The Arabidopsis ALDP protein homologue COMATOSE is instrumental in peroxisomal acetate metabolism

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INTRODUCTION

The classic gluconeogenic model of lipid mobilization is the conversion of fatty acids into sucrose during establishment in order to support the growing seedling. Acetyl-CoA from \( \beta \)-oxidation enters the glyoxylate cycle for conversion into organic acids for transportation to mitochondria, which subsequently produce sucrose through the TCA (tricarboxylic acid) cycle and gluconeogenesis [1]. Establishing the path of carbon flow through the glyoxylate cycle has relied almost exclusively on feeding radiolabelled acetate to seeds and seedling tissues [2–4]. Gluco- and neogenesis [1]. Establishing the path of carbon flow through the glyoxylate cycle has relied almost exclusively on feeding radiolabelled acetate to seeds and seedling tissues [2–4]. Gluco-

The Arabidopsis acn (acetate non-utilizing) mutants were isolated by fluoroacetate-resistant germination and seedling establishment. We report the characterization of the acn2 mutant. Physiological analyses of acn2 showed that it possessed characteristics similar to those of the mutants cts (COMATOSE)-1 and pxa [peroxisomal ABC (ATP-binding-cassette) transporter]. The acn2 locus was mapped to within 3 cM of the CTS gene on the bottom arm of chromosome IV using CAPS (cleavage amplification polymorphism) and SSLP (simple sequence-length polymorphism) markers. Crossing acn2 and cts-1 failed to restore the fluoroacetate-sensitive phenotype, suggesting that these mutations were allelic. Sequencing of the ACN2 locus revealed a C \( \rightarrow \) T nonsense mutation in exon 13, which would have resulted in the elimination of the C-terminal hemitransporter domain of the encoded protein. Neither the full-length CTS protein nor the truncated protein was detected on immunoblots using either C-terminal- or N-terminal-specific anti-CTS antibodies respectively, demonstrating the absence of the entire CTS protein in acn2 mutants. Emerged seedlings of both cts-1 and pxa1 mutants displayed increased resistance to FAc (monofluoroacetic acid) compared with the corresponding wild-type seedlings. Complementation studies showed that mutation of the CTS gene was responsible for the FAc-resistant phenotype, as when the wild-type protein was expressed in both the cts-1 and pxa1 mutant lines, the strains became FAc-sensitive. Feeding studies confirmed that both acn2 and cts-1 mutants were compromised in their ability to convert radiolabelled acetate into soluble carbohydrate. These results demonstrate a role for the ABC protein CTS in providing acetate to the glyoxylate cycle in developing seedlings.

Key words: acetyl-CoA, Arabidopsis, COMATOSE, glyoxylate cycle, lipid mobilization, \( \beta \)-oxidation.

Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxybutyric acid; AAE, acyl-activating enzyme; ABC, ATP-binding-cassette; ABCD, D subgroup of ABC protein family; ACN, acetate non-utilizing; ALD, adrenoleucodystrophy; ALDP, ALD protein; CAPS, cleavage amplification polymorphism; CTS, COMATOSE; FAc, monofluoroacetic acid; IBA, indole-3-butyric acid; JA, jasmonic acid; LACS, long-chain acyl-CoA synthetase; LeR, Landsberg erecta ecotype; MS, Murashige and Skoog; NBF, nucleotide-binding fold; pxxa, peroxisomal ABC transporter; RT, reverse transcription; SSLP, simple sequence-length polymorphism; TAG, triacylglycerol; TMD, transmembrane domain; ttxg, transparent testa glabra; Vlcs, very-long-chain acyl-CoA synthetase.

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The *cts* mutant of *Arabidopsis* was isolated in a forward genetic screen to identify lines that possessed reduced germination potential [18]. Footitt et al. [19] reported that the mutation responsible for the reduced germination potential phenotype resided in the gene encoding the ABC protein homologue of human ALDP. Just prior to this, Zolman et al. [20] described a mutation in the same gene, which was isolated from a screen to identify mutants resistant to IBA (indole-3-butyric acid), an auxin analogue [21]. Mutant alleles for this gene, *ped* (peroxisome-defective) 3, were isolated in a screen for mutants resistant to 2,4-DB (2,4-dichlorophenoxyacetic acid), an auxin analogue, as early as 1998 [22], but the mutant loci were not reported until later [23]. The nature of the screens by which the mutants were isolated, the reduced lipid mobilization and the accumulation of acyl-CoAs in young seedlings [19] suggested that the function of the protein was to supply fatty acid and/or acyl-CoAs for peroxisomal β-oxidation. Evidence that JA (jasmonic acid) levels were reduced in both basal and wounded *cts-1* led Theodoulou et al. [24] to conclude that CTS is responsible for transporting fatty acid/CoA substrates of at least four carbons in length, encompassing a wide variety of straight-chain or cyclic derivatives. The isolation of the FAc-resistant mutant *acn2*, which exhibited characteristics similar to *cts-1* [19], *pxa* (peroxisomal ABC transporter) 1 [21] and *ped3* [23], raised the possibility that the protein may also function as an acetate transporter. FAc exerts its toxicity through the inhibition of aconitase on conversion into fluorocitrate by the sequential action of AcetCS and citrate synthase [25]. Therefore a mutation that would prevent FAc entering the glyoxylate cycle would result in enhanced tolerance to FAc. Using a physiological, genetic and biochemical approach, we demonstrate that CTS is integral to acetate metabolism in glyoxysomes in developing seedlings, and present evidence to support the speculation that unesterified acetate is the compound transported.

**EXPERIMENTAL**

**Plant material**

All seed batches used in the experiments were surface-sterilized and were allowed to imbibe in the dark at 4°C for 4 days before being sown on to agar plates. For all experimental procedures, seeds were germinated at 20°C with constant illumination at 70 μmol of photons/m² per s, except for the immunoblot analysis, where seeds were germinated and grown with a day length of 9 h. Standard agar medium plates contained 0.8% agar and half-strength MS (Murashige and Skoog) salts [26], to which sucrose was added to a concentration of 20 mM where specified. Prior to the addition of agar and subsequent autoclaving, all media were adjusted to pH 5.7 using 0.1 M KOH. To test FAc resistance, seeds were sown on to standard agar medium plates containing 0.5 mM sodium FAc (Sigma–Aldrich). FAc was prepared as a concentrated stock solution, filter-sterilized and added to autoclaved standard agar medium. The auxin analogues 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4-DB were prepared as concentrated stocks in ethanol and added to autoclaved standard agar medium. The concentrations used experimentally were 250 mM, 500 nM, 1 μM, 3 μM and 5 μM for 2,4-DB, and 20 nM, 80 nM, 120 nM and 240 nM for 2,4-D. For root growth measurements, surface-sterilized seeds were sown on to standard agar medium plates containing 20 mM sucrose. After 4 days of incubation in the dark at 4°C, the plates were transferred to the growth room. After 4 further days in the growth room, ungerminated seeds of *acn2* and *cts-1* were moved to selective plates and damaged by nicking the testa with forceps. Wild-type seeds were moved from the cold to growth conditions at this time.

All growth and feeding studies were conducted on *acn2* lines that had been back-crossed three times. Each successive round of back-crossing entailed screening the F2 generation for sucrose-dependent germination in the presence of 0.5 mM FAc. Whole plants were grown at 20°C on a 9 h light/15 h dark cycle.

**Characterization of the *acn2* locus and gene and protein expression analysis**

For mapping purposes, F2 generation *acn2* plants homozygous for FAc resistance were crossed to Ler (Landsberg erecta ecotype) reciprocally. FAc-resistant F2 generation seedlings (approx. 120) were rescued by sowing on to soil and growing in order to harvest leaf material for DNA extraction. Genomic DNA was isolated from leaf material using the PUREGENE® DNA isolation kit (Gentra Systems). Primer combinations and thermocycling conditions for analysing CAPS (cleavage amplification polymorphisms) were performed as specified by Konieczny and Ausubel [27] and Baumbusch et al. [28]. The analysis of SSLPs (simple sequence-length polymorphisms) was conducted as described by Bell and Ecker [29]. PCR set-up and thermocycling was conducted on an MWG Automated Biosystem RoboSeq™ 4204 with an integrated 96-well thermocycler.

To sequence the *acn2* locus, primer pairs were designed to amplify overlapping regions of the *ACN2* gene (At4g39850) from the sequence in the TAIR (*The Arabidopsis Information Resource*) database website (http://www.arabidopsis.org). The resulting PCR products were isolated from agarose gels, purified and sequenced commercially by the John Innes Genome Laboratory. A portion of exon 13 from both Col-0 and *ACN2* DNA and cDNA templates was amplified using the forward primer 5′-AAGTGTAGTGTTGCCTCGTTTTC-3′ and the reverse primer 5′-AAAGAGGCTATTCGGTCAGAGAT-3′. *ACN2* cDNA was prepared by reverse transcription from total RNA isolated from newly emerged seedlings at PGS (principal growth stage) 0.7 [30], which had been germinated on standard agar medium containing 20 mM sucrose. Total RNA was isolated using the PURESCRIPT® RNA isolation kit (Gentra Systems), modified with the insertion of a phenol/chloroform/isooamyl alcohol (25:24:1) wash step prior to isopropanol precipitation.

For expression analysis of *CTS* in wild-type and mutant seedlings, vernalized seeds were sown on to standard agar medium plates with or without 20 mM sucrose. Germinated seedlings were removed after 4 days, and the remaining seeds damaged by nicking the seed coat with forceps under a microscope. PolyA mRNA was isolated from either ten seeds from mutant lines plated on to standard agar medium, or from five PGS 0.7 seedlings plated on to standard agar medium containing 20 mM sucrose. PolyA mRNA was isolated using Dynabeads (Dynal AS) according to the manufacturer’s instructions. Reverse transcription was performed as described by Laval et al. [31]. PCR was conducted using the *CTS* specific forward primer 5′-ACGGATGTGAAATTTGATCAGAT-3′ and the reverse primer 5′-TGCTGATTCCACCTTGTTTCT-3′.

For the immunoblot analysis of CTS expression, vernalized seeds were plated on to standard agar medium plates containing 20 mM sucrose. Germinated seeds were removed after 4 days, and ungerminated mutant seeds were damaged by nicking the testa. Protein was extracted from PGS 0.7 seedlings as described by Footitt et al. [19], except the quantities of PMSF and Sigma protease inhibitor cocktail used were increased to 5 mM and to 5% (v/v) respectively. Protein aliquots of 20 μg were resolved by SDS/PAGE (7.5% gel), blotted on to a PVDF membrane and stained with Ponceau S to ensure equal protein loading. The membranes were then probed with either an antibody against
the C-terminal portion of CTS (raised as a recombinant protein containing amino acids 1112–1337 of CTS) or the N-terminal of CTS (raised using a peptide containing amino acids 10–25 of CTS). The C-terminal antibody was purified by affinity chromatography before use. After probing with the C-terminal antibody, the blot was stripped in 60 mM Tris/HCl, pH 6.7, 2% (w/v) SDS and 0.7% (v/v) 2-mercaptoethanol at 50°C for 30 min and reprobed with the N-terminal antibody. Detection of immunoreactive proteins was performed using the ECL® (enhanced chemiluminescence) + PlusTM Western blotting detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

Radiolabelled acetate feeding

The [14C]acetate feeding experiments were adapted from those described by Eastmond et al. [3] with the following modifications. A total of 100 PGS 0.7 Arabidopsis seedlings were bubbled for 4 h in a 1.5 ml microcentrifuge tube containing 0.2 ml of 1 mM sodium [2-14C]acetate (20.5 MBq/mmol) and 50 mM Mes, pH 5.2. Two consecutive 150 µl aliquots of 5 M KOH were used to trap the released CO2. Both fractions were combined for liquid-scintillation counting. After 4 h of bubbling, the seedlings were washed, extracted and fractionated [2]. The proportion of radioactivity in each component was determined on a Wallac 1409 liquid-scintillation counter using 10 ml of PerkinElmer Optiphase HiSafe3 liquid-scintillation cocktail. The ethanol-insoluble material was combusted with a Biological Material Oxidizer OX400 (R. J. Harvey Instrument Corporation). The CO2 was trapped in 15 ml of OxosolTM C14 (National Diagnostics) and counted directly.

Subcellular fractionation and enzyme assays

Fractionation of seedling organelles was conducted as described by Eastmond et al. [3] with the modifications specified by Turner et al. [7]. AcetCS activity was measured spectrophotometrically in a coupled reaction with citrate synthase and malate dehydrogenase as described by Miller and Bonner [32]. Short-chain acyl-CoA oxidase activity was measured as described by Hyrb and Hogg [33].

RESULTS

Phenotypic characteristics of acn2

The synthetic auxin analogues 2,4-DB and IBA have been used to select for defects in glyoxysomal function, including biogenesis and fatty acid β-oxidation [21,22]. A general effect of auxin analogues on sensitive genotypes is a reduction in root growth, because the compounds are able to be converted into their more bioactive derivatives [20,22]. Therefore we compared the root growth of acn2 seedlings (Col-0 background) with that of the 2,4-DB resistant cts-1 seedlings (Ler background), in the presence of increasing concentrations of 2,4-DB (Figure 1A). In the absence of 2,4-DB, root growth of the mutants was less than that of the corresponding wild-type seedling. Mutant root growth decreased with increasing concentrations of 2,4-DB, but not as dramatically as either wild-type strain. Significantly greater root growth for the mutant strains compared with wild-type strains was observed for both acn2 and cts-1 starting at 1 and 3 µM 2,4-DB respectively. At 5 µM 2,4-DB, root growth of the wild-type seedlings was abolished, whereas a large number of mutant seedlings exhibited substantial root growth. Like cts-1, acn2 was found to be sensitive to the degradation product 2,4-D, consistent with the possibility that conversion of 2,4-DB into 2,4-D is disrupted in acn2 (Figure 1B).

The presence of short-chain fatty acids had been reported to remove dormancy and instigate germination of cts-1 [19]. For cts-1 germination, we found a significant effect of propionate and butyrate at concentrations of 0.01 and 0.1 mM respectively. Neither acetate nor other concentrations of propionate or butyrate tested had any effect (results not shown). No effect on acn2 seed germination was apparent in the presence of fatty acids (results not shown). Some of the vegetative phenotypes observed for acn2 are exhibited by pxa1 [20], such as poor initiation of lateral root formation and smaller rosettes with fewer leaves. In addition, the leaves were crinkled and waxy, a phenotype reported previously for the ped3 alleles [23]. The delay in flowering time was not as dramatic for acn2 as observed for pxa1 (results not shown). Such phenotypic variation for cts alleles is not unusual and has been discussed recently [17].

Allelism of acn2 and cts-1

The chromosomal position of the mutant locus was determined by a map-based PCR approach using CAPS markers [27,28] and
Table 1 Phenotypic analysis of the F1 generation of acn2 and cts-1 crosses

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MS+ (%)</th>
<th>MS/Suc+ (%)</th>
<th>FAcR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (n = 3)</td>
<td>45 ± 3</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Ler (n = 3)</td>
<td>100</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>cts-1 (n = 3)</td>
<td>11 ± 4</td>
<td>53 ± 2</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>acn2 (n = 3)</td>
<td>&lt; 1</td>
<td>35 ± 4</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>cts-1 × acn2 (n = 6)</td>
<td>5 ± 2</td>
<td>49 ± 17</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Ler × acn2 (n = 3)</td>
<td>100</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Ler × cts-1 (n = 3)</td>
<td>100</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Col-0 × acn2 (n = 3)</td>
<td>96 ± 2</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

* Approx. 100 seeds were sown on to agar plates containing half-strength MS salts, pH 5.7. The plates were incubated in the dark for 4 days before being transferred to the growth cabinet.
† Seeds that had not germinated were transferred on to agar plates containing half-strength MS salts and 20 mM sucrose.
‡ Seeds were sown directly on to agar plates containing half-strength MS salts, pH 5.7, 20 mM sucrose and 0.5 mM FAc. Seeds from mutants and mutant crosses were nicked 24 h after transfer to the growth cabinet.

The plates were incubated in the dark for 4 days before being transferred to the growth cabinet.

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The phenotypes tested were the proportion of total seeds germinated by 4 days after transfer to the growth chamber and establishment in the presence of FAc. FAcR, fluorocarboxylate resistance; NA, not applicable; suc, sucrose.

SSLPs [29]. The acn2 locus was found to lie within 2 cM of the CAPS marker DHS1, and to within 3 cM of the SSLP marker NGAl107. These map distances placed the acn2 locus very close to the position of the CTS gene on chromosome IV. No other gene in the vicinity of CTS would be expected to give the phenotypes observed for acn2 when disrupted by mutation. Reciprocal crosses of acn2 with cts-1 were conducted in order to test complementation of the genotypes. Crosses with cts-1 as the pollen donor were more successful and produced more seeds, which corresponded to a reduced fertility of acn2. The same occurred with Ler and acn2 crosses. The F1 generation of the cts-1 × acn2 and Ler × acn2 crosses were tested for dormancy and FAc resistance (Table 1). A small proportion of seeds from the cts-1 × acn2 crosses germinated in the absence of sucrose, similar to that observed for either the cts-1 or acn2 single mutant, whereas 100% of the seeds from the Ler × acn2 and Col-0 × acn2 crosses germinated. As with the single mutants, a substantial increase in the proportion of seeds from the mutant crosses germinated when transferred on to sucrose plates, and F1 generation seed from the crosses established well in the presence of FAc and sucrose once the testa was damaged or removed. Although listed in Table 1 as being FAc-sensitive, the F1 generation Ler × acn2 crosses possessed a slightly elevated FAc resistance, as previously reported for the F1 generation of the backcross of acn2 to its parental wild-type strain [5].

DNA was isolated from acn2 plants exhibiting sucrose-dependent establishment in the presence of FAc, to ensure that only a homozygous locus would be sequenced. Comparison of the first-pass sequences of overlapping PCR-amplified fragments of the CTS gene with the full-length genomic sequence present in the TAIR database, At4g39850, revealed a single mutation, which was a C → T substitution in exon 13 (Figure 2A). When compared with the coding sequence for At4g39850, the mutation lies at nucleotide position 2284, which introduces a stop codon at Gln762, truncating the protein in the first predicted TMD of the second hemitransporter domain. A detailed description of the gene and a comprehensive comparison of the protein primary sequence with human and Saccharomyces cerevisiae ABC transporters is presented in Zolman et al. [20].

RT (reverse transcription)–PCR analysis of the expression of CTS in acn2 seedlings was performed compared with cts-1 and their respective wild-type parents (Figure 2B). The expression of CTS in wild-type seedlings from seeds germinated in the presence of sucrose, and in seeds left on standard agar medium plates for 24 h (no radical emergence) is similar to that previously reported by Footitt et al. [19]. No CTS mRNA was expected to be present in cts-1, since the choice of primers used for PCR amplification reside downstream of the chromosome V translocation site within exon 10 [19]. Using the same primer pair, CTS mRNA was expressed within the acn2 mutant in both newly emerged seedlings and un-germinated seeds that had been allowed to imbibre. Although ACN2 was expressed, no immunoreactive protein was observed using CTS-specific polyclonal antibodies [19] raised against either the C-terminal or N-terminal portion of CTS (Figures 2C–2E). Although the specificity of the N-terminal antibody is less than that of the affinity-purified C-terminal one, it is apparent that the lack of an approx. 84 kDa protein (predicted molecular mass of the truncated protein) indicated that the entire
Acetate metabolism and signalling

Figure 3  FAc resistance of CTS mutants

Seeds of wild-type and complemented mutants were not nicked. The labels 27H10 and 87C13 represent clone designations from the GeTCID (Gene Transfer Clone Identification and Distribution Service) (John Innes Centre), provided for in planta expression as part of the GARNet (Genetic Arabidopsis Resource Network) facilities. pxa1/35SPXA1 represents the mutant line expressing the PXA1 cDNA under the control of the CaMV (cauliflower mosaic virus) 35S promoter [26]. (A) Seeds were treated as described in the Experimental section, except that spontaneously germinated mutants were not removed prior to nicking the testa. All seeds were sown on to standard agar medium containing 20 mM sucrose with (+) or without (−) 0.5 mM FAc. Photographs were taken 8 days after transfer to growth conditions. Root growth of Col-0-related genotypes (B) and Ler-related genotypes (C) in the presence of increasing FAc concentrations. Seeds were treated with FAc as described in the Experimental section. Root lengths were measured 6 days after nicking the testae of the mutants. Results are means ± S.D. for at least ten seedlings. Lines have been included to clarify connections for obscured data points. Based on Student’s t test statistics, wild-type root growth was significantly less than the mutant seedling (P < 0.0001) at FAc concentrations of 0.1 mM and greater as indicated by *. Average root growth for complementation genotypes cts-1/155A23 and cts-1/159N01 was intermediate between Ler and the complementation genotypes shown.

CTS protein is missing in acn2. All protein bands observed in the mutant strains between 72 and 95 kDa were present also in the wild-type strains, suggesting either non-specific binding of the anti-CTS antibody or the presence of contaminating antibodies within the antiserum.

FAc resistance of CTS mutants

The acn2 mutant was isolated based on FAc-resistant germination and emergence [5]. Interestingly, the subsequent generation of seed from the initial plant did not establish, except after the seed coat was damaged or removed in the presence of sucrose. The discovery of the mutant was quite fortuitous, because we did not select specifically for sucrose-dependent germination and establishment, and isolation of the mutant resulted from spontaneous germination owing to the inclusion of FAc-containing standard agar medium plates. We also reported that acn2 appeared to be dominant, with F1 generation seeds from the Col-0 × acn2 backcrosses showing slightly elevated resistance to FAc. Seedlings from the F2 generation seed batches exhibited three different levels of FAc resistance, which is indicative of the three possible genotypes at the acn2 locus. This mirrors the dominance reported for the various ped3 alleles [22] and the pxa1 allele [20] for resistance to 2,4-DB and IBA respectively. In contrast with FAc, the phenotype of sucrose-dependent establishment was recessive in acn2, as reported for the other CTS mutants. The FAc-resistance phenotypes of acn2, pxa1 and cts-1 are demonstrated in Figure 3. Signs which were indicative of resistance include greening, expansion of the cotyledons and, in particular, root growth, which does not occur with either wild-type strain. The effects of FAc on root growth of each genotype are shown in Figures 3(B) and 3(C). Significant differences between the mutant and wild-type seedlings were observed at FAc concentrations as low as 0.1 mM. Interestingly, the spontaneously germinated seeds of undamaged homozygous acn2 mutants exhibited a wild-type FAc-sensitive phenotype, as shown by yellow cotyledons and no root growth (results not shown). The resistance of cts-1 is in stark contrast with the sensitive phenotypes of its wild-type parent, Ler, and the mutant that had been transformed with the wild-type gene [19], i.e. cts-1/27H10 and cts-1/87C13 (Figure 3A). The pxa1 mutant, expressing the full-length PXA1 cDNA (pxa1/35SPXA1), was also sensitive to FAc (Figures 3A and 3B). These results demonstrate that CTS plays a role in mediating FAc sensitivity in wild-type plants.
in seedlings that spontaneously germinate. The reduction in the acetate may explain why wild-type sensitivity to FAc is observed in the cts-1 mutants, as suggested by FAc disruption to glyoxysomal acetate metabolism, as observed for the ttg1-1 (transparent testa glabra) double mutant and Lcrtg1-1, which is similar to that for Col-0 (~6 nmol/mg of protein per h [13]). A trial of cts-1 gave an activity of 18 nmol/mg of protein per h. It was clear that to obtain sufficient material, thousands of cts-1 seeds needed to be damaged, which would not permit the precise staging of dark-growth development. Therefore the cts-1/ttg (transparent testa glabra) double mutant was also tested, since the ttg mutation facilitates normal germination of seeds in the ttg background [19]. AcetCS activity was also apparent in the cts-1/ttg1-1 double mutant at 40 ± 16 nmol/mg of protein per h (n = 3). The greater apparent specific activity of the cts-1/ttg1-1 double mutant may be attributed to differences in the background genotype in which the ttg1-1 mutation resides. Short-chain ACX (acyl-CoA oxidase) specific activities were similar between the cts-1/ttg1-1 double mutant and Ler, and did not suggest a differential stability of glyoxysomes (results not shown). These results indicate that the decrease in carbohydrate labelling is not caused by compromised glyoxysomal AcetCS activity in plants lacking CTS.

DISCUSSION

Mutant CTS alleles have been identified from a number of forward genetic screens based on the tolerance to fatty acid analogues, including IBA [21] and 2,4-DB [22]. cts was first identified as a germination factor, whose expression overcame dormancy-promoting programmes [18]. Analysis of fatty acid and acyl-CoA profiles of germinating cts seeds revealed that the mobilization of TAG (triaclyglycerol) is greatly compromised in the mutants; high levels of long-chain fatty acids in TAG remain and long-chain acyl-CoAs accumulate [19]. These observations led to the conclusion that CTS is involved in the transport of fatty acyl-CoAs into peroxisomes for \( \beta \)-oxidation, in a manner analogous to that of the yeast Pxa1/Pxa2 and mammalian ABCD1 and ABCD3 (for recent reviews, see [17,35]) proteins. A recent important finding is that the level of JA is substantially reduced in cts alleles, thereby also implicating CTS in the transport of OPDA ([9S, 13S]-12-oxygenodienoic acid), which is converted into JA within peroxisomes [24]. Alterations in fatty acid and acyl-CoA profiles and disruption of the metabolism of JA, 2,4-DB and IBA in cts alleles was the basis for speculation that substrates for CTS

speculation for the incorporation of amino acids into proteins cannot be made, since the relative labelling of amino acid pools between cts-1 and Ler was reversed compared with acn2 and Col-0. It is evident that both the cts-1 and acn2 mutants retain the capability to assimilate radiolabelled acetate by other mechanisms.

Some reports have suggested that the human ALDP protein may function by stabilizing VLCS (very-long-chain acyl-CoA synthetase), since the defect demonstrated in X-linked ALD1 cells is the activation of long-chain fatty acids to very-long-chain acyl-CoA [15]. Although characterized VLCS mutations are not associated with X-ALD [34] and current evidence suggests that ALDP does not stabilize or physically associate with VLCS [17], we decided to verify that the labelling results observed for cts were not a result of a reduction in AcetCS activity. Glyoxysomes were isolated from mutant and wild-type seedlings on sucrose density gradients and assayed for AcetCS activity

![Figure 4 Comparison of exogenous [2-14C]acetate utilization by acn2 and cts-1 seedlings](image-url)
transport were at least four carbons long, and could include ring moieties [20,24]. Our results suggest that CTS has the potential to facilitate the transport of compounds as small as acetate.

Although mammalian and yeast models suggest that CoA derivatives are transported, and the accumulation of long-chain acyl-CoAs in cts-1 is consistent with the concept that the Arabidopsis CTS transports acyl-CoAs [19], there is evidence that CTS may transport fatty acids as well. Single or multiple gene mutations in every step of peroxisomal β-oxidation, acyl-CoA oxidase [36,37], multifunctional protein [38] or thiolute [22,39], produces the phenotype of sucrose-dependent germination and accumulation of fatty acyl-CoA [37–39]. Thus the acyl-CoA accumulation observed for cts alleles is not unique to disruption of a particular transport step, and no direct conclusion about substrate transport can be drawn [17]. Elimination of the glyoxysomal LACSs (long-chain acyl-CoA synthetases) LACS6 and LACS7 in Arabidopsis, produces sucrose-dependent establishment, similar to that observed for the CTS mutants [40]. Such a severe phenotype at this step suggests that the majority of fatty acids are activated into their CoA esters directly within glyoxysomes. Fulda et al. [40] presented two models for fatty acid supply to LACS6/LACS7, one which involved CTS/PXA1/PED3 and the other which used a currently unidentified fatty acid transporter. Although distinction between the two models is not possible for unesterified long-chain fatty acids, our results support the CTS/PXA1/PED3 model for the transport of unesterified acetate. The reduction in the amount of radiolabelled acetate appearing in the soluble carbohydrate fraction for the two cts alleles is similar to the reduction observed for the acn1 mutant [7]. If acetate was activated into acetyl-CoA within the cytosol and subsequently transported into peroxisomes by CTS, then it would not be possible to explain the similarly large reduction in labelled soluble carbohydrate observed for acn1. We cannot exclude the possibility that acetate or FAc is activated to (F)acetyl-CoA in the cytosol, and that the CoA moiety is removed during the transport process. In either case, it would be unesterified acetate that is transported. The presence of AcetCS activity in glyoxysomes of seedlings lacking CTS eliminates the alternative explanation of a negative effect on ACN1 activity, thus the explanation of compromised acetate transport is more plausible. The model for the involvement of CTS and AA7/ACN1 in the glyoxylate cycle is given in Scheme 1. It shows that acetate (either exogenous or endogenous recycled acetate) enters the glyoxysome via CTS, is activated by AA7/ACN1 and subsequently partitioned between malate synthase and citrate synthase.

It has been proposed that CTS functions as a signalling molecule during the initiation of germination and metabolic pathways during seedling establishment [24]. During germination, CTS may either produce a germination-promoting compound or remove a germination inhibitor, and its role subsequently alters to facilitate the transport of compounds as small as acetate.

The broken arrow indicates the hypothesis that acetate is transported in the free, unesterified, form. Ac: acetate; AcCoA, acetyl-CoA; Ac<sup>ex</sup>, exogenously supplied acetate; Cit, citrate; CSY, citrate synthase; Glyox, glyoxylate; Mal, malate; MLS, malate synthase; OAA, oxaloacetate.

CTS and the glyoxylate cycle have a specific secondary function to re-assimilate acetate, as well as to facilitate lipid mobilization? Despite extensive research into reserve mobilization within oilseeds, surprisingly little is known about the intermediate steps in carbon reserve partitioning and potential carbon recycling during seedling development. A comparative investigation of time-course labelling patterns appearing in metabolite fractions and specific intermediates from isotopically labelled acetate and fatty acids, which employs the existing glyoxylate cycle and other mutants at various stages of germination and establishment, is essential in order to remedy our lack of knowledge concerning seedling carbon nutrition.

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