies demonstrate that *dbr5* and *chy1* seedlings are reduced in valine catabolism. These data support the hypothesis that CHY1 plays a key role in peroxisomal valine catabolism and that disruption of this enzyme results in accumulation of a toxic intermediate, methacrylyl-CoA, that inhibits 3-ketoacyl-CoA thiolase activity and thus blocks peroxisomal β-oxidation. We also show that *CHY1* is repressed in seedlings grown on sugars, which suggests that branched chain amino acid catabolism is transcriptionally regulated by nutritional status.

Keywords: β-Hydroxyisobutryl-CoA hydrolase; 3-Ketoacyl-CoA thiolase; 2,4-DB; Fatty acid β-oxidation; Valine

1. Introduction

The transition of seeds to photosynthetically competent seedlings is a critical phase in the higher plant life cycle. To achieve photoautotrophism, seedlings must adapt both developmental and metabolic programs to the prevailing environmental conditions [1]. Soluble carbohydrates that are present in many seeds are sufficient to support germination (defined as radicle emergence from the seed coat), whereas post-germinative growth is primarily supported by insoluble seed storage reserves in the form of starch, protein or oil [2]. From the onset of germination, the seed storage reserves are converted to soluble metabolites that can be transported around the seedling and used to support growth and respiration. The composition of seed storage reserves and the organs in which they are deposited differ depending on the species. In oilseeds such as castor, sunflower, oilseed rape and *Arabidopsis*, oil in the form of triacylglycerol is the major storage reserve [2]. Extensive biochemical studies primarily in castor and more recent molecular genetic studies in *Arabidopsis* have led to a comprehensive understanding of the biochemical processes involved in the conversion of storage oil to soluble sugars or in their provision as substrate for respiration [3–5]. Many oilseeds also contain significant protein storage reserves, but much less is known about the mechanisms of storage protein breakdown and the fate of the resulting amino acids in developing seedlings.

Fatty acids released from storage lipids are degraded by oxidation at the β-carbon and the removal of two-carbon units. In plants fatty acid β-oxidation occurs primarily in specialised peroxisomes called glyoxysomes, which also house the glyoxylate cycle [3,6]. Degradation of the short fatty acid moiety on the branched chain amino acids (BCAAs), valine, leucine and isoleucine, also involves reactions of β-oxidation. The end products of these reactions enter the TCA cycle and fuel respiration [7–9]. Although the mobilisation of leucine has been reported previously [7], very little is known about the degradation of iso-leucine and valine in plants.

The intra-cellular location of BCAA breakdown in plants remains a topic of debate with evidence for the involvement of both mitochondria and peroxisomes (reviewed in [5]). Branched chain amino acids are first oxidatively transaminated to form α-keto acids, which undergo oxidative decarboxylation and esterification to produce acyl-CoA esters. The branched chain α-ketoacid dehydrogenase that carries out this first step is located in mitochondria in mammalian cells [10]. The presence of mitochondrial targeting signals on the corresponding *Arabidopsis* proteins suggests this is also the case in plants [5]. Biochemical characterisation of acyl-CoA dehydrogenases associated with mitochondria from maize and sunflower and an isovaleryl-CoA dehydrogenase (IVD) from potato showed that these enzymes exhibit significant activity with isovaleryl-CoA and isobutyryl-CoA, which are intermediates of isoleucine and valine catabolism, respectively, [11,12]. Localisation of an *Arabidopsis* IVD::Green Fluorescent Protein reporter to mitochondria in *N. tabaccum* protoplasts has confirmed the in vivo location of the AtIVD enzyme [13,14]. Thus, it would appear that the first steps of BCAA catabolism are located in mitochondria. However, work performed on peroxisomes and mitochondria from mung bean hypocotyls [14] demonstrated that the production of BCAA associated acyl-CoA esters occurred almost exclusively in peroxisomes. However, one intermediate of leucine catabolism, methyl-crotonyl-CoA, was only detected if the peroxisomes in the assay mixture were replaced by a cell-free extract as the enzyme source [15].

* Corresponding author. Fax: +44-1904-328762.
E-mail address: iag1@york.ac.uk (I.A. Graham).

[1] Present address: Max-Planck-Institute for Molecular Plant Physiology, 14424 Potsdam, Germany.

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Consequently, it was concluded that in intact tissue either a second, extra-peroxisomal pathway of leucine catabolism exists or that intermediates of the peroxisomal pathway are metabolised after leaving the organelle.

The catabolism of valine involves its conversion to isobutyryl-CoA, which is desaturated to form the α,β-unsaturated thioester methacrylyl-CoA. Hydration to β-hydroxyisobutyryl-CoA (HIBYL-CoA) and thioester hydrolysis forms the diffusible and transportable compound β-hydroxy-isobutyrate. This β-hydroxy acid is oxidised to methyl-malonyl semialdehyde and thioesterified to form propionyl-CoA [14]. An *Arabidopsis* mutant *chy1*, which is disrupted in a peroxisomal β-hydroxyisobutyryl-CoA hydrolase gene, has recently been described [16]. CHY1 is 43% identical to a human valine catabolic enzyme that hydrolyses β-hydroxyisobutyryl-CoA. This human HIBYL-CoA hydrolase, when targeted to peroxisomes, complements the indole butyric acid (IBA) resistance of *chy1* [16]. The conversion of IBA to the auxin phytohormone indole acetic acid (IAA) by β-oxidation formed the basis of the screen that resulted in the isolation of *chy1* and other indole butyric acid resistant (*ibr*) mutants [17]. IBA resistance of *chy1* and the linked phenotype of compromised hypocotyl elongation in the absence of exogenous sucrose led to the suggestion that this mutant indirectly disrupts β-oxidation and the conversion of IBA to IAA, because a toxic intermediate of valine catabolism, methacrylyl-CoA, could accumulate in peroxisomes [16].

Arabidopsis mutants disrupted in each of the three major steps of straight chain fatty acids β-oxidation exhibit varying degrees of 2,4-dichlorophenoxybutyric acid (2,4-DB) resistance. This is due to the mutant's compromised β-oxidation of the pro-herbicide 2,4-DB to the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) [18–21]. As a result of a genetic screen for resistance to 2,4-DB, we isolated a number of other 2,4-DB resistant (*dbr*) mutants [22]. Here, we show that one of these, *dbr5*, is allelic to *chy1*. Characterisation of *dbr5* provides the first direct evidence that CHY1 plays a major role in the catabolism of valine and that storage fatty acid breakdown is impaired in *dbr5* seedlings due to inhibition of the β-oxidation enzyme 3-ketoacyl-CoA thiolase. Furthermore, gene expression studies indicate that the carbohydrate status plays an important role in the regulation of BCAA catabolism during post-germinative seedling growth.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh Columbia-0 (Col), Wassilewskia (Ws) and Landsberg *erecta* (Ler) were used. EMS mutagenised seeds (Col, *glabrous1*) were obtained from Lehle Seeds (Round Rock, TX, USA). *chy1* seeds were generously provided by B. Bartel (Rice University, Houston, TX, USA). Seeds were surface sterilised and aseptically grown on media containing *1/2* strength Murashigge and Skoog salts [23], 0.25 mM 2-[*N*-morpholino] ethanesulfonic acid (MES), pH 5.7 (KOH), 1% (w/v) sucrose and 0.8 % (w/v) agar. 2,4-D or 2,4-DB was added to or sucrose omitted from the media where indicated. Seeds were imbibed at 5 °C in complete darkness for four nights and grown in a 16 h photoperiod (photon flux density, 160 μmol m^{-2} s^{-1}, 22 °C light/18 °C dark). Dark-grown seedlings were given a 30 min light pulse after imbibition and transferred to continuous darkness at 22 °C. The plates were placed in a vertical position. Light-grown seedlings were selected for genetic analysis four days after imbibition (DAI). For phenotypic analysis, the root length of seedlings was measured seven DAI. The hypocotyl of dark-grown seedlings was measured five DAI.

2.2. Mapping of mutant loci

Mutants were mapped after out-crossing into Landsberg *erecta* plants. The resulting F2 seeds were plated on media containing 2,4-DB. DNA was isolated from resistant plants. Mutants were mapped using published simple sequence length polymorphisms (SSLP) [24] and insertion/deletion (INDEL) markers [25]. The INDEL markers CER 452263 (5′-tat ggt tta ctc tct ccg gt-3′/5′-caa aat tct gat ctc tag tc-3′) and CER454435 (5′-cag aca cga agc aca atc tt-3′/5′-ttg aag aaa ggt acg aac tt-3′) were designed using the CEREON database (http://www.arabidopsis.org/Cereon/index.html).

Identification of the defects in the dbr5 mutants. Genomic DNA isolated from *dbr5* mutant plants was amplified using two previously described pairs of oligonucleotides, K14B20-1/K14B20-2 and K14B20-3/K14B20-4 [16]. These primers amplify overlapping fragments that cover the gene from 110 bp upstream of the putative translation start site to 40 bp downstream of the stop codon. Amplification products were sequenced directly by Lark Technologies Inc. (Saffron Waldon, Essex, UK) using the primers employed in the PCR amplification and internal sequencing primers.

2.3. Fatty acid analysis

Total fatty acids were measured according to the method of Browse et al. [26] with adaptations as previously detailed [27].

2.4. Enzyme assays and Western blot analysis

To determine enzyme activities in whole seedlings, total protein extracts were prepared as described by Hooks et al. [28]. Isocitrate lyase and 3-ketoacyl-CoA thiolase assays were performed as previously described [29]. Acyl-CoA oxidase assays were performed according to the method of Hryb and Hogg [30]. NAD-dependent malate dehydrogenase assays were performed according to the method of Davies [31]. PEPCK assays were performed according to the method of Walker and Leegood [32]. Western blot analysis was carried out on total protein using an antibody raised against the *Arabidopsis* KAT2 protein as previously described [33].

2.5. Reverse transcriptase-PCR

Total RNA was isolated using the RNeasy kit from Qiagen. The synthesis of single stranded cDNA was carried out using SuperScript™ II RNase H^- reverse transcriptase from Invitrogen (Paisley, PA4 9RF, UK). The primers used were: ACT2S and ACT2A (5′-gttgggatgaaccagaagga-3′ and 5′-cttacaatttcccgctctgc-3′) and CHY1S and CHY1A (5′-cagagtgcttaccgcaggtt-3′ and 5′-ctttgcgatccctaaagcag-3′). Semi-quantitative reverse transcriptase-PCR (RT-PCR) was performed as previously described [34,35].

2.6. Radiolabel feeding

Between 20 and 100 *Arabidopsis* seedlings were incubated in 0.5 ml of 50 mM MES (pH 5.2) containing 5 mM L-[U-^{14}C] valine or L-[U-^{14}C] proline (20 MBq $mmol^{-1}$) for 3 h at 22 °C. The reactions were stopped by the addition of 0.5 ml of 6 M formic acid. Each reaction was conducted in a sealed vial and $^{14}CO_2$ was collected in a well within the vial containing 0.1 ml of 15% (w/v) KOH. The ^{14}C content of the KOH was determined by liquid scintillation counting.

3. Results

3.1. Isolation of 2,4-DB resistant mutants

We previously employed a screen using 2,4-DB to isolate a number of 2,4-DB resistant (*dbr*) mutants from an M2 population of EMS mutagenised *Arabidopsis* seeds [22]. Backcross and complementation analysis of seven *dbr* mutants revealed them all to be recessive and to represent five independent mutants with the *dbr5* mutant having three alleles [22]. The *dbr5* mutant alleles are resistant to much higher concentrations (5–10 μM) of 2,4-DB than the other *dbr* mutants [22] (Fig. 1A). This high level of resistance to 2,4-DB is similar to that exhibited by the *kat2/ped1* mutant [18,33] that is disrupted in a germination-induced isoform of the β-oxidation enzyme 3-ketoacyl-CoA thiolase (Fig. 1A). Sucrose dependent seedling

growth as monitored by hypocotyl length in dark-grown seedlings is also similar for the *dbr5* alleles and *kat2* (Fig. 1B). Backcross and complementation analysis of *kat2* with *dbr5-1* and *dbr5-2* was carried out. The segregation analysis, although made difficult by the low germination frequency of *kat2* [33,36], was consistent with two separate loci (data not shown).

3.2. *dbr5 seedlings are impaired in storage fatty acid breakdown*

kat2 seedlings are blocked in the breakdown of triacylglycerol storage reserve, even when seedling growth is rescued by an exogenous sugar supply [33]. We determined the amount of total fatty acids in seeds and four-day-old *kat2*, *dbr5-1*, *dbr5-2* and wild-type seedlings and compared the eicosenoic acid content (Table 1), which can be used as a marker for triacylglycerol in *Arabidopsis* [36]. In agreement with previous reports [33,37], there is no detectable decrease in the eicosenoic acid content of *kat2* seedlings compared to dry *kat2* seeds, whereas in both wild-type accessions eicosenoic acid is reduced to levels below 38% after four days (Table 1). In *dbr5-1* and *dbr5-2* seedlings, eicosenoic acid levels are intermediate between *kat2* and the wild-types (Table 1), indicating that storage fatty acid breakdown is partially inhibited in *dbr5*.

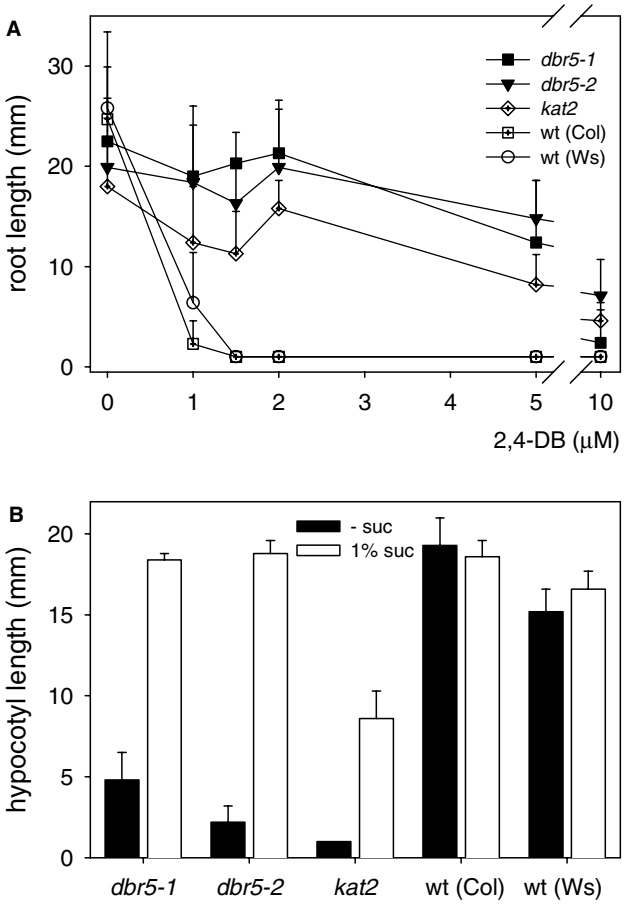

Fig. 1. Response of *dbr* mutants to 2,4-DB and exogenous sugar. (A) Seeds were plated on media containing various concentrations of 2,4-DB. After imbibition for four days, seedlings were grown for seven days under long day light conditions (16 h light). Each value is the mean of root length measurements made on at least 20 seedlings. (B) Seeds were plated on media without sucrose. After imbibition, the plates were given a light pulse of 30 min. Thereafter, seedlings were grown in continuous darkness for five days. Each value represents the mean of hypocotyl measurements made on at least 20 seedlings.

Table 1
Ecosenoic acid levels of four-day-old seedlings

	Eicosenoic acid (%)
dbr5-1	50.9
dbr5-2	60.9
kat2	94.0
wt (Col)	37.8
wt (Ws)	30.9

Seedlings were grown for four DAI. Seeds were cultivated under long daylight conditions on media containing 1% sucrose. The total fatty acid content of seedlings was determined using the method described by Browse et al. [26] with adaptations as detailed in Larson and Graham [27]. Each value is the mean taken of three samples. Values of the ecosenoic acid content are expressed as a percentage of the amount in dry seeds.

3.3. *dbr5 seedlings have reduced 3-ketoacyl-CoA thiolase activity*

The mRNA and activities of many enzymes involved in the conversion of storage lipid to sucrose peak approximately 2 days after the onset of germination [38]. Activities of various such enzymes were assayed in extracts from two-day-old seedlings grown in the presence of light plus 1% sucrose (Table 2). Acyl-CoA oxidase (ACX) and 3-ketoacyl-CoA thiolase are involved in peroxisomal β-oxidation, isocitrate lyase (ICL) is unique to the glyoxylate cycle and phospho*enol* pyruvate carboxykinase performs a key step in gluconeogenesis. The peroxisomal isoform of NAD-dependent malate dehydrogenase has been proposed to play a key role in the regeneration of NAD, which is used as a reductant in peroxisomal β-oxidation. This enzyme was previously shown to be required for growth of yeast cells on media containing fatty acids as sole carbon source [39].

We found that all the enzymes assayed were present at significant levels in two-day-old *dbr5* and *kat2* mutant seedlings (Table 2). However, there was a major reduction of 3-ketoacyl-CoA thiolase activity in the *dbr5* alleles to approximately 30% of the wild-type, which is the same low level as present in *kat2* (Table 2). Although *dbr5* phenocopies *kat2*, we have shown that these two mutants are not allelic. At least two other thiolase isogenes are present in *Arabidopsis* [33] and these are likely to account for the remaining 30% activity in *kat2*. However, *dbr5* exhibits a much larger decrease in thiolase activity than could be accounted for by a mutation in one of the remaining thiolase genes.

Two-day-old *dbr5* seedlings contain wild-type levels of KAT2 protein, whereas it is completely absent in *kat2* as determined by Western blot analysis (Fig. 2A). To establish whether the decrease in 3-ketoacyl-CoA thiolase activity seen in *dbr5* is specific for the period corresponding to storage reserve mobilisation, a time course over 11 days after imbibition was carried out (Fig. 2B). 3-ketoacyl-CoA thiolase activity of wild-type seedlings increases to a peak 2–3 DAI and thereafter declines to a constant level by day 5. In *kat2*, 3-ketoacyl-CoA thiolase activity is compromised for the first three days following imbibition due to the absence of the KAT2 protein but by day 5 the activity is comparable to wild-type levels (Fig. 2B). This indicates that other 3-ketoacyl-CoA thiolase isoforms predominate following seedling establishment. 3-ketoacyl-CoA thiolase activity of *dbr5*, however, is significantly

Table 2
Enzyme activities in two-day-old seedlings

	ACX (6:0)	ACX (10:0)	PEPcK	Thiolase	ICL	NAD-MDH
dbr5-1	532 ± 83	203 ± 72	984 ± 115	513 ± 84	1507 ± 412	60 898 ± 8727
dbr5-2	514 ± 74	199 ± 66	519 ± 64	252 ± 31	1124 ± 300	58 447 ± 6679
kat2	751 ± 120	423 ± 96	575 ± 377	326 ± 93	1259 ± 276	67 850 ± 6248
wt (Col)	602 ± 200	351 ± 160	918 ± 115	1337 ± 287	1753 ± 408	74 167 ± 8116
wt (Ws)	618 ± 75	392 ± 55	904 ± 270	1914 ± 336	1718 ± 401	70 547 ± 13 070

Seedlings were grown on media containing 1% (w/v) sucrose under long daylight conditions (16 h light). Acyl-CoA oxidase (ACX) activity was assayed using hexanoyl- and decanoyl-CoA. The activities of phospho*enol* pyruvate carboxykinase (PEPcK), 3-ketoacyl-CoA thiolase (Thiolase), isocitrate lyase (ICL) and NAD-dependent malate dehydrogenase (NAD-MDH) were determined using the substrates phospho*enol* pyruvate, acetoacetyl-CoA, isocitrate and oxalacetate, respectively. Values are expressed as mU min^{-1} g fresh $weight^{-1}$. Values are the mean of measurements on three samples ± S.E. Each sample was assayed three times.

decreased both during early post-germinative growth and following seedling establishment (Fig. 2B). From two to eleven days after imbibition *dbr5* 3-ketoacyl-CoA thiolase activity does not exceed 30% of the wild-type activity. It is important to note that although 3-ketoacyl-CoA thiolase activity is decreased overall in *dbr5*, the activity pattern is similar to that of wild-type seedlings. These results suggest that the decrease in 3-ketoacyl-CoA thiolase activity of *dbr5* is due to some form of post-translational regulation that affects more than one thiolase isozyme.

3.4. Identification and characterisation of the dbr5 locus

In order to map the EMS-mutagenised *DBR5* locus, *dbr5-2* plants were crossed with the Landsberg *erecta* accession and a recombinant F2 population was screened for 2,4-DB resistant seedlings. 64 of these seedlings were tested for the occurrence of recombination events between polymorphic sites using 20 SSLP markers [24]. A further two INDEL markers were designed to cover areas on chromosomes III and V for which no suitable SSLP markers had been published. The INDEL markers CER 452263 and CER 454435 were generated using the CEREON database [25].

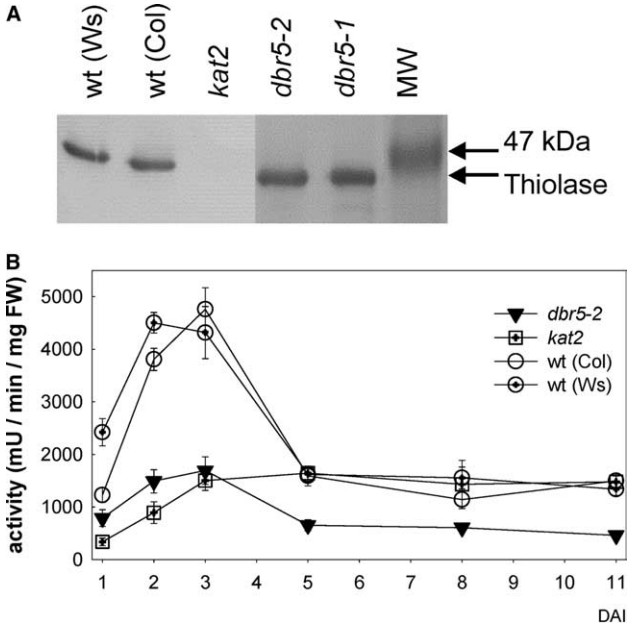

Fig. 2. Western blot analysis of thiolase from two-day-old seedlings and determination of the 3-ketoacyl-CoA thiolase activity during post-germinative growth. (A) Whole plant extracts of two-day-old seedlings grown under long daylight conditions on media containing 1% sucrose were subjected to SDS–PAGE. Proteins were transferred to a nitrocellulose membrane and probed with anti-*Arabidopsis* KAT2 antiserum. Each lane corresponds to the total protein extracts of five seedlings. (B) 3-Ketoacyl-CoA thiolase activity was assayed on whole plant extracts of seedlings grown under long daylight conditions on media containing 1% sucrose. Samples were taken after one, two, three, five, eight and 11 DAI. Values are means of measurements on three samples. Each sample was assayed three times. 3-Ketoacyl-CoA thiolase activity was determined using the substrate acetoacetyl-CoA.

We found that the mutation responsible for the *dbr5* phenotype closely linked to the INDEL marker CER 454435 from the lower arm of chromosome V, since all 64 seedlings were homozygous for Col-2 at this site (Fig. 3A). This marker is also closely linked to the *CHY1* locus (At5g65940). Previously a *chy1* mutant was isolated on the basis of resistance to IBA [17]. The *chy1* mutant is disrupted in the peroxisomal enzyme β-hydroxyisobutyryl-CoA hydrolase (*CHY1*), which may act in valine catabolism. The *chy1* mutant also has defects in peroxisomal fatty acid β-oxidation. Allelism tests showed that all *dbr5* × *chy1-2* F1 hybrid plants were both 2,4-DB resistant and sugar dependent and F2 seedling analysis further confirmed that *dbr5* and *chy1* are allelic (data not shown). Sequencing of the *CHY1* locus showed that G to A substitutions had occurred at the last base of introns 6 and 11 in *dbr5-1* and *dbr5-2*, respectively, in each case resulting in disruption of the 3′ splice site. (Fig. 3B). The nature of the lesion in *dbr5-3* was not determined.

Expression analysis of *CHY1* using RT-PCR showed that two-day-old *chy1-2*, *dbr5-1* and *dbr5-2* seedlings lack *CHY1* mRNA (Fig. 3C). It is, therefore, likely that the disruption of splice sites in *dbr5-1* and *dbr5-2* leads to the premature degradation of *CHY1* mRNA in these mutants. As a positive control for the expression analysis in *chy1* and *dbr5*, the constitutively expressed gene coding for actin, *ACT2* was used.

3.5. CHY1 expression is induced during post-germinative growth and is repressed by sugar

Using semi-quantitative RT-PCR, the mRNA expression levels of *CHY1* were determined throughout the first days after seed imbibition and in several tissues of mature plants. Genes involved in the breakdown of seed storage products are often strongly induced during the first few days after seed germination and then return to basal levels when seedlings become photoauxotrophic (reviewed in [5]). *CHY1* expression is also induced during the first few DAI but, in contrast to *KAT2* [33] or *ACX3* [20], expression remains at a high level after 5 days when seedlings are photoauxotrophic (Fig. 4A). It is also interesting to note that *CHY1* mRNA could be detected in imbibed seeds and also in all tissues of mature plants (Fig. 4A).

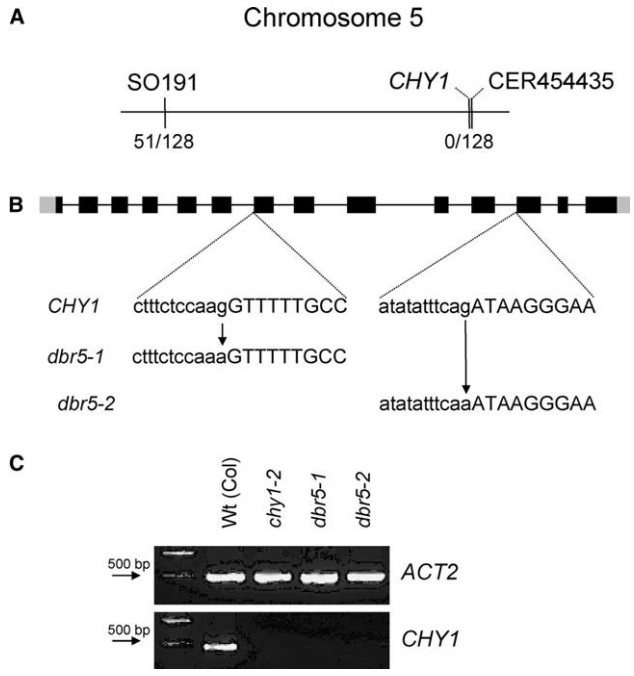

Fig. 3. Mapping and sequencing of *dbr5* and expression analysis of *CHY1*. (A) Positions of the PCR-based markers SO191 and CER454435 and the mutant *chy1* are shown above the chromosome. The recombination frequency (number of recombinants/number of chromosomes) for *dbr5-2* is shown below the chromosome. (B) *CHY1* (At5g65940) exons are shown by black boxes, introns by thin lines and untranslated 5′ and 3′ regions by stippled boxes. *dbr5-1* has a G to A substitution at position 1331 (MIPS genomic sequence for At5g65940; http://mips.gsf.de/proj/thal/db/index.html) that alters the last base and 3′ splice site of intron 6. *dbr5-2* has a G to A substitution at position 2404 that alters the last base and 3′ splice site of intron 11. (C) mRNA expression levels of the constitutively expressed gene actin-2 (*ACT2*) and *CHY1* were determined by RT-PCR. The first lane of each gel is DNA size markers as indicated.

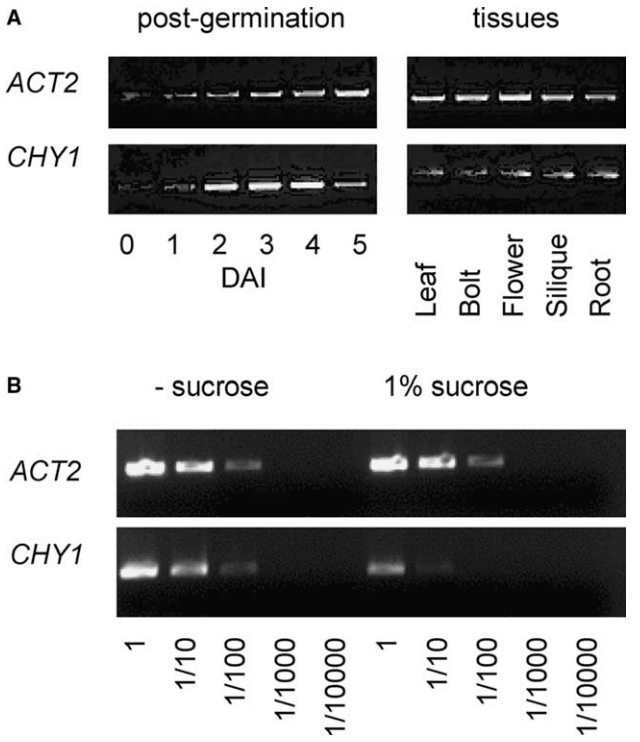

Fig. 4. Expression analysis of *CHY1*. (A) RNA was isolated from developing seedlings after zero to five DAI and from mature tissues. Expression levels of *CHY1* and *ACT2* were determined using semi-quantitative RT-PCR [35]. (B) Seedlings were cultivated for 5 days on media with or without 1% (w/v) sucrose under long daylight conditions. Total RNA was isolated from these seedlings and the expression levels of *CHY1* and *ACT2* were determined using RT-PCR. In order to quantify the relative expression levels, the cDNA template was diluted 10-, 100-, 1000- and 10 000-fold and the detection limit compared [34].

It has previously been demonstrated that genes involved in the breakdown of seed storage products are repressed by the presence of exogenous sugars [40,41]. Consistent with this, *Arabidopsis* seedlings grown in the presence of 1% sucrose showed a significant repression of the *CHY1* expression levels compared to seedlings grown in the absence of exogenous sugar (Fig. 4B).

3.6. Valine catabolism is impaired in dbr5/chy1

Zolman et al. [16] hypothesised that *CHY1* acts in peroxisomal valine catabolism based on the demonstration that the heterologously expressed CHY1 protein hydrolyses β-hydroxyisobutyryl-CoA and that *chy1* can be complemented by a mammalian valine catabolic enzyme with 43% identity to *CHY1*. However, a direct effect on valine catabolism was not proven in the *chy1* mutant. Wild-type, *dbr5* and *chy1-2* seedlings were incubated in the presence of radio-labelled [^{14}C] valine or proline. Amino acid catabolism was determined by the release of [^{14}C] CO_2. In comparison to wild-type, two-day-old *chy1-2* and *dbr5* seedlings show a significant reduction of valine catabolism (Fig. 5). This difference persists at day 5 but is much less pronounced. In contrast, the metabolism of proline is comparable between wild-type and mutant seedlings at both day 2 and day 5 after imbibition.

4. Discussion

The aim of this work was to characterise the *Arabidopsis dbr5* mutant, which shows a high level of resistance to the pro-herbicide 2,4-DB and a dependence on exogenous sugar for seedling growth. Genetic analysis revealed that *dbr5* is allelic to the previously published *Arabidopsis* mutant *chy1* [16], which is defective in a peroxisomal β-hydroxyisobutyryl-CoA hydrolase. The current work represents a significant advance in our understanding of the mutant phenotype and the role of the β-hydroxyisobutyryl-CoA hydrolase enzyme in plant BCAA and fatty acid metabolism.

Firstly, our work demonstrates that the *dbr5* mutant is significantly reduced in 3-ketoacyl-CoA thiolase enzyme activity during germination and post-germinative seedling growth and consequently fatty acid breakdown is also reduced. This provides the first direct evidence to support the hypothesis that the accumulation of a toxic intermediate, methacrylyl-CoA, causes the *chy1* phenotypes (IBA/2,4-DB resistance and sucrose dependence for seedling establishment) [16] by inhibiting the peroxisomal β-oxidation 3-ketoacyl-CoA thiolase. Secondly, we show that valine catabolism is disrupted during post-germinative growth of *dbr5* seedlings, thus providing direct evidence for the involvement of CHY1 in the valine catabolic pathway. Thirdly, we demonstrate that the *CHY1* gene is regulated by carbohydrate status in young

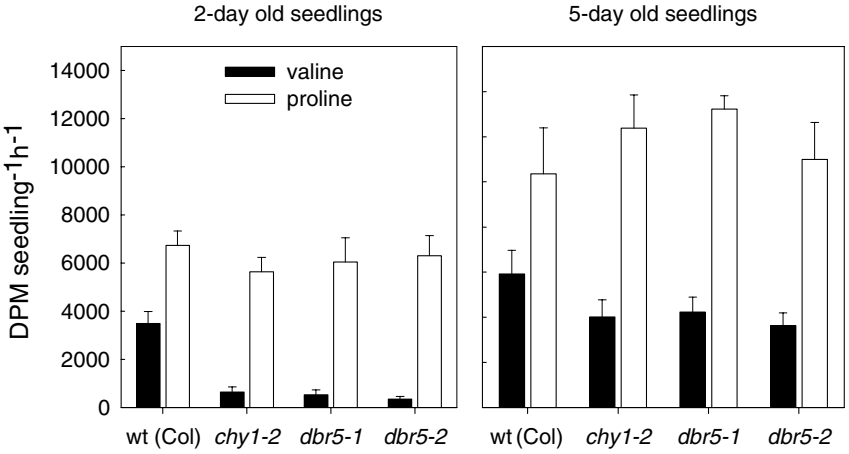

Fig. 5. Amino acid catabolism in young seedlings. Two- and five-day-old seedlings were incubated for 3 h with 5 mM [^{14}C] valine or proline. Catabolism of amino acids was measured as the release of [^{14}C] CO_2. Values are means ± S.E. of three separate reactions.

seedlings, indicating a potential mechanism for the global control of the catabolism of storage lipids and storage proteins in young seedlings.

Assays of marker enzymes involved in β-oxidation, the glyoxylate cycle and gluconeogenesis revealed that 3-ketoacyl-CoA thiolase activity was selectively reduced in *dbr5* even though thiolase protein levels, as determined by Western blot analysis, were similar to those of wild-type seedlings. In fact, the reduced 3-ketoacyl-CoA thiolase activity was comparable to that of a mutant completely disrupted in the germination specific KAT2/PED1 thiolase isozyme [18,33]. However, in contrast to the *kat2* mutant, which shows a recovery in 3-ketoacyl-CoA thiolase activity to wild-type levels following seedling establishment 5 DAI, the 3-ketoacyl-CoA thiolase activity of *dbr5* remained at a reduced level beyond this period. Two conclusions can be drawn from these data. First, at least two thiolase isoforms are active in *Arabidopsis* plants. KAT2 is expressed early during germination and post-germinative growth. Following seedling establishment, at least one other thiolase isoform is active. This is consistent with the fact that the *kat2* mutant exhibits a sucrose dependent growth phenotype during post-germinative growth but once seedlings are established and transferred to soil they develop and grow normally [33]. Second, given that the repression of 3-ketoacyl-CoA thiolase activity in *dbr5* extends beyond that period observed in *kat2*, it can be concluded that more than one isoform of thiolase is subject to repression in the *dbr5* mutant background. These conclusions are consistent with and support the hypothesis put forward by Zolman et al. [16] that in the *chy1* mutant background fatty acid β-oxidation is indirectly disrupted because the toxic compound methacrylyl-CoA can accumulate.

The low but detectable level of thiolase activity remaining in extracts from the *dbr5* mutants is most likely due to the fact that the acetoacetyl-CoA used as substrate can also be acted on by the cytosolic acetoacetyl-CoA thiolases as previously discussed [33]. These enzymes differ significantly from their peroxisomal counterparts in terms of primary amino acid sequence and structure and are presumably not inhibited by methacrylyl-CoA.

Previous reports demonstrated [42,43] that methacrylyl-CoA acts as a potent Michael acceptor rapidly reacting with nucleophiles such as cysteine, glutathione and coenzyme A. Inhibition of 3-ketoacyl-CoA thiolase activity by methacrylyl-CoA was previously shown in studies using thiolase isolated from pig heart by Salam and Bloxham [44]. We used a highly sensitive method developed in our laboratory for the quantitative detection of medium and long-chain acyl-CoAs [27,45,46] but found that we were unable to identify short-chain acyl-CoAs due to poor ionisation responses in the Mass Spectrometer. This along with the fact that we have been unable to obtain a standard sample of methacrylyl-CoA from a commercial supplier or through enzymatic synthesis has meant that we have been unable to directly measure methacrylyl-CoA ester from extracts of *Arabidopsis* tissue.

Given that the observed phenotypes of sucrose dependent seedling growth and 2,4-DB resistance could be due to an indirect effect caused by disruption of valine catabolism, it was essential to establish if valine catabolism is altered in the *dbr5*/*chy1* mutant. Feeding experiments using the [^{14}C] labelled amino acids valine and proline clearly demonstrated that the *dbr5* and *chy1* alleles are specifically impaired in valine catabolism. Two-day-old mutant seedlings showed a significant decrease compared to wild-type, whereas in five-day-old seedlings the difference was much less, suggesting that alternate isozymes or enzymatic routes for valine catabolism may operate in the older seedlings. It is interesting to note that both two- and five-day-old seedlings have a significant capacity to break down both valine and proline to CO_2, thus indicating that amino acid catabolism can operate as a source of respiratory substrate in young seedlings.

The fact that valine catabolism is disrupted in the *dbr5*/*chy1* mutant further confirms the hypothesis that in the mutant methacrylyl CoA accumulates, which in turn inhibits 3-ketoacyl-CoA thiolase activity. The observation that the thiolase protein remains inactive in cell extracts that have undergone a significant dilution compared with the intracellular concentration suggests that the binding of the inhibitor is irreversible.

We found that *CHY1* gene expression increases during the first three days following seed imbibition and remains at a high level thereafter. This is consistent with the fact that the indirect effect on thiolase activity in *dbr5* persists in older seedlings. The *CHY1* gene is also expressed at significant levels in all tissues analysed, suggesting that BCAA catabolism is active during

most parts of the plant life cycle. However, apart from seedling establishment, there were no other obvious phenotypic effects throughout the life cycle of *dbr5*. There are a number of genes that show significant homology with *CHY1* in the *Arabidopsis* genome including two that, like CHY1, encode proteins with a peroxisomal targeting signal [5]. One or other of these may compensate at other stages in development of the mutant.

Interestingly, *CHY1* expression is repressed when seedlings are cultivated in the presence of exogenous sugar. The *Arabidopsis* isovaleryl-CoA dehydrogenase gene, the product of which exhibits substrate specificity with intermediates of both leucine and valine catabolism, and the genes encoding the E1 and E2 subunits of the BCKDH which catalyses the second step in leucine catabolism are also repressed by sucrose in *Arabidopsis* [47,48]. Taken together, these data suggest that the genes and related pathways of BCAA catabolism are subject to carbohydrate repression, and that these amino acids could be used to provide respiratory substrate under low carbohydrate conditions.

Storage lipid is mainly used to provide carbon skeletons for respiration or as substrate for gluconeogenesis in young seedlings. Storage protein could also contribute respiratory or gluconeogenic substrate through amino acid catabolism in young seedlings as well as providing building blocks for new protein synthesis. Gluconeogenesis from leucine has in fact been shown to occur in the endosperm of germinating castor bean [49,50]. It is, therefore, likely that metabolic regulation of genes involved in the catabolism of both fatty acids and amino acids will coordinate their ultimate utilisation in anabolic or catabolic pathways depending on the nutritional status of the young seedling at a specific point time.

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