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Arabidopsis Peroxisomal Citrate Synthase Is Required for Fatty Acid Respiration and Seed Germination [W]

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We tested the hypothesis that peroxisomal citrate synthase (CSY) is required for carbon transfer from peroxisomes to mitochondria during respiration of triacylglycerol in *Arabidopsis thaliana* seedlings. Two genes encoding peroxisomal CSY are expressed in Arabidopsis seedlings, and seeds from plants with both *CSY* genes disrupted were dormant and did not metabolize triacylglycerol. Germination was achieved by removing the seed coat and supplying sucrose, but the seedlings still did not use triacylglycerol. The mutant seedlings were resistant to 2,4-dichlorophenoxybutyric acid, indicating a block in peroxisomal β-oxidation, and were unable to develop further after transfer to soil. The mutant phenotype was complemented with a cDNA encoding CSY with either its native peroxisomal targeting sequence (PTS2) or a heterologous PTS1 sequence from pumpkin (*Cucurbita pepo*) malate synthase. These results suggest that peroxisomal CSY in Arabidopsis is not only a key enzyme of the glyoxylate cycle but also catalyzes an essential step in the respiration of fatty acids. We conclude that citrate is exported from the peroxisome during fatty acid respiration, whereas in yeast, acetylcarnitine is exported.

INTRODUCTION

β-Oxidation of long-chain fatty acids in plants and fungi occurs in peroxisomes, whereas in animals, it occurs in both peroxisomes and mitochondria (Kunau et al., 1995). The complete respiration of such fatty acids requires oxidation of the acetyl-CoA produced by β-oxidation. In plants and fungi, this requires transfer of carbon from the peroxisome to the mitochondrion. Studies in yeast have established that acetyl units are transferred to the mitochondrion as acetylcarnitine (van Roermund et al., 1995, 1999). This is represented as route 1 in Figure 1. A single gene encodes both peroxisomal and mitochondrial carnitine acetyl-CoA transferase (Elgersma et al., 1995), and a mitochondrial carnitine-acylcarnitine carrier is apparently responsible for import of acylcarnitine from the cytosol into the mitochondrion (van Roermund et al., 1999). The mechanism of export of acylcarnitine from the peroxisome in yeast remains to be established.

In higher plants, the pathway of carbon transfer is unclear. Studies of fatty acid respiration in germinating sunflower (*Helianthus annuus*) seeds showed that acetyl-CoA is converted to citrate by peroxisomal citrate synthase (CSY) even in the absence of the glyoxylate cycle (Raymond et al., 1992). It was proposed that carbon from the β-oxidation of fatty acids is exported from the peroxisome as citrate for subsequent respiration in the mitochondrion (Raymond et al., 1992). This is represented by route 2 in Figure 1. It was subsequently established that citrate is normally exported from the peroxisome during operation of the glyoxylate cycle because aconitase is cytosolic (Courtois-Verniquet and Douce, 1993; De Bellis et al., 1994; Hayashi et al., 1995). Thus, a plausible model for the operation of the citric acid cycle among peroxisome, cytosol, and mitochondrion during fatty acid respiration has been formulated (Eastmond and Graham, 2001; Cornah and Smith, 2002; Figure 1). However, an acylcarnitine carrier-like enzyme (A BOUT DE SOUFFLE [BOU]) has been located in the *Arabidopsis thaliana* mitochondrial membrane, and mutations that eliminate this protein slow the rate of triacylglycerol (TAG) breakdown in germinating seeds and block the transition from germinated seed to seedling (Lawand et al., 2002). It was hypothesized that BOU may be part of a pathway for carbon transfer from peroxisome to mitochondrion during fatty acid β-oxidation. However, BOU activity is required for seedling establishment in the light but not in the dark, which does not seem consistent with the involvement of BOU in the metabolism of products of peroxisomal β-oxidation because TAG breakdown and glyoxylate cycle activity are similar throughout light and dark. The *bou* mutant is blocked in the synthesis of polar lipids, suggesting a role in membrane biogenesis in the light (Lawand et al., 2002).

Mutations that block peroxisomal fatty acid β-oxidation have marked effects on Arabidopsis seed germination and plant growth. For example, the *peroxisome deficient-1* (*ped1*) or *3-ketoacyl CoA thiolase-2* (*kat2*) mutation that eliminates the major 3-ketoacyl thiolase produces seeds that germinate but then require sucrose to develop into seedlings (Hayashi et al., 1998; Germain et al., 2001). The seedlings do not metabolize

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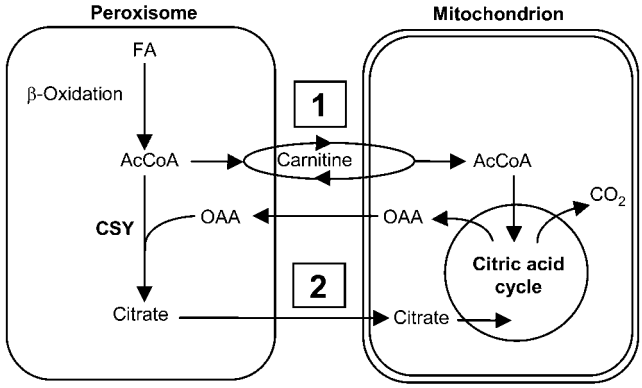

Figure 1. Model Showing Two Routes of Carbon Transport from Peroxisome to Mitochondrion during Respiration of Fatty Acids.

Route 1 has been demonstrated in *Saccharomyces cerevisiae*. Route 2 has been proposed to function in plants. FA, fatty acid; AcCoA, acetyl-CoA; OAA, oxaloacetate.

their TAG, and peroxisome development is abnormal (Germain et al., 2001). The *COMATOSE* (*CTS*) gene encodes an ABC-type transporter that is thought to import acyl-CoAs into the peroxisome for β-oxidation (Footitt et al., 2002). *cts* mutant seeds are dormant but can be made to germinate by removing the seed coat and providing sucrose. Such seedlings do not metabolize their TAG (Footitt et al., 2002). Mutants lacking peroxisomal long-chain acyl-CoA synthetase are similar to *kat2* mutants, in that they require sucrose to progress from germinated seed to seedling, and they do not metabolize TAG (Fulda et al., 2004). The *abnormal inflorescence meristem1* (*aim1*) mutation, which eliminates one of the two multifunctional proteins (MFP), indicates the importance of β-oxidation during floral development (Richmond and Bleecker, 1999). A double mutant in which two members of the acyl-CoA oxidase (ACX) gene family are mutated (*acx3* and *acx4*) is embryo lethal, showing that β-oxidation is essential for seed development as well as germination (Rylott et al., 2003). Thus, it is clear from studies in which fatty acid β-oxidation is blocked that distinct phenotypes are obtained.

The hypothesis that an acylcarnitine shuttle transfers carbon from peroxisome to mitochondrion during TAG metabolism in plants is not easily tested because an acyl-CoA carnitine acetyltransferase has not yet been found and the function of BOU is unclear. However, the hypothesis that carbon is exported from the peroxisome in the form of citrate predicts an essential role for peroxisomal CSY in the respiration of TAG. In yeast, elimination of peroxisomal CSY has no effect on fatty acid respiration (van Roermund et al., 1995) because of the operation of the acetylcarnitine shuttle (van Roermund et al., 1999). Here, we test the hypothesis that CSY is required for TAG respiration in Arabidopsis.

RESULTS

Identification of Peroxisomal CSYs

The Arabidopsis genome contains five genes predicted to encode CSY. Phylogenetic analysis shows that they fall into two groups, with two and three members each (see Supplemental Figure 1 online). The intron/exon structure of the genes is highly conserved within the two groups but not between groups. One group comprises two genes, At2g44350 and At3g60100, which have predicted mitochondrial targeting sequences at their N termini. At2g44350 has also been identified in the mitochondrial proteome (Heazlewood et al., 2004). The three proteins in the other group are more similar in sequence to peroxisomal CSY of pumpkin (*Cucurbita pepo*) (Kato et al., 1996) and contain predicted type-2 peroxisomal targeting sequences (PTS2) at their N termini. Such PTS2 sequences are characterized by a conserved $R(X)_6HL$ motif. We named these putative peroxisomal *CSY* genes *CSY1* (At3g58740), *CSY2* (At3g58750), and *CSY3* (At2g42790).

To determine which of the putative peroxisomal *CSY* genes are expressed in germinating seeds and developing seedlings, gene-specific oligonucleotide primers were designed so that transcripts of each gene could be detected by RT-PCR. Because of the high degree of sequence similarity between peroxisomal *CSY* genes, the specificity of the primers was determined in two ways. First, the RT-PCR products obtained with each primer combination were digested with restriction endonucleases that could distinguish different gene products. The results obtained showed that RT-PCR products obtained with each primer pair were of the predicted size and were digested with specific endonucleases to products of the expected sizes (Figure 2A). Second, the nucleotide sequences of the RT-PCR products were determined and shown to correspond to the predicted gene in each case (data not shown).

RNA was isolated from a range of organs of flowering Arabidopsis plants for RT-PCR analysis. *CSY2* and *CSY3* genes are expressed throughout the shoot, but *CSY1* RNA is detected only in siliques (Figure 2B, top panel). *CSY1* is expressed in developing seeds during the period when TAG accumulates in the embryo (Figure 2B, middle panel). To examine expression of *CSY* genes in seedlings, RNA was isolated at daily intervals from day 1 to day 8 (seedlings reaching growth stage 1.02 according to Boyes et al., 2001). *CSY2* and *CSY3* are both expressed throughout seedling growth, whereas *CSY1* RNA was hardly detectable (Figure 2B, bottom panel). Expression of *CSY2* and *CSY3* could also be detected in imbibed (day 0) seeds (data not shown). Although RT-PCR is not quantitative, we deduce that both *CSY2* and *CSY3* are expressed strongly throughout seedling development and in the mature shoot.

To confirm the peroxisomal targeting of Arabidopsis CSY2 and CSY3, constructs were made encoding the putative PTS2 sequences of each gene fused to green fluorescent protein (GFP) and the *Cauliflower mosaic virus* (CaMV) 35S promoter (Figure 3A). To confirm the identity of peroxisomes, a further construct was made encoding monomeric red fluorescent protein (mRFP) with a PTS1 sequence at the C-terminal end (Figure 3A). This PTS1 comprised the 10 C-terminal amino acids of pumpkin malate synthase that had previously been verified to transport RFP to peroxisomes in Arabidopsis (Lin et al., 2004). Plants were transformed with GFP and mRFP transgenes and then crossed and fluorescence observed in the root hairs of seedlings expressing both fluorescent proteins (Figure 3B). Both CSY2 and CSY3 putative PTS2 sequences target GFP to the

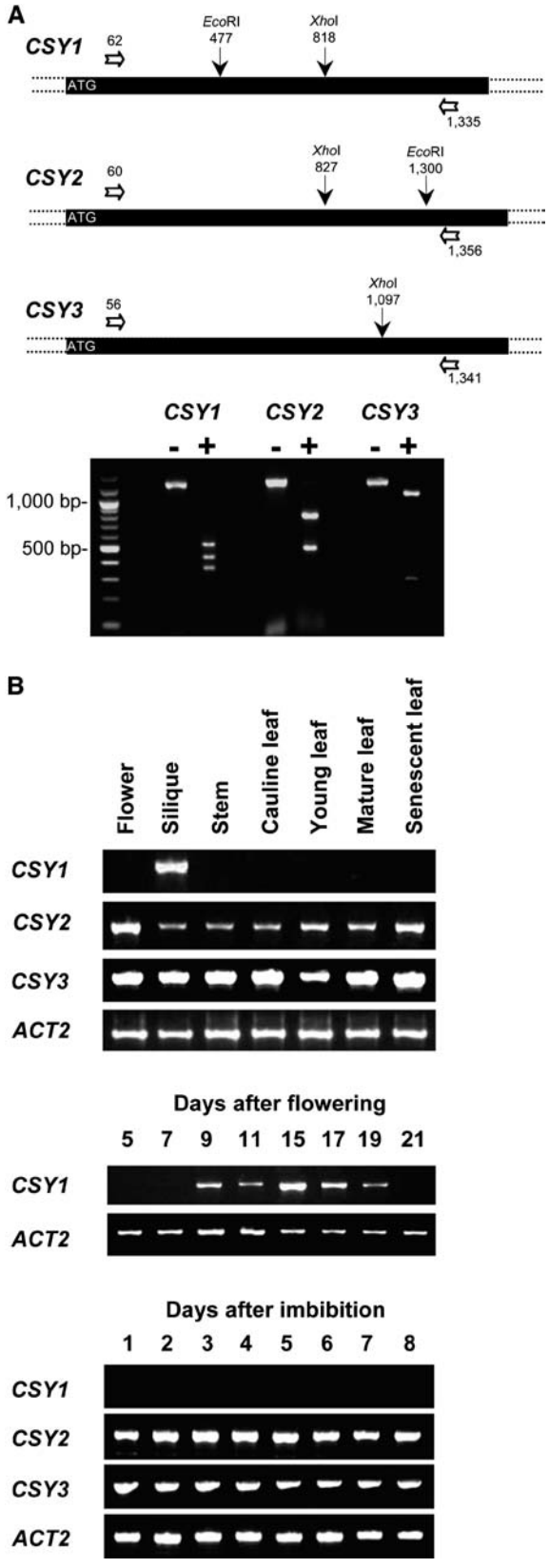

Figure 2. Characterization of Three Arabidopsis Peroxisomal CSY Genes.

(A) Schematics of the primer locations (open arrows) and unique restriction endonuclease sites (closed arrows) in the *CSY1*, *CSY2*, and *CSY3* cDNAs (top panel). Ethidium bromide–stained gel of the unique RT-PCR products produced from each *CSY* mRNA using primers illustrated, with (+) and without (−) subsequent digestion with restriction endonucleases (bottom panel).

(B) Gene expression patterns of peroxisomal *CSY* genes detected by RT-PCR in different organs of 6-week-old Arabidopsis plants (top panel); *CSY1* in maturing Arabidopsis seeds (middle panel); *CSY* genes in developing seedlings (bottom panel). The amount of cDNA template in each RT-PCR reaction was normalized to the signal from the *ACT2* gene.

same microbodies to which PTS1 targets mRFP (Figure 3B). No evidence for targeting of GFP to other organelles was observed. This confirms the peroxisomal targeting of CSY2 and CSY3.

Isolation and Characterization of *csy* Knockout Mutants

To determine the function of CSY during TAG mobilization, two independent T-DNA insertion mutants each were isolated for *CSY2* and *CSY3* (Figure 4A). In all cases, the T-DNA was inserted within the structural gene and expected to completely disrupt expression of the functional product. Isolation of homozygous knockout mutants was confirmed by PCR analysis (data not shown), and absence of RNA was demonstrated by RT-PCR analysis using RNA from day 2 seedlings as template (Figure 4B). The mutants are named *csy2-1*, *csy2-2*, *csy3-1*, and *csy3-2*. Mutant seeds germinated normally, and seedling growth was similar to that of the wild type, although such seedlings were slightly smaller than the wild type at day 5 (Figure 4C). When grown in the presence of 1% (w/v) sucrose, mutant and wild-type seedlings are indistinguishable (Figure 4C). Furthermore, growth and development of *csy* mutants in soil beyond the seedling stage are indistinguishable from the wild type (data not shown).

Total CSY activity was measured in wild-type, *csy2-1*, and *csy3-1* seedlings at daily intervals (Figure 4D). CSY activity is highest at days 0 to 2 when TAG mobilization begins and is thereafter lower. Both mutants had reduced CSY activity at days 0 to 2 relative to the wild type, and although this difference is small, it is seen consistently. It is reported that 5,5′-dithio-bis (2-nitrobenzoic acid) inhibits peroxisomal CSY activity but not mitochondrial CSY in castor bean (*Ricinus communis*) endosperm (Schnarrenberger et al., 1980), but we could not reliably distinguish these enzymes in Arabidopsis using 5,5′-dithio-bis(2-nitrobenzoic acid) (data not shown).

The *csy* Double Knockout Has a Dormant Seed Phenotype

Mutants of *csy2* and *csy3* were crossed to make double mutants. The F1 double heterozygotes appeared to grow and develop normally, and after selfing, seedlings and plants heterozygous for one *csy* gene and homozygous mutant for the other *csy* gene were readily obtained. Thus, only one functional copy of either *CSY2* or *3* is sufficient for plant growth and development. By contrast, no *csy2 csy3* homozygous double mutant seedlings could be detected in the F2 generation. However, a small

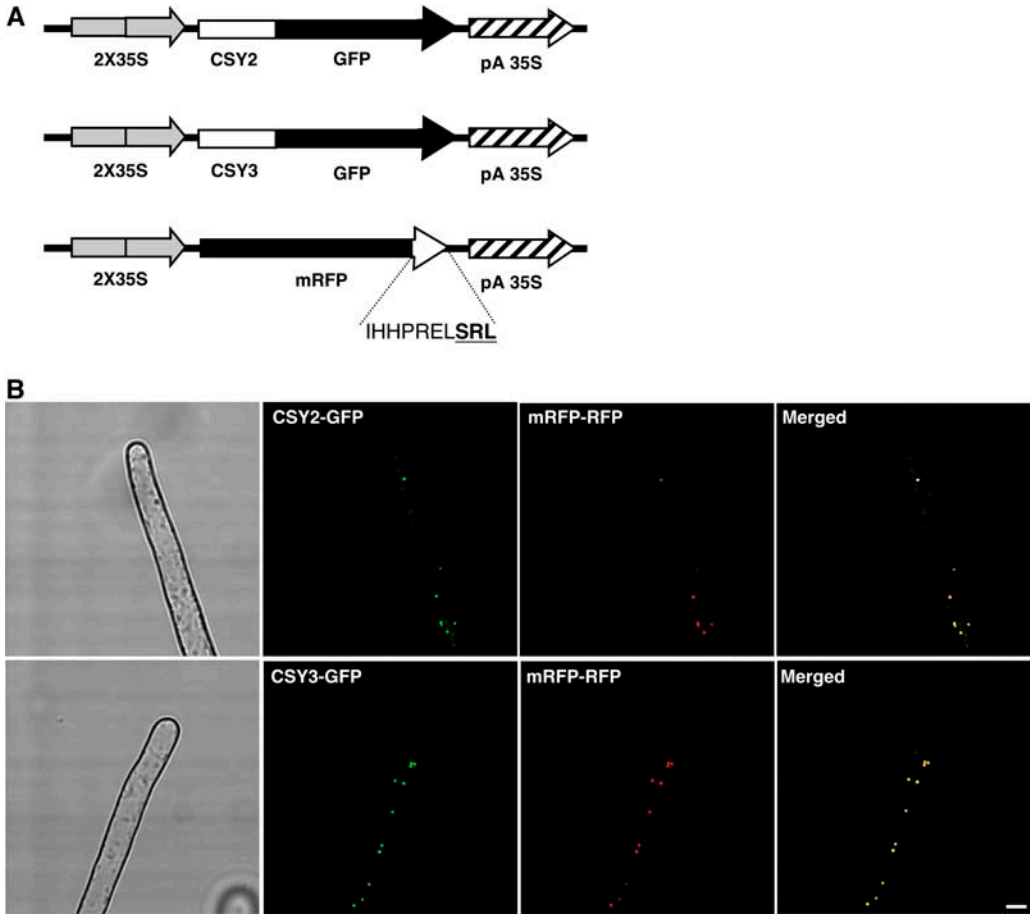

Figure 3. Peroxisomal Targeting of CSY2 and CSY3.

(A) Constructs comprising the putative PTS2 signal sequences of CSY2 or CSY3 fused to GFP. The mRFP construct has the pumpkin PTS1 sequence introduced at the C terminus.

(B) Confocal images of root hair cells of transgenic Arabidopsis plants expressing both GFP and mRFP. Bright-field, GFP signal, mRFP signal, and merged GFP and mRFP images are shown. Bar = 10 μm.

proportion of seeds did not germinate, even when provided with exogenous sucrose. The *cts* mutant germinates at a very low frequency even when provided with sucrose but can be induced to germinate by removal of the seed coat (Footitt et al., 2002). To determine if *csy2 csy3* mutants have a similar dormant phenotype, seeds from the F1 heterozygotes that failed to germinate after 5 d of incubation in the presence of sucrose were surgically disrupted to expose or remove the embryo and incubated further in the presence of 3% (w/v) sucrose. Such embryos grew and developed into seedlings, although they grew more slowly than the wild type, and they contained anthocyanins indicative of stress (Figure 5A). PCR analysis on DNA from such seedlings (data not shown) and RT-PCR analysis on RNA (Figure 5B) demonstrated that they were homozygous *csy2 csy3* double mutants.

Seeds of the *cts* mutant can be induced to germinate by incubation with propionic or butyric acid (Footitt et al., 2002) or by prolonged stratification (e.g., 4 weeks) at 4°C (Russell et al., 2000). Although weak organic acids did not apparently increase the frequency of germination of homozygous *csy2 csy3* mutant seed, prolonged stratification (4 weeks) at 4°C did increase the number of seeds germinating after transfer to 20°C (data not shown), and some seed of the double mutant did spontaneously germinate after 1 or 2 weeks of incubation on sucrose at 20°C (see Supplemental Figure 2 online).

The dormancy of homozygous *csy2 csy3* double mutant seeds could be due to a deficiency in the embryo itself, which might be overcome when the physical constraints of the seed coat are removed, or to a maternal effect of the surrounding endosperm or seed coat inhibiting embryo metabolism or growth. Dormant double mutant seed was obtained whether the maternal genotype was heterozygous for *csy2* and *csy3* or was homozygous for either *csy2* or *csy3* and heterozygous for the other gene.

Double mutant seedlings, whether obtained by surgical treatment or by rare spontaneous germination, failed to establish as mature plants either when maintained on sucrose in vitro or when transferred to soil. By contrast, the *cts* mutant flowered and

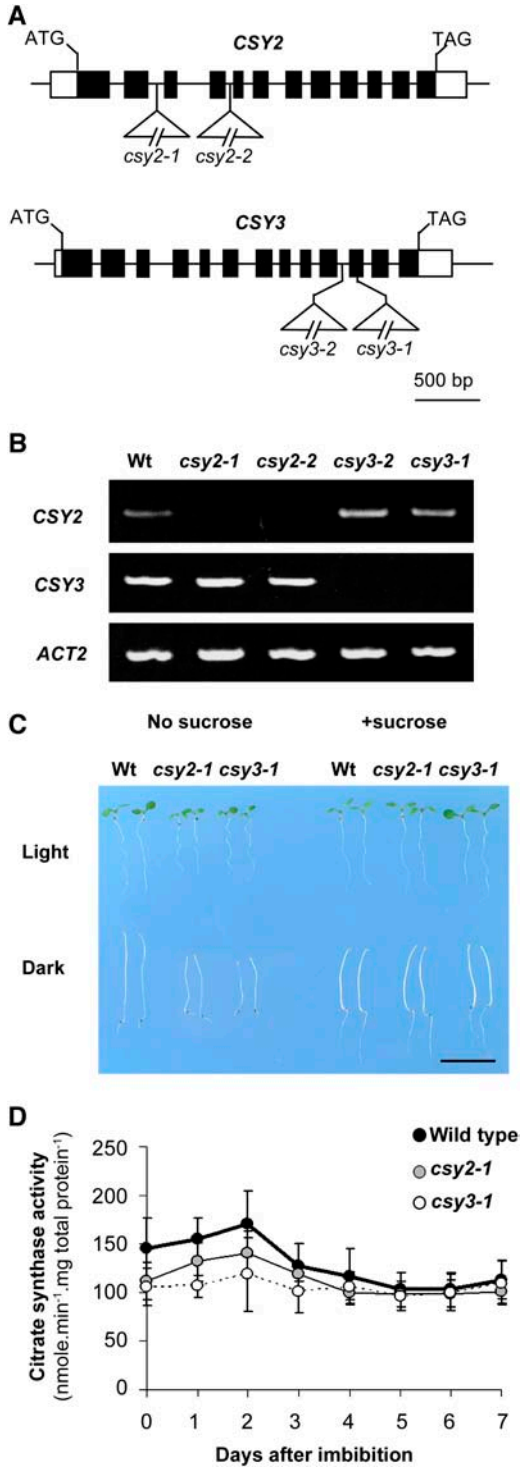

Figure 4. Isolation and Characterization of CSY2 and CSY3 T-DNA Knockout Mutants.

(A) Gene structure of *CSY2* and *CSY3* showing positions of the T-DNAs in individual mutants. Exons are black boxes, introns are lines, and untranslated regions are white boxes.

(B) RT-PCR with RNA isolated from 2-d-old seedlings. The amount of cDNA template in each RT-PCR reaction was normalized to the signal from the *ACT2* gene.

(C) Phenotypes of *csy* mutants. Seedlings were grown vertically on plates in continuous light or dark in the presence or absence of 1% (w/v) sucrose. Bar = 1 cm.

(D) CSY enzyme activity in total cell extracts of wild-type and *csy* mutant seedlings from 0 to 7 d after imbibition. Data plotted are mean ± SD ($n = 3$), where closed circles are the wild type, shaded circles are *csy2*, and open circles are *csy3*.

produced seed readily when grown in soil (data not shown). Thus, we could not obtain supplies of seeds from homozygous *csy2 csy3* double mutant plants. Instead, double mutant seed was routinely obtained from plants homozygous for *csy2* and heterozygous for *csy3*. Approximately 25% of seed failed to germinate after several days of incubation at 20°C, of which a very high proportion was *csy2 csy3* homozygous double mutant seed. Such seeds could be used for analysis of the double mutant phenotype. Seeds that did not germinate after 2 d of incubation on plates were induced to germinate by seed coat removal. Two days later, they were assayed for total CSY activity, together with 2-d-old wild-type, *csy2*, and *csy3* seedlings that had germinated normally. The results show that the double mutant seedlings contain only ~25% of CSY activity compared with the wild type (Figure 5C). The remaining CSY activity is assumed to be mitochondrial.

The Mutant Phenotype Is Caused Specifically by the Lack of Peroxisomal CSY

To confirm that the dormant seed phenotype observed in the *csy2 csy3* double mutant is due to mutations in the *CSY* genes and not due to secondary mutations caused during transformation by T-DNA, double mutants were made in all four possible combinations involving the two mutant alleles of each gene. In all cases, a dormant seed phenotype was observed (see Supplemental Table 1 online). To confirm that the phenotype is not due to inherent disruption of expression of other specific genes caused as a consequence of T-DNA insertion in a *CSY* gene, transgenic complementation was undertaken. The transgene employed comprised the 35S promoter linked to a CSY3 cDNA. Plants that were homozygous *csy3/csy3* and heterozygous *CSY2/csy2* were subjected to the floral dipping transformation procedure using *Agrobacterium tumefaciens* (Clough and Bent, 1998). The seeds from these plants were plated on hygromycin to select for transformants. Several of the transformants obtained were double homozygous *csy2 csy3* mutants containing the transgene (data not shown), which grew normally (Figure 5D).

To establish that CSY functions specifically in the peroxisome and not in the mitochondrion because of possible dual targeting of the PTS2 sequence (Lee et al., 2000), complementation was also performed with a transgene encoding CSY3 targeted to the peroxisome by a PTS1 sequence. The coding information for the 20 N-terminal amino acids of the PTS2 sequence was removed, and the 10 amino acids from the C-terminal end of pumpkin malate synthase that includes the PTS1 sequence (Hayashi et al.,

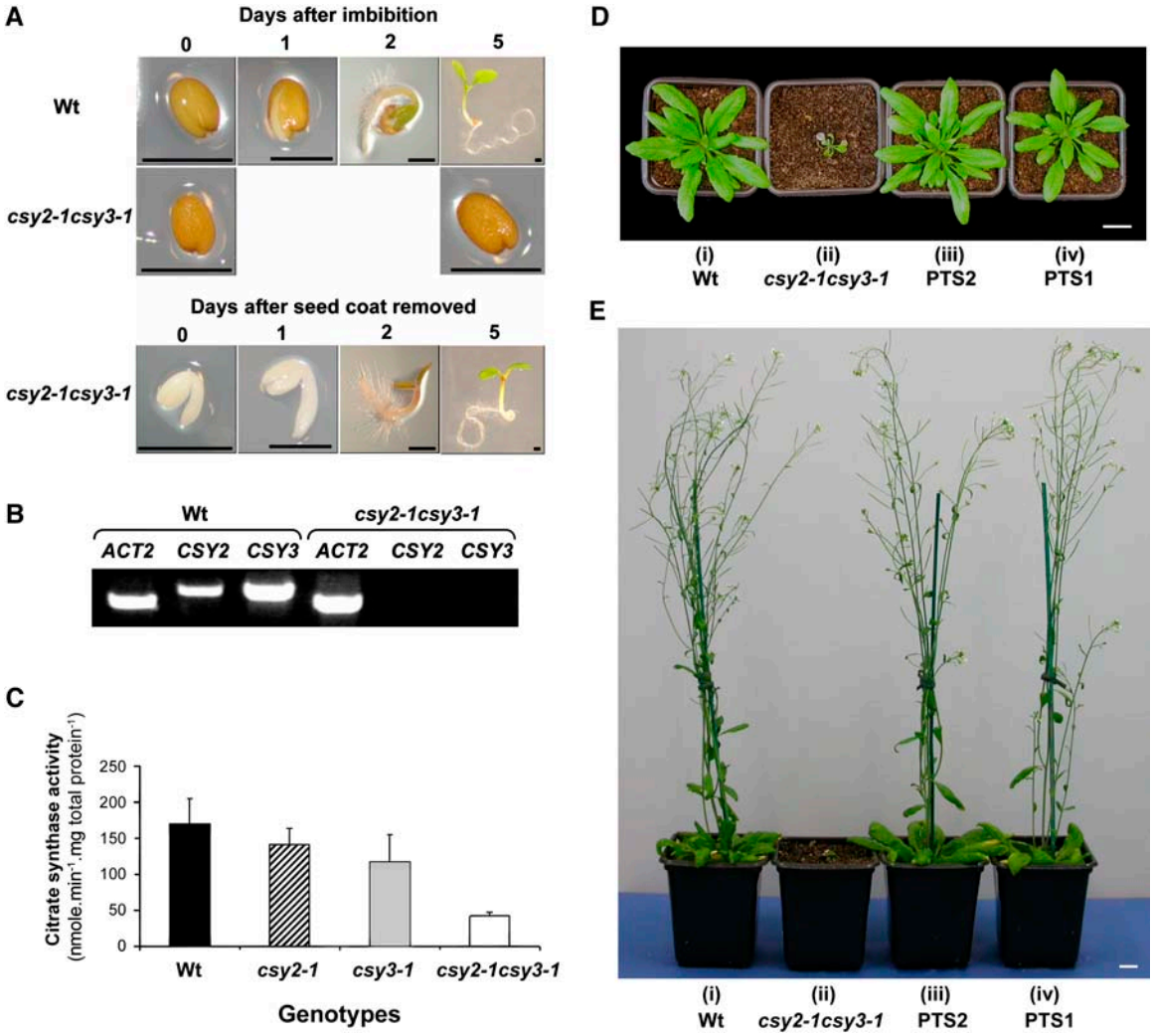

Figure 5. Characterization of *csy2 csy3* Double Mutants.

(A) Top panel, wild-type seedling during first 5 d after imbibition compared with *csy2-1 csy3-1* double mutant at days 0 and 5 in the light on half-strength MS medium plus 1% (w/v) sucrose. Bottom panel, *csy2-1 csy3-1*after seed coat removal and culture on half-strength MS medium plus 3% (w/v) sucrose. Bars = 1 mm.

(B) RT-PCR of *CSY2* and *CSY3* RNAs from 2-d-old wild-type and *csy2-1 csy3-1* seedlings.

(C) CSY enzyme activity in 2-d-old seedlings grown in continuous light conditions. Data are mean ± SD ($n = 3$), where black is the wild type, hatched is *csy2*, shaded is *csy3*, and white is *csy2-1 csy3-1*.

(D) Phenotypes after 4 weeks of growth of (i) the wild type, (ii) *csy2-1 csy3-1*, (iii) *csy2 csy3* complemented with CSY3 cDNA with authentic PTS2 sequence, (iv) *csy2 csy3* complemented with CSY3 cDNA with PTS2 sequence replaced by PTS1. Bar = 1 cm.

(E) Same as **(D)** but after 7 weeks of growth. Bar = 1 cm.

1996) were added to the *CSY3* cDNA. Again, plants that were homozygous *csy3/csy3* and heterozygous *CSY2/csy2* were subjected to the floral dipping transformation procedure using Agrobacterium. The seeds from these plants were plated on hygromycin to select for transformants. Several of the transformants obtained were double homozygous *csy2 csy3* mutants containing the transgene (data not shown). Such plants grew normally (Figures 5D and 5E). We conclude that the dormant seed and impaired plant growth phenotype is caused by the absence of peroxisomal CSY.

The *csy* Double Mutant Seeds Fail to Mobilize TAG Because of a Block in Fatty Acid β-Oxidation

Mutants blocked in β-oxidation fail to mobilize TAG and are compromised in germination or seedling growth (Hayashi et al., 1998; Germain et al., 2001; Footitt et al., 2002; Fulda et al., 2004). To determine if *csy* double mutants can mobilize TAG, embryos were removed from dormant seeds at day 1.5 and grown for 2, 3, 4, and 5 d. The TAG content of such seedlings was determined by the quantitation of eicosenoic acid (20:1) by gas

chromatography–mass spectrometry. In *csy* double mutants, the amount of TAG does not change from day 1.5 to day 5 (Figure 6), whereas that in wild-type seedlings declines rapidly from day 2 such that it is almost absent by day 5 (Figure 6). It is not possible to identify homozygous mutant seed before 1.5 d, so we cannot determine TAG content at day 0. However, because TAG does not decline after day 1.5, and is similar in amount to that of wild-type seedlings at day 0, we assume that TAG does not decline between day 0 and day 1.5.

Transmission electron microscopy was performed on thin sections prepared from the rare *csy2 csy3* double mutant seedlings that spontaneously germinate. The genotype of such seedlings 5 d after germination was confirmed by RT-PCR analysis of one cotyledon, whereas the other was used for electron microscopy. Although lipid bodies are not present in wild-type seedlings at day 5 (Figures 7A and 7B), they persist in mutant seedlings (Figures 7C and 7D). These results are similar to those observed with *kat2/ped1* (Hayashi et al., 1998; Germain et al., 2001) and *cts1* seedlings (Footitt et al., 2002). In cells of *kat2/ped1* cotyledons at day 5, extremely large peroxisomes are seen (Hayashi et al., 1998; Germain et al., 2001). By contrast, the peroxisomes of *cts1* appear to be normal in size (Footitt et al., 2002). The peroxisomes in the *csy2 csy3* double mutant seedlings at day 5 are enlarged relative to those of the wild type, and they contain inclusions (Figure 7D), although these features are not as pronounced as in *kat2/ped1* seedlings.

To test if the block in TAG mobilization is due to a block in β-oxidation, we employed the proherbicide 2,4-dichlorophenoxybutyric acid (2,4-DB). This is converted by peroxisomal β-oxidation to the herbicide 2,4-D, leading to severe inhibition of root growth; thus, seedlings blocked in β-oxidation are resistant to 2,4-DB (Hayashi et al., 1998, 2002; Zolman et al., 2001; Footitt et al., 2002). Homozygous *csy2 csy3* double mutant embryos obtained by surgical removal from dormant seeds were grown on medium containing 2,4-DB. The results show that the double mutant seedlings are resistant to 2,4-DB, but sensitive to 2,4-D, whereas wild-type seedlings are sensitive to both (Figure 8A). The degree of resistance of the double mutant seedlings is similar to that observed with known β-oxidation mutants, including *cts1* (Footitt et al., 2002) and *ped1* (Hayashi et al., 1998). We deduce that peroxisomal β-oxidation is blocked in the *csy* double mutant. Genes encoding enzymes of all key steps in β-oxidation are expressed normally in such seedlings (Figure 8B), indicating that there is not a generalized depression of expression of such genes. Expression of *CSY4* encoding the major mitochondrial CSY is also normal (Figure 8B). The difficulty in obtaining sufficient quantities of homozygous double mutant seeds precluded detailed biochemical studies of β-oxidation enzyme activities.

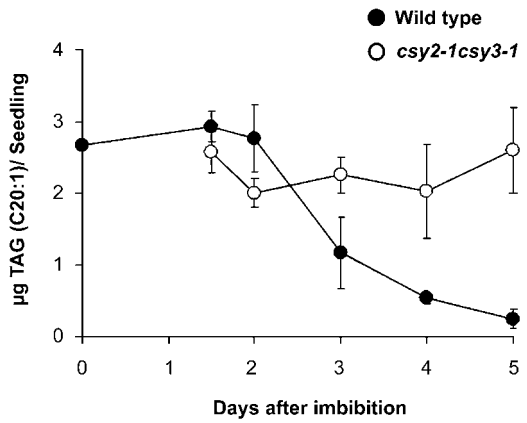

Figure 6. Quantification of TAG in Wild-Type and *csy2 csy3* Double Mutant Seedlings during Post-Germinative Growth.

C20:1, used as a marker for TAG, was detected by gas chromatography–mass spectrometry in triplicate batches of 20 seedlings grown in continuous light on half-strength MS medium plus 3% (w/v) sucrose. Data are mean ± SD ($n = 3$). Closed circles, the wild type; open circles, *csy2 csy3*.

Expression of genes encoding enzymes of β-oxidation can be increased during carbon starvation or senescence of plant cells and tissues (Dieuaide et al., 1992; Hooks et al., 1995). To determine if this is true of *CSY2* and *CSY3*, fully expanded source leaves were detached from plants at growth stage 3.90 (Boyes et al., 2001) and incubated in the dark for 0, 2, and 4 d, after which RNA was isolated. RT-PCR analysis showed that the RNA encoding CSY3 increased slightly in response to carbon starvation and that CSY2 RNA increased markedly. By comparison, RNA for long-chain acyl-CoA synthetase (LACS7) and MFP2 increased little, whereas that for thiolase (KAT2) and acyl-CoA oxidase (ACOX2) increased markedly in response to dark treatment (Figure 9). Malate synthase and isocitrate lyase gene expression was not detected in these leaves, indicating that the glyoxylate cycle is not active (Figure 9). These results are consistent with a role for peroxisomal CSY in the respiration of fatty acids.

DISCUSSION

We have shown that peroxisomal CSY is an absolute requirement for the metabolism of TAG in germinating Arabidopsis. Double mutant *csy2 csy3* seedlings are unable to break down their TAG and are resistant to 2,4-DB, indicating that they are blocked in β-oxidation. The phenotype of *csy* double mutants is not simply due to a block in the glyoxylate cycle because glyoxylate cycle mutants respire their TAG, are sensitive to 2,4-DB, and have mild phenotypes that can be easily rescued with exogenous sugar (Eastmond et al., 2000; Cornah et al., 2004). We conclude that peroxisomal CSY is required for the β-oxidation of fatty acids from TAG. In the absence of CSY, acetyl-CoA presumably cannot be further metabolized and so β-oxidation is blocked. Thus, CSY is required for carbon export from the peroxisome.

In yeast, mutation of peroxisomal CSY does not prevent the respiration of fatty acids because yeast contains an acylcarnitine shuttle to transfer acetyl units to the mitochondrion for respiration (van Roermund et al., 1995, 1999). It was proposed that carbon from fatty acids can be transferred to the mitochondrion either as acetylcarnitine or as succinate after passage through the glyoxylate cycle (van Roermund et al., 1999). We suggest that carbon from β-oxidation could potentially be transferred as citrate in yeast as well as in Arabidopsis. Citrate is normally exported from the peroxisome in both yeast and plants as part of

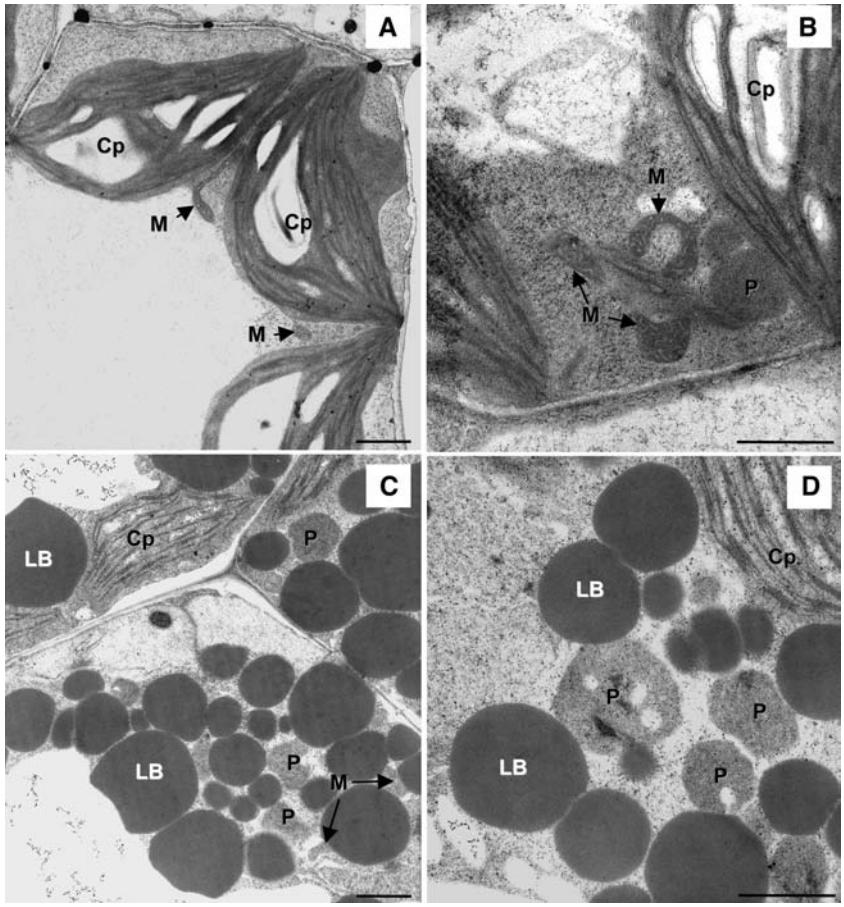

Figure 7. Electron Micrographs of 5-d-Old Wild-Type and *csy2 csy3* Double Mutant Seedlings from Rare Spontaneous Germination Events.

(A) and **(B)** Wild-type seedlings.
(C) and **(D)** *csy2-1 csy3-1* seedlings.
P, peroxisomes; M, mitochondria; Cp, chloroplasts; LB, lipid bodies. Bars = 1 μm.

the glyoxylate cycle because aconitase is cytosolic. Such citrate or isocitrate could either be imported into mitochondria in exchange for oxaloacetate by a carboxylic acid transporter in the mitochondrial membrane (Picault et al., 2002) or it could be metabolized by the glyoxylate cycle. It is not yet known if oxaloacetate is imported into the peroxisome to maintain CSY activity or if it is converted to aspartate before import (Mettler and Beevers, 1980). The partitioning of citrate between the glyoxylate cycle and respiratory pathway may be determined by the relative demands for sugar or energy by the seedling.

Whereas yeast can employ the acylcarnitine shuttle, the data presented here indicate that Arabidopsis does not contain this alternative mechanism because double mutant *csy* seedlings are completely unable to break down their TAG. The role of BOU, the acylcarnitine carrier-like protein in the Arabidopsis mitochondrial membrane (Lawand et al., 2002), has still to be elucidated, but our data indicate that this pathway is not responsible for a major transfer of carbon from peroxisomes to mitochondria in seedlings.

Our data support the original proposal of Raymond et al. (1992) that peroxisomal CSY participates together with mitochondrial citric acid cycle enzymes in the respiration of acetyl units from peroxisomal fatty acid β-oxidation. The proposed requirement for peroxisomal CSY in fatty acid β-oxidation is consistent with the observed expression throughout the plant of *CSY* genes (Figure 2B) and β-oxidation genes (Hooks, 2002). Peroxisomal CSY activity is detected in a range of plant tissues, rather than just those in which the glyoxylate cycle is active (Cornah and Smith, 2002). Furthermore, *CSY* and β-oxidation genes increase in expression in senescing leaves, whereas glyoxylate cycle genes do not (Figure 9).

Although there are three peroxisomal *CSY* genes in the Arabidopsis genome, we have demonstrated that only two of these (*CSY2* and *3*) are active during seed germination and seedling development. Knockout mutations in each of these genes reveal that there is a substantial degree of functional overlap between the two isozymes because they only have a mild phenotype in the absence of sucrose. Indeed, a single copy of either of these genes is sufficient for normal germination and plant growth. The severe phenotype of *csy2 csy3* double mutants indicates that CSY1 is unable to compensate for the lack of CSY2

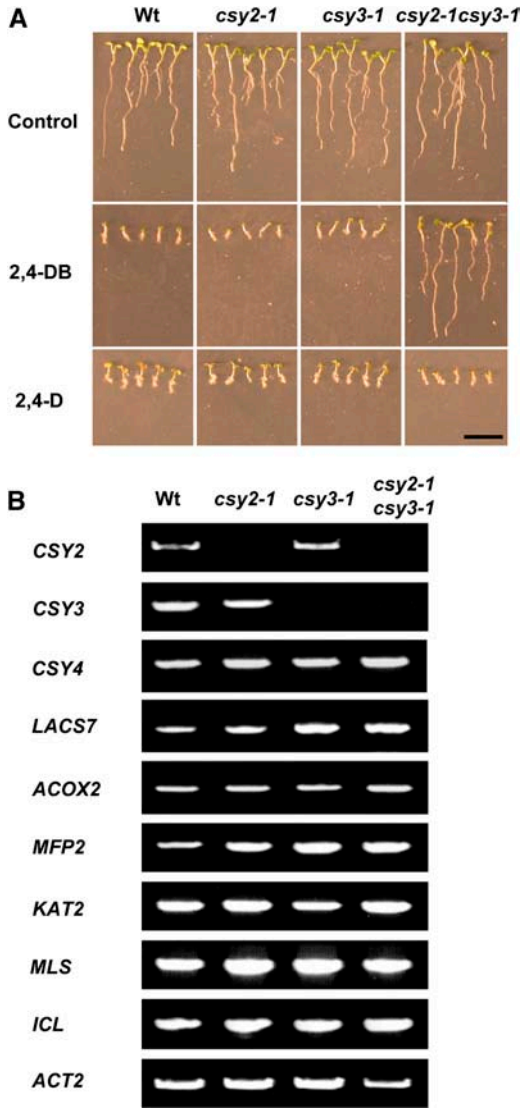

Figure 8. Characterization of β-Oxidation Function and Gene Expression in *csy* Mutants.

(A) Seedlings on half-strength MS plus 3% (w/v) sucrose (control) and plus 800 nM 2,4-DB or 230 nM 2,4-D. Seedlings were grown vertically on plates in continuous light. Bar = 1 cm.

(B) Gene expression of key enzymes of β-oxidation and glyoxylate cycle detected by RT-PCR. RNA was isolated from 2-d-old light-grown seedlings. The amount of cDNA template in each RT-PCR reaction was normalized to the signal from the *ACT2* gene. CSY4, mitochondrial isoform of CSY At2g44350; LACS7, peroxisomal long-chain acyl-CoA synthetase; ACOX2, acyl-CoA oxidase 2; KAT2, 3-ketoacyl CoA thiolase; MLS, malate synthase; ICL, isocitrate lyase.

and 3 in the peroxisome. This is explained by the expression of *CSY1* only in siliques and specifically in developing seeds (Figure 2B, top and middle panels). Several studies have shown β-oxidation activity in developing oilseeds (Eccleston and Ohlrogge, 1998; Hooks, 2002) and the *acx3 acx4* double mutant has an embryo-lethal phenotype, showing the importance of β-oxidation in seed development (Rylott et al., 2003). We propose that the requirement for peroxisomal CSY in fatty acid β-oxidation during seed development is fulfilled by the CSY1 isoform in *csy2 csy3* double mutants.

The failure of *csy2 csy3* double mutants to grow into mature plants and to flower suggests an essential role for β-oxidation. No β-oxidation mutants so far described are compromised to such an extent as *csy2 csy3*, but in all cases this can be explained by redundancy of genes (Richmond and Bleecker, 1999; Germain et al., 2001; Hooks, 2002; Fulda et al., 2004). An alternative explanation is that CoA is sequestered as acetyl-CoA in the *csy2 csy3* mutant and that either the lack of free CoA or an inhibitory effect of acetyl-CoA may inhibit growth. Similar explanations have been advanced to explain resistance to indol-3-butyric acid in ACX mutants (Adham et al., 2005).

The seed dormancy of double *csy* mutants may result because they are unable to produce enough energy to initiate the processes of germination. However, like the *cts* mutant (Footitt et al., 2002), exogenous sucrose cannot trigger germination unless the seed coat is disrupted. This suggests that fatty acid β-oxidation plays a role beyond that of energy or carbon provision to the germinating seed. Similarly, a block in β-oxidation in the peroxisome prevents lipase action in the lipid body and can lead to abnormal peroxisome development (Germain et al., 2001). All of these observations suggest a role for lipid signaling in the

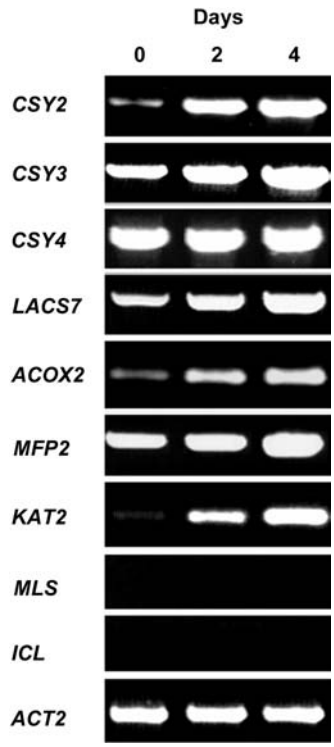

Figure 9. Expression of β-Oxidation Genes in Starved Leaves Detected Using RT-PCR.

RNA was isolated from detached leaves after 0, 2, and 4 d of incubation in the dark. The abbreviations are as described in Figure 8.

control of germination and metabolism. Whether this is due to β-oxidation removing a particular signaling molecule or creating one is unknown. Nevertheless, β-oxidation appears to provide an essential trigger for seed germination in Arabidopsis.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana seeds were surface-sterilized, stratified, and germinated as described previously (Sherson et al., 2000). Seedlings were grown on half-strength MS medium (Sigma-Aldrich, Poole, Dorset, UK) with or without sucrose in continuous light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C unless otherwise stated. Double mutant seedlings were obtained from the embryos of dormant seeds and grown on half-strength MS medium containing 3% (w/v) sucrose for 7 d before transfer to soil.

Phylogenetic Analysis

The amino acids sequences of Arabidopsis, pumpkin (*Curcurbita pepo*), and *Saccharomyces cerevisiae* CSYs were aligned using ClustalX and then edited using Bioedit software (version 4.8.4; http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html). Protdist was used to calculate a distance matrix and then Neighbor was used to build a phylogentic tree (both programs part of PHYLIP software package, version 3.63; http://evolution.genetics.washington.edu/phylip.html). Bootstrap analysis was then performed with 1000 replicates using the same combination of programs.

Screening of T-DNA Insertion Lines and Isolation of *csy* Knockout Mutants

Arabidopsis seed lines containing T-DNA insertions in the *CSY2* and *3* genes were obtained from the Syngenta SAIL collection (*csy2-1*, *csy2-2*, and *csy3-1*; Sessions et al., 2002) and the SALK collection (*csy3-2*; Alonso et al., 2003). PCR-based screening was used to identify individuals homozygous for T-DNA insertions in *CSY2* (At3g58750) and *CSY3* (At2g42790) genes. The gene-specific primers CSY2_For (5′-GTCTGATACCGTCGGATTGG-3′), CSY3_For (5′-CGGAAGGAAAACAGGATTCTCC-3′), and CSY_comRev (5′-CCAGTGTGACAAGTATCCAGC-3′) were used in combination with T-DNA left border primers (Krysan et al., 1999; Alonso et al., 2003).

RT-PCR

RNA was isolated from seedlings using the Qiagen RNAeasy kit (Crawley, West Sussex, UK) and used to generate cDNA with either the Qiagen Omniscript or Qiagen Sensiscript RT-PCR kit. PCR was performed for 35 cycles with gene-specific primers (5′–3′) as follows: *ACT2* (CAACCAATCGTGTGTGA and CTGTGAACGATTCCTGGA), *CSY1* (CTGAGCCGAATCAGGTGTTGC and CCAGTGTGACAAGTATCCAGC), *CSY2* (GTCTGATACCGTCGGATTGG and CCAGTGTGACAAGTATCCAGC), *CSY3* (CGGAAGGAAAACAGGATTCTCC and CCAGTGTGACAAGTATCCAGC), *CSY4* (AGCAGGACCGTCTGAAGAAA and CGGAATAACCTTGCCACTGT), *LACS7* (GTATGGTGGTGTTGCTGTCG and ATGGTTCTGGCACCAAAGTC), *ACOX2* (CTTCCAACTCATGATTCCAAAGGAGTC and CAGCAGCCACCTGTTGCAGAAGTACAG), *MFP2* (CCTTGACATAGTCGGGAGGA and GGCATTCCAAACTTGCTGAT), *KAT2* (GAGTCCATGACTACCAATCCAATGCC and CCCAAGAGAAGCAAGAGTTGTGGTTG), *MLS* (ATGGAGCTCGAGACCTCAGTTTATC and GAGCCTTGAGACATTGATAGGGTAG), *ICL* (GCAGAGGGAGGCAAGAATGAGCATG and TAACACTCGGCCTTGCTCATTTGAC).

Plasmid Construction

The plasmids used in this study are all based on the double CaMV 35S promoter and CaMV terminator cassette inserted into the pGreen binary vector p0179, which confers hygromycin resistance, except p35S:mRFP-SRL, which was cloned into pGreen0045, which confers kanamycin resistance (Hellens et al., 2000). A *CSY3* cDNA was obtained from Riken (pda02633; Seki et al., 2002) and subcloned within the CaMV cassette to generate p35S:CSY3. A modified *CSY3* cDNA was generated in which the first 60 bases, corresponding to the PTS2 signal sequence, were deleted and a PTS1 sequence, corresponding to the last 10 amino acids of the pumpkin MLS gene (Hayashi et al., 1996; Lin et al., 2004) were fused at the 3′ end. This construct was called p35S:ΔCSY3-SRL.

GFP constructs p35S:CSY2-GFP and p35S:CSY3-GFP were generated by fusing the leader sequence, corresponding to the first 44 and 40 amino acids of CSY2 and CSY3, respectively, to the coding sequence for GFP and subcloning these fragments into the CaMV cassette. A peroxisomally targeted mRFP construct, p35S:mRFP-SRL, was generated by addition of the pumpkin MLS PTS1 sequence at the 3′ end. The fragment was subcloned into the CaMV cassette and cloned into pGreen0045.

Arabidopsis Transformation

The plasmid constructs were transfected in *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986), and Arabidopsis was subjected to transformation by the floral dip method (Clough and Bent, 1998). Seed collected from dipped plants was screened on the appropriate antibiotic and resistant seedlings selected and verified by PCR.

Microscopy

Confocal microscopy was performed to observe GFP and mRFP in root hair cells as previously described (Lin et al., 2004). For transmission electron microscopy, cotyledons from seedlings germinated for 5 d on half-strength MS medium supplemented with 1% (w/v) sucrose were processed as previously described (Germain et al., 2001)

Biochemical Analysis

For enzyme assays, tissue extracts were prepared from Arabidopsis seedlings, and CSY activity was assayed as described (Kato et al., 1995).

Protein content was determined as reported (Bradford, 1976) using BSA as the standard. Total fatty acids were extracted and quantified by gas chromatography–mass spectrometry (Browse et al., 1986) using C20:1 as a marker for TAG.

Arabidopsis Genome Initiative numbers of the five putative CSY sequences from Arabidopsis are At3g58740, At3g58750, At2g42790, At2g44350, and At3g60100. The CSY gene name has been registered with The Arabidopsis Information Resource.

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