

MONODEHYROASCORBATE REDUCTASE4 Is Required for Seed Storage Oil Hydrolysis and Postgerminative Growth in *Arabidopsis*^W

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Hydrogen peroxide is a major by-product of peroxisomal metabolism and has the potential to cause critical oxidative damage. In all eukaryotes, catalase is thought to be instrumental in removing this H₂O₂. However, plants also contain a peroxisomal membrane-associated ascorbate-dependent electron transfer system, using ascorbate peroxidase and monodehydroascorbate reductase (MDAR). Here, I report that the conditional seedling-lethal *sugar-dependent2* mutant of *Arabidopsis thaliana* is deficient in the peroxisomal membrane isoform of MDAR (MDAR4). Following germination, *Arabidopsis* seeds rely on storage oil breakdown to supply carbon skeletons and energy for early seedling growth, and massive amounts of H₂O₂ are generated within the peroxisome as a by-product of fatty acid β -oxidation. My data suggest that the membrane-bound MDAR4 component of the ascorbate-dependent electron transfer system is necessary to detoxify H₂O₂, which escapes the peroxisome. This function appears to be critical to protect oil bodies that are in close proximity to peroxisomes from incurring oxidative damage, which otherwise inactivates the triacylglycerol lipase SUGAR-DEPENDENT1 and cuts off the supply of carbon for seedling establishment.

INTRODUCTION

In eukaryotes, the oxidative metabolism that takes place in peroxisomes generates hydrogen peroxide (H₂O₂), which must be detoxified to prevent damage to proteins, lipids, and DNA (van den Bosch et al., 1992; Singh, 1997; Willekens et al., 1997). This important task is performed universally by catalase, which catalyzes the decomposition of H₂O₂ into molecular oxygen and water within the peroxisomal matrix. Defects in catalase cause peroxisome dysfunction and are detrimental in mammals, plants, and yeast (Zhang et al., 1993; Willekens et al., 1997; Sheikh et al., 1998; Horiguchi et al., 2001).

Uniquely, in addition to catalase, higher plant peroxisomes possess a membrane-bound ascorbate-dependent electron transfer system that is also able to remove H₂O₂ (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996; Mullen and Trelease, 1996; Karyotou and Donaldson, 2005). This system is thought to rely on the cooperative action of ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDAR). APX initiates electron transfer from two molecules of ascorbate to convert H₂O₂ to water, and the monodehydroascorbate is then recycled to reduced ascorbate by MDAR via electron transfer from NADH. Monodehydroascorbate can also spontaneously disproportionate to ascorbate and dehydroascorbate, and the latter can be

reduced back to ascorbate by glutathione-dependent dehydroascorbate reductase (Jiménez et al., 1997; del Rio et al., 1998).

Interestingly, although catalase is highly active in plant peroxisomes, it has a much lower affinity for H₂O₂ than APX, and this has led to the suggestion that at low concentrations, H₂O₂ might be preferentially scavenged by the APX/MDAR system (Bunkelmann and Trelease, 1996; Mullen and Trelease, 1996). It has been hypothesized that the membrane association of APX/MDAR allows the system to protect membrane lipids and integral proteins from oxidative damage and act as a cordon to limit the escape of H₂O₂ into the cytosol (Yamaguchi et al., 1995; Mullen and Trelease, 1996; Karyotou and Donaldson, 2005). MDAR may also play a role in reductant balance within the peroxisome by recycling NAD⁺ (Bowditch and Donaldson, 1990; Mullen and Trelease, 1996).

The physiological importance of catalase in maintaining redox balance in plant peroxisomes has been demonstrated in several studies using mutants or antisense suppression (Kendall et al., 1983; Takahashi et al., 1997; Willekens et al., 1997; Vandenabeele et al., 2004), but less attention has been paid to peroxisomal APX and MDAR. Overexpression of *APX3*, encoding a peroxisomal isoform from *Arabidopsis thaliana*, has been reported to increase protection against oxidative stress (Wang et al., 1999). However, Narendra et al. (2006) have recently shown that a null mutant in *APX3* is healthy under normal growth conditions. It therefore remains unclear whether the peroxisomal APX/MDAR system is required for plant growth and development and whether it plays a different role from that of catalase.

In this study, I provide molecular genetic evidence for the function of the ascorbate-dependent electron transfer system by characterizing an *Arabidopsis* mutant in the peroxisomal membrane isoform of MDAR (MDAR4; Lisenbee et al., 2005). This mutant was

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originally designated *sugar-dependent2* (*sdp2*; Eastmond, 2006), and it exhibits a conditional seedling-lethal phenotype because its seeds are unable to break down storage oil to provide carbon skeletons and energy for early seedling growth. In oilseeds such as *Arabidopsis*, massive amounts of H₂O₂ are generated within the peroxisome as a by-product of fatty acid β-oxidation (Graham and Eastmond, 2002). My data suggest that without MDAR4, the escaping H₂O₂ inactivates the lipases on the oil body surface that are responsible for catalyzing the first step in storage oil breakdown.

RESULTS

SDP2 Is Required for Storage Oil Hydrolysis in Germinating Arabidopsis Seeds

In *Arabidopsis* seeds, the breakdown of stored triacylglycerol (TAG) following germination is essential to drive the initial phase of seedling growth and allow photosynthetic establishment (Hayashi et al., 1998). To investigate this process, a forward genetic screen was used to isolate a collection of *Arabidopsis* mutants that require an alternate exogenous source of carbon (sucrose) to support postgerminative growth (Eastmond, 2006). Screening these *sdp* mutants for defects in TAG hydrolysis revealed that *sdp1*, *sdp2*, and *sdp3* are defective in the lipase activity that is associated with the oil body membranes. I have previously characterized the patatin domain TAG lipase encoded by *SDP1* (Eastmond, 2006). Here, I investigate *SDP2*.

The *sdp2* mutant germinates normally (see Supplemental Table 1 online), but the cotyledons fail to expand or green, and seedling growth arrests (Figure 1A). Providing sucrose rescues

the growth phenotype (Figure 1A), and once *sdp2* seedlings establish photosynthetic competence, they grow normally and complete their life cycle. Analysis of the fatty acid content of *sdp2* seeds germinated in the presence of sucrose revealed that the mutant is severely impaired in its ability to breakdown TAG (Figures 1B and 1C). Eicosenoic acid is specifically found in TAG in *Arabidopsis* (Lemieux et al., 1990); therefore, it can be used as a convenient marker to monitor TAG breakdown (Eastmond et al., 2000a). The levels of both total fatty acids and eicosenoic acid (20:1) decline by <20% over the course of the first 5 d of postgerminative growth in *sdp2*, while in wild-type seedlings, they fall by 60 and 95%, respectively.

SDP2 Encodes a Component of the Peroxisomal Antioxidant System

The *SDP2* locus was mapped to a region on chromosome 3 near *GLABRA1* and between PCR-based markers *ciw11* and *snap77* (Figure 2A). Further mapping reduced the interval to an ~80-kb region, containing 22 open reading frames. Sequencing candidate genes within this region revealed that three independent ethyl methanesulfonate *sdp2* alleles contained mutations in At3g27820 (Figure 2B). This gene encodes an isoform of MDAR (MDAR4) that is associated with the peroxisomal membrane (Lisenbee et al., 2005). MDAR4 forms part of an ascorbate-dependent electron transfer system on the peroxisomal membrane that has been proposed to play a role in scavenging H₂O₂, produced as a by-product of oxidative metabolism (Yamaguchi et al., 1995; Mullen and Trelease, 1996; Karyotou and Donaldson, 2005).

In the *sdp2-1* mutant allele, a T-to-C mutation causes a Val-to-Ala amino acid substitution at position +14 in the polypeptide

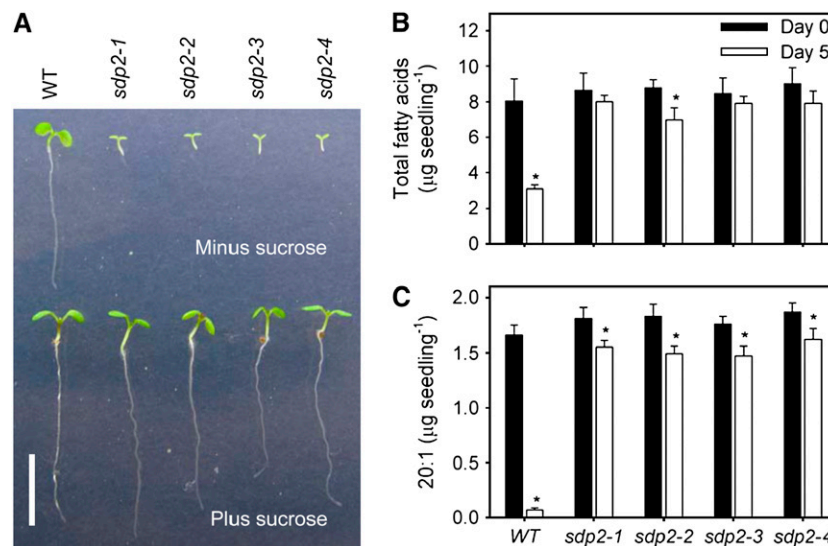


Figure 1. Postgerminative Growth and Fatty Acid Breakdown in *sdp2*.

(A) Images of 5-d-old *sdp2* and wild-type seedlings grown on medium with and without 1% (w/v) sucrose. Bar = 1 cm.

(B) and **(C)** Total fatty acid **(B)** and eicosenoic acid (20:1) **(C)** content of *sdp2* and wild-type seeds and 5-d-old seedlings grown on medium with 1% (w/v) sucrose. Values are the mean ± SE of measurements on five batches of 20 seeds or seedlings. *Significantly different from seeds (P < 0.05).

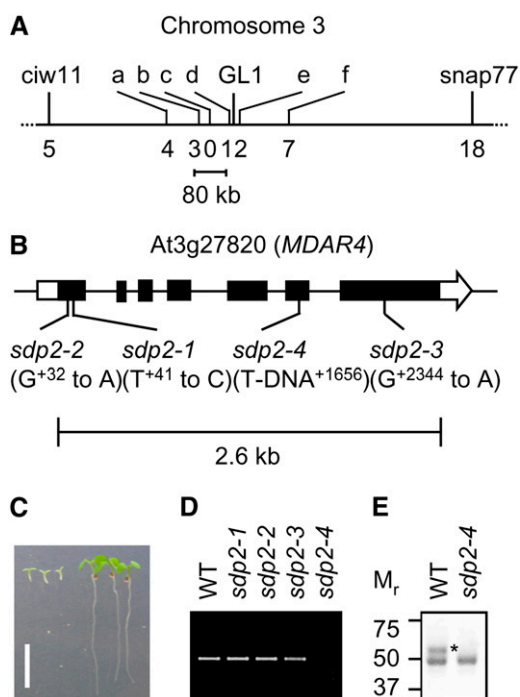


Figure 2. Molecular Characterization of *SDP2*.

(A) Mapping of *SDP2*. PCR-based simple sequence polymorphism, cleaved amplified polymorphic sequence, and single nucleotide amplified polymorphism markers were used to map *SDP2* to an 80-kb region on the top arm of chromosome 3 near *GL1*. The positions of markers a to f (listed in Supplemental Table 2 online) are denoted by bars, and the number of recombination events/total number of chromosomes (380) is listed below each.

(B) Schematic diagram of the *SDP2* locus showing mutations in *At3g27820* (*MDAR4*). Black bars are exons, and the white bar and arrow represent the 5' and 3' untranslated regions, respectively. Insertion/substitution positions are numbered relative to the ATG. A full-length cDNA clone of *SDP2* has previously been published (GenBank accession number AY039980).

(C) Complementation of *sdp2-4* by transformation with a genomic copy of *MDAR4*. Images of 5-d-old *sdp2-4* (left) and *sdp2-4 MDAR4* (right) seedlings grown on medium without sucrose. Bar = 1 cm.

(D) *SDP2* transcript levels in 2-d-old wild-type and *sdp2* seedlings detected using RT-PCR. The PCR primers amplify a 568-bp product from the 3' end of the cDNA.

(E) *SDP2* protein in peroxisomes purified from wild-type and *sdp2-4* seedlings detected using protein gel blotting. *The larger polypeptide band corresponding to *MDAR4* is 54 kD.

sequence (Figure 2B). In the *sdp2-2* and *sdp2-3* mutant alleles, A-to-G mutations give rise to Gly-to-Glu amino acid substitutions in the polypeptide sequence at positions +11 and +386, respectively (Figure 2B). An amino acid sequence alignment indicates that the Gly residues at positions +11 and +386 in *MDAR4* are conserved among all *Arabidopsis* *MDAR* isoforms (Obara et al., 2002). The Val residue at position +14 is also conserved in *MDAR1*, 2, 3, and 4 but not in *MDAR5* and 6 (Obara et al., 2002). Plant *MDAR* polypeptide sequences contain two putative nucleotide binding regions with the consensus motif V/IXGXGXX-

GXXXG/A (Drew et al., 2006). These motifs are likely to form Rossmann folds and interact with the adenine moieties of either the cofactor flavin adenine dinucleotide or the electron donor NADH (Drew et al., 2006). The mutations in *sdp1-1* and *sdp1-2* give rise to amino acid substitutions within the first putative nucleotide binding region. A T-DNA allele of *MDAR4* (*sdp2-4*) that contains an insertion in exon 6 (Figure 2B) was also obtained from the SALK collection (Alonso et al., 2003). This line exhibits the same phenotype as the ethyl methanesulfonate alleles (Figure 1A). To confirm that the phenotype of *sdp2-4* is caused by a defect in *MDAR4*, the mutant was complemented by transformation with a construct carrying a genomic copy of the gene (Figure 2C).

RT-PCR performed on RNA from 2-d-old seedlings showed that *MDAR4* transcripts are present in the wild type, *sdp2-1*, *sdp2-2*, and *sdp2-3* but are absent from *sdp2-4* (Figure 2D). To investigate whether *MDAR4* protein is present in the *sdp2-4* allele, protein gel blots were performed on peroxisome-enriched fractions obtained by sucrose gradient centrifugation of crude seedling extracts (Eastmond et al., 2000b). A polyclonal antibody was used that was raised against a 47-kD *MDAR* from cucumber (*Cucumis sativus*). This antibody has been shown previously to recognize two polypeptides in purified *Arabidopsis* peroxisomes (Lisenbee et al., 2005). The 47-kD band corresponds to the matrix isoform *MDAR1*, and the 54-kD band corresponds to the membrane isoform *MDAR4*. Both bands were present in peroxisomal fractions from the wild type, but the 54-kD band was specifically absent in peroxisomal fractions from *sdp2-4* (Figure 2E).

Peroxisomes and Oil Bodies Cluster Together in *sdp2* Seedlings

It has been proposed that the APX/*MDAR* system on the peroxisomal membrane acts as a cordon to intercept H_2O_2 that is not detoxified by catalase and prevent it from damaging membrane lipids and integral proteins and escaping from the immediate vicinity of the peroxisome surface out into the cytosol (Yamaguchi et al., 1995; Mullen and Trelease, 1996). The lack of oil body lipase activity in germinating *sdp2* seeds (Eastmond, 2006) might therefore be a symptom of oxidative damage. Confocal microscopy was used to investigate peroxisome morphology and proximity to oil bodies in living cells of *sdp2* seedlings. In epidermal cells from the cotyledons of 5-d-old *sdp2-4* seedlings, green fluorescent protein (GFP)-labeled peroxisomes formed large (5 to 10 μ m) clusters that are associated with groups of transparent spherical undegraded oil bodies (Figure 3A). By contrast, in the wild type, essentially no oil bodies remained by this stage of development, and the individual peroxisomes were dispersed throughout the cytosol (Figure 3A). Electron microscopy images of sections through parenchyma cells from the cotyledons of 5-d-old seedlings also suggest an association between oil bodies and peroxisomes (Figure 3B). The striking peroxisome clustering phenotype in *sdp2-4* was only observed in seedling tissues that contained undegraded oil bodies and was not obvious in tissues from other stages in plant development under the growth conditions used in this study (data not shown).

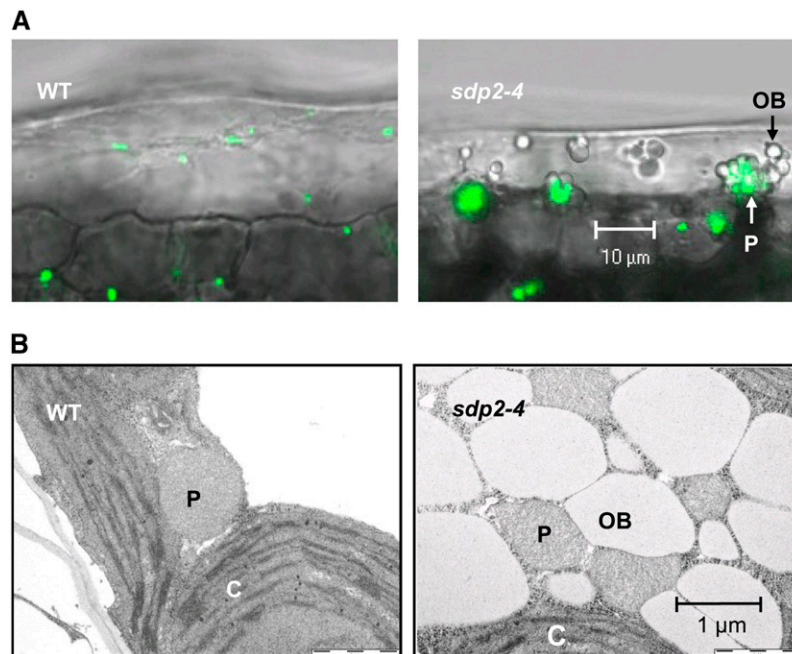


Figure 3. Peroxisome Clustering and Proximity to Oil Bodies in *sdp2* Seedlings.

Confocal images of epidermal cells (**A**) and electron microscopy images of parenchyma cells (**B**) of cotyledons from 5-d-old *sdp2-4* and wild-type seedlings grown on medium containing 1% (w/v) sucrose. For confocal microscopy, peroxisomes were visualized using a genetic background containing PTS1-targeted GFP driven by the 35S promoter (Cutler et al., 2000). P, peroxisome; OB, oil body; C, chloroplast. Bars = 10 μm in (**A**) and 1 μm in (**B**).

β -Oxidation Causes Oxidative Damage to Oil Bodies in *sdp2* Seedlings

During the postgerminative growth of oilseeds, acyl-CoA oxidase, which is the first enzyme of peroxisomal fatty acid β -oxidation, is likely to be the major source of H_2O_2 production in the cell (Graham and Eastmond, 2002). To determine whether fatty acid β -oxidation inhibits oil body lipase activity in *sdp2* seedlings, the *sdp2-4* mutant was crossed into the fatty acid catabolism-deficient mutant *pxa1*. The PXA1/CTS/PED3 protein is an ABC transporter, which is required to import substrate (fatty acids or acyl-CoAs) into the peroxisome for β -oxidation (Zolman et al., 2001). TAG lipase activity was almost undetectable in oil body membranes prepared from 2-d-old *sdp2-4* seedlings (Figure 4A). However, lipase activity was recovered to wild-type levels in the *sdp2-4 pxa1* double mutant (Figure 4A). To investigate whether oil bodies from *sdp2* incur oxidative damage, lipid peroxidation was estimated using the ferrous oxidation xylenol orange assay (Griffiths et al., 2000), and protein oxidation was monitored by detecting protein carbonyls (Levine et al., 1990). Both lipid hydroperoxides (LOOHs) and protein carbonyl levels were significantly elevated in oil bodies purified from 2-d-old *sdp2-4* seedlings versus the wild type, and in both cases, these increased levels were suppressed in the *pxa1* background (Figures 4B and 4C).

SDP1 Is a Target of Oxidative Damage in *sdp2* Seedlings

A large proportion of the TAG lipase activity that is associated with oil body membranes is attributable to SDP1 (Eastmond, 2006), suggesting that this is a likely target of oxidative damage.

In vitro incubation of purified recombinant SDP1 with H_2O_2 showed that the protein can be inactivated by concentrations >0.5 mM (Figure 5A). The loss of catalytic activity caused by H_2O_2 was accompanied by oxidation of the protein, as indicated by a 20-fold increase in the level of carbonylation (Figure 5A). Enzyme assays showed that recombinant SDP1 is still able to hydrolyze oil bodies purified from 2-d-old *sdp2* seedlings but at a significantly lower rate than from the wild type (Figure 5B). This suggests that peroxidation of substrate and/or oxidation of additional oil body proteins also forms a partial barrier to oil hydrolysis by SDP1.

To investigate whether SDP1 suffers oxidative damage in *sdp2-4* seedlings, the protein was purified from oil body membranes by immunoprecipitation using affinity-purified polyclonal antibodies raised against an SDP1 peptide. The purified protein was then reacted with 2,4-dinitrophenylhydrazine (DNP) to derivatize carbonyl groups, and the carbonyl groups were detected with anti-DNP antibodies. Carbonyls could only be detected in SDP1 purified from *sdp2-4* oil body proteins and not from the wild type (Figure 5C). The polyclonal antibodies raised against the SDP1 peptide detected similar amounts of SDP1 in oil body protein samples from *sdp2-4* and the wild type (Figure 5D). Together, these data suggest that SDP1 is transcribed, translated, and targeted to oil bodies in *sdp2* mutant seedlings but that it is then inactivated by oxidative damage.

An Initial Increase in H_2O_2 Levels in *sdp2* Is Likely to Result from a Failure to Recycle Ascorbate

The *sdp2* mutant is defective in the second component of the APX/MDAR system (MDAR4). This mutation might be expected

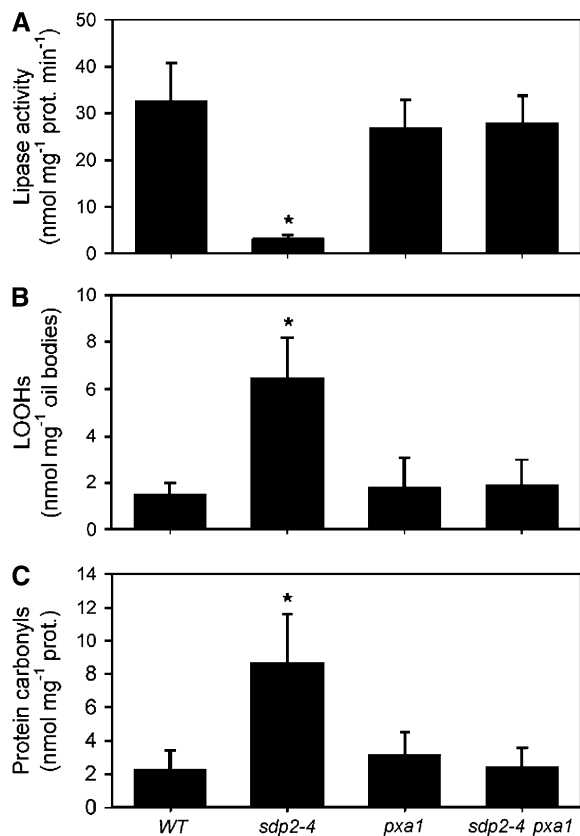


Figure 4. β -Oxidation-Dependent Oxidative Damage to Oil Bodies in *sdp2* Seedlings.

Rate of [¹⁴C]triolein hydrolysis (A), LOOH levels (B), and protein carbonyl levels (C) in oil bodies purified from 2-d-old *sdp2-4*, *pxa1*, *sdp2-4 pxa1*, and wild-type seedlings. The concentration of triolein was equivalent to 10 mM. Values are the mean \pm SE of measurements on five separate preparations. *Significantly different from the wild type ($P < 0.05$).

to reduce the capacity of seedlings to recycle ascorbate immediately following germination and lead to a shortage of substrate for APX and consequently cause a rise in H_2O_2 levels. To test this hypothesis, H_2O_2 , the total ascorbate pool (ascorbate + dehydroascorbate), and the percentage of the ascorbate pool that is reduced were measured in 1- and 3-d-old *sdp2-4* seedlings grown on medium with sucrose (Huang et al., 2005). At day 1, the total ascorbate pool in *sdp2-4* was similar to the wild type, but the percentage of reduced ascorbate was significantly lower, and the level of H_2O_2 was increased (Figure 6). By contrast, at day 3, H_2O_2 levels in *sdp2-4* were lower than those of the wild type (Figure 6A). The production of H_2O_2 in *sdp2-4 pxa1* and *pxa1* seedlings was strongly suppressed relative to *sdp2-4* and the wild type, respectively, at both days 1 and 3 (Figure 6A). These data are consistent with a model in which immediately following *sdp2* germination, the production of H_2O_2 by fatty acid β -oxidation leads to a negative feedback loop whereby the H_2O_2 inactivates SDP1 and progressively cuts off the supply of substrate for β -oxidation, which in turn reduces H_2O_2 production.

Seedlings of *sdp2* Retain the Capacity to β -Oxidize 2,4-Dichlorophenoxybutyric Acid

By detoxifying H_2O_2 that escapes the peroxisome, the ascorbate-dependent electron transfer system has the potential to protect components within the peroxisomal matrix and membrane. MDAR4 may also play a specific role in fatty acid catabolism by recycling NAD^+ within the peroxisome to support the L-3-hydroxyacyl-CoA dehydrogenase reaction of β -oxidation (Bowditch and

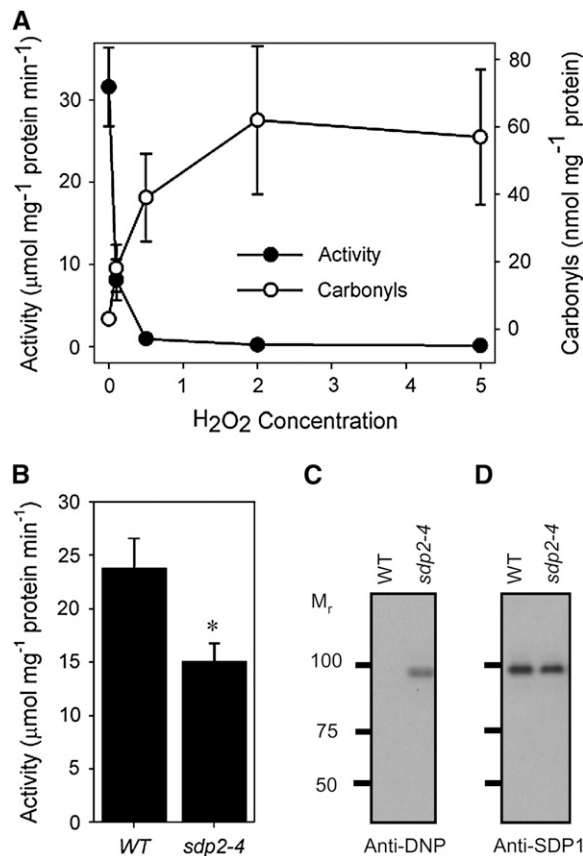


Figure 5. Carbonylation and Inactivation of SDP1.

(A) Effect of H_2O_2 concentration on recombinant SDP1 activity and level of carbonylation. Purified recombinant SDP1 was preincubated with H_2O_2 for 2 h at 22°C before the protein was assayed for either lipase activity or carbonyl levels. Lipase assays were performed using 10 mM [¹⁴C]triolein as a substrate. Prior to the addition of the substrate, H_2O_2 was removed from the samples by adding 10 units mL⁻¹ of catalase and incubating for 10 min at 22°C. Catalase assays confirmed that essentially all the H_2O_2 is removed by this treatment. Values are the mean \pm SE of measurements from four separate incubations.

(B) Activity of recombinant SDP1 on oil bodies purified from 2-d-old *sdp2-4* and wild-type seedlings. Values are the mean \pm SE of measurements from four separate incubations. *Activity significantly different from the wild type ($P < 0.05$).

(C) Detection using anti-DNP antibodies of carbonyl groups in SDP1 immunoprecipitated from oil body membranes of 2-d-old *sdp2-4* and wild-type seedlings.

(D) Detection of SDP1 protein in purified oil body membranes from 2-d-old *sdp2-4* and wild-type seedlings using anti-SDP1 antibodies.

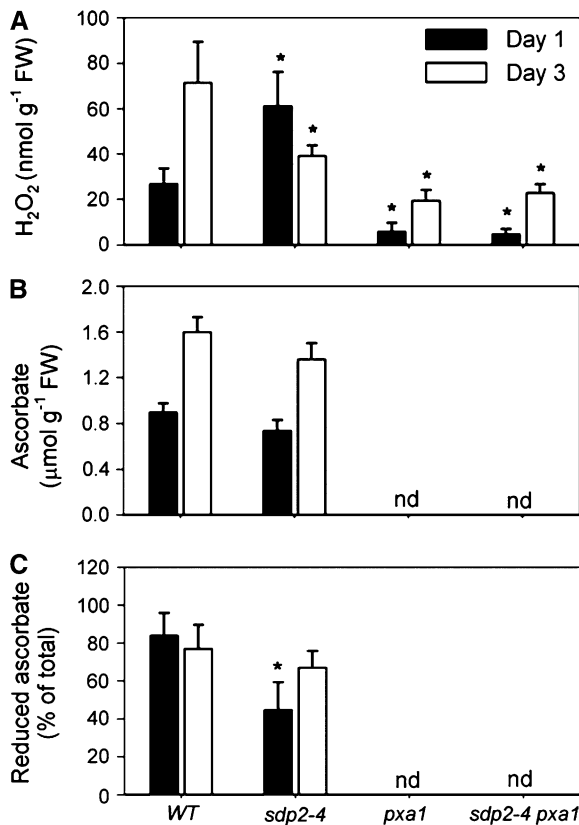


Figure 6. Metabolite Levels in Germinating *sdp2* Seedlings. H₂O₂ (A), total ascorbate (ascorbate + dehydroascorbate) (B), and reduced ascorbate (C) as a percentage of the total in 1- and 3-d-old *sdp2-4*, *pxa1*, *sdp2-4 pxa1*, and wild-type seedlings germinated on medium containing 1% (w/v) sucrose. nd, not determined. Values are the mean ± SE of measurements on five separate extracts. *Significantly different from the wild type (P < 0.05).

Donaldson, 1990; Mullen and Trelease, 1996). To investigate whether either of these factors is likely to play a major role in the phenotype of *sdp2*, the mutant was grown on medium containing 2,4-dichlorophenoxybutyric acid (2,4-DB). This compound is converted to the herbicide 2,4-D by a single cycle of β-oxidation (Hayashi et al., 1998). *Arabidopsis* mutants in many genes that are either directly required for fatty acid β-oxidation or for general peroxisome function exhibit a 2,4-DB-resistant phenotype (Baker et al., 2006). To date, this list of mutants includes *pxa1/cts/ped3*, *acx1*, *acx3*, *acx4*, *aim1*, *ped1/kat2*, *cys2*, *cys3*, *chy1*, *ped2/pex14*, *pmdh1* *pmdh2*, *pex4*, *pex5*, and *pex6*. However, unlike these mutants, root growth in *sdp2-4* seedlings does not exhibit increased resistance to 2,4-DB (Figure 7), indicating that many peroxisomal membrane and matrix proteins remain at least partially functional. In vitro assays performed on extracts of 2-d-old *sdp2-4* seedlings also show that the activities of peroxisomal marker enzymes, acyl-CoA oxidase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, isocitrate lyase, malate synthase, and catalase, are all similar to the wild type (Table 1).

The activity of the cytosolic marker enzyme NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured in 2-d-old seedlings of *sdp2-4* grown on medium containing sucrose (Table 1). This enzyme is an indicator of oxidative damage caused by H₂O₂ in *Arabidopsis* (Hancock et al., 2005; Job et al., 2005). The activity of GAPDH was not affected, suggesting that the deficiency in MDAR4 is unlikely to have caused a general increase in oxidative damage to cytosolic proteins.

Catalase Deficiency Has a Comparatively Small Effect on Storage Oil Breakdown

To compare the effect of MDAR4 deficiency with that of catalase, a series of *Arabidopsis* CATALASE2 antisense lines (Vandenabeele et al., 2004) were screened for their ability to breakdown storage

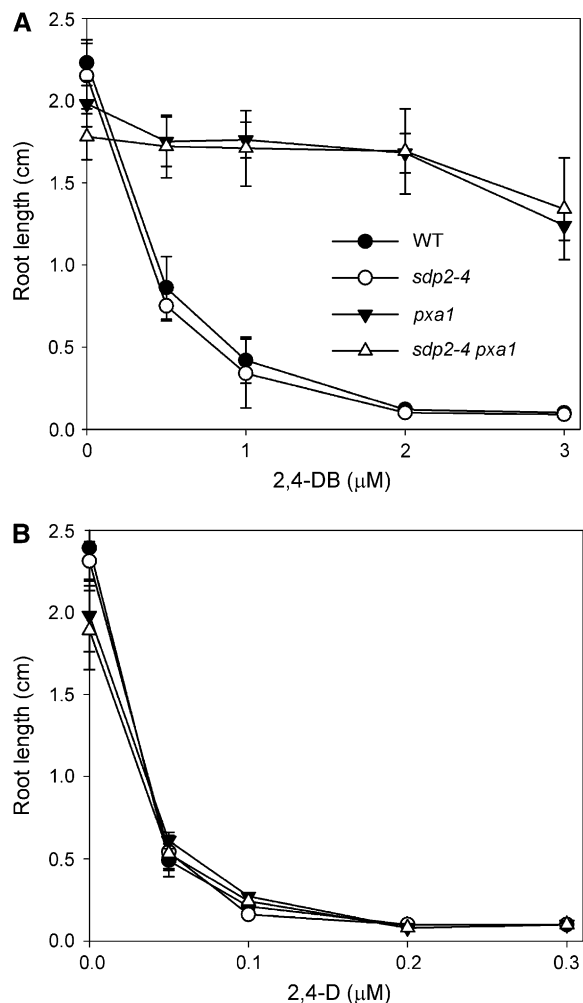


Figure 7. β-Oxidation of 2,4-DB by *sdp2* Seedlings. Effect of 2,4-DB concentration (A) and 2,4-D concentration (B) on root length of 5-d-old *sdp2-4*, *pxa1*, *sdp2-4 pxa1*, and wild-type seedlings grown on medium containing 1% (w/v) sucrose. Values are the mean ± SE of measurements on five batches of 20 seedlings.

Table 1. Peroxisomal and Oil Body Membrane Enzyme Activities in Wild-Type, *sdp2*, and CAT2HP2 Seedlings

Enzyme	Activity (nmol mg ⁻¹ Protein min ⁻¹)			
	Wild Type (Col-0)	<i>sdp2-4</i>	Wild Type (Col4)	CAT2HP2
Peroxisome				
ACX (16:0)	4.1 ± 0.7	3.6 ± 0.5	4.6 ± 0.3	4.0 ± 0.6
ACX (10:0)	10.7 ± 1.7	8.9 ± 0.9	11.9 ± 2.0	7.9 ± 3.3
ACX (4:0)	42.9 ± 3.8	40.1 ± 4.2	36.7 ± 2.9	34.8 ± 4.8
ECH (4:0)	46.3 ± 3.7	47.5 ± 4.9	48.6 ± 5.2	43.1 ± 5.0
HAD (4:0)	36.5 ± 3.8	39.0 ± 2.7	41.7 ± 4.4	37.6 ± 3.6
KAT (4:0)	54.9 ± 7.2	58.4 ± 9.4	57.9 ± 7.0	44.1 ± 11
ICL	79.6 ± 6.6	81.7 ± 5.2	87.3 ± 7.9	16.5 ± 2.0*
MLS	47.2 ± 2.0	52.6 ± 3.7	50.0 ± 5.3	20.5 ± 1.9*
CAT	2302 ± 245	2459 ± 319	2511 ± 304	211 ± 29*
Oil body				
Lipase	21.9 ± 3.9	0.4 ± 0.1*	23.9 ± 4.0	17.8 ± 3.5
Cytosol				
GAPDH	179 ± 24	166 ± 31	nd	nd

Seeds were germinated on medium containing 1% (w/v) sucrose. Peroxisomal enzyme activities were measured in whole extracts from 2-d-old seedlings. Acyl-CoA oxidase (ACX) activity was assayed using palmitoyl-CoA, decanoyl-CoA, and butyryl-CoA. Enoyl-CoA hydratase (ECH) was assayed using crotonyl-CoA. L-hydroxyacyl-CoA dehydrogenase (HAD) and 3-ketoacyl-CoA thiolase (KAT) activities were assayed using acetoacetyl-CoA. Lipase activity was measured in purified oil body membranes using 10 mM [¹⁴C]triolein as a substrate. Values are the mean ± SE of measurements on three separate extracts. *Activity significantly different from the wild type ($P < 0.001$). ICL, isocitrate lyase; MLS, malate synthase; CAT, catalase; nd, not determined.

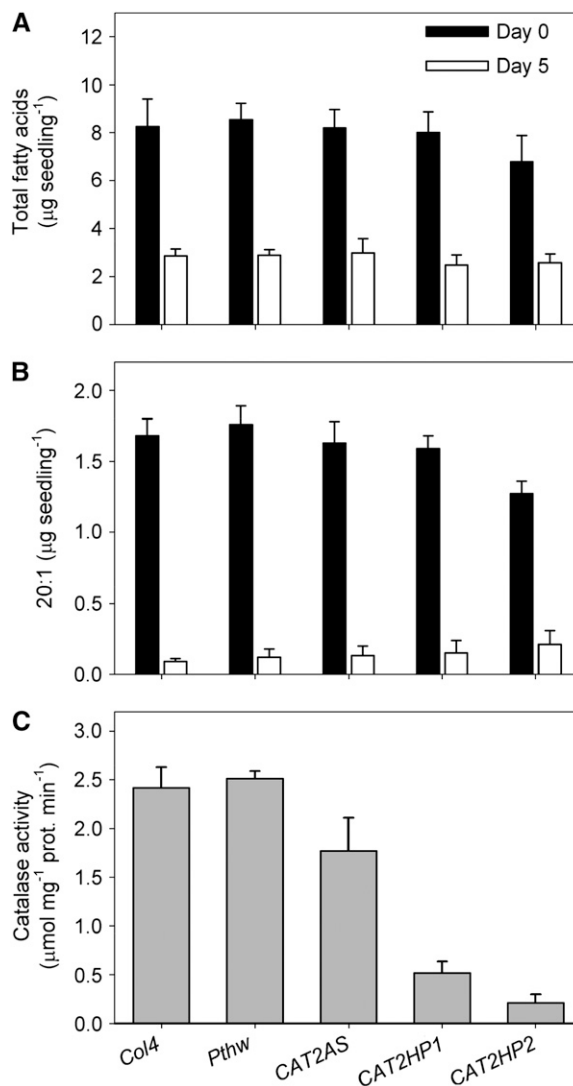
oil following germination. Surprisingly, unlike *sdp2*, none of these lines were strongly impaired in total fatty acid or eicosanoic acid breakdown when grown on medium containing sucrose (Figure 8). Enzyme assays confirmed that the lines had reduced catalase activity following germination, with CAT2HP2 exhibiting the lowest level (only 10% of the wild type; Figure 8C). Further analysis of CAT2HP2 seedlings revealed that the activities of specific peroxisomal enzymes (isocitrate lyase and malate synthase) are significantly reduced in comparison with the wild type, while oil body membrane lipase activity is not affected (Table 1). This indicates that a 90% reduction in catalase activity does impact negatively on peroxisome function, albeit insufficiently to prevent fatty acid β -oxidation from occurring when seedlings are provided with sucrose.

DISCUSSION

Plant peroxisomes contain two major enzymatic antioxidant mechanisms capable of removing the highly toxic H₂O₂ that is generated as a by-product of oxidative metabolism (Mullen and Trelease, 1996). The first mechanism exploits catalase in the matrix and the second uses a membrane-bound ascorbate-dependent electron transfer system, requiring the cooperative action of APX and MDAR. Although the importance of catalase is well established, the physiological requirement for a component of the APX/MDAR system has yet to be demonstrated. In this study, I

provide genetic evidence to show that a component of this system plays an essential role during the life cycle of *Arabidopsis*.

The seeds of many plants contain oil that serves as an essential source of carbon to drive postgerminative growth and allow photosynthetic establishment (Hayashi et al., 1998). The breakdown of this oil is accompanied by the generation of massive amounts of H₂O₂ within the peroxisome because H₂O₂ is formed as a by-product of the acyl-CoA oxidase step of fatty

**Figure 8.** Effect of Catalase Deficiency on Fatty Acid Breakdown during Postgerminative Growth.

(A) and (B) Total fatty acid (A) and eicosanoic acid (20:1) (B) content of seeds and 5-d-old seedlings grown on medium with 1% (w/v) sucrose. Col4, the wild type; Pthw, empty vector control; CAT2AS, CAT2HP1, and CAT2HP2, catalase-deficient lines (Vandenabeele et al., 2004). Values are the mean ± SE of measurements on five batches of 20 seeds or seedlings.

(C) Catalase activities in extracts from 2-d-old seedlings of Col4, Pthw, CAT2AS, CAT2HP1, and CAT2HP2. Values are the mean ± SE of measurements on three separate extracts.

acid β -oxidation (Graham and Eastmond, 2002). To identify genes that are required for storage oil breakdown in *Arabidopsis*, I previously used a genetic screen in which mutants that require sucrose to support postgerminative growth were isolated (Eastmond, 2006). Three of these *sdp* mutants (*sdp1*, *sdp2*, and *sdp3*) were found to lack the lipase activity that is associated with their oil body membranes, explaining why they are unable to breakdown storage oil. *SDP1* encodes a TAG lipase (Eastmond, 2006). Here, I show that *SDP2* is the peroxisomal membrane isoform of MDAR (MDAR4), which constitutes one of the components of the ascorbate-dependent electron transfer system (Lisenbee et al., 2005).

It has previously been reported that during periods of rapid β -oxidation, mammalian peroxisomes can leak H_2O_2 (Mueller et al., 2002). The most plausible explanation for the phenotype of *sdp2* is that without the APX/MDAR system some of the H_2O_2 produced by acyl-CoA oxidase following seed germination escapes from the peroxisome and causes oxidative damage to oil bodies, inactivating *SDP1* (Figure 9). Substantial data were obtained to support this hypothesis. Analysis of *sdp2* seedlings immediately following germination confirmed that they have elevated levels of H_2O_2 and that their oil body proteins and lipids become oxidized. Furthermore, when *sdp2* was introduced into a *pxa1* background, which cannot β -oxidize fatty acids (Zolman et al., 2001), H_2O_2 levels, oxidative damage to oil bodies, and loss of lipase activity were all suppressed. The activity of *SDP1* can be inhibited by H_2O_2 in vitro. Finally, oxidized *SDP1* can be detected in oil bodies from *sdp2* seedlings but not from the wild type. Inactivation of *SDP1* is sufficient to account for much of the sugar-dependent phenotype of *sdp2* (Eastmond, 2006). However, it cannot be discounted that additional proteins, which are necessary for oil hydrolysis, might also be damaged. Proteomic analysis by Job et al. (2005) has established that in wild-type

Arabidopsis seeds and seedlings, proteins from many subcellular compartments exhibit a significant level of oxidation. However, no oxidized oil body proteins were identified in their study.

Unlike oil bodies, peroxisomes do not appear to depend so greatly on the APX/MDAR system for protection against H_2O_2 . Seedlings of *sdp2* are able to β -oxidize 2,4-DB. This capability implies that numerous proteins in the peroxisomal membrane and matrix retain at least part of their normal capacity (Baker et al., 2006). The activities of seven peroxisomal marker enzymes are also not adversely affected in *sdp2*. It is possible that a defect in the APX/MDAR system is not severely detrimental to peroxisomes because catalase remains active within the matrix. However, I cannot exclude the possibility that peroxisomes in *sdp2* are damaged. Analysis of catalase antisense lines (Vandenabeele et al., 2004) showed that following *Arabidopsis* seed germination, certain peroxisomal enzyme activities are reduced, while lipase activity on oil body membranes is unaffected. In particular, the activity of the key glyoxylate cycle enzyme isocitrate lyase (Eastmond et al., 2000a) is reduced by 80% in the strongest catalase antisense line. This observation is consistent with recent in vitro data, which show that isocitrate lyase from castor bean (*Ricinus communis*) endosperm is readily inactivated by H_2O_2 and that this enzyme physically associates with catalase in the peroxisome (Nguyen and Donaldson, 2005; Yanik and Donaldson, 2005).

A number of studies have previously presented electron micrographic evidence to suggest that associations exist in oil-seed cells between oil bodies and other subcellular compartments, principally peroxisomes (e.g., Wanner and Theimer, 1978; Hayashi et al., 2001). Interestingly, Chapman and Trelease (1991) have also provided biochemical evidence that in cotton (*Gossypium hirsutum*) seedlings, neutral lipids are transferred directly from the oil body into the peroxisomal membrane. It is therefore possible that physical contact plays a role in storage oil breakdown in oilseeds by facilitating the transfer of fatty acids from oil bodies to peroxisomes so that they can be β -oxidized. Using confocal microscopy, I was able to show that peroxisomes and oil bodies do cluster together in the cotyledon cells of living *sdp2* seedlings and that this association persists as long as the oil bodies remain undegraded. It is not known whether peroxisome/oil body associations are influenced by H_2O_2 production, but it is noteworthy that H_2O_2 and UV-B light treatments have previously been reported to cause peroxisomes to cluster together with one another in animal and plant cells (Lingard and Trelease, 2006).

An association between oil bodies and peroxisomes would help explain why the deficiency in MDAR4 is so detrimental to storage oil hydrolysis since it would result in a close proximity between *SDP1* and acyl-CoA oxidases, which generate H_2O_2 . In yeast, a close association between oil bodies and peroxisomes has also recently been reported, and evidence has been provided that proteins involved in β -oxidation (e.g., acyl-CoA oxidase) are localized close to the inner surface of the peroxisomal membrane at sites of contact with the oil body (Binns et al., 2006). The concentration of H_2O_2 required to inhibit *SDP1* in vitro (~ 0.5 mM) is 16-fold higher than the estimated cytosolic concentration in 1-d-old *sdp2* seedlings (~ 0.03 mM). This concentration was calculated using the data from Figure 6A, assuming that the cytosol constitutes $\sim 20\%$ of the seedling volume. However, H_2O_2 concentrations are unlikely to be uniform

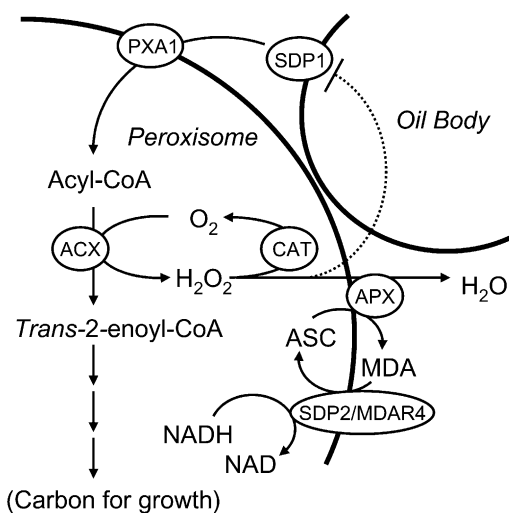


Figure 9. Diagram Illustrating the Proposed Role of MDAR4 in Storage Oil Breakdown in Germinating *Arabidopsis* Seeds.

ASC, ascorbate; MDA, monodehydroascorbate; *SDP1*, lipase; *PXA1*, ABC transporter; *ACX*, acyl-CoA oxidase; *CAT*, catalase.

and could be heightened at, or near, the peroxisomal membrane, particularly if acyl-CoA oxidases are situated there.

The *sdp2* mutant is defective in the second enzyme in the APX/MDAR system (MDAR4). Metabolite measurements suggest that in *sdp2*, the monodehydroascorbate produced at the peroxisomal membrane cannot be recycled efficiently, causing the availability of ascorbate to diminish and the APX/MDAR system to collapse. There are several alternative ways in which ascorbate could still be replenished for APX in the absence of MDAR4. In addition to MDAR4, *Arabidopsis* peroxisomes also contain the soluble matrix isoform MDAR1 (Lisenbee et al., 2005). Monodehydroascorbate can also disproportionate to ascorbate and dehydroascorbate, and biochemical studies have suggested that glutathione-dependent dehydroascorbate reductase activity is present in plant peroxisomes and therefore could convert dehydroascorbate back to ascorbate (Jiménez et al., 1997; del Rio et al., 1998). Finally, it has been argued that ascorbate and monodehydroascorbate are likely to be shuttled across the peroxisomal membrane since the catalytic site of MDAR4 is situated on the matrix side of the peroxisomal membrane, while the catalytic site of APX3 is on the cytosolic side (Lisenbee et al., 2005). Despite these possibilities, the phenotype of *sdp2* suggests that none can complement the function of MDAR4 in protecting oil bodies against β -oxidation-dependent oxidative damage.

The predominant APX isoform from the peroxisomal membranes of *Arabidopsis* is APX3 (At4g35000). Surprisingly, Narendra et al. (2006) have recently reported that the *APX3* gene is dispensable for growth and development. Therefore, a deficiency in APX activity might not give rise to the same phenotype as *sdp2*. Redundancy cannot be dismissed as an explanation for the apparent disparity, considering that there are nine APX genes in the *Arabidopsis* genome (Lisenbee et al., 2003). Specifically, there is a homolog of APX3 (*APX5*; At4g35970) with sequence similarity throughout the polypeptide sequence, including the C-terminal transmembrane domain and targeting motif. However, the level of expression of *APX5* is very low relative to *APX3* (Narendra et al., 2006).

My data do not preclude the possibility that MDAR4 functions independently of APX. Sattler et al. (2004) have recently shown that a deficiency in the lipid-soluble antioxidant tocopherols (vitamin E) also leads to lipid peroxidation after the germination of *Arabidopsis* seeds and that this adversely affects storage oil breakdown and seedling growth. Furthermore, sucrose can partially relieve the postgerminative growth defect. Tocopherols can scavenge lipid peroxy radicals, yielding a tocopheroxyl radical that could be recycled by reacting with ascorbate to produce monodehydroascorbate (Liebler, 1993). It is therefore possible that MDAR4 functions in a one-electron redox cycle that regenerates tocopherol from the tocopheroxyl radical at the peroxisomal membrane. However, it is unlikely that MDAR4 can operate solely by this mechanism since the *sdp2* mutant has a more severe postgerminative growth arrest phenotype than the tocopherol-deficient *vte2* mutant under normal growth conditions (Sattler et al., 2004). It is also possible that ascorbate could scavenge reactive oxygen species directly or that MDAR4 might be capable of recycling the oxidation products of other powerful antioxidants, such as phenolics (Sakihama et al., 2000). Indeed MDAR is unique in that it is the only enzyme known to use organic

radicals as substrates (Hossain et al., 1984). Apart from its enzyme activity, MDAR4 might also act as an anchor for proteins associated with the peroxisomal inner membrane that lack a transmembrane domain, such as the long-chain acyl-CoA synthases LACS6 and LACS7 (Fulda et al., 2004).

Peroxisomal MDAR has also been implicated in fatty acid catabolism through the provision of NAD^+ cofactor for L-3-hydroxyacyl-CoA dehydrogenase and malate dehydrogenase (Bowditch and Donaldson, 1990; Mullen and Trelease, 1996). These enzymes are required for β -oxidation and glyoxylate cycle function, respectively; theoretically, if H_2O_2 was detoxified entirely by APX/MDAR, the system could recycle sufficient NAD^+ for both pathways (Mullen and Trelease, 1996). However, Mettler and Beevers (1980) have proposed an alternative scheme in which peroxisomal malate dehydrogenase operates in reverse to supply NAD^+ for L-3-hydroxyacyl-CoA dehydrogenase via a metabolite shuttle, coupled to the mitochondrial electron transport chain. Malate dehydrogenase has since been shown to perform this role in yeast (van Roermund et al., 1995). Analysis of *sdp2* suggests that MDAR4 is not essential for providing NAD^+ in *Arabidopsis* peroxisomes since the mutant can β -oxidize 2,4-DB. By contrast, Pracharoenwattana et al. (2007) have shown that the glyoxylate cycle can still function in *Arabidopsis pmdh1 pmdh2* mutant seedlings, which lack peroxisomal malate dehydrogenase activity, but that this double mutant is impaired in both the β -oxidation of 2,4-DB and oil breakdown. These data provide evidence to suggest that the metabolite shuttle proposed by Mettler and Beevers (1980) is necessary to supply NAD^+ for fatty acid β -oxidation (Pracharoenwattana et al., 2007). Within the peroxisome, MDAR4 (and MDAR1) activity must regenerate some NAD^+ . However, it is likely that a proportion of the H_2O_2 generated by acyl-CoA oxidase is detoxified by catalase, and in this event, the regeneration of NAD^+ by the APX/MDAR system alone could not sustain both L-3-hydroxyacyl-CoA dehydrogenase and forward malate dehydrogenase activities. The result would be a negative feedback loop, which could stall β -oxidation. The reversible activity of peroxisomal malate dehydrogenase might be essential for β -oxidation, not because it supplies all the NAD^+ but because it can balance the shortfall.

In addition to fatty acid β -oxidation, the photorespiratory pathway also generates large quantities of H_2O_2 in plant peroxisomes as a result of the activity of glycolate oxidase (Willekens et al., 1997). Catalase has been shown to play a major role in detoxifying this H_2O_2 . Antisense suppression of catalase results in oxidative damage and triggers cell death in tobacco (*Nicotiana tabacum*) and *Arabidopsis* plants that are subjected to high light treatment (Willekens et al., 1997; Vandenabeele et al., 2004). Although public microarray data show that *MDAR4* is expressed in leaves, *sdp2* plants do not exhibit more obvious phenotypic symptoms of oxidative damage than the wild type when they are subjected to high light (data not shown). Further studies will be required to determine if MDAR4 plays a role associated with photorespiration. Reactive oxygen species also play important signaling roles in plants (Hancock et al., 2005), and it is conceivable that inhibition of SDP1 activity might be a significant mechanism in the regulation of oil hydrolysis during postgerminative growth.

In conclusion, catalase and the APX/MDAR system are both important parts of the peroxisomal antioxidant machinery during

the postgerminative growth of *Arabidopsis* seedlings. Our data suggest that their roles are physiologically different and that neither can fully compensate for the loss of the other. Catalase protects constituents of the peroxisomal matrix from oxidative damage, while the main role of MDAR4 appears to be to prevent H₂O₂ from escaping beyond the outer surface of the peroxisomal membrane. The consequences of H₂O₂ escape appear to be fatal primarily because inactivation of TAG hydrolysis on closely associated oil bodies prevents the seedling from releasing the carbon skeletons and energy that it needs for initial postgerminative growth.

METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* (ecotypes Columbia-0 and Landsberg *erecta*) were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). The *sdp2-4* mutant (SALK_068667) was obtained from T-DNA express (Alonso et al., 2003). The PTS1-targeted GFP line A5 (Cutler et al., 2000), the *pxa1* mutant (Zolman et al., 2001), and catalase-deficient lines (Vandenabeele et al., 2004) were kindly provided by Chris Somerville (Carnegie Institution, Stanford University, CA), Bonnie Bartel (Rice University, Houston, TX), and Frank Van Breusegem (Ghent University, Flanders Interuniversity Institute for Biotechnology, Belgium), respectively. Seeds were surface-sterilized, applied to agar plates containing half-strength Murashige and Skoog salts (Sigma-Aldrich), and imbibed in the dark for 4 d at 4°C. The plates were then transferred to a growth chamber set to 21°C (16 h light/8 h dark; PPFD = 150 μmol m⁻² s⁻¹). In some experiments, 1% (w/v) sucrose, 2,4-DB, or 2,4-D were added to the agar medium.

Metabolite Analysis

The fatty acid composition of seed and seedling lipids was measured by gas chromatography analysis after combined digestion and fatty acid methyl ester formation from frozen tissue using the method of Browse et al. (1986). H₂O₂ and ascorbate (ascorbate and dehydroascorbate) were extracted from 0.1 g fresh weight of seedlings and measured spectrophotometrically using the methods described by Huang et al. (2005).

Mapping

The *sdp2-1* mutant was outcrossed to the wild-type ecotype Landsberg *erecta*. F1 plants were allowed to self-fertilize, and the F2 progeny were screened for the sugar-dependent phenotype. Genomic DNA was isolated from 190 F2 *sdp2-1* lines using the Extract-N-Amp Plant PCR kit (Sigma-Aldrich). Mapping was performed using simple sequence polymorphisms (Bell and Ecker, 1994), cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993), and single nucleotide amplified polymorphisms (Drenkard et al., 2000) using information from the Monsanto *Arabidopsis* polymorphism collection (Jander et al., 2002). New markers used in this study are listed in Supplemental Table 2 online. Candidate genes within the mapping interval were amplified from *sdp2-1*, *sdp2-2*, and *sdp2-3* genomic DNA by PCR and sequenced to identify mutations.

Transcript and Protein Analysis

DNase-treated total RNA was isolated from *Arabidopsis* seedlings using the RNeasy kit from Qiagen. The synthesis of single-stranded cDNA was performed using SuperScript II RNase H⁻ reverse transcriptase from Invitrogen. *SDP2* transcripts were detected by PCR using primers SDP2F (5'-CAAAGACGGGAGCCACTTAC-3') and SDP2R (5'-CTGCTGACTCA-

CAACCGTGT-3'). Measurement of total protein levels, SDS-PAGE, and protein gel blot analysis were performed as described previously (Eastmond, 2004). Rabbit anti-cucumber MDAR antiserum, anti-DNP antibody (Millipore), and anti-SDP1 antibody were used as primary antibodies at dilutions of 1 in 1000, 1 in 30, and 1 in 500, respectively. The SDP1 antibody was raised against the SDP1-specific peptide SSEDGLQEPVSGSVC by Eurogentec and affinity purified.

Microscopy

Transmission electron microscopy was performed as described previously (Eastmond, 2006). Five-day-old *Arabidopsis* seedlings were fixed for 2 h in 2.5% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 100 mM phosphate buffer, pH 7.0, with a secondary fixation of 1% (w/v) osmium tetroxide in phosphate buffer for 1 h. The tissue was embedded in Spurr's resin, sectioned, and stained with uranyl acetate and Reynolds lead citrate. Confocal microscopy was performed using a Zeiss LSM 510 Meta on an Axioplan 2M, fitted with a ×63 PlanApo lens (numerical aperture of 1.4). The sample was excited with a 488-nm argon laser and GFP emission collected via a 505- to 530-nm band-pass filter. Bright-field images were captured simultaneously with the transmission detector.

Organelle Purification, Protein Purification, and Measurement of Oxidative Damage

Oil bodies and oil body membranes were purified from 2-d-old *Arabidopsis* seedlings, and recombinant N-terminal His₆-tagged SDP1 was expressed and purified using protocols that were described previously (Eastmond, 2006). Peroxisomal fractions were obtained from homogenates of 5-d-old etiolated *Arabidopsis* seedlings using sucrose density gradient centrifugation as described previously (Eastmond et al., 2000b). The levels of LOOHs in purified oil body lipids were estimated using the ferrous oxidation xylene orange assay following the protocol described by Sattler et al. (2004). The only modification to the method was that the lipids were extracted from oil bodies in 1.5-mL tubes without homogenization. The levels of protein carbonyls in oil body membranes and purified recombinant SDP1 were determined using the spectrophotometric quantification method described by Nguyen and Donaldson (2005). SDP1 was immunoprecipitated from oil body membranes using the IP50 Protein G Immunoprecipitation kit (Sigma-Aldrich), and carbonyl groups were detected using the OxyBlot protein oxidation detection kit (Millipore) as described by Nguyen and Donaldson (2005).

Enzyme Assays

TAG lipase activity was measured in purified oil body membranes and recombinant SDP1 using an emulsion of [¹⁴C]triolein as described previously (Eastmond, 2006). Purified oil bodies were also used as a substrate for recombinant SDP1 using the assay procedure described by Eastmond (2006). For assays of peroxisomal enzymes, 2-d-old seedlings grown on medium containing 1% (w/v) sucrose were ground in a pestle and mortar with 1 mL of buffer (150 mM Tris/HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 10 mM flavin adenine dinucleotide, and 10% [v/v] glycerol). The extract was clarified by centrifugation at 15,000g for 30 min at 4°C, and the supernatant was desalted using a Sephadex G-50 spin column. The extract was assayed spectrophotometrically for acyl-CoA oxidase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, isocitrate lyase, malate synthase, catalase, and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase activities according to methods described previously (Takahashi et al., 1997; Eastmond et al., 2000a; Hancock et al., 2005; Rylott et al., 2006).

Complementation of *sdp2-4*

A region of genomic DNA containing *MDAR4* was amplified from *Arabidopsis* using primers 5'-ATCTCATGATTGAGTGGGTGATTGGTTG-3' and

5'-AGCTTCTTCGAGGGTTAGGGATGAGATT-3', and the product was cloned into the pCR2.1-TOPO vector from Invitrogen. Using standard molecular biology techniques, *MDAR4* was excised and cloned into the pGREENII vector (Hellens et al., 2000). The *MDAR4* construct was transformed into *Agrobacterium tumefaciens* strain GV3101 containing the pSOUP vector (Hellens et al., 2000) by electroporation and into *Arabidopsis sdp2-4* ecotype Columbia by the floral dip method (Clough and Bent, 1998). Transformants containing the T-DNA were selected by screening for loss of a sugar-dependent phenotype, and the presence of wild-type *MDAR4* transcripts was confirmed by RT-PCR.

Accession Number

Sequence data for *SDP2* (*MDAR4*) cDNA can be found in the GenBank/EMBL data libraries under accession number AY039980.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Frequency of *sdp2* Germination.

Supplemental Table 2. New SSLP and CAPS Markers Used to Map *SDP2*.

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