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

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#### Abbreviations:

The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxybutyric acid; AcetCS, acetyl-CoA synthetase; *acn*, acetate non-utilizing; CTS, COMATOSE, FAc, monofluoroacetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; PGS, Principal Growth Stage

## Summary

The *Arabidopsis acn* (acetate non-utilizing) mutants were isolated based on 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 the mutants *cts-1* (COMATOSE) and *pxa1* (peroxisomal ABC transporter 1). The *acn2* locus was mapped to within 3 cM of the COMATOSE gene on the bottom arm of chromosome IV using CAPS and SSLP markers. Crossing *acn2* and *cts-1* failed to restore the fluoroacetate sensitive phenotype suggesting allelism of the mutations. Sequencing of the *ACN2* locus revealed a C-T nonsense mutation in exon 13, which would result in elimination of the C-terminal hemitransporter domain of the encoded protein. Neither full-length CTS protein nor truncated protein was detected on immunoblots using C-terminal or N-terminal specific anti-CTS antibodies, respectively, demonstrating the absence of the entire CTS protein in *acn2*. Emerged seedlings of both *cts-1* and *pxa1* alleles displayed increased resistance to FAc compared to corresponding wild-types. Both *cts-1* and *pxa1* mutant lines expressing the wild-type protein through complementation were FAc sensitive demonstrating that mutation of the COMATOSE gene was responsible for the FAc resistant phenotype. Feeding studies confirmed that both *acn2* and *cts-1* were compromised in their ability to convert radiolabelled acetate into soluble carbohydrates. These results demonstrate a role for the ABC protein COMATOSE in providing acetate to the glyoxylate cycle in developing seedlings.

## INTRODUCTION

The classic gluconeogenic model of lipid mobilization is to convert fatty acids to sucrose during establishment to support the growing seedling. Acetyl-CoA from  $\beta$ -oxidation enters the glyoxylate cycle to be converted to organic acids for transport to mitochondria, which subsequently produce sucrose by steps of the TCA cycle and gluconeogenesis [1]. Establishing the path of carbon flow through the glyoxylate cycle has relied almost exclusively on feeding radiolabeled acetate to seeds and seedling tissues [2,3,4]. Gluconeogenic models derived from the use of exogenous acetate instead of labeled fatty acids must include the activation of acetate to acetyl-CoA and the process of acetate and/or acetyl-CoA entry into glyoxysomes. However, the specific proteins involved have remained unknown until recently. Through characterization of an Arabidopsis mutant resistant to the toxic acetate analogue FAc [5], we determined that the AMP-binding protein AAE7/ACN1 [6] was a glyoxysomal short-chain acetyl-CoA synthetase responsible for the activation step [7]. This finding suggested that free acetate had to enter glyoxysomes for activation, but it remained unknown if it diffused into glyoxysomes, was drawn into them by an ion trapping mechanism, or was actively transported by some protein component.

COMATOSE (CTS) is a member of the superfamily of ATP-Binding Cassette (ABC) proteins that is ubiquitous to cellular organisms. CTS is a full-sized transporter characterised by two nucleotide binding folds (NBF) and two transmembrane spanning domains (TMD) in a forward TMD1-NBF1-TMD2-NBF2 orientation. In surveys of the ABC protein family of Arabidopsis, it was noted that CTS is similar to the yeast and mammalian peroxisomal membrane proteins (PMPs, [8,9]). The ABCD subfamily of yeast contains two members PXA1 and PXA2, which are half-transporters that heterodimerise [10] to facilitate transport of long-chain fatty acyl-CoA across the peroxisomal membrane [11]. The ABCD subfamily of peroxisomal transporters in humans is comprised of four members ABCD1-4, of which ABCD3 (PMP70) and ABCD1 (ALDP) appear to transport, respectively, long-chain [12] and very long-chain fatty acyl-CoA [13] for chain-shortening by  $\beta$ -oxidation. Elimination of ALDP in humans by mutation results in the progressive genetic illness X-linked adrenoleukodystrophy [14], which results in increased levels of very long-chain fatty acids in nerve cells due to impaired  $\beta$ -oxidation [15]. The health consequences can be severe with those afflicted with childhood cerebral ALD perhaps surviving only into their teens [16]. Peroxisomal ABC transporters have been the focus of a recent review [17].

The *COMATOSE* mutant of Arabidopsis was isolated in a forward genetic screen to identify lines that possessed reduced germination potential [18]. Footitt et al. [19] reported the mutation responsible for the phenotype resided in the gene encoding the ABC protein homologue of the 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 the auxin analogue indole-3-butyric acid [21]. Mutant alleles for this gene, *ped3*, were isolated in a screen for mutants resistant to the auxin analogue 2,4-dichlorophenoxy butyric acid 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 either fatty acid and/or acyl-CoAs for

peroxisomal  $\beta$ -oxidation. Evidence that 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 the mutants, *cts-1* [19], *pxa1* [21], and *ped3* [23], raised the possibility that the protein may also function as an acetate transporter. FAc exerts its toxicity through inhibition of aconitase upon conversion to fluorocitrate via the sequential actions of acetyl-CoA synthetase and citrate synthase [25]. Therefore, a mutation that would prevent FAc entering the glyoxylate cycle would result in enhanced FAc tolerance. Through a physiological, genetic and biochemical study we demonstrate that CTS is integral to acetate metabolism in glyoxysomes in developing seedlings, and we present evidence to support speculation that unesterified acetate is the compound transported.

## EXPERIMENTAL

### Plant Material

All seed batches used in the experiments were surface-sterilized and imbibed in the dark at 4 °C for 4 days before sowing onto agar plates. For all experimental conditions seeds were germinated at 20 °C at 70  $\mu\text{mol}$  of photons ( $\text{m}^2$ )<sup>-1</sup> s<sup>-1</sup> constant illumination, except for the immunoblot analysis where seeds were germinated and grown at a day length of 9 h. Standard agar media plates contained 0.8 % agar and 1/2-strength MS salts [26] to which sucrose was added to a concentration of 20 mM where specified. Prior to addition of agar and subsequent autoclaving, all media were adjusted to pH 5.7 with 0.1 N KOH. For tests of resistance to FAc, seeds were sown onto standard agar media plates containing 0.5 mM sodium FAc (Sigma-Aldrich Ltd., Poole, Dorset UK). FAc was prepared as concentrated stock solution, filter sterilized and added to autoclaved standard agar media. The auxin analogues 2,4-D and 2,4-DB were prepared as concentrated stocks in ethanol and added to autoclaved standard agar media. For root growth measurements, surface-sterilized seeds were sown onto standard agar media plates containing 20 mM sucrose. After four days in the dark at 4 °C, plates were transferred to the growth room. After 4 more days, 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/15 h, light/dark cycle.

### Characterisation of the *acn2* locus and gene and protein expression analysis

For mapping purposes, F2 generation *acn2* plants homozygous for resistance to FAc were crossed to *Ler* reciprocally. Approximately 120 FAc resistant F2 generation seedlings were rescued onto soil and grown for harvesting leaf material for DNA extraction. Genomic DNA was isolated from leaf material using the PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN USA). Primer combinations and thermocycling conditions for analysing Cleaved Amplified Polymorphic Sequences are specified in Konieczny and Ausubel [27] and Baumbusch et al. [28]. The analysis of Simple Sequence Length Polymorphisms was conducted according to Bell and Ecker [29]. PCR reaction set-up and thermocycling were conducted on an MWG Automated Biosystem RoboSeq™ 4204 with an integrated 96-well thermocycler.

For sequencing the *acn2* locus, primer pairs were designed to amplify overlapping regions of the *ACN2* gene (At4g39850.1, full genomic sequence) from the sequence from The Arabidopsis Information Resource website. 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 sense primer 5'-AAGTGTAGTGTTCCTCGTTTTTC-3' and

antisense primer 5'-AAAGAGGCTATTCGGTCAGAGAT-3'. *ACN2* cDNA was prepared by reverse transcription from total RNA isolated from newly emerged seedlings at principle growth stage (PGS) 0.7 [30], which had been germinated on standard agar media containing 20 mM sucrose. Total RNA was isolated using the Gentra PURESCRIPT® RNA Isolation Kit, modified with a phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) wash step inserted prior to isopropanol precipitation.

For the expression analysis of *CTS* in wild-types and mutants, vernalized seeds were sown onto standard agar media plates with or without 20 mM sucrose. Germinated seedlings were removed after four days and the remaining seeds damaged by nicking the seed coat with forceps under a microscope. PolyA mRNA was isolated from either 10 seeds from mutant lines plated onto standard agar media or from 5 PGS 0.7 seedlings if plated onto standard agar media plus sucrose. PolyA mRNA was isolated using the Dynabeads (Dynal AS, Oslo, Norway) according to the manufacturer's instructions. Reverse transcription was conducted as described in Laval et al. [31]. PCR was conducted using the *CTS* specific sense primer 5'-ACGGATGCTGAAATTGATTCAGT-3' and antisense primer 5'-TGCTGAGTTCACTCTGTTGTCT-3'.

For the immunoblot analyses of *CTS* expression, vernalized seeds were plated on standard agar media plates containing 20 mM sucrose. Germinated seeds were removed after four days and ungerminated mutant seeds were damaged by nicking the testa. Proteins were extracted from PGS 0.7 seedlings, as in Footitt et al. [19], except the quantities of PMSF and Sigma protease inhibitor cocktail were increased to 5 mM and to 5% (v/v), respectively. Protein aliquots of 20 µg were separated by SDS-PAGE on a 7.5% gel, blotted onto PVDF membrane and stained with Ponceau S to ensure equal loading. They were then probed with either an antibody recognising 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 1-25 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 60mM Tris pH 6.7 containing 2% SDS and 0.7% (v/v) β-mercaptoethanol at 50°C for 30 minutes and reprobed with the N-terminal antibody. Detection of immunoreactive proteins was by chemiluminescence using the ECL+Plus™ Western blotting detection kit (Amersham Pharmacia) according to manufacturer's instructions.

### Labeled acetate feeding

The [<sup>14</sup>C]acetate feeding experiments were adapted from Eastmond et al. [3] with the following modifications. One hundred 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-<sup>14</sup>C]acetate (20.5 MBq·mmol<sup>-1</sup>) and 50 mM MES, pH 5.2. Two consecutive 0.15 ml aliquots of 5N KOH were used to trap released CO<sub>2</sub>. Both fractions were combined for scintillation counting. After 4 h, 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 Perkin Elmer™ Optiphase HiSafe3 liquid scintillation cocktail. The ethanol-insoluble material was combusted using a Biological Material Oxidizer OX400 (R. J. Harvey Instrument Corporation, New Jersey,



USA). The CO<sub>2</sub> was trapped in 15 ml of Oxosol™ <sup>14</sup>C (National Diagnostics, Hesse, East Riding of Yorkshire, UK) 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 in Turner et al. [7]. Acetyl-CoA synthetase activity was measured spectrophotometrically in a coupled reaction with citrate synthase and malate dehydrogenase according to Miller and Bonner [32]. Short-chain acyl-CoA oxidase activity was measured according to Hyrb and Hogg [33].

## RESULTS

### Phenotypic characteristics of *acn2*

The synthetic auxin analogues 2,4-dichlorophenoxybutyrate (2,4-DB) and indole-3-butyric acid have been used to select for defects in glyoxysomal function, including biogenesis and fatty acid  $\beta$ -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 root growth of *acn2* (Col-0 background) to *cts-1* (*Ler* background), which is 2,4-DB resistant, in the presence of increasing concentrations of the compound (Figure 1A). In the absence of 2,4-DB, root growth of the mutants was less than the corresponding wild-type. Mutant root growth decreased with increasing concentrations of 2,4-DB, but not as dramatically as either wild-type. Significantly greater root growth compared to wild-types was observed for both *acn2* and *cts-1* starting at 1  $\mu$ M and 3  $\mu$ M, respectively. At 5  $\mu$ M, root growth of the wild-types was abolished, whereas a large number of mutant seedlings exhibited substantial root growth. Like *cts-1*, *acn2* was found sensitive to the degradation product 2,4-D, consistent with the possibility that conversion of 2,4-DB to 2,4-D was 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 for propionate and butyrate at concentrations of 0.01 mM and 0.1 mM, respectively. Neither acetate nor the other concentrations of propionate and butyrate tested had any affect. No effect of fatty acids on *acn2* seed germination was apparent (data not shown). Some of the vegetative phenotypes we 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 for the *ped3* alleles [23]. The delay in flowering time was not as dramatic for *acn2* as it is for *pxa1* (data 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 map-based PCR approach using CAPS markers [27,28] and SSLPs [29]. The *acn2* locus was found to lie within 2 cM of the CAPS marker *DHS1* and to within 3 cM of the SSCP marker *NGA1107*. These map distances place the *acn2* locus very close to the position of chromosome IV known to hold the *CTS* gene. No other gene in the vicinity of *CTS* would be expected to give the phenotypes observed for *acn2* upon disruption by mutation. Reciprocal crosses of *acn2* to *cts-1* were conducted in order to test complementation of genotypes. Crosses with *cts-1* as the pollen donor were more successful and produced more seed, which corresponded with a reduced fertility of *acn2*. The same occurred with *Ler* and *acn2* crosses. The F1 generation of the *cts-1* x *acn2* and *Ler* x *acn2* crosses were tested for dormancy and FAc resistance (Table 1). A small proportion of seed of the *cts-1* x *acn2* crosses germinated in the absence of sucrose similar to that of either *cts-1* or *acn2* single mutants, whereas

100% of the seed from the *Ler* x *acn2*, and *Col-0* x *acn2* crosses germinated. As with the single mutants, a substantial increase in the proportion of seeds from the mutant crosses germinated upon transfer to sucrose plates and F1 generation seed from the crosses established well in the presence of FAc (sucrose included) once the testa was damaged or removed. Although listed in Table 1 as being FAc sensitive, the F1 generation *Ler* x *acn2* crosses possessed the slightly elevated resistance previously reported for the F1 generation of the backcross of *acn2* to its parental wild-type [5].

DNA was isolated from *acn2* plants exhibiting sucrose-dependent establishment in the presence 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 At4g39850.1 present in the TAIR database [www.arabidopsis.org] revealed a single mutation, which was a C-T substitution in exon 13 (Figure 2A). Referring to the CDS for At4g39850.1, the mutation lies at nucleotide position 2284, which introduces a stop codon at <sup>762</sup>Gln that would truncate the protein in the first predicted transmembrane spanning region of the second hemitransporter domain. A detailed description of the gene and a comprehensive comparison of the protein primary sequence with human and *S. cerevisiae* ABC protein transporters is presented in Zolman et al. [20].

RT-PCR analysis of the expression of *CTS* in *acn2*, compared to *cts-1* and their respective wild-type parents, is given in 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 media plates for 24 h (no radical emergence) is similar to that 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 within exon 10 [19]. Using the same primer pair, *CTS* mRNA was expressed within the *acn2* mutant in both newly emerged seedlings and imbibed, un-germinated seeds. Although *ACN2* was expressed, no immunoreactive protein was observed using *CTS* polyclonal antibodies [19] raised against either the C-terminal and N-terminal portions of the protein (Figure 2C-E). Although the specificity of the un-purified N-terminal antibody is less than that of the affinity-purified C-terminal one, it is apparent that the lack of a protein of approximately 84 kDa -- the predicted molecular mass of the truncated protein -- indicates that the entire *CTS* protein is missing in *acn2*. All protein bands observed in the mutants between 72 and 95 kDa are present also in the wild-types and suggest either non-specific binding of the anti-*CTS* antibody or 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 upon damaging or removal of the seed coat in the presence of sucrose. It may be that finding the mutant was quite fortuitous, because we did not look specifically for sucrose-dependent germination and establishment, and isolation of the mutant resulted from spontaneous germination due to sucrose being included within FAc-containing standard agar

media plates. We also reported that *acn2* appeared to be dominant with F1 generation seeds from the Col-0 x *acn2* backcrosses showing slightly elevated resistance to FAc. Seedlings from F2 generation seeds batches exhibited three different levels of FAc resistance, which would be 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, 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. Indicative of resistance was greening and expansion of the cotyledons and particularly root growth, which does not occur with either wild-type. The effects of FAc on root growth of each genotype are shown in Figures 3B and 3C. Significant differences between mutants and wild-types were observed at FAc concentrations as low as 0.1 mM. Interestingly, the spontaneously germinated seeds of undamaged homozygous *acn2* exhibited a wild-type FAc sensitive phenotype as shown by yellow cotyledons and no root growth (data not shown). The resistance of *cts-1* is in stark contrast to 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*) also was sensitive to FAc (Figure 3A and 3B). These results demonstrate that CTS plays a role in mediating FAc sensitivity in wild-type plants.

### CTS mutants show reduced incorporation of acetate into soluble carbohydrate

In order to establish the metabolic consequences due to disruption to glyoxysomal acetate metabolism suggested by FAc resistance, we conducted [<sup>14</sup>C]acetate feeding studies on *acn2* and *cts-1* compared to parental wild-types. The profiles of radiolabel recovered within the different analytical fractions are characteristic of the disruption of acetate entry into the glyoxylate cycle as observed for the *icl* [3,7] and *acn1* mutants [7]. Dramatic reductions were observed in radiolabel measured within the soluble carbohydrate fraction whereas only minor differences were observed in the other fractions. The proportion of radiolabel in the carbohydrate fraction of the mutants was, on average, less than 20% of that present in the same fraction of wild-type seedlings. The ability of *cts-1* and *acn2* to assimilate some acetate may explain why wild-type sensitivity to FAc is observed in seedlings that spontaneously germinate. The reduction in the conversion of exogenous acetate to carbohydrate in the *acn2* and *cts-1* mutant was similar to that for *acn1* [7]. The reduced radioactivity observed within the ethanol insoluble fractions for *cts-1* and *acn2* likely resulted from less radiolabelled carbohydrates going into complex structural and storage polysaccharides. The small, but significant, increase in the labelled organic acid pools within the mutants probably reflects the difference in acetate assimilation. Similar 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 to *acn2* and Col-0. It is evident that both mutants retain the capability to assimilate radiolabelled acetate by other mechanisms.

Some reports have suggested that human ALDP protein may function by stabilizing very long-chain acyl-CoA synthetase (VLCS), since the defect in X-linked adrenoleukodystrophy cells is the activation of

long-chain fatty acids to very-long chain acyl-CoA [15]. Although characterized VLCS mutations are known not to be associated with X-ALD [34] and current evidence suggests that ALDP does not stabilize or physically associate with VLCS [17], we wanted to verify that the labelling results observed for *cts* were not due to lower AcetCS activity. Therefore, glyoxysomes were isolated from mutants and wild-type on sucrose density gradients and assayed for AcetCS activity [7]. Short-chain acyl-CoA oxidase activity was used as a co-localisation control. The glyoxysomes from the bottom fraction of the sucrose gradient were gently disrupted either by a freeze/thaw cycle or by passage through a small bore syringe needle. Thus, any physical interaction between CTS and ACN1, which is the only enzyme contributing AcetCS activity in seedling glyoxysomes [7], was unlikely to be disrupted. AcetCS activity was detected at a level of  $7.5 \pm 3.8$  nmol mg protein<sup>-1</sup> h<sup>-1</sup> (n=3) for wild-type *Ler*, which was similar to that for Col-0 (~ 6 nmol mg protein<sup>-1</sup> h<sup>-1</sup>, [13]). A trial of *cts-1* gave an activity of 18 nmol mg protein<sup>-1</sup> h<sup>-1</sup>. It was clear that damaging the thousands of *cts-1* seeds needed to obtain sufficient material would not permit precise staging of dark-grown development. Therefore, the *cts1 ttg1-1* double mutant was also tested, since the *TRANSPARENT TESTA* mutation facilitates normal germination of seeds in the *cts* background [19]. AcetCS activity was also apparent in the *cts-1 ttg1-1* double mutant at  $40 \pm 16$  nmol mg protein<sup>-1</sup> h<sup>-1</sup> (n=3). The greater apparent specific activity of the *cts-1 ttg1-1* double mutant may be attributable to differences in the background genotype in which the *ttg1-1* mutation resides. Short chain ACX specific activities were similar between *cts-1 ttg1-1* double mutant and *Ler* and did not suggest differential stability of glyoxysomes (data not shown). These results indicate that the decrease in carbohydrate labelling is not due to compromised glyoxysomal AcetCS activity in plants lacking CTS.

## DISCUSSION

Mutant CTS alleles have been identified from a number of forward genetic screens based on tolerance to fatty acid analogues, including to IBA [21] and 2,4-DB [22]. *COMATOSE* was first identified as a germination factor, whose expression overcame dormancy promoting programs [18]. Analysis of fatty acid and acyl-CoA profiles of germinating *cts* seeds revealed that the mobilization of TAG is greatly compromised in the mutants, and high levels of long-chain fatty acids in TAG remain and long-chain acyl-CoAs accumulate [19]. These results led to the conclusion CTS is involved in the transport of fatty acyl-CoAs into peroxisomes for  $\beta$ -oxidation in a manner analogous to the yeast PXA1/PXA2 and mammalian ABCD1 and ABCD3 [for recent reviews see 17 and 35]. A recent important finding is that jasmonic acid levels are substantially reduced in *cts* alleles, thereby implicating CTS in the transport of (9S, 13S)-12-oxo-phytodienoic acid (OPDA), which is converted to jasmonic acid within peroxisomes [24]. Alterations of 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 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  $\beta$ -oxidation, acyl-CoA oxidase [36,37], multifunctional protein [38] or thiolase [22,39] produce the phenotype of sucrose-dependent germination and accumulate fatty acyl-CoA [37,38,39]. Thus, the acyl-CoA accumulation observed for *cts* alleles is not unique to a transport step, and thus no direct conclusion about substrate transport can be drawn [17]. Elimination of the glyoxysomal long-chain acyl-CoA synthetases LAC6 and LAC7 in Arabidopsis produces sucrose-dependent establishment similarly to the *CTS* mutants [40]. Such a severe phenotype at this step suggests that the majority of fatty acids are activated to their CoA esters directly within glyoxysomes. Fulda et al. [40] presented two models for fatty acid supply to LAC6/LACS7, one involving CTS/PXA1/PED3 and the other utilizing an unknown 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 radiolabel acetate appearing in the soluble carbohydrate fraction for the two *cts* alleles is similar to that for the *acn1* mutant [7]. If acetate was activated to 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 probable. The model for the involvement of CTS and AAE7/ACN1 in the glyoxylate cycle

is given in Figure 5. It shows that either exogenous or recycled (endogenous) acetate that enters the glyoxysome via CTS is activated by AAE7/ACN1 to be partitioned between malate synthase and citrate synthase.

It has been proposed that the function of CTS is signaling during the initiation of germination and metabolic 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 provide fatty acids for  $\beta$ -oxidation during seedling establishment. Comparative germination studies of *cts-1*, *cts-2*, and *pxa1-1* with other  $\beta$ -oxidation and glyoxylate cycle deficient mutants indicate that a lack of CTS results in reduced germination potential as opposed to increased dormancy, since *cts* seeds after-ripen normally [41]. Comparison of the germination potential of *cts* with *kat2-1* -- a mutant lacking a peroxisomal 3-ketoacyl-CoA thiolase -- led to the conclusion that reduced germination was not specifically a consequence of compromised  $\beta$ -oxidation and that CTS likely serves other metabolic roles. Our results support the conclusion of potential multiple metabolic roles for CTS. They emphasize the potential involvement of CTS in distinct metabolic pathways, since acetate would bypass  $\beta$ -oxidation and enter the glyoxylate cycle directly upon activation to acetyl-CoA. Is it possible that the 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 detail is known about the intermediate steps in carbon reserve partitioning and potential carbon recycling during seedling development. A comparative investigation of time-course labeling 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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## Figure Legends

### Figure 1 Comparison of 2,4-DB resistance of mutants and wild-type

Seeds were treated as described in Experimental. Root lengths were measured 6 days after transfer to growth conditions. Symbols and error bars represent the average  $\pm$  sd of at least 10 seedlings. **A.** Root growth in the presence of 2,4-DB. Student's T-tests were conducted on data from roots at concentrations of 1, 3, and 5  $\mu$ M between each mutant and respective wild-type. Symbols:  $\blacktriangledown$ ,  $P < 0.01$  *acn2* and  $P < 0.1$  *cts-1* vs *Ler*; \*,  $P < 0.0001$  for both *acn2* and *cts-1*. **B.** Root growth in the presence of 2,4-D. Col-0 and *acn2* were significantly different ( $P < 0.0001$ ) at all concentrations. *Ler* and *cts-1* were statistically different at concentration below 0.12  $\mu$ M ( $P < 0.0001$ ) but statistically non-significant at concentration of 0.12  $\mu$ M and 0.24  $\mu$ M ( $P > 0.1$ ).

### Figure 2 Molecular characterisation of *acn2*

**A.** The *acn2* locus. DNA and total RNA were isolated from Col-0 and *acn2* mutants. All exons of the *acn2* locus were sequenced once and the mutation confirmed by resequencing of Exon 13 and the corresponding region of the cDNA. Exons and introns are represented by open and filled bars, respectively. **B.** An ethidium bromide-stained agarose gel showing the expression of *CTS* as determined by RT-PCR. *rbcS* represents the small subunit of ribulose biphosphate carboxylase. **C-E.** Analysis of *CTS* protein in PGS 0.7 seedlings germinated on standard agar media plates plus 20 mM sucrose using C-terminal (**C**) or N-terminal (**D**) specific antibodies. *Ler*, Lane 1; *cts-1*, Lane 2; Col-0, Lane 3; *acn2*, Lane 4. Twenty  $\mu$ g of protein was loaded into each well, and equal loading was verified by staining with Ponceau S (**E**).

### 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 (John Innes Centre), provided for *in planta* expression as part of GARNet facilities. *pxa1/35SPXA1* represents the mutant line expressing the *PXA1* cDNA under the control of the CaMV 35S promoter (26). **(A).** Seeds were treated as described in Experimental, except that spontaneously germinated mutants were not removed prior to nicking the testa. All seeds were sown onto standard agar media containing 20 mM sucrose with (+) or without (-) 0.5 mM FAc. Photos 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 Experimental. Roots lengths were measured 6 days after nicking the testas of the mutants. Symbols and error bars represent the averages  $\pm$  sd of at least 10 seedlings. Lines have been included to clarify connections for obscured data points. Based on T-Test statistics, wild-type root growth was significantly less than mutant ( $P < 0.0001$ ) at FAc concentrations of 0.1 mM and greater as indicated by asterisks. Average root growth for complementation genotypes *cts-1/155A23* and *cts-1/159N01* was intermediate between *Ler* and the complementation genotypes shown.

**Figure 4 Comparison of exogenous [2-<sup>14</sup>C]acetate utilization by *acn2* and *cts-1* seedlings**

Component designations: CO<sub>2</sub>, KOH-trapped label; CHO, soluble carbohydrates (neutral fraction); AA, amino acids (basic fraction); OA, organic acids (acidic fraction); Lipid, ether-soluble label; SO, ethanol-insoluble label from Sample Oxidizer. The values and error bars represent the average  $\pm$  sd of 3 independent experiments for Col-0 and *Ler* and 6 independent experiments for *acn2* and *cts-1*. The seedlings were maintained at the growth conditions of light and temperature mentioned in Experimental during the incubation period. The total amounts of radiolabel taken up over the 4 h incubation period ( $\times 10^3$  DPM) were  $53.0 \pm 14.2$ ,  $65.8 \pm 29.7$ ,  $50.3 \pm 10.3$  and  $37.8 \pm 10.4$  for Col-0, *acn2*, *Ler*, and *cts-1*, respectively. The symbols represent T-test probability values between mutant and wild-type: \*,  $P < 0.0001$ ; ▼,  $P < 0.01$ . All other differences were insignificant at  $P > 0.05$ .

**Figure 5 Scheme of acetate transport and activation for entry into the glyoxylate cycle in developing seedlings**

Abbreviations: Ac, acetate; Ac<sup>ex</sup>, exogenously supplied acetate; Ac<sup>en</sup>, acetate from endogenous sources; AcCoA, acetyl-CoA; Glyox, glyoxylate; OAA, oxaloacetate; MLS, malate synthase; CSY, citrate synthase; Mal, malate; Cit, citrate. The dashed line indicates the hypothesis that acetate is transported in the free, un-esterified form.

**TABLE 1 Phenotypic analysis of the F1 generation of *acn2* and *cts-1* crosses**

The phenotypes tested were the proportion of total seeds germinating by 4 d after transfer to growth chamber and establishment in the presence of FAc. The letter 'n' gives the number of independent seed batches tested for appearance of phenotype. NA: not applicable.

Genotype	MS <sup>1</sup>	MS + Suc <sup>2</sup>	FAc <sup>R3</sup>
<i>Col-0</i> (% , n=3)	99 ± 1	NA	0
<i>Ler</i> (% , n=3)	100	NA	0
<i>cts-1</i> (% , n=3)	11 ± 4	53 ± 2	> 99
<i>acn2</i> (% , n=3)	< 1	35 ± 4	> 99
<i>cts-1</i> x <i>acn2</i> (% , n=6)	5 ± 2	49 ± 17	> 99
<i>Ler</i> x <i>acn2</i> (% , n=3)	100	NA	0
<i>Ler</i> x <i>cts-1</i> (% , n=3)	100	NA	0
<i>Col-0</i> x <i>acn2</i> (% , n=3)	96 ± 2	NA	0

<sup>1</sup> Approximately 100 seeds were sown onto agar plates containing 1/2-strength MS salts, pH 5.7. The plates were incubated in the dark for 4 days before being transferred to the growth cabinet.

<sup>2</sup> Seeds that had not germinated were transferred onto agar plates containing 1/2-strength MS salts plus 20 mM sucrose.

<sup>3</sup> Seeds were sown directly onto agar plates containing 1/2-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. FAc<sup>R</sup> indicates fluoroacetate resistance.

Figure 1.

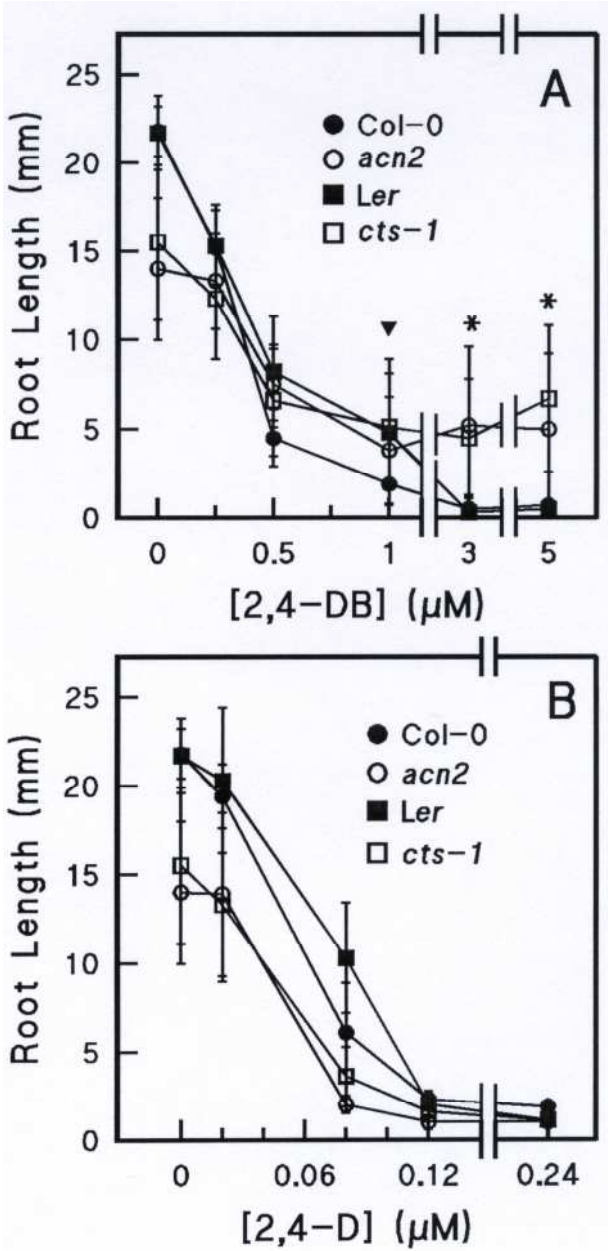


Figure 2.

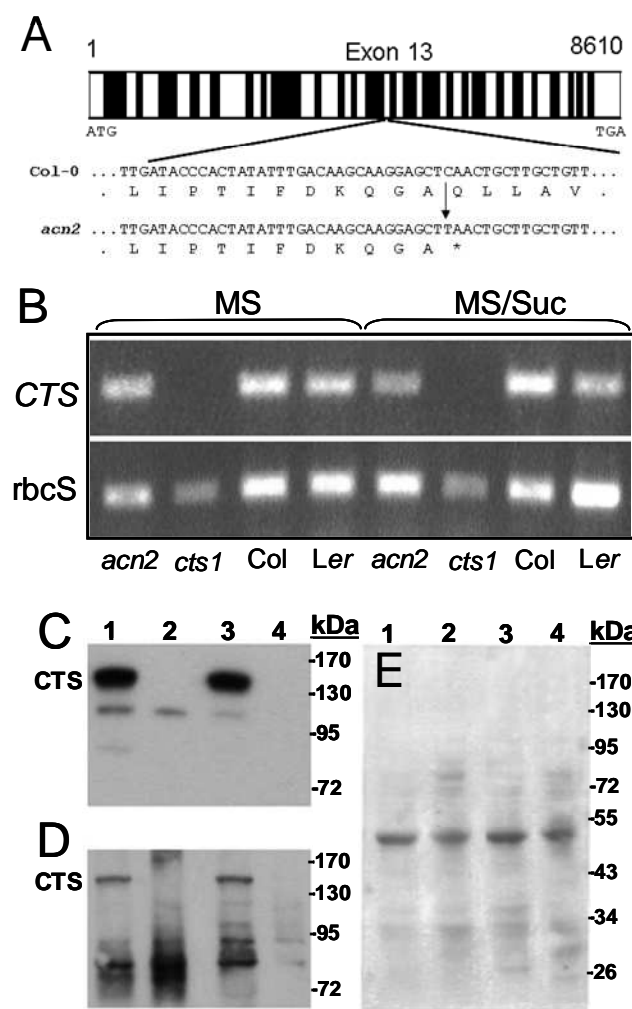




Figure 3.

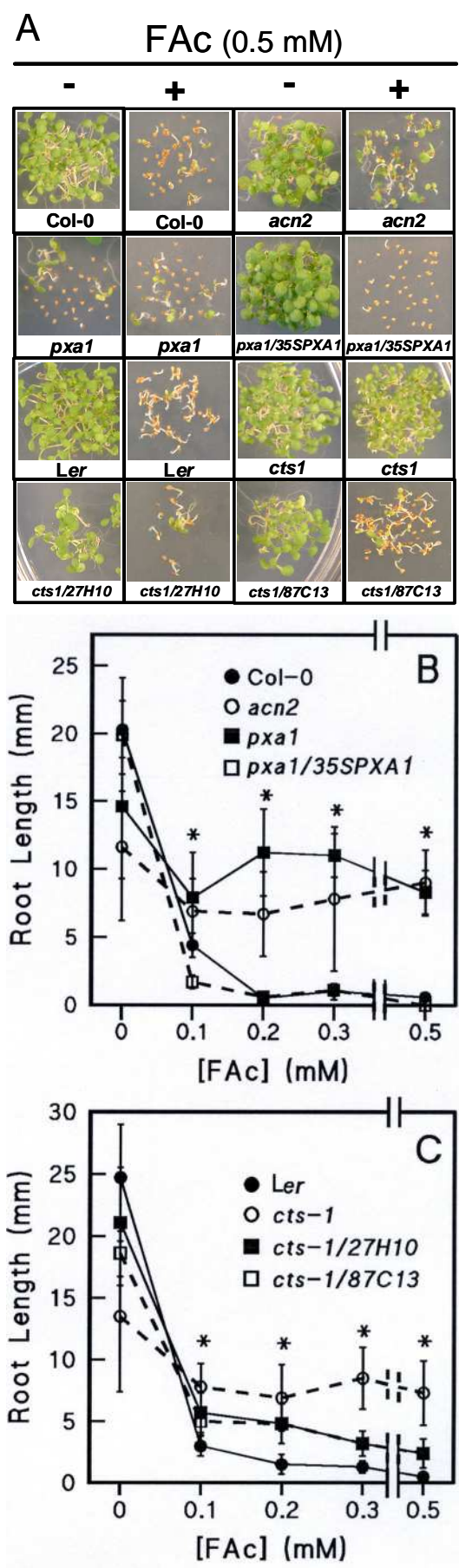


Figure 4.

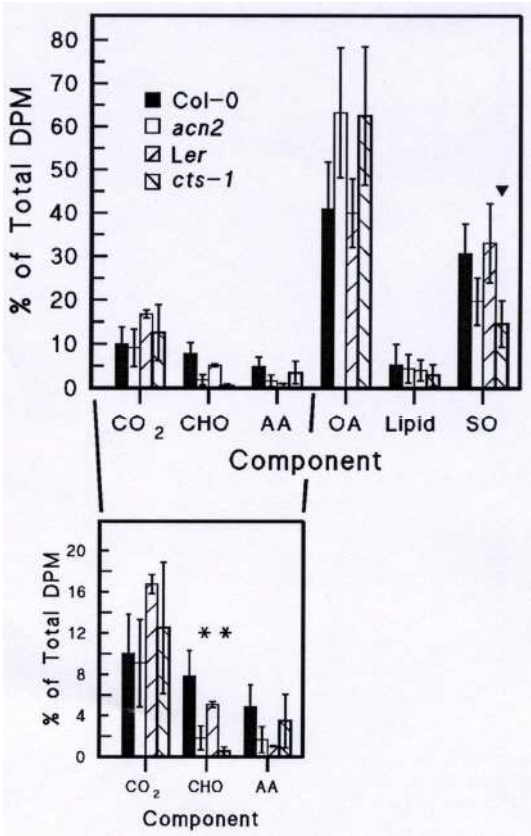


Figure 5.

