Characterization of Arabidopsis Fluoroacetate-resistant Mutants Reveals the Principal Mechanism of Acetate Activation for Entry into the Glyoxylate Cycle*

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The toxic acetate analogue monofluoroacetic acid was employed to isolate Arabidopsis tDNA-tagged plants deficient in their ability to utilize or sense acetate. Several tDNA-tagged lines were isolated, including two that were determined to be allelic to an EMS-mutagenized line denoted acn1 for ae non-utilizing. Following conventions, the tDNA-tagged mutants were designated acn1-2 and acn1-3. Both mutants displayed identical behavior to acn1-1 on a variety of fluorinated and nonfluorinated organic acids, indicating that resistance was specific to fluoroacetate. Thermal asymmetric interlaced PCR identified the sites of tDNA insertion in both mutants to be within different exons in a gene, which encoded a protein containing an AMP-binding motif. Reverse transcription-PCR confirmed that the gene was not expressed in the mutants, and quantitative reverse transcription-PCR showed that the gene is expressed in imbibed seeds and increases in amount during establishment. The wild type AMP-binding protein cDNA was cloned and expressed in Escherichia coli, and the expressed protein was purified by nickel chelate chromatography. The enzyme was identified as an acyl-CoA synthetase that was more active with acetate than butyrate and was not active with fatty acids longer than C-4. The enzyme was localized to peroxisomes by enzymatic analysis of organelar fractions isolated by sucrose density gradient centrifugation. Labeling studies with [14C]acetate showed that acn1 seedlings, like those of the isocitrate lyase mutant icl-1 (isocitrate lyase), are compromised in carboxylate synthesis, indicating that this enzyme is responsible for activating exogenous acetate to the coenzyme A form for entry into the glyoxylate cycle.

During seed germination and establishment, catabolism of fatty acids by glyoxysomal β-oxidation produces large quantities of acetate as acetyl-CoA, which is converted to sucrose via the sequential actions of the glyoxylate cycle, trichloroacetic acid cycle, and gluconeogenesis (1, 2). In seeds, in which oil is stored in the endosperm, storage lipid is converted completely to sucrose (3). Recent reports (4) suggest that acetate utilization during seedling establishment of some oilseed species may be more complex than described by classical gluconeogenic models. In Arabidopsis, exogenous acetate is respired within mitochondria as well as being converted to sucrose (5). An Arabidopsis mutant lacking a mitochondrial carnitine acyl carrier (CAC) protein cannot establish itself, indicating that alternative mechanisms of acyl-CoA or acetyl-CoA metabolism during seedling establishment are required (6).

The importance of acetate as a respiratory metabolite has been demonstrated also by acetate/glucose diauxic growth studies of rice cell cultures (7), where acetate was found to be utilized preferentially by inhibiting glucose uptake (8). The preferential use of acetate was accompanied by an increase in the activity of glyoxylate cycle enzyme isocitrate lyase, which supported the proposal that acetate was a positive regulator of glyoxylate cycle enzyme and gene expression in plants (9, 10). Acetate has also been shown to inhibit photosynthetic gene expression and may help regulate the switch from heterotrophy to autotrophy in developing seedlings (11).

We have undertaken a program to identify Arabidopsis mutants disrupted in their ability to sense acetate as a regulatory metabolite affecting gene expression (12). We have adapted for Arabidopsis a method employing the biotin monofluoroacetic acid, which was successfully used to isolate the acetate regulatory gene facB from Emericella (Aspergillus) nidulans (13, 14). In addition to identifying an important acetate regulatory gene, studies of fluoroacetate-resistant mutants of filamentous fungi have revealed many acetate metabolism genes required for growth on acetate as a sole carbon source, such as those encoding glyoxylate cycle enzymes (15, 16). The acetate non-utilizing mutants, in conjunction with fatty acid nonutilizing mutants (17), have proven to be valuable tools used to develop detailed models of acetate utilization in filamentous fungi (18). By employing the screening strategy of resistance to FAc, we predicted that we would also identify plant acetate utilization genes as well as potential regulatory genes. Characterization of two tDNA-tagged mutants revealed independent mutations in a short-chain acyl-CoA synthetase gene of unknown biological function (19). Based on resistance to FAc, we hypothesized that the enzyme may activate exogenously supplied acetate for entry into the glyoxylate cycle in establishing seedlings (12). We demonstrate that the enzyme is responsible for this process, and we discuss the function of the enzyme relating to potential sources of acetate production within cells.

EXPERIMENTAL PROCEDURES

Plant Material and Genetic Screen—Eighty three seed batches from the Weigel, tDNA mutagenized collection (20) were obtained from the

* The abbreviations used are CAC, carnitine acyl carrier; AcetCS, acetyl-CoA synthetase; FAc, monofluoroacetic acid; RT, reverse transcription; Ni-NTA, nickel-nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid; EMS, ethylmethanesulfonate.
Acetate Metabolism and Signaling

Arabidopsis Biological Resource Center (Ohio State University, Columbus). All seeds were surface-sterilized and imbibed in the dark at 4 °C for 3 days before sowing onto agar plates. For all experimental conditions, seeds were germinated at 20 °C at 70 µmol of photons (m²·s)⁻¹ constant illumination. Standard agar media plates contained 0.8% agar, half-strength Murashige and Skoog salts (21), and 20 mM sucrose. The media, prior to the addition of agar and subsequent autoclaving, were adjusted to pH 5.7 with 0.1 N KOH. For the KOH genetic screen, 200 seeds from each batch were sown onto standard agar media plates containing 0.5 mM sodium FAc acid (Sigma). FAc was prepared as a concentrated stock solution, filter-sterilized, and added to the standard agar media after autoclaving. After 7 days, resistant seedlings were rescued onto standard agar media plates minus FAc. Surviving seedlings were transferred to soil after 4 days. Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Comparative Germination and Growth Studies—Comparative germination tests of the mutants using the various selective agents were conducted as described in Hooks et al. (12). Segregation of progeny for glufosinate resistance was conducted at a concentration of 30 µg ml⁻¹. Seedling growth studies were conducted by using standard agar plates with the specific concentrations of acetate and butyrate replacing sucrose. Standard agar plates were used for purposes of normalizing seedling weight. Not all seedlings within the wild-type and mutant seedlings gave equivalent seedling weights under standard conditions. After 7 days of growth, seedlings were harvested, counted, and the average fresh weight per seedling determined.

Identification of Flanking Sequences and Characterization of Mutant Loci—Genomic DNA was isolated from leaf material from acn1-2 (agar-utilizing) and acn1-3 using the Puregene® DNA isolation kit (Gentra Systems, Minneapolis, MN). The Thermal Asymmetric Interface PCR (22) was performed on a PerkinElmer Life Sciences 9700 thermal cycler or MJ Research MiniCycler™. Reaction conditions and cycling times were identical to those reported, except that a denaturation cycle of 93 °C for 1 min and 95 °C for 1 min was inserted at the start of the secondary and tertiary PCR sequences. Only TAIL PCR primers AD2 and AD3 were used in conjunction with the tDNA-specific primers. PCR (22) was performed on a PerkinElmer Life Sciences 9700 thermal cycler or MJ Research MiniCycler™. Reaction conditions and cycling times were identical to those reported, except that a denaturation cycle of 93 °C for 1 min and 95 °C for 1 min was inserted at the start of the secondary and tertiary PCR sequences. Only TAIL PCR primers AD2 and AD3 were used in conjunction with the tDNA-specific primers. Detection of the transgene was conducted by using the library by PCR using the primers 5'-TAAACAAATCGGAGGATCCGATATG-3' and 5'-TTATCATATTGAAAGTGACACACAAG-3', which have integral BamHI and SalI restriction sites, respectively. The fragment was directionally cloned into the BamHI and SalI sites of the expression vector pQE31, and the resulting construct was transformed into E. coli M15[pREP4] cells. Protein was expressed and column-purified on Ni-NTA resin according to manufacturer’s instructions (Qiagen Ltd.).

Tissue Extraction and Subcellular Fractionation—Fractionation of seedling organelles was conducted as described by Eastmond et al. (5) with the following modifications. The tissue used consisted of ~7000 4-day-old dark-grown seedlings, which were ground in a smooth mortar with pestle in 5 ml of grinding buffer. Two ml of filtered extract were layered onto an 8-ml, 55 to 30% linear sucrose density gradient sitting on a 1-ml cushion of 55% sucrose. The gradient was centrifuged at 20,000 × g for 3 h in a Beckman L8-M ultracentrifuge using an SW-28 swing out rotor.

Enzyme Assays—Acyl-CoA synthetase activity was measured either spectrophotometrically in a coupled reaction with citrate synthase and malate dehydrogenase according to Millerd and Bonner (27), or radioactively by using [14C]-labeled short-chain fatty acids according to Huang (28). The radioassay was conducted in a manner that monitored the spectrophotometric assay. The radioassay was an end point assay that required separation of product from substrate by TLC. Product losses from the recovery process and subsequent scintillation counting could cause a substantial underestimation of the amount of product formed. Other procedural factors, such as enzyme dilution, may have also contributed to the lower rates. Therefore, each assay protocol (radio- or spectrophotometric) was separately optimized to ensure that enzyme was running at maximum activity, and the proportion of radioactive in each component was determined according to Tolbert (30). Total protein amount was measured according to Bradford (31) using bovine serum albumin as quantification standard. Chlorophyll amounts were determined according to Wintermans and de Mots (32).

Labelled Acetate Feeding—The [14C]acetate feeding experiments were adapted from Eastmond et al. (5) with the following modifications. One hundred 2-day-old 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·mM⁻¹) and 50 mM MES, pH 5.2. Two consecutive 0.15-ml aliquots of 5 × KOH were used to trap respired CO₂. Both fractions were combined for scintillation counting. After 4 h, the seedlings were bubbled, extracted, and fractionated as described above, except that the continual monitoring of the spectrophotometric assay. The radioassay was an end point assay that required separation of product from substrate by TLC. Product losses from the recovery process and subsequent scintillation counting could cause a substantial underestimation of the amount of product formed. Other procedural factors, such as enzyme dilution, may have also contributed to the lower rates. Therefore, each assay protocol (radio- or spectrophotometric) was separately optimized to ensure that enzyme was running at maximum activity, and the proportion of radioactive in each component was determined according to Tolbert (30). Total protein amount was measured according to Bradford (31) using bovine serum albumin as quantification standard. Chlorophyll amounts were determined according to Wintermans and de Mots (32).

RESULTS

Acid Screen of tDNA Mutagenized Populations—A screen of 83 seed pools of tDNA activation-tagged lines (20) yielded, among others, two seed lines that were well established in the presence of 500 µM FAc. The established resistant seedlings were evident among the background of seeds exhibiting delayed germination (Fig. 1). From seed stock numbers N21328 and N21343 (NASC, Nottingham, UK) one and seven resistant...
seedlings, respectively, were rescued. PCR analyses of the tDNA/genomic junctions of each mutant showed that those from batch N21343 were siblings. A genetic analysis of reciprocal crosses of the mutants to the parental wild type Col-7 (Columbia) demonstrated that each was recessive with respect to FAC resistance, and, therefore the phenotype was because of a loss of function mutation. Reciprocal crosses of the mutants to the EMS mutant acn1 demonstrated allelism of the mutations with all F1 and F2 progeny being Fac-resistant (12). Therefore, the EMS mutant allele was referred to as acn1-1 and the tDNA-tagged alleles as acn1-2 (N21343) and acn1-3 (N21328), respectively.

The mutants were examined for the specificity of FAC sensitivity by growth analyses on a variety of compounds through which FAC toxicity may be manifested (Fig. 2). The compounds were tested at concentrations equivalent to that of FAC used in the screen, except for fluorocitrate, which was used at 1 mM in order to give 0.5 mM of the L stereoisomer. Wild type responses of acn1-2 and acn1-3 to the FAC equivalent concentrations of sodium acetate and sodium butyrate were observed for both mutants showing that enhanced tolerance to cytosolic acidification was not the basis of FAC resistance (Fig. 2). Wild type-like sensitivity of both mutants to fluorocitrate demonstrated that aconitase activity or mechanisms of citrate transport were not compromised in the mutants and thus were not the basis of FAC resistance. Only with FAC was any difference observed between wild type and mutants, which indicated that resistance to FAC was a specific metabolic phenomenon.

Seedling fresh weights of acn1-2 were compared with those for the wild types, Col-7 and Col-0, and the EMS mutant acn1-1 in the presence of increasing concentrations of the weak acids acetate and butyrate (Fig. 3). Both wild types and both mutants exhibited no effects to the exogenous acetate at concentrations of 1 and 2 mM. At 3.5 mM acetate, wild type growth was decreased 30–40% whereas that of the mutants was decreased 50–60% (Fig. 3A). At this acetate concentration, chlorophyll levels in the mutants were ~30% those in the wild types (data not shown). These results indicate that the mutants are more sensitive to exogenous acetate than wild type and may be disrupted in an important mechanism of acetate utilization and detoxification. Higher steady-state acetate levels in the mutant would lead to a relatively lower cytosolic pH because of the weak acid properties of acetate. Arabidopsis seedlings were also sensitive to exogenous sodium butyrate (Fig. 3B). With butyrate, a decrease in average seedling weight comparable with that on 3.5 mM acetate was achieved for both wild types at an exogenous butyrate concentration of only 1 mM. However, a significant effect of the mutation on growth was not apparent at this concentration and was only observed upon increasing the concentration to 3.5 mM. Chlorophyll levels were unaffected at the two lower butyrate concentrations and were not able to be determined in the mutants at 3.5 mM (data not shown). A reduced tolerance of seedlings to butyrate relative to acetate would reflect a lesser ability to detoxify it.

 Identification and Characterization of tDNA Insertion Sites for acn1-2 and acn1-3—Thermal Asymmetric InterLaced PCR
(22) was performed on acn1-2 and acn1-3 to generate cDNA fragments containing the junction of the tDNA left border and genomic sequence. This technique utilized a series of three PCRs involving a set of nested tDNA-specific and degenerate primers in order to amplify a cDNA product that spanned the tDNA/genomic junction. The product from the third amplification was cloned and sequenced to obtain the genomic sequence flanking the tDNA. BLAST (23) analysis of the flanking genomic sequences revealed that tDNAs for both lines resided in a putative AMP-binding protein located on the top arm of chromosome III (At3g16910). This is the same gene termed AAE7 (acyl-activating enzyme) by Shockey et al. (19). Mutants acn1-2 and acn1-3 resulted from insertions in exons 3 and 2, respectively (Fig. 4A). The acn1-2 locus had a tandem tDNA insertion. Both inserts were lying in the same orientation with the left border facing the start of transcription. The upstream tDNA was intact, whereas the left border of the downstream tDNA had been truncated by −1 kbp. The acn1-3 locus had a triple repeat with a left border at each tDNA/genomic junction. Southern hybridization data and segregation analysis of glufosinate resistance for each of the mutants, backcrossed to Col-7, showed that there was only one insertion site within each genome (data not shown). The orientation of the tDNAs within acn1-3, such that left borders are at both genomic junctions, demonstrated that resistance was not likely due to expression of a genomic sequence driven by the cauliflower mosaic virus 35S enhancers, which lie at the right borders of intact tDNAs.

Expression analysis of ACN1 was done in order to verify the lack of transcript in the mutants and to determine the relative transcript levels in developing seedlings, leaves, and roots of Col-7. PCR was conducted on the ABI Prism 7000 thermocycler using SYBR®-green PCR master mix. Amounts of RT-PCR products were normalized against the amount of RT-PCR product from 18S RNA. The values and error bars represent the average ± S.D. of three independent experiments.

Fig. 3. Effects of acetate and butyrate on seedling growth of mutants acn1-1 and acn1-3. Symbols are given in B. Seeds were germinated on standard agar plates or agar plates containing half-strength Murashige and Skoog salts, pH 5.7, and increasing concentrations of either sodium acetate (A) or sodium butyrate (B). Normalized fresh weights were calculated by dividing the fresh weight per seedling (mg) of each replicate from the organic acid plates by the average mg per seedling values (n = 3) for each seed batch germinated on standard agar plates. The values and error bars represent the average ± S.D. of three values, respectively.

Fig. 4. Molecular characterization of mutant and wild type plants. A, map of tDNA insertions within the ACN1 locus. TAIL PCR was used to determine the flanking genomic sequences. The number of tDNAs inserted was determined by Southern blot analysis of genomic DNA restricted with EcoRI. The orientation of the tDNAs was determined by PCR using combinations of tDNA border- and gene-specific primers. B, an ethidium bromide-stained gel of RT-PCR products showing no ACN1 transcript in acn1-2 leaves and seedlings. The abbreviations used are as follows: L, 1-kb DNA ladder (Promega); C, Col-7; M, mutant acn1-2; P, full-length ACN1 cDNA. The sizes of the bands in the ladder are given on the left. Identical results were obtained for acn1-1 and acn1-3. C, quantitative RT-PCR analysis of ACN1 expression in developing seedlings, leaves (L), and roots (R) of Col-7. PCR was conducted on the ABI Prism 7000 thermocycler using SYBR®-green PCR master mix. Amounts of RT-PCR products were normalized against the amount of RT-PCR product from 18S RNA. The values and error bars represent the average ± S.D. of three independent experiments.
monitor relative transcript levels throughout early seedling development (Fig. 4C). Levels of ACN1 transcript were compared with the 18 S RNA (25, 33). Compared with 18 S RNA, the commonly used RT-PCR control actin-2 had an expression profile that mirrored that of ACN1 (data not shown). The observation that ACN1 was expressed in imbibed seeds was consistent with the observed ability of FAc to delay seed germination. The expression increased up to day 8 and stayed relatively high in mature organs as shown by the expression levels in leaves and roots from 2-week-old plants.

Acyl-CoA Synthetase Activity and Substrate Specificity of ACN1—The disrupted gene encodes a predicted protein of 571 amino acids with a molecular mass of 57 kDa. From amino acids 49–486, the predicted protein showed high similarity to the family of AMP-dependent synthetases and ligases (PFAM, PF00501) with the characteristic S/T/G-rich domain at region 204–216 and the conserved PKG tripeptide at position 214–216 (34). The S/T/G-rich domain is required for the adenylate activation of carboxylic acids (35, 36). ACN1 had the PTS I-type terminal peptide SRL indicating that it is targeted to peroxisomes. By using an E. coli-based membrane preparation, Shockey et al. (19) had shown that the enzyme possessed a relatively low level of acyl-coenzyme A synthetase activity with acetate. A bioinformatic analysis of the primary sequence of ACN1 suggested that it was likely to be a soluble enzyme and thus would be amenable to overexpression in E. coli and native purification on Ni-NTA resin. This was confirmed by PCR cloning of the ACN1 and overexpressing it in E. coli as a His tag conjugate. Protein determination assays and SDS-PAGE showed that overexpression and purification yielded between 3 and 4% of the total E. coli protein at a purity greater than 95%.

The purified protein showed AcetCS activity in a coupled assay with malate dehydrogenase and citrate synthase. Specific activities from the spectrophotometric assay were routinely greater than 100 μmol of acetate converted to acetyl-CoA in 1 h by 1 mg of protein. The substrate specificity of ACN1 for short-chain fatty acids was determined by using a radioisotope assay and 14C-labeled short-chain fatty acids (Fig. 5). The enzyme was active with both acetate (C-2) and butyrate (C-4) at 100 and 70% activity, respectively, but was not active with hexanoate (C6). Although the calculated specific activities with acetate as substrate were substantially lower than those determined spectrophotometrically, increasing the concentration of either substrate did not increase rates. This indicates that the relative rates are a true reflection of substrate specificity.

Localization of ACN1 to Peroxisomes—The PTS-1 targeting tripeptide suggested that the enzyme would be located in peroxisomes. This was confirmed by an analysis of AcetCS activity in peroxisomes from both wild type and mutant seedlings isolated on sucrose density gradients (Fig. 6). The 30–55% linear gradient gave fundamentally similar results. B, inset, the migration profiles of triose-phosphate isomerase (TIM) and Chlorophyll (Chl) in fractionated, greened seedlings are shown.
activities were determined in order to compare levels of activity in preparations from both wild types and mutants and to normalize activities among the different organellar fractions. As expected, AcetCS activity was found in the plastidial fraction of wild type seedlings (37), but no activity was observed to be in the mitochondrial fractions in wild types or mutants. In fact, AcetCS-specific activity was higher in the plastidial fraction of the mutant, indicating that this activity was not eliminated in the mutant. The specific activity of peroxisomal AcetCS in the wild type seedlings was approximately equal to that in plastids but completely lacking in the mutant, which showed that the mutations in ACN1 eliminated peroxisomal AcetCS activity.

Most interestingly, the fractionation experiments provided evidence for an independent cytosolic AcetCS activity. The specific activity of short-chain acyl-CoA oxidase in the cytosol was less than 2-fold that in intact peroxisomes. However, the specific activity of AcetCS in the cytosolic fraction was equal to or greater than that in intact peroxisomes. Because short-chain acyl-CoA oxidase activity was an estimate of cytosolic contamination by peroxisomal content, we could calculate that a corresponding release of peroxisomal AcetCS activity did not account for the entire cytosolic AcetCS activity. The greater AcetCS-specific activity in the cytosolic fraction was not because of contamination by plastidial AcetCS activity, because the plastids appeared to remain virtually intact during the isolation procedure. This was evident from a lack of chlorophyll within the cytosolic fractions, when the experiment was conducted on 4-day-old dark-grown seedlings that were allowed to green before harvesting by exposing them to light for 12 h (Fig. 6B, inset). Substantial conversion of etioplasts to chloroplasts was evident from a partial shift of triose-phosphate isomerase activity and chlorophyll to fractions of density intermediate between mitochondria and peroxisomes (30). Chlorophyll contaminating the cytosol would have been evident from released thylakoids forming a diffuse band in fractions containing less than 35% sucrose (38, 39). These results, obtained using a gentle grinding procedure and single centrifugation step, were not inconsistent with a yield approaching the upper end of 50–95% integrity estimated for plastids isolated by classical gradient centrifugation (40).

**Acetate Utilization by ACN1**—The localization of ACN1 to peroxisomes indicated that this enzyme is responsible for activating exogenous acetate for entry into the glyoxylate cycle. We examined this by comparing the metabolism of [2-14C]acetate by acn1-1 with the isocitrate lyase mutant, icl-1 (isocitrate lyase) (5). The acn1-1 mutant was used, because it shares with icl-1 the same parental wild type Columbia isolate. Nearly identical results were obtained for the acn1-2 mutant when compared with the parent isolate Col-7 (data not shown). The proportions of radiolabel appearing in each component of fed icl-1 were similar to those obtained by Eastmond et al. (5) (Fig. 7). For icl-1, ~80% less of the radiolabel appeared in the soluble sugar component of the fractionated extract compared with Col-0, whereas the proportion of label appearing in CO₂, amino acids, and organic acids increased. The amount of radiolabel appearing in the soluble sugar component of acn1-1 was reduced by at least 60% with a small increase in the proportion of label appearing in the organic acid component, similar to icl-1. Some differences between acn1-1 and icl-1 were observed. For example, no increase was observed for radiolabel in evolved CO₂ and the amount of radiolabel in the amino acid fraction was not increased but was reduced compared with that observed in Col-0. Furthermore, the amount of radiolabel in the lipid fraction was 2–3-fold greater than in either Col-0 or the icl-1 mutant. Some differences between acn1 and icl-1 in the assimilation of labeled acetate into the various components were not unexpected, because steps preceding the missing ICL step in the icl-1 mutant would still have access to labeled acetyl-CoA within the glyoxysome. In addition, the overall metabolism of exogenous acetate by icl-1 was ~50% less than by either acn1-1 or wild type. The reason for this difference is not immediately apparent. The differences among our values and those of Eastmond et al. (5) for the icl-1 mutant, such as the lower amount of radiolabel in evolved CO₂ and the relatively greater proportions of radiolabel in the organic acid and ethanol-insoluble fractions, likely reflected our use of [2-14C]-acetate, whereas they employed [1-14C]acetate.

**DISCUSSION**

In both fungi and higher plants, the assimilation of acetate via the glyoxylate cycle requires its activation to acetyl-CoA. The original FAC screens conducted by Apirion (13) isolated the facA mutant, which was deficient in the acetyl-CoA synthetase activity necessary to feed exogenous acetate into the glyoxylate cycle. The demonstration that seed tissues of plants assimilate exogenous acetate via the glyoxylate cycle (3, 41) implied that plants possessed an equivalent enzyme to activate acetate. The plant enzyme had never been identified. In a pilot study, we employed the toxic acetate analogue FAC to screen for EMS-mutagenized Arabidopsis mutants exhibiting Fac-resistant germination, and we isolated a mutant, acn1-1, completely tolerant of the toxin (12). Because of its sensitivity to elevated levels of exogenous sodium acetate, acn1-1 was the likely candidate to have the metabolic lesion at this synthetase step.

Screening a tDNA-mutagenized population produced two mutants (Fig. 1) with physiological characteristics nearly identical to acn1-1 (Figs. 2 and 3). A genetic analysis demonstrated both tDNA mutants were allelic to acn1-1. TAIL PCR revealed the sites of insertion to be within the same AMP-binding protein (At3g16910), which contains a peroxisomal targeting signal (42). The peroxisomal location of ACN1 was confirmed in this study (Fig. 6). Characterization of the E. coli expressed and purified protein demonstrated short-chain acyl-CoA synthetase activity with acetate being a better substrate than butyrate.

**FIG. 7. Comparison of exogenous [2-14C]acetate utilization by acn1-1 and icl1-1 seedlings.** Component designations are as follows: CO₂, 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 ± S.D. of four independent experiments for Col-0 and icl1-1 and three independent experiments for icl-1. The seedlings were maintained at the growth conditions of light and temperature mentioned under “Experimental Procedures” during the incubation period. The total amounts of radiolabel taken up over the 4-h incubation period (×10^5 dpm) were 54.1 ± 6.5, 43.7 ± 8.3, and 20.4 ± 5.5 for Col-0, acn1-1 and icl1-1, respectively.
This result contrasts with the substrate specificity for this enzyme reported for AAET by Shockey et al. (19), which showed the enzyme more active with butyrate than acetate. The difference in substrate specificity may be due to their use of an artificial E. coli-based membrane system to investigate activity, whereas we used the purified, soluble protein. It is apparent that the acn1 mutation had a more profound effect on acetate metabolism (Fig. 3), which coincides with the substrate specificity (Fig. 5). The enhanced sensitivity of wild type seedlings to butyrate probably reflects fewer, or less efficient, mechanisms for its detoxification, whereas acetate may be metabolized directly in plastids (43) as well as in glyoxysomes.

Similarities in acetate metabolism between Arabidopsis and filamentous fungi are apparent. From studies with E. nidulans, Apirion (13) concluded that two distinct pathways of acetate assimilation exist with the major one mediating FAC sensitivity. Our results from the [2-14C]acetate feeding studies suggest other mechanisms of acetate assimilation within establishing Arabidopsis seedlings (Fig. 7). The exclusivity of the glyoxylate pathway to acetate and acetyl-CoA assimilation in Arabidopsis had been questioned by the finding that the Arabidopsis mutant icl-1 mobilized lipid reserves and respired exogenous acetate (5). This implied a second mechanism, besides the succinate shuttle, for moving acetyl units from glyoxysomes to mitochondria for gluconeogenesis. A mechanism for this route may not be selectable using FAC and may form part of the FAC-insensitive pathway of acetate assimilation.

Eastmond and Graham (4) concluded that the glyoxylate cycle serves essentially an anaplerotic role in Arabidopsis seedling development to facilitate lipid mobilization. Although an exclusively anaplerotic role has been disputed (44), provision of carbon from lipid or acetate to the trichloroacetic acid cycle by routes involving steps of the glyoxylate cycle is possible and may be a function of the cycle. Currently, the defining function of the complete glyoxylate cycle is to provide the majority of carbon for gluconeogenesis (44). In this context, our results ascribe a function to ACN1 as the principal component in the metabolism of exogenous acetate by the glyoxylate cycle. If 80% of exogenous acetate ending up in soluble carbohydrates passes through an intact glyoxylate cycle (see Ref. 5 and this study), then we can estimate that at least 75% of that acetate is converted to acetyl-CoA by ACN1. Although we have identified a major point of acetate activation for entry into the glyoxylate cycle, the assimilation of exogenous acetate is not eliminated in the mutant. It is possible to infer other routes of acetate utilization. One involves the mitochondrial CAC-like protein with the acetyl-CoA serving as substrate being provided by the cytosolic ATP-citrate lyase, which would cleave citrate exported from glyoxysomes (45). However, this mechanism would not appear to be possible within the acn1 mutants that lack glyoxysomal AcetCS activity. It is interesting to note that although we did not observe AcetCS activity in glyoxysomal fractions from etiolated seedlings of the mutants, another glyoxysomal AcetCS-like enzyme was identified by a proteomic analysis of glyoxysomes from etiolated cotyledons (46). The AcetCS activity of this enzyme must be confirmed. However, we did observe that the cytosol may possess a substantial AcetCS activity, which provides a mechanism whereby exogenous acetate may be activated directly to acetyl-CoA for import into the mitochondria by the CAC-like protein. This mechanism would bypass the glyoxylate cycle entirely and may explain why exogenous acetate continues to be respired and assimilated in both the acn1 and icl1 mutants. This mechanism is analogous to the model for E. nidulans presented by De Lucas et al. (18), whereby acetate is activated to acetyl-CoA in the cytosol to be converted to acetylcarbinone for transport into mitochondria. It is evident that the situation regarding acetate assimilation during germination and establishment of Arabidopsis seedlings is certainly more complex than the classic gluconeogenic model involving the glyoxylate cycle.

A distinct question remains as to the generation of endogeneous acetate during germination that would warrant the existence of ACN1. A potential need to scavenge free acetate has been proposed because it is produced during the synthesis of cysteine and ornithine and upon deacetylation of histones (47). We are currently examining the effects of the acn1 mutation on the assimilation of acetate using radiolabeled feeding and metabolite profiling by 1H NMR. Preliminary results show that low levels of free acetate (~50 nmol g-1 fresh weight) are present in Col-7 seedlings, which may be as much as 10-fold greater than levels of acetyl-CoA (48). The difference in amounts raises the question of free acetate contributing substantially to acetyl-CoA formation in seedlings, a process that may become more important as lipid reserves expire. Recycling of acetate may serve two purposes; one may be to conserve carbon liberated during the processes mentioned above, and the other may be to eliminate acetate as a potentially toxic free acid acting to lower intracellular pH.

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