The Role of Acetyl-Coenzyme A Synthetase in Arabidopsis

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The acs1 knockout mutant that has a disruption in the plastidic acetyl-coenzyme A (CoA) synthetase (ACS; At5g36880) gene was used to explore the role of this protein and plastidic acetate metabolism in Arabidopsis (Arabidopsis thaliana). Disruption of the ACS gene decreased ACS activity by 90% and largely blocked the incorporation of exogenous 14C-acetate and 14C-ethanol into fatty acids. Whereas the disruption had no significant effect on the synthesis of bulk seed triacylglycerols, the acs1 plants were smaller and flowered later. This suggests that the pyruvate dehydrogenase bypass provided by the aerobic fermentation pathway that converts pyruvate to acetate and probably on to fatty acids is important to the plants during normal growth. The role of ACS in destroying fermentative intermediates is supported by the increased sensitivity of the acs1 mutant to exogenous acetate, ethanol, and acetaldehyde compared to wild-type plants. Whereas these observations suggest that flux through the aerobic fermentation pathway is important, the reason for this flux is unclear. Interestingly, acetate is able to support high rates of plant growth on medium and this growth is blocked in the acs1 mutant.

Acetyl-CoA is the carbon source for plastidic fatty acid biosynthesis. Historically, two reactions have been proposed to supply this acetyl-CoA, the plastidic pyruvate dehydrogenase complex (PDHC; Reid et al., 1977) and plastidic acetyl-CoA synthetase (ACS; Smirnov, 1960). ACS was the first enzyme implicated in acetyl-CoA synthesis in plastids. In addition, intact plants and isolated plastids are readily capable of converting exogenously supplied acetate into fatty acids (e.g. Roughan, 1978; Roughan et al., 1976, 1979; Schulze-Siebert and Schultz, 1987; Springer and Heise, 1989; Heintze et al., 1990). Nevertheless, biochemical and molecular evidence has converged to show that ACS is not a major source of acetyl-CoA for fatty acid biosynthesis in leaves or seeds. Bao et al. (1998) provided metabolic data showing that there was insufficient acetate to account for rates of fatty acid biosynthesis. Ke et al. (2000) isolated the cDNA clones for ACS and for the E1 subunit of PDHC and showed that, whereas PDHC expression in the plastid correlated in time and place with triacylglycerol accumulation in developing Arabidopsis (Arabidopsis thaliana) seeds, the temporal and spatial expression of ACS mRNA did not. Recently, Schwender et al. (2003, 2006) have performed flux analyses of lipid biosynthesis in canola embryos. These data quantitatively demonstrate that most of the carbon directed to fatty acid synthesis in this organ comes from glycolysis and enters the acetyl-CoA pool through plastidic pyruvate dehydrogenase. Finally, Behal et al. (2002) developed a population of Arabidopsis plants using sense and antisense expression of ACS that had 10% to 500% of wild-type ACS activity. Whereas these plants showed a nearly linear relationship between ACS level and their capacity to convert 14C-acetate into fatty acids, there was no relationship between ACS activity and 14CO2 incorporation into fatty acids. In contrast, disruption of the E2 subunit of PDHC resulted in an early embryo-lethal phenotype (Lin et al., 2003). All of these studies show that acetyl-CoA made from acetate by ACS is not a major substrate for bulk fatty acid biosynthesis, leaving that honor to the plastidic PDHC reaction.

What, then, is the function of ACS in the plastid? Kuhlemeier’s group has suggested that plants undergo aerobic fermentations, particularly in floral tissue (op den Camp and Kuhlemeier, 1997; Tadege and Kuhlemeier, 1997; Mellema et al., 2002; Gass et al., 2005). This pathway would draw pyruvate out of glycolysis where it would be converted to acetaldehyde. The acetaldehyde could then be either reduced to ethanol, as in standard renditions of anaerobic fermentation, or oxidized to acetate probably by a mitochondrial isoform of aldehyde dehydrogenase (Liu and Schnable, 2002). ACS would be involved in processing the acetoacetate to acetate for fatty acid biosynthesis (Fig. 1). This pathway acts to bypass the PDHC converting glycolytic pyruvate to fatty acids without using that enzyme. They have postulated that this pathway is of particular importance during fertilization when pollen is undergoing very high metabolism and growth in the low oxygen environment of the style. Indeed, they have shown that a mutant in pyruvate
decarboxylase activity produces pollen that competes poorly against wild-type pollen and that this bypass can become essential when PDHC activity is inhibited (Gass et al., 2005).

Other short-chain acyl-CoA synthetases occur in plants and are capable of converting acetate to acetyl-CoA. Turner et al. (2005) have identified a peroxisomal enzyme (ACN1) that appears to be an important entryway for acetate into the glyoxylate cycle.

In this article, we provide evidence that the PDHC bypass pathway exists in Arabidopsis plants. The pathway might have a role in the detoxification of ethanol, acetaldehyde, and/or acetate because we were unable to detect any of this protein immunologically (data not shown). Rather, the activity probably represents acetate fixation by other acyl-CoA synthetases in Arabidopsis, including peroxisomal ACN1 (Shockey et al., 2003; Turner et al., 2005). Earlier, we showed that once we corrected for enzyme loss from plastids during organelle isolation, ACS is completely plastidic (Ke et al., 2000). Putting these data together suggests that about 90% of the in vitro acetate fixation capacity is catalyzed by plastidic ACS and 10% is peroxisomal ACN1.

To verify biochemically that the T-DNA knockout of the ACS gene did block ACS activity in situ, the rates of 14C-acetate and 14C-ethanol incorporation into fatty acids in Arabidopsis leaves were measured (Fig. 3, A and B). With both substrates, the acs1 knockout decreased the rate of 14C incorporation into fatty acids by about 90%. Clearly, the ACS gene is responsible for the vast majority of acetate incorporation into fatty acids by leaves (Fig. 3) and intact seedlings (data not presented) as well as the bulk of the in vitro ACS activity.

Whereas we were able to demonstrate that ACS was not important in leaf lipid synthesis from 14CO2 using the antisense-ACS plants (Behal et al., 2002), these plants could not be used to evaluate the role of ACS in seed triacylglycerol levels. This was addressed using acs1. Seeds from wild-type Columbia and acs1 grown under identical conditions were analyzed for total lipid content. There was no significant difference in lipid content of the seeds, suggesting that ACS has no detectable role in their synthesis (Table I).

Despite the lack of involvement in bulk fatty acid biosynthesis, the acs1 mutation does have a phenotype.

RESULTS
Identification and Characterization of an ACS Knockout Mutant

Whereas antisense plants had been useful in demonstrating that ACS did not have a predominant role in fatty acid biosynthesis in plastids (Behal et al., 2002), variability between lines and within a line between generations made them of limited use for metabolic studies. To circumvent these limitations, we obtained a knockout mutant for ACS in Arabidopsis (acs1). The mutant was in the Salk collection and Salk_015522 contained a single T-DNA insert in the penultimate intron of the ACS gene (Fig. 2A). The T-DNA insertion results in an apparent destabilization of the ACS mRNA, which does not accumulate to detectable levels (Fig. 2C). In vitro ACS activity was decreased by over 90% in the acs1 plants (Fig. 2B). The residual activity probably is not catalyzed by ACS because we were unable to detect any of this protein immunologically (data not shown). Rather, the activity probably represents acetate fixation by other acyl-CoA synthetases in Arabidopsis, including peroxisomal ACN1 (Shockey et al., 2003; Turner et al., 2005). Earlier, we showed that once we corrected for enzyme loss from plastids during organelle isolation, ACS is completely plastidic (Ke et al., 2000). Putting these data together suggests that about 90% of the in vitro acetate fixation capacity is catalyzed by plastidic ACS and 10% is peroxisomal ACN1.

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Figure 1. Pathway for aerobic fermentation and acetate metabolism in plants. The pathway can bypass plastidic pyruvate dehydrogenase by oxidizing pyruvate to acetaldehyde and acetate. Acetate is converted into acetyl-CoA by ACS. Acetyl-CoA formed in the plastid by this reaction can be used for fatty acid biosynthesis. It is possible that the carbon may be converted to carbohydrates by the glyoxylate shunt.

Figure 2. Molecular and biochemical analysis of the acs1 knockout mutation. A, The structure of the ACS gene showing the site of the T-DNA insertion in the penultimate intron. B, In vitro ACS enzyme activity in wild-type and acs1 plants. C, Northern blot showing the ACS mRNA in the wild-type and acs1 plants.
Compared to the Columbia wild type, the *acs1* plants grew more slowly on soil (data not shown) and on medium with or without added Suc (Fig. 4). The growth rate on Suc (Fig. 5, A and B, white circles) shows that, during the first 2 weeks following germination, there was no significant difference between the *acs1* and wild-type plants. During the remaining 2 weeks of the experiment, however, the *acs1* plants grew substantially slower than the wild-type plants. The slower growth resulted in smaller stature up to plant maturity (data not shown) and a 2- to 3-d delay in bolting in soil-grown plants (Fig. 6). Therefore, while not of primary importance in the bulk of the plant growth and development, lipid synthesis in leaves and seeds, ACS clearly has an important function in plant growth and development. The delayed bolting phenotype was specifically important function in plant growth and development.

Role of ACS in Aerobic Fermentation

One possible role for ACS in plants is that it is involved in the detoxification of acetate, acetaldehyde, and ethanol produced by fermentation. All of these compounds have the potential to be deleterious to plant growth either by direct toxicity, cytosolic acidification, or altered gene expression (Shulaev and Oliver, 1990). It is reasonable, therefore, that blocking their detoxification would slow plant growth.

This idea was tested by growing wild-type and *acs1* plants on medium containing acetaldehyde, acetate, and ethanol (Table II). In the presence of 1 mM acetate, growth of wild-type plants was only inhibited about 5%; in 1 mM acetaldehyde, growth was inhibited 39%; and in 10 mM ethanol, growth was inhibited 35%. Whereas these two carbon compounds inhibited the growth of wild-type plants, they inhibited the growth of the *acs1* mutant plants more. Growth of the *acs1* plants was inhibited 63% by acetate, 71% by acetaldehyde, and 90% by ethanol. This clearly suggests that, by blocking the ability to detoxify acetate, acetaldehyde, and ethanol through the fermentation pathway, the disruption of the ACS gene made the plants more sensitive to these pathway intermediates. These data support the idea that the smaller size and growth delay in the *acs1* plants resulted from an inability to detoxify intermediates in the fermentation pathway.

If this model for delayed flowering in *acs1* is correct, we would expect to see increased levels of ethanol and acetate in *acs1* plants compared to wild type. Despite repeated efforts, however, we have not been able to measure such increases.

Lipid peroxidation and protein oxidation products were measured in the *acs1* and wild-type plants to see whether a portion of the flowering delay resulted from increased oxidative stress (Shulaev and Oliver, 2006). There were no differences in either thiobarbituric acid-reactive lipid peroxidation products or in protein oxidation resulting in carbonyl groups detected with 2,4-dinitrophenylhydrazine between *acs1* and the wild-type plants (data not shown).

Expression of ACS in Tissues

Northern analyses suggest that ACS is expressed at a moderate level in all of the organs tested (Fig. 7A). Expression levels were highest in flowers, young siliques, and seedlings. To get a look at ACS expression at the tissue level, a promoter-GUS expression construct was used. ACS has a very long 5′-untranslated region (UTR) so a 2-kb sequence upstream from the ATG site was used to drive GUS expression in the ACS-GUS vector. The GUS expression supports the northern analyses with stronger GUS activity in flowers and seedlings. In the newly germinated seedlings, GUS was found in the cotyledons and the hypocotyl.

Table 1. Triacylglycerol levels in seeds of wild-type and ACS knockout plants

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<th>Plants</th>
<th>Triacylglycerols in Seeds % DW</th>
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<tr>
<td>Wild type</td>
<td>48.6 ± 0.6</td>
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<tr>
<td><em>acs1</em></td>
<td>49.8 ± 0.6</td>
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Levels in roots were low in light-grown seedlings during the first few days except in the root tip (Fig. 7B). Older and dark-grown seedlings showed more root expression (Fig. 7B). In flowers, the highest levels of GUS expression were in pollen and in the upper portion of the style (Fig. 7C). So, whereas our results suggest that ACS expression and aerobic fermentation occur throughout the plant, these results strongly support the extensive work by Kuhlemeier’s group that there is a high level of expression of the pyruvate bypass pathway in flowers where it is important in pollen germination and growth (Gass et al., 2005).

Role of ACS in Acetate-Dependent Growth by Arabidopsis Plants

During the course of our studies on the effects of two carbon fermentation intermediates on plant growth, we were surprised to observe that Arabidopsis plants can partially substitute acetate for Suc in the growth medium. In our standard growth experiments, Arabidopsis seeds (Columbia, acs1, and acs1-ACS) were placed on 0.5× Murashige and Skoog medium containing 2% (w/v) Suc, no Suc, or no Suc plus 1 mM sodium acetate. The plants were grown in Magenta boxes for 21 d before they were harvested and weighed. The results are the means ± SD for 50 plants per treatment. Numbers with different letters are significantly different from each other ($P < 0.01$) using a paired t test.

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DISCUSSION

Because ACS was the first enzyme identified that would produce acetyl-CoA in plastids, it was for many years considered the primary source of acetyl-CoA for fatty acid biosynthesis. Both metabolic and genetic studies, however, have shown this is not the case (Bao et al., 1998; Ke et al., 2000; Behal et al., 2002; Lin et al., 2003; Schwender et al., 2006) and this conclusion has been confirmed with our knockout mutant, acs1. The leaves of the acs1 plants fixed $^{14}\text{CO}_2$ into fatty acids at the same rate as wild type (data not shown) and the seed of the mutant contained the same amount of triacylglycerols as the wild type. This does not mean that carbon does not flow through ACS into fatty acids. On the contrary, exogenously provided acetate and ethanol are readily incorporated into fatty acids and of Suc resulted in decreased growth rates following germination. In Figure 4, removal of the Suc from the medium decreased plant growth for the wild-type, acs1, and complemented acs1-ACS line to 29% to 46% of the amount in Suc-containing medium. The inclusion of 1 mM sodium acetate to the Suc-free medium restored growth in the wild-type and complemented line to 64% to 70% of the rate on Suc medium during the 21 d of this experiment (Fig. 4). Thus, lines with normal ACS activity show increased growth that is dependent on the added acetate. This was not the case with the acs1 mutant that did not show significantly greater growth in acetate-containing medium than it did in the medium without Suc (Fig. 4). These results demonstrate that Arabidopsis plants were capable of incorporating the carbon from acetate into the diverse cellular components necessary for plant growth and suggest that ACS has a role in that process.

The time course for acetate-dependent growth by wild-type plants is informative (Fig. 5A). During the first 2 weeks after germination, growth on acetate was substantially slower than growth on Suc. Thus, by 16 d, the acetate-grown plants were only one-half the size of Suc-grown plants. Following this lag period, however, growth of the acetate-grown plants was similar to those grown on Suc. With the acs1 plants, growth on acetate lagged throughout the entire growth period (Fig. 5B).
the rate of incorporation is about 90% inhibited in \textit{acs1}. Rather, these data show that the total amount of fatty acid normally produced by this route is small compared to the total amount of fatty acid synthesized.

Although the flux of carbon through ACS is not large compared to the amount of plastidic acetyl-CoA produced by PDHC, the production of plastidic acetyl-CoA by ACS is essential for optimal plant growth. Disruption of this activity in the \textit{acs1} mutant resulted in plants with decreased biomass and delayed flowering. Taken together, these two observations—that the loss of ACS does not seem to deprive the plants of necessary acetyl-CoA while at the same time the disruption of ACS does have a negative effect on plant growth—suggest to us that the purpose of the enzyme may be to remove its substrate, acetate, rather than supplying its product, acetyl-CoA.

Under this model, the actual role of ACS would be to detoxify the products of aerobic fermentation. This is supported by the observation that the \textit{acs1} mutants are much more sensitive to exogenously added acetate, acetaldehyde, and ethanol than wild-type plants. Without ACS to sweep these intermediates into acetyl-CoA and eventually fatty acids, their toxicity was enhanced. Just as the ACS knockout makes \textit{acs1} more sensitive to added fermentation intermediates, it would also be more sensitive to the endogenous two carbon intermediates. The observations that the \textit{acs1} plants are smaller than wild-type Columbia and flower later under normal growth conditions suggest that there is sufficient flux through the fermentative pathway under nonstress conditions to retard plant growth. Thus, aerobic fermentation appears to be a normal part of plant metabolism.

We were unable to confirm one prediction of this model that \textit{acs1} plants had more ethanol and acetate in their tissue than wild type. This might not be surprising given that 1 mM exogenous acetate and 10 mM ethanol inhibited growth of \textit{acs1} 63% and 90%, respectively. The amount of these two carbon molecules needed to delay flowering 2 d would be very low. In addition, they might be limited to a very small portion of the plant, such as meristems. More sensitive measures of these intermediates, such as Zuckermann et al. (1997), might be capable of detecting these differences. It is also possible that the phenotype results from loss of acetyl-CoA in some specific tissues that do not result in a change in total fatty acid synthesis and not from the toxic effects of acetate as proposed.

It is interesting that potentially toxic fermentation intermediates are metabolized in healthy aerial plant tissues where the low oxygen levels that normally induce fermentation are not expected. Some groups have demonstrated that ethanol produced in the roots where anaerobic conditions are more prevalent is transported to the leaves where it is converted to acetaldehyde and acetate (Kreuzwieser et al., 1999, 2000; Karl et al., 2002). Thus, this pathway found in aerobic tissue might be metabolizing compounds generated by hypoxic or postanoxic conditions (Zuckermann et al., 1997) in the roots. Alternatively, acetaldehyde could be produced in the leaves as a bypass of the pyruvate dehydrogenase reaction. In fungi, this metabolic bypass allows high rates of glycolytic metabolism under aerobic conditions where Glc oxidation exceeds the capacity of PDHC (Starai and Escalante-Semerena, 2004; Wolfe, 2005). Kuhlemeier’s group has argued for several years that plants perform aerobic fermentation particularly in germinating pollen. Gass et al. (2005) provide evidence that the pathway can bypass a blockage in PDHC activity and allow pollen germination to proceed under these conditions.

**Table II. Effect of fermentation intermediates on growth of wild-type and \textit{acs1} plants**

Plants were grown on medium with Suc or with Suc supplemented with 1 mM acetate, 1 mM acetaldehyde, or 10 mM ethanol. Plant growth was then measured 14 d after the chilling treatment when the weights of about 100 individual plants were determined. The data presented are the means ± sd. Numbers with different superscript letters are significantly different from each other at \( P < 0.01 \) using a paired \( t \) test.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Suc + Acetate % of Suc Only</th>
<th>Suc + Acetaldehyde % of Suc Only</th>
<th>Suc + Ethanol % of Suc Only</th>
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<tr>
<td>Wild type</td>
<td>0.082 ± 0.018 (^a)</td>
<td>0.078 ± 0.0009 (^a)</td>
<td>0.050 ± 0.002 (^b)</td>
</tr>
<tr>
<td></td>
<td>95%</td>
<td>61%</td>
<td>65%</td>
</tr>
<tr>
<td>\textit{acs1}</td>
<td>0.062 ± 0.012 (^c)</td>
<td>0.023 ± 0.004 (^d)</td>
<td>0.018 ± 0.008 (^d)</td>
</tr>
<tr>
<td></td>
<td>37%</td>
<td>29%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>( g/plant )</td>
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The ability of eukaryotes to grow on acetate is limited to some plants and fungi. Biochemically, the only pathway that can take two carbon molecules like acetate to carbohydrates needed for growth is the glycogen cycle. In most oil seeds (like Arabidopsis), the enzymes of the glycogen cycle are expressed during germination where they are essential for the conversion of storage lipids into four carbon organic acids for gluconeogenesis. The expression of these enzymes is usually transient and they disappear after the stored triacylglycerols have been metabolized. Interestingly, in some of these plants, like cucumber (Cucumis sativus), but apparently not Arabidopsis (Charlton et al., 2005), the glycogen cycle enzymes are induced to reappear by starvation either from Suc depletion in cell cultures or in the dark (Ismail et al., 1997; Eastmond et al., 2000; Eastmond and Graham, 2001; Baker et al., 2006). In this situation, the absence of Suc induces the glycogen cycle to allow acetate derived from fatty acids released from membrane lipid degradation to contribute to cell growth.

Having observed that Suc-starved plants were able to incorporate acetate into cell biomass, it was possible to use the *acs1* knockout mutant to determine the role of ACS in the activation of this acetate to acetyl-CoA. Figures 4 and 5 show that, whereas acetate was able to support growth of Arabidopsis on Suc-free medium in wild-type plants, *acs1* mutants were not able to use acetate as a carbon source. There was no significant weight difference in *acs1* seedlings grown with or without acetate in Suc-free medium.

There are at least two potential reasons for the essential nature of ACS in acetate-dependent growth. First, it is possible that ACS is preventing acetate toxicity, thus allowing plants to be healthy enough to use acetate as a carbon source for growth. The second possibility is that the carbon in acetyl-CoA produced in the chloroplast by ACS is entering the glycogen cycle. Whereas ACN1 has an important role in this process, about 40% of the carbohydrate synthesized from acetate continues in the *asn1*-1 mutant (Turner et al., 2005) and this likely represents ACS activity. Should ACS have a role in providing acetate for the glycogen cycle, this will require an as-yet unidentified pathway for moving the fixed acetate from the plastid to the peroxisome. Whereas pathways can be envisioned for fatty acids, triacylglycerides, and carriers like citrate, their relative roles will require further study.

The role of ACS in aerobic fermentation seems straightforward, although questions remain about the function (if any) for the pathway. Is carbon drawn from glycolysis for a specific purpose or does the pathway exist under aerobic conditions to metabolize the products from a low residual level of pyruvate decarboxylase activity? The ability of Arabidopsis plants to use acetate to support growth is particularly surprising and it is not clear whether this serves an as-yet undiscovered physiological function or represents
standard reactions involved in lipid turnover associated with autophagy.

MATERIALS AND METHODS
Plant Materials and Growth Conditions
Arabidopsis (Arabidopsis thaliana) Columbia plants were grown on soil or in petri dishes or Magenta boxes under continuous light at 22°C. Growth medium consisted of 0.5X Murashige and Skoog salts and 0.8% agar with or without 2% (w/v) Suc and/or other chemicals as noted. To assure uniform germination, seeds were treated at 4°C for 4 d before beginning growth experiments.

An Arabidopsis T-DNA insertion line, Salk_015522, in the ACS gene, At5g36880, (acs1) was obtained from the Arabidopsis Biological Resource Center (ABRC). The location of the T-DNA insertion was confirmed by PCR (Lin et al., 2003). The primers used for confirming the T-DNA localization within ACS in Salk_015522 were ACSqF1, 5'-tcttacctacaaactctac-3' and ACSct6, 5'-ggcagctattctatggaggagaagattctccg at-3', with primers ACSnt6, 5'-tcttacctacaaactctac-3' and ACSnt5, 5'-ggcagctattctatggaggagaagattctccg at-3', and ACSct6, 5'-ggcagctattctatggaggagaagattctccg at-3', were used for detecting T-DNA in the line. The ACS transcripts were detected by reverse transcription (RT)-PCR or northern blotting. The primers for RT-PCR were ACSexF13, 5'-tcttacctacaaactctac-3' and ACSexR17, 5'-taccaagtacctctgct-3', RNA blots were probed with ACS cDNA J9 (Ke et al., 2000).

Biochemical and Physiological Characterization of T-DNA Insertion Mutants
In vitro ACS activity, measured as 14C-acetate conversion into 14C-acetyl-CoA, and 14C-acetate, 14C-etanol, and 14C-CO₂ fixation into fatty acids were determined as described earlier (Behal et al., 2002).

Lipid content in Arabidopsis seeds was estimated by determining residual weight after extracting lipids into organic solvents. About 0.1 g dried seeds were ground with a mortar and pestle in 1.5 mL hexane: isopropanol (3:2 [v/v]) and the extract transferred to a 2-ml Eppendorf tube. The lipids were extracted by vortexing overnight at room temperature. The tubes were centrifuged at 12,000 g for 10 min and the organic supernatant discarded. The remaining pellets were dried overnight at 60°C and the oil-free residue weighed.

Lipid peroxidation was measured as in Mihara et al. (1980) and protein oxidation as in Davletova et al. (2005).

Complementation of acs1 Mutant and Promoter-GUS Fusion Expression in Arabidopsis
A vector for expressing the ACS cDNA in the acs1 mutant was constructed by modifying pCambia3301 (http://www.Cambia.org). pCambia 3301 was digested with HindIII and BstEII to remove the LacA-35S promoter-GUS fragment, the overlaps of the two ends were filled in, and the resulting blunt ends were religated. A 2-kb fragment of genomic DNA containing the promoter and 5'-UTR of the ACS gene was amplified by PCR with primers ACSpF-2014b, 5'-agacctgctggctaaacagaagagaaacg-3' and ACSpR-1, 5'-atcttttagcttttgagagagagagact-3'. These primers placed SacI and Xbal sites at the 5' and 3' ends of the ACS promoter sequence. The ACS coding sequence region was amplified from the ACS cDNA clone J9 (Ke et al., 2000) with PCR by primers ACSnt6, 5'-tgctgtagtatgccttcctc-3' and ACSnt5, 5'-aagctctcgtacatcaagcg-3', which introduced Xbal and PstI sites into the 5' and 3' ends of the ACS cDNA. The ACS promoter and the ACS cDNA were then cloned into the modified pCambia vector. The ACS promoter-GUS vector was also constructed from the modified pCambia3301. The GUS open reading frame was amplified by PCR with GUSSnt11, 5'-cccaaatctgtgctctcgtgaga-3' and GUSSnt1, 5'-ggcagctattcttgcctctc-3', which placed HindIII and BstEII sites at the 5' and 3' ends, and cloned into the modified pCambia 3301 containing the ACS promoter fragment. Agrobacterium strain GV3101 was used to transform the vectors into wild-type (Columbia) and acs1 plants by the floral-dip method (Clough and Bent, 1998).

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LITERATURE CITED
Lin M, Behal RH, Oliver DJ (2003) Disruption of pEL2, the gene for the E2 subunit of the plastid pyruvate dehydrogenase complex, in Arabidopsis causes an early embryo lethal phenotype. Plant Mol Biol 52: 865–872