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SUGAR-DEPENDENT1 Encodes a Patatin Domain Triacylglycerol Lipase That Initiates Storage Oil Breakdown in Germinating *Arabidopsis* Seeds [W]

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Triacylglycerol hydrolysis (lipolysis) plays a pivotal role in the life cycle of many plants by providing the carbon skeletons and energy that drive postgerminative growth. Despite the physiological importance of this process, the molecular mechanism is unknown. Here, a genetic screen has been used to identify *Arabidopsis thaliana* mutants that exhibit a postgerminative growth arrest phenotype, which can be rescued by providing sugar. Seventeen *sugar-dependent* (*sdp*) mutants were isolated, and six represent new loci. Triacylglycerol hydrolase assays showed that *sdp1*, *sdp2*, and *sdp3* seedlings are deficient specifically in the lipase activity that is associated with purified oil bodies. Map-based cloning of *SDP1* revealed that it encodes a protein with a patatin-like acyl-hydrolase domain. SDP1 shares this domain with yeast triacylglycerol lipase 3 and human adipose triglyceride lipase. In vitro assays confirmed that recombinant SDP1 hydrolyzes triacylglycerols and diacylglycerols but not monoacylglycerols, phospholipids, galactolipids, or cholesterol esters. *SDP1* is expressed predominantly in developing seeds, and a SDP1–green fluorescent protein fusion was shown to associate with the oil body surface in vivo. These data shed light on the mechanism of lipolysis in plants and establish that a central component is evolutionarily conserved among eukaryotes.

INTRODUCTION

Seed germination is followed by a phase of rapid growth as the seedling strives to establish a root system and achieve photosynthetic competence. This growth is fuelled by the mobilization of storage reserves that were laid down during seed maturation (Bewley and Black, 1994). Oil is arguably the most common storage compound in seeds (Levin, 1974), and it is sequestered in oil bodies surrounded by a phospholipid monolayer (Huang, 1992; Murphy, 1993). The initial step in oil breakdown is catalyzed by lipase (EC: 3.1.1.3), which hydrolyzes triacylglycerol (TAG) at the oil/water interface to yield free fatty acids and glycerol. The free fatty acids are then transferred to the glyoxysome and activated to acyl-CoAs for subsequent catabolism by β-oxidation. Most of the acetyl-CoA produced is ultimately converted to sugars by the glyoxylate cycle and gluconeogenesis. These latter pathways have been well documented in plants, and many of the genes concerned have been cloned and characterized (Cornah and Smith, 2002; Graham and Eastmond, 2002; Baker et al., 2006). By contrast, remarkably little is known about the mechanism and regulation of lipolysis, in spite of the pivotal role that it plays in supporting postgerminative growth.

Lipase activities have been studied at the biochemical level in a variety of seeds (Huang, 1983; Mukherjee, 1994). In most, the activities are only detectable upon germination and increase concomitantly with the disappearance of TAG. These lipase activities are often membrane associated and can be found in the oil body, glyoxysome, or microsomal fractions of seed extracts, depending upon the species (Huang, 1983; Mukherjee, 1994). Since lipases are interfacial enzymes, those that reside on the surface of the oil body might logically be expected to play a role in TAG breakdown. However, studies using electron microscopy have indicated that oil bodies are in close proximity with other organelles (particularly glyoxysomes), and it has long been hypothesized that an association between them may be required to facilitate fatty acid release and transfer (Wanner and Theimer, 1978; Hayashi et al., 2001). Lipases have been purified from the seeds of plants such as maize (*Zea mays*) (Lin and Huang, 1984), castor (*Ricinus communis*) (Maeshima and Beevers, 1985; Fuchs et al., 1996), oilseed rape (*Brassica napus*) (Fuchs and Hansen, 1994), and ironweed (*Vernonia galamensis*) (Ncube et al., 1995). More recently, several genes have been cloned from plants that encode proteins with TAG lipase activity (Eastmond, 2004; Matsui et al., 2004; El-Kouhen et al., 2005). However, to date, no lipase gene has been proven to play a physiological role in storage oil hydrolysis in germinating seeds.

In *Arabidopsis thaliana*, oil constitutes 35 to 40% of the seed fresh weight. The disruption of several genes required for fatty acid catabolism has been shown to block TAG use during *Arabidopsis* germination (Hayashi et al., 1998; Baker et al., 2006). The development of these mutants is arrested either prior to germination or early in postgerminative growth, demonstrating that oil mobilization is critical for the completion of this stage of the plant's life cycle. Simply providing an alternative source of carbon, such as sucrose, can rescue their postgerminative growth (Hayashi et al., 1998; Baker et al., 2006). Here, this sugar-dependent phenotype has been used in a forward genetic screen to identify new *Arabidopsis* mutants that cannot mobilize

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their storage oil. Positional cloning of *sugar-dependent1* (*sdp1*) has revealed the molecular identity of an oil body–associated TAG lipase that is responsible for catalyzing the initial step in storage oil mobilization in *Arabidopsis* seeds.

RESULTS

Isolation of *sdp* Mutants

Arabidopsis seeds rely on their stored oil reserves to support postgerminative growth until they achieve photosynthetic competence. Mutants that fail to deposit storage oil during seed maturation, or are unable to mobilize it upon germination, exhibit arrested seedling growth (Focks and Benning, 1998; Hayashi et al., 1998). Providing an alternative source of carbon, such as sucrose, can rescue the phenotype. Here, this selection strategy has been used in a forward genetic screen to isolate a collection of *sdp Arabidopsis* mutants. Approximately 240,000 ethyl methanesulfonate (EMS)–mutagenized M2 ecotype Columbia-0 (Col-0) seeds were screened according to the procedure described in Methods. Lines that inherited an *sdp* phenotype, but did not exhibit obvious defects in seed filling (Focks and Benning, 1998), were selected for further analysis in this study. The lines are all recessive and fall into 17 complementation groups. Six of the groups represent new loci (*sdp1* to *6*). The remaining lines are allelic to previously characterized mutants. Table 1 contains a summary of the *sdp* mutant collection.

Three *sdp* Mutants Are Deficient in Oil Body Lipase Activity

To investigate whether any of the *sdp* mutants are deficient in TAG lipase activity, enzyme assays were performed on extracts from germinating seeds, using emulsified triolein as a substrate (Hills and Murphy, 1988). Control experiments performed on wild-type *Arabidopsis* seeds show that the rate of triolein hydrolysis increases >10-fold after germination (Figure 1A) and that a proportion of this activity (10 to 20% on a per seedling basis) is associated with purified oil body membranes (Figure 1B). Similar results have also been reported by El-Kouhen et al. (2005). When extracts from the *sdp* mutants were assayed, it was discovered that *sdp1*, *sdp2*, and *sdp3* are defective specifically in oil body membrane lipase activity (Figure 1B). These data suggest that SDP1, 2, and 3 are likely to play an important role in TAG hydrolysis. In this study, SDP1 has been cloned and characterized.

sdp1 Is Impaired in Storage Oil Breakdown during Postgerminative Growth

The *sdp1* mutant is not significantly impaired in germination (see Supplemental Table 1 online). However, *sdp1* exhibits a much slower rate of postgerminative growth than the wild type, and this retarded growth is rescued by the provision of 1% (w/v) sucrose (Figure 2A). Once *sdp1* seedlings develop photosynthetic activity, they grow normally and are indistinguishable from the wild type throughout the rest of their life cycle. Although 5-d-old *sdp1-1* seedlings grown on sucrose appear morphologically normal, both light and electron microscopy revealed that the cells retain clusters of oil bodies (Figures 2B and 2C). By contrast, essentially no oil bodies remain in wild-type cotyledon cells by this stage of development (Figures 2B and 2C).

Measurement of the TAG content of *sdp1* seeds germinated in the presence of sucrose confirmed that the mutant is severely impaired in its ability to breakdown TAG. The level of TAG declined by <20% over the course of the first 5 d of postgerminative growth in *sdp1-1*, while in wild-type seedlings, it fell by 98% (Figure 3A). Similar results were also obtained for the other *sdp1* alleles (data not shown). In germinating seedlings, the fatty acids that are released from the oil body are activated to acyl-CoAs before they are catabolized by peroxisomal fatty acid β-oxidation (Fulda et al., 2004). Measurements performed on 2-d-old *sdp1-1* seedlings show that levels of long-chain acyl-CoAs are significantly reduced relative to the wild type (Figure 3B). Together, these data support the hypothesis that *sdp1* is specifically impaired in storage oil hydrolysis. Enzyme assays

Table 1. *Arabidopsis sdp* Mutants

Mutant	Alleles	Identity	Reference
sdp1	5	Patatin-like TAG lipase	This study
sdp2	11	—	
sdp3	2	—	
sdp4	3	—	
sdp5	2	—	
sdp6	8	—	
ped1/kat2	4	3-Ketoacyl-CoA thiolase	Hayashi et al. (1998)
ped2/pex14	2	Peroxin 14	Hayashi et al. (2000)
pex5	2	Peroxin 5	Zolman et al. (2000)
icl	22	Isocitrate lyase	Eastmond et al. (2000)
chy1	2	β-Hydroxyisobutyryl-CoA hydrolase	Zolman et al. (2001a)
pxa1/ped3/cts	3	Peroxisomal ABC transporter	Zolman et al. (2001b)
mls	4	Malate synthase	Cornah et al. (2004)
pck1	9	Phosphoenolpyruvate carboxykinase	Penfield et al. (2004)
pex6	1	Peroxin 6	Zolman and Bartel (2004)
mfp2	5	Multifunctional protein	Rylott et al. (2006)
pex4	1	Peroxin 4	Zolman et al. (2005)

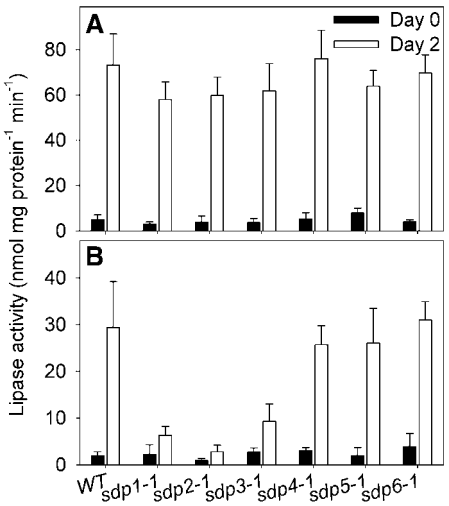

Figure 1. TAG Lipase Activity in Germinating *sdp* Seeds.

Crude cell extracts **(A)** and purified oil body membranes **(B)** were prepared from imbibed seeds and 2-d-old seedlings of *sdp* mutants and the wild type, and their capacity to hydrolyze [^{14}C]triolein was measured. Seedlings were grown on agar plates containing half-strength Murashige and Skoog (MS) salts plus 1% (w/v) sucrose. The values are the mean ± SE of measurements on four separate preparations.

performed on extracts from germinating *sdp1-1* seedlings also suggest that despite the reduced availability of substrate in vivo, the enzymic capacity of fatty acid β-oxidation, the glyoxylate cycle, and gluconeogenesis are not reduced (see Supplemental Table 2 online).

SDP1 Encodes At5g04040

High-resolution mapping was used to locate the *SDP1* locus. The *sdp1-1* mutant was outcrossed to wild-type ecotype Landsberg *erecta* and 768 *sdp1-1* lines selected from the F2 progeny. PCR-based polymorphic markers were then used to localize *SDP1* to an ~12-kb region on the north arm of chromosome 5, situated between markers CTR1 and ciw15 (Figure 4A). This region contains four genes. The genomic sequence of each candidate was sequenced from three (of the five) EMS *sdp1* alleles, and point mutations were found in the coding region of At5g04040 (Figure 4B). In *sdp1-1*, *sdp1-2*, and *sdp1-3* plants, point mutations lead to single amino acid substitutions in the protein (Glu^{438} to Lys, Pro^{456} to Ser, and Gly^{241} to Asp). Two null alleles of *sdp1* were also obtained from T-DNA Express (Alonso et al., 2003). The *sdp1-4* (SALK_102887) and *sdp1-5* (SALK_076697) alleles contain single T-DNAs inserted in the first exon of At5g04040 (Figure 4B), and both exhibit the same phenotype as the EMS alleles (Figure 2A).

SDP1 Is a Patatin-Like Protein

A full-length cDNA encoding At5g04040 (GenBank accession number AY136470) has previously been isolated by the SSP Consortium (Yamada et al., 2003). The SDP1 polypeptide is 825 amino acid residues long and has a calculated molecular mass of 92 kD. Querying the National Center for Biotechnology Information (NCBI) Conserved Domain Database (Marchler-Bauer et al., 2005) revealed that the sequence contains a predicted patatin-like domain (Pfam01734) between amino acid residues 243 and 390. Patatin is an abundant storage protein from potato (*Solanum tuberosum*) tubers that is known to exhibit lipolytic activity (Shewry, 2003). Patatin is classified as a phospholipase type A_2 and forms the prototype for a calcium-independent phospholipase A_2 (iPLA$_2$) subfamily with many members (Andrews et al., 1988). The patatin-like domain of SDP1 has a conserved Ser esterase motif (GXSXG) (Figure 5A) but lacks the nucleotide (GXGXXG) and Asp active site (DX[G/A]) signatures that are generally considered to be characteristic of the iPLA$_2$ class (Jenkins et al., 2004).

Although patatin can hydrolyze a variety of substrates in vitro, it is not active against TAG (Andrews et al., 1988; Anderson et al., 2002). However, patatin-like proteins from yeast and animals have recently been discovered, which have bona fide TAG lipase activity. Triacylglycerol lipase 3 (TGL3), TGL4, and TGL5 are involved in oil breakdown in *Saccharomyces cerevisiae* (Athenstaedt and Daum, 2003, 2005), and adipose triglyceride lipase (ATGL/desnutrin/iPLA$_2$-ζ) and Brummer lipase are necessary for fat hydrolysis in humans and *Drosophila melanogaster*, respectively (R. Zimmermann et al., 2004; Gronke et al., 2005). Interestingly, beyond the patatin-like domain, very little sequence similarity exists between the yeast and animal lipases. *Arabidopsis* SDP1 is most closely related to the family of three yeast lipases (Figures 5B and 5C), although the overall level of amino acid sequence similarity is relatively small (<30%).

Including SDP1, there are 12 proteins with a predicted patatin-like (Pfam01734) domain in *Arabidopsis*. Of these proteins, only At3g57140 (SDP1-like) shares significant sequence similarity with SDP1 (Figure 5C) (~74% amino acid identity). Interestingly, *Arabidopsis* also contains a single patatin-like protein (At1g33270) that shares ~28% amino acid similarity with ATGL and Brummer (Figure 5C). A BLAST search of the publicly available sequence databases showed that SDP1 homologues are distributed widely in plants. For example, the monocot *Oryza sativa*, the legume *Medicago truncatula*, and the moss *Physcomitrella patens* all contain proteins that share >60% amino acid identity with SDP1.

SDP1 Hydrolyzes TAG

To investigate whether SDP1 has TAG lipase activity, a His$_6$-tagged recombinant version of the protein (rSDP1) was expressed in *S. cerevisiae* under the control of the *GAL1* promoter and purified using nickel-chelating resin (Figure 6A). In vitro assays showed that rSDP1 can hydrolyze [^{14}C]triolein readily, with a specific activity of ~40 μmol mg^{-1} protein min^{-1} under the conditions used in this study (Figure 6B). The rate of hydrolysis was proportional to the amount of protein (data not shown) and also to the amount of emulsified substrate, up to ~9 mg mL^{-1} (equivalent to ~10 mM) (Figure 6C). When the pH of the assay medium was varied, rSDP1 was found to have a broad optimum around pH 8.0 (Figure 5D).

To further characterize rSDP1, its activity was tested against a variety of substrates (Table 2). rSDP1 could hydrolyze TAGs

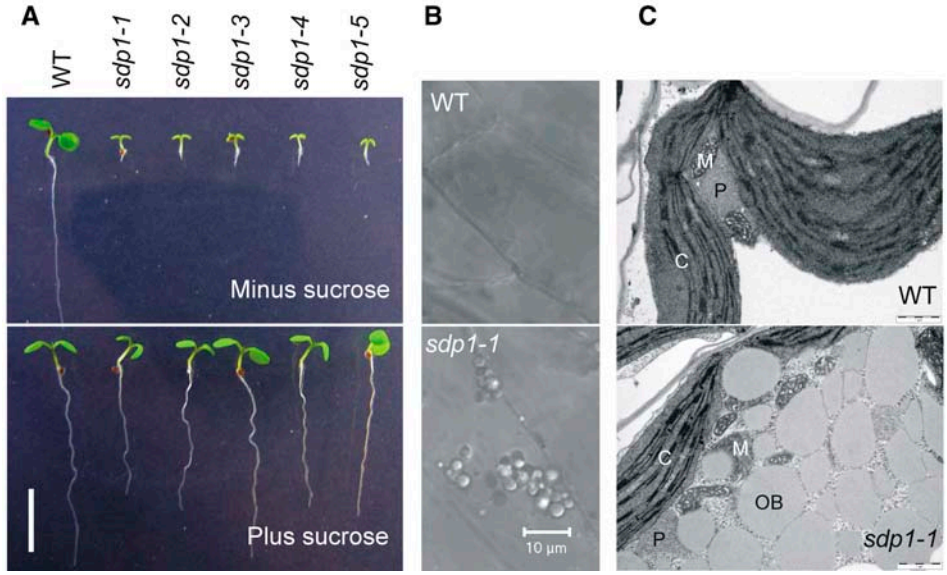

Figure 2. Postgerminative Growth and Cell Ultrastructure in *sdp1*.

(A) Five-day-old *sdp1* and wild-type seedlings grown in the light on agar plates containing half-strength MS salts plus or minus 1% (w/v) sucrose. Bar = 1 cm.

(B) Light microscopy of hypocotyl cells from whole 5-d-old *sdp1-1* and wild-type seedlings that were grown on agar plates containing half-strength MS plus 1% (w/v) sucrose. Bar = 10 μm.

(C) Electron microscopy images of cotyledon cells from 5-d-old *sdp1-1* and wild-type seedlings that were grown on agar plates containing half-strength MS plus 1% (w/v) sucrose. P, peroxisome; OB oil body; M, mitochondrion; C, chloroplast. Bar = 1 μm.

containing long- and medium-chain saturated fatty acids and long-chain polyunsaturated fatty acids. The enzyme was much less active on DAG [*sn*-1,2(2,3) diolein and *sn*-1,3 diolein] and could not hydrolyze MAG (*sn*-1 monoolein). The selectivity of rSDP1 for TAG was supported by analysis of the products of [^{14}C]triolein hydrolysis (Figure 6B). The major products were oleic acid and diolein. Relatively little monoolein was produced. rSDP1 was unable to hydrolyze a representative phospholipid (1,2-dioleoyl-3-phosphatidylcholine), a galactolipid (monogalactosyldiacylglycerol), a sterol ester (cholesteryl-oleate), an acyl-CoA (oleoyl-CoA), or an artificial ester (*p*-nitrophenyl palmitate) (Table 2).

The effect of known inhibitors of lipases was investigated using triolein as a substrate (Table 2). The activity of rSDP1 was sensitive to preincubation with the classical Ser esterase inhibitors diethyl-*p*-nitrophenyl phosphate and diisopropyl fluorophosphate. The mechanism-based iPLA$_2$ inhibitor [*E*]-6-[bromoethylene]-3-[1-naphthalenyl]-2*H*-tetrahydropyran-2-one (Jenkins et al., 2004) also inhibited rSDP1 activity at submicromolar concentrations. These data lend experimental evidence to suggest that SDP1 uses the same catalytic mechanism as patatin (Rydel et al., 2003).

SDP1 Is Expressed in Seeds

The expression of *SDP1* was investigated by searching the publicly available microarray database, using Genevestigator (P. Zimmermann et al., 2004). This search suggested that *SDP1* is expressed almost exclusively in late seed development, a pattern that is more normally associated with genes involved in storage reserve synthesis rather than breakdown. More detailed analysis using real-time PCR confirmed that *SDP1* transcript abundance increases at the beginning of seed maturation, when oil is actively being deposited, and remains high right up until seed desiccation is completed (Figure 7A). *SDP1* mRNA levels are also high early in seed imbibition, but they decline as the seed germinates and remain relatively low throughout postgerminative growth, when oil catabolism is most active (Figure 7A).

SDP1–Green Fluorescent Protein Associates with the Oil Body Membrane in Vivo

The fact that oil body membranes from germinating *sdp1* seeds are deficient in lipase activity strongly suggests that SDP1 is an oil body–associated protein. To investigate the subcellular location of SDP1 in vivo, an SDP1–green fluorescent protein (GFP) fusion was transiently expressed in 5-d-old *sdp1-1* seedlings, under the control of the 35S promoter. Confocal microscopy showed that in living cells, SDP1-GFP associates with the surface of oil bodies (Figure 7B). Fractionation of a seedling homogenate by centrifugation confirmed that SDP1-GFP is predominantly localized to the oil body fraction (Figure 7C), and lipase assays performed on purified oil body membranes also showed that the fusion protein is catalytically active (Figure 7C).

DISCUSSION

In germinating oilseeds, TAG lipase initiates the mobilization of oil, which ultimately provides the carbon skeletons and energy

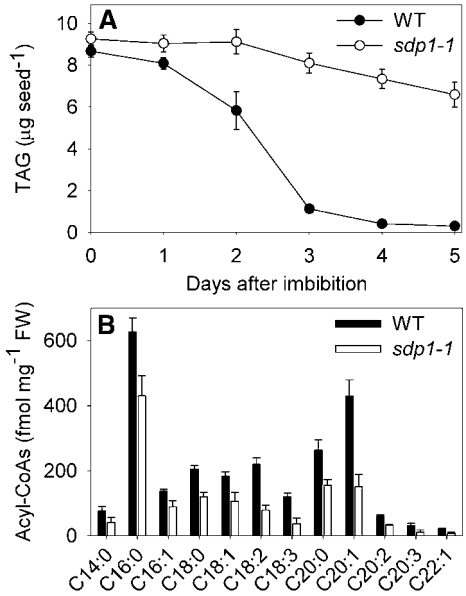

Figure 3. TAG Breakdown and Acyl-CoA Levels in *sdp1.*

(A) Total TAG content of *sdp1-1* and wild-type seeds germinated and grown in the light on agar plates containing half-strength MS salts plus 1% (w/v) sucrose. The values are the mean ± SE of measurements from eight separate batches of 20 seeds or seedlings.

(B) Acyl-CoA levels in 2-d-old *sdp1-1* and wild-type seedlings germinated and grown in the light on agar plates containing half-strength MS salts plus 1% (w/v) sucrose. The values are the mean ± SE of measurements from five separate samples, each containing ~10 mg fresh weight (FW) of seedlings.

necessary to drive postgerminative growth (Bewley and Black, 1994). In spite of the pivotal role that this enzyme plays in the life cycle of many plants, the protein (or proteins) responsible have not been identified previously. In this study, a simple genetic screen has been used to isolate a collection of *Arabidopsis* mutants that are impaired in storage oil mobilization and therefore require sugar to support early seedling growth. From this collection of *sdp* mutants, three loci were identified that are required for oil body lipase activity during germination. Positional cloning and characterization of *SDP1* revealed that it encodes the lipase that initiates oil breakdown in germinating *Arabidopsis* seeds.

SDP1 is not a member of the conventional lipase superfamily, which includes many well-characterized TAG lipases from mammals (such as gastric lipase and hormone sensitive lipase). These hydrolases are all canonical α/β-fold proteins containing a catalytic triad composed of Ser, His, and Asp or Glu (Ollis et al., 1992). Instead, SDP1 is related to the $iPLA_2$ family, of which potato patatin is the founding member (Andrews et al., 1988). Proteins from this family are also Ser esterases, but their folding topology is different and their catalytic mechanism relies on a Ser-Asp catalytic dyad (Rydel et al., 2003). Patatin cannot hydrolyze TAG, but it has recently emerged that several patatin-like proteins from yeast and animals are TAG lipases. Furthermore, genetic evidence has shown that these unorthodox patatin-like TAG lipases (PTLs) are required for oil breakdown (Athenstaedt and Daum, 2003; R. Zimmermann et al., 2004; Gronke et al., 2005). SDP1 is the first member of this family to be characterized from plants, and its discovery suggests that PTLs play a conserved role in initiating oil breakdown in all eukaryotes.

SDP1 is an oil body–associated protein, like the other PTLs that have been characterized to date (Athenstaedt and Daum, 2003, 2005; R. Zimmermann et al., 2004; Gronke et al., 2005). This location is entirely consistent with its role in initiating oil breakdown. Assuming that the specific activity of rSDP1 is similar to that of the native protein, it is possible to estimate that SDP1 constitutes <0.1% of total oil body protein. Interestingly, although many germinating oilseeds are known to possess lipase activities associated with their oil bodies, in several species, no activity has been detected. These species include peanut (*Arachis hypogoea*), soybean (*Glycine max*), and cucumber (*Cucumis sativus*) (Huang, 1983). It remains to be established whether the mechanism of storage oil breakdown is appreciably different in these plants. However, it would seem likely that PTLs are involved considering the remarkable conservation of function among *Arabidopsis*, yeast, humans, and insects.

Oleosin is the most abundant (and best characterized) protein on the oil body surface in seeds (Huang, 1992; Murphy, 1993). Studies have shown that this protein is targeted to the oil body via the endoplasmic reticulum and uses a single 70–amino acid residue hydrophobic domain for anchorage (Frandsen et al., 2001). Hydropathicity predictions using a standard algorithm

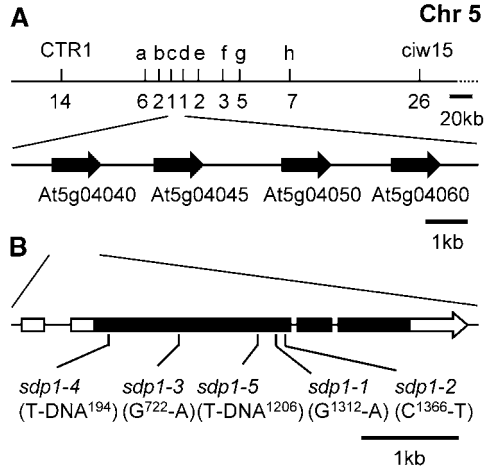

Figure 4. Positional Cloning of *SDP1*.

(A) A schematic diagram to illustrate how PCR-based simple sequence length polymorphism and cleaved-amplified polymorphic sequence markers were used to map *SDP1* to a 12-kb region on chromosome 5 that contains four genes. The positions of markers a to h (listed in Supplemental Table 3 online) are denoted by bars, and the number of recombination events/total number of chromosomes (1536) is listed below each.

(B) A representation of the *SDP1* locus (At5g04040) showing the positions of mutations that were identified in five independent *sdp1* alleles. Insertion/substitution positions are numbered relative to the initiation codon. Black bars are exons, and the white bars and arrow indicate the 5′ and 3′ untranslated regions.

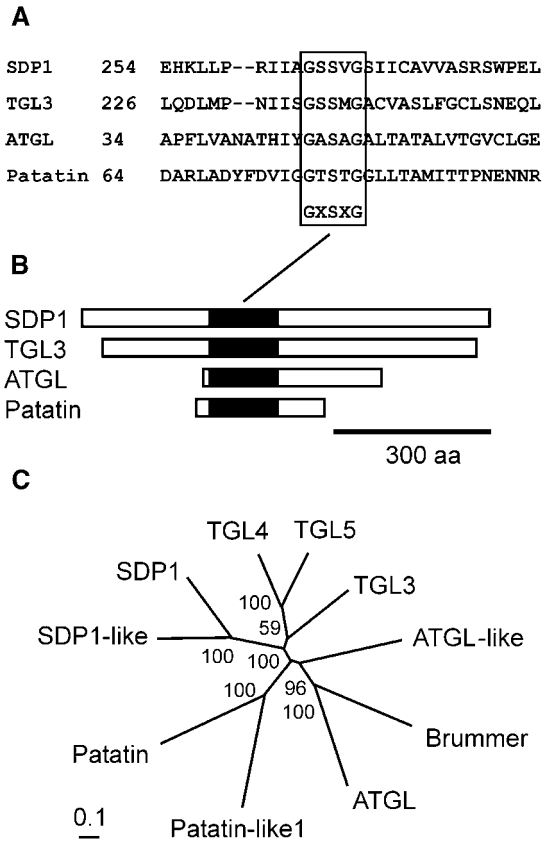

Figure 5. Sequence Similarity between SDP1 and Patatin-Like Proteins.

(A) An amino acid sequence alignment of the sequence surrounding the lipase (GXSXG) motif found in the patatin-like domains of *Arabidopsis* SDP1, yeast TGL3, human ATGL, and potato patatin. The alignment was performed using ClustalX (version 1.83).

(B) A schematic diagram showing the positions of the patatin-like (Pfam01734) domains in *Arabidopsis* SDP1, yeast TGL3, human ATGL, and potato patatin. The domains (marked in black) were identified by querying the NCBI Conserved Domain Database (Marchler-Bauer et al., 2005). aa, amino acids.

(C) A phylogenetic tree illustrating the relationship between patatin-like domains found in TAG lipases from yeast (TGL3, 4, and 5), *Arabidopsis* (SDP1, SDP1-like, and ATGL-like), *Drosophila* (Brummer), and humans (ATGL). The prototype patatin domain from potato patatin and the most closely related of the 12 patatin-like proteins in *Arabidopsis* (Patatin-like1) are also included. The phylogenetic analysis was performed on the alignment shown in Supplemental Figure 1 online using MEGA (version 2.1). Bootstrap values were determined from 1000 trials and are shown at each node. The length of the branch line indicates the extent of divergence according to the scale (relative units) at the bottom.

(Kyte and Doolittle, 1982) indicate that SDP1 contains only short hydrophobic stretches. Although this feature differs from oleosin, it is apparently common among oil body proteins from yeast and mammals (Athenstaedt and Daum, 2003; Subramanian et al., 2004). It has been suggested that major oil body proteins like oleosin or caleosin might play a role in regulating lipolysis by facilitating or blocking lipase access to its substrate (Frandsen et al., 2001). In mammals, perilipins are known to perform this function (Subramanian et al., 2004). However, oil bodies from *S. cerevisiae* apparently have no structural proteins and yet TGL3, 4, and 5, ATGL, and rSDP1 can all associate with the oil body surface in vivo (Athenstaedt and Daum, 2003, 2005; Kurat et al., 2006; data not shown). Further work will be required to understand how SDP1 (and other PTLs) are transferred to the oil body surface and whether additional proteins are involved.

The catalytic properties of SDP1 are broadly consistent with those reported for the TAG lipase activity from oil body membranes of oilseed rape (Lin and Huang, 1983; Hills and Murphy, 1988). This species is a relative of *Arabidopsis*. Both activities have a neutral to alkaline pH optima and can hydrolyze a range of TAGs containing long-chain saturated and unsaturated fatty acids. The lipase activity measured in purified oil body membranes from oilseed rape is able to hydrolyze TAG, DAG, and MAG (Lin and Huang, 1983). By contrast, SDP1 exhibits strong specificity toward TAG and is significantly less active against DAG and inactive on MAG. These data support the hypothesis that SDP1 is responsible for the first hydrolytic attack on the TAG molecule in germinating *Arabidopsis* seeds. A similar preference for TAG has been reported for human ATGL and yeast TGL4 (R. Zimmermann et al., 2004; Kurat et al., 2006). By analogy with

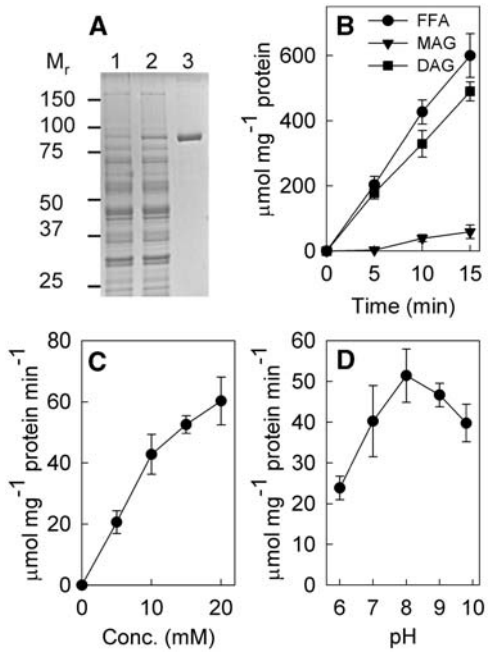

Figure 6. Expression of SDP1 in *S. cerevisiae* and Determination of TAG Lipase Activity.

(A) A Coomassie-stained SDS-PAGE gel of His_6-tagged SDP1. Lane 1, total protein from uninduced cells; lane 2, total protein from induced cells; lane 3, affinity-purified rSDP1.

(B) to **(D)** The effect of time **(B)**, substrate concentration **(C)**, and pH **(D)** on [^{14}C]triolein hydrolysis catalyzed by purified rSDP1. The values are the mean ± SE of measurements on four separate incubations. The standard amount of emulsified substrate used was 9 mg mL^{-1} (~10 mM), except in **(C)**, where the amount of substrate was varied. FFA, free fatty acids.

Table 2. Substrate Specificity of rSDP1 and Effect of Inhibitors

Substrate (plus inhibitor)	Activity (μmol mg^{-1} protein min^{-1})
Triolein	41.3 ± 4.0
Trieicosenoin	49.1 ± 7.5
Trilinolein	28.8 ± 3.5
Trilinolenin	24.1 ± 4.9
Tripalmitin	33.4 ± 2.3
Trilaurin	25.9 ± 3.7
sn-1,2(2,3) Diolein	6.6 ± 0.9
sn-1,3 Diolein	5.9 ± 2.0
sn-1 Monoolein	ND
1,2-Dioleoyl-3-phosphatidylcholine	ND
Monogalactosyldiacylglycerol	ND
Cholesteryl-oleate	ND
Oleoyl-CoA	ND
p-Nitrophenyl palmitate	ND
Triolein (plus 10 mM E_{600})	ND
Triolein (plus 10 mM DFP)	ND
Triolein (plus 1 μM BEL)	ND

The values are the mean ± SE of measurements from four separate incubations. The final concentration used was equivalent to 10 mM for all substrates, with the exception of oleoyl-CoA and *p*-nitrophenyl palmitate, which were 100 μM. Inhibitors were incubated with rSDP1 for 15 min before the substrate was added. ND, not detected; E_{600}, diethyl-*p*-nitrophenyl phosphate; DFP, diisopropyl fluorophosphate; BEL, [*E*]-6-[bromoethylene]-3-[1-naphthalenyl]-2*H*-tetrahydropyran-2-one.

lipolysis in human adipocytes (R. Zimmermann et al., 2004), it is likely that DAG and MAG lipases work in concert with SDP1 to hydrolyze TAG to fatty acids and glycerol. These lipases may also be associated with the oil body membrane (Lin and Huang, 1983), and their identity remains to be resolved.

Although germinating *sdp1* seeds are impaired in TAG breakdown, a small amount of storage oil mobilization does occur. The rate can be calculated from Figure 3A and is <20% of the wild type. This probably explains why postgerminative growth of *sdp1* is retarded but not arrested. Defects in peroxisomal fatty acid import, activation, and β-oxidation can cause more severe postgerminative growth arrest phenotypes and in some cases dramatically reduce germination frequency (Baker et al., 2006). It has been argued that these phenotypes are not entirely due to carbon shortage but to additional roles of peroxisomal β-oxidation in germination (Baker et al., 2006). Like *Arabidopsis sdp1*, the yeast *tgl3*Δ deletion strain also retains a small capacity for storage oil breakdown (Athenstaedt and Daum, 2003). However, TAG hydrolysis is completely blocked when a *tgl3*Δ *tgl4*Δ *tgl5*Δ triple mutant is created (Athenstaedt and Daum, 2005). *Arabidopsis* contains one SDP1 homologue (SDP1-like) and a single protein that is distantly related to human ATGL (ATGL-like). Public microarray data suggest that these two genes are also expressed in seeds; therefore, they could account for the small residual rate of TAG hydrolysis that occurs in germinating *sdp1* seedlings. In contrast with *SDP1*, microarray data shows that *SDP1-like* is strongly expressed in pollen and flowers. Oil bodies are known to be abundant in pollen and in the tapetal cells of the anther (Kim et al., 2002). The oil bodies in pollen rapidly disappear following germination (Rodriguez-Garcia et al., 2003); therefore, SDP1-like might play an important role in fertilization by providing carbon to support stigma penetration and pollen tube growth.

Because SDP1 catalyzes the initial step in TAG breakdown, it is the prime target for the regulation of this important process. Generally, very little lipase activity is detected in imbibed seeds prior to germination, and the activity increases dramatically following germination, coinciding with the onset of TAG mobilization (Huang, 1983; Hills and Murphy, 1988; El-Kouhen et al.,

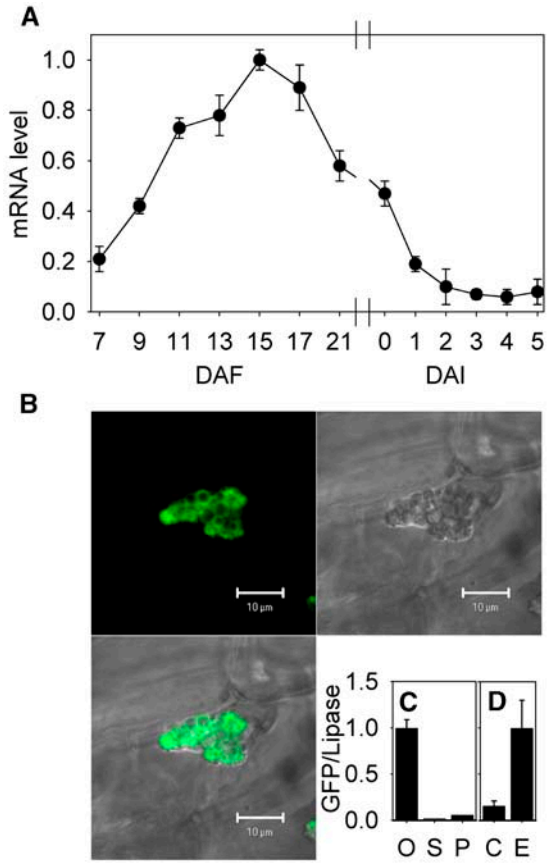

Figure 7. Expression and Localization of SDP1.

(A) Real-time PCR analysis of the expression of *SDP1* during seed development, germination, and early seedling growth. Values are the mean ± SE of measurements on four separate RNA extractions. Equal quantities of total RNA were used from each sample. DAF, days after fertilization; DAI, days after imbibition.

(B) A confocal image of a cell from a 5-d-old *sdp1-1* seedling transiently expressing the SDP1-GFP fusion protein. Top left, GFP; top right, bright field; bottom left, superimposed images. Bar = 10 μm.

(C) GFP fluorescence measured in different fractions from crude extracts of 5-d-old *sdp1-1* seedlings expressing SDP1-GFP that were centrifuged at 10,000*g* for 15 min. Values are the mean ± SE of measurements on three separate samples. O, oil pad; S, supernatant; P, pellet.

(D) TAG lipase activity in oil body membranes purified from 5-d-old *sdp1-1* seedlings (C) and 5-d-old *sdp1-1* seedlings expressing SDP1-GFP (E). Values are the mean ± SE of measurements on three separate samples.

2005). Surprisingly, *SDP1* transcript levels do not correlate positively with enzyme activity since they are highest throughout seed maturation, and in imbibed seeds, expression actually declines prior to radicle emergence. The likely explanation for this discrepancy is that SDP1 is regulated at the posttranscriptional level. Very little is known about the regulation of PTLs from any organism at present. However, ATGL and TGL4 are both phosphorylated (R. Zimmermann et al., 2004; Kurat et al., 2006) and in the case of TGL4, the protein has been identified as a substrate for cyclin-dependent protein kinase, Cdc28/CDK1 (Ubersax et al., 2003). Biochemical studies on oil body lipase activity from germinating seeds have also suggested that the enzymes could be subject to various forms of regulation. These include stimulation by Ca^{2+} (Hills and Beevers, 1987), inhibition by acyl-CoAs (plus CoA) (Hills et al., 1989), and gibberellic acid induced relocation from the vacuole to the oil body (Fernandez and Staehelin, 1987). To date, studies on *Arabidopsis* mutants lend support for a feedback mechanism by which acyl-CoA utilization could control the rate of lipolysis. Mutants that are defective in peroxisomal fatty acid/acyl-CoA import, activation, or β-oxidation accumulate long-chain acyl-CoAs following germination and do not breakdown their storage oil, which remains in oil bodies (Hayashi et al., 1998; Baker et al., 2006). Further work will be required to elucidate precisely how SDP1 activity and subcellular localization are regulated in seeds, and the identification of SDP2 and SDP3, which are also required for oil body lipase activity, may prove helpful in this respect.

Finally, although oil breakdown occurs predominantly after germination, recent work has shown that some oil is also turned over during late seed development (Chia et al., 2005). It has been suggested that the role of this oil breakdown is to provide carbon for continued metabolism during seed desiccation, after the tropic connection with the mother plant has been severed (Chia et al., 2005). The extent of oil turnover could potentially influence the level of oil accumulation in developing seeds. It has already been established that in yeast and animals disruption of PTLs leads to the overaccumulation of oil (R. Zimmermann et al., 2004; Athenstaedt and Daum, 2005; Gronke et al., 2005; Kurat et al., 2006). It will be interesting to determine whether a complete block in TAG hydrolysis in developing *Arabidopsis* seeds can also cause an increase in oil yield.

METHODS

Materials

Wild-type *Arabidopsis thaliana* (ecotypes Col-0 and Landsberg *erecta*) and the T-DNA Express lines (SALK_102887 and SALK_076697) were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). EMS-mutagenized Col-0 seed was purchased from Lehle Seeds. Ian Graham (University of York, UK) and Bonnie Bartel (Rice University, Houston, TX) kindly provided the mutants used for complementation tests. All reagents were obtained from Sigma-Aldrich, except for glycerol tri[1-^{14}C]oleate, which was from Amersham Biosciences.

Mutant Selection

For the sugar-dependent mutant screen, EMS M2 seeds >250 μM in diameter were selected using a sieve. This procedure removed poorly developed seeds that otherwise greatly increase the background of the screen. Approximately 240,000 seeds were sterilized, applied to agar plates containing half-strength MS salts, pH 5.7, and imbibed at 4°C for 4 d. The plates were then exposed to light (PPFD = 150 μmol m^{-2} s^{-1}) for 30 min and incubated for 5 d in a vertical orientation in the dark at 21°C. Seeds that germinated but did not develop hypocotyls longer than ~1 cm were selected and rescued onto agar plates containing half-strength MS salts plus 1% (w/v) sucrose, pH 5.7. The initial selection was performed on dark-grown seedlings because this treatment accentuates the seedlings dependence on their storage reserves and therefore increases the stringency of the screen (Eastmond et al., 2000). The rescued seedlings were grown on plates in continuous light (PPFD = 150 μmol m^{-2} s^{-1}) at 21°C until they were photosynthetically competent and then transferred to soil and grown to seed in the glasshouse. The lines were rescreened under the same conditions in the presence and absence of sucrose in the dark (and in the light). Those lines that inherited a requirement of sucrose to support postgerminative growth were retained for further analysis. The EMS *sdp* alleles used in this study were backcrossed three times to wild-type Col-0.

Tissue Extraction and Oil Body Membrane Purification

The extraction and purification of oil body membranes from germinating *Arabidopsis* seeds was adapted from the method of Hills and Murphy (1988), and all steps were performed at 4°C. Approximately 0.5 g fresh weight of imbibed seeds or 2-d-old seedlings were ground gently with a mortar and pestle in 3 mL of grinding medium. The homogenate was centrifuged at 100*g* for 5 min to pellet cell debris. A 200-μL sample of the supernatant (crude extract) was kept for lipase assays. The remaining supernatant was overlaid with a cushion of 60% (w/v) sucrose/1 mM EDTA, pH 7.5, and centrifuged at 10,000*g* for 15 min. The oil pad (above the cushion) was removed and dispersed in 10% (w/v) sucrose/1 mM EDTA, pH 7.5. The emulsion was overlaid with 1 mM EDTA, pH 7.5, and centrifuged for 15 min at 10,000*g*. This procedure was repeated twice more before the oil pad was delipidated by extracting three times with an equal volume of diethyl ether. Any remaining traces of diethyl ether were removed under a stream of N_2 gas.

Enzyme Assays

Lipase assays were performed on plant extracts and on purified recombinant protein using the methods described by Eastmond (2004). The only modification was that the standard reaction mixture was adapted from Hills and Murphy (1988) and consisted of 50 mM Bis-Tris propane/HCl, pH 8, 2 mM DTT, 2 mM $CaCl_2$, and substrate. Protein content was determined as described by Bradford (1976) using BSA as a standard. The preparation of seedling extracts for measurements of β-oxidation, glyoxylate cycle, and gluconeogenesis enzymes and the details of the assay protocols have been described previously (Eastmond et al., 2000; Rylott et al., 2006).

Lipid and Acyl-CoA Analysis

TAGs were extracted from seeds and seedlings and their fatty acid content measured by gas chromatography analysis essentially as described by Tonon et al. (2002), except that the tissue was homogenized in 1.5-mL tubes using a pestle attached to a drill. Acyl-CoAs were extracted and measured as described by Larson and Graham (2001).

Microscopy

Transmission electron microscopy was performed as described previously (Eastmond, 2004). 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. Light microscopy was performed using a Zeiss LSM 510 Meta on an Axioplan 2M, fitted with a ×63 Plan Apo lens (numerical aperture 1.4). Samples containing GFP were excited with a 488-nm argon laser and emission collected via a 505- to 530-nm band-pass filter. Bright-field images were captured simultaneously with the transmission detector.

Mapping

The *sdp1-1* mutant was outcrossed to 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 768 F2 *sdp1-1* lines using the Extract-N-Amp plant PCR kit (Sigma-Aldrich). Mapping was performed using simple sequence length polymorphism (Bell and Ecker, 1994) and cleaved-amplified polymorphic sequence markers (Konieczny and Ausubel, 1993). Appropriate markers were designed using information from the Monsanto *Arabidopsis* polymorphism collection (Jander et al., 2002) and are listed in Supplemental Table 3 online. Candidate genes within the mapping interval were amplified from *sdp1-1*, *sdp1-2*, and *sdp1-3* genomic DNA by PCR and sequenced to identify mutations.

Protein Expression and Purification

For the construction of N-terminal His_6-tagged SDP1 under a GAL1 promoter (selectable marker TRP^+), the open reading frame was amplified by PCR from *Arabidopsis* cDNA using gene-specific primers (5′-GTACCGATGCATCATCATCATCATCATGATATAAGTAATGAGGCTAGT-3′ and 5′-TCTAGACTAAGCATCTATAACACTACCAGACA-3′). The PCR product was cloned into the pCR2.1-TOPO vector from Invitrogen. Using standard molecular techniques (Sambrook et al., 1989), *SDP1* was then excised with *Kpn*I and *Xba*I and the fragment inserted into the multiple cloning site of the pYES2 plasmid (Invitrogen). The plasmid was transformed into yeast INVSc1 cells using the Invitrogen *S. c.* EasyComp kit. Protein expression was induced and yeast cells were grown to early stationary phase as described by Athenstaedt and Daum (2003). rSDP1 protein was purified under native conditions using the ProBond purification system (Invitrogen). The procedure was as described by the manufacturer except that 2 mM sodium taurodeoxycholate was included in the buffers. For SDS-PAGE, the protein samples were first solubilized in 0.1% (w/v) SDS and 0.1% (w/v) NaOH at 37°C (Athenstaedt and Daum, 2003) and then separated as described previously (Eastmond, 2004). The gels were stained using 0.1% (w/v) Coomassie Brilliant Blue R 250 in methanol:acetic acid:water (4:1:5 [v/v/v]).

RNA Extraction, cDNA Synthesis, and Quantitative PCR

DNase-treated total RNA was isolated from *Arabidopsis* seeds using the method of Wu et al. (2002), which combines extraction in a borate buffer plus proteinase K with the RNeasy kit spin columns from Qiagen. The synthesis of single-stranded cDNA was performed using SuperScript II RNase H^- reverse transcriptase from Invitrogen. Steady state levels of *SDP1* mRNA were measured by real-time quantitative PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems) and the SYBR green PCR master mix as described by Penfield et al. (2004). The primers used were SDP1F (5′-AAATGGCTTACCGGAGGAAGTT-3′), SDP1R (5′-TGAGCCCATTCCTCATAAGTCA-3′), 18SF (5′-TCCTAGTAAGCGCGAGTCATC-3′), and 18SR (5′-CGAACACTTCACCGGATCAT-3′).

Transient Expression of SDP1-GFP

SDP1 was amplified by PCR from *Arabidopsis* seed cDNA using primers 5′-CCATGGATATAAGTAATGAGGCTAGTGTCGAT-3′ and 5′-CCATGGCATCTATAACACTACCAGACACCGGTTC-3′. The PCR product was cloned into the pCR2.1-TOPO vector from Invitrogen. Subsequent cloning steps were performed using standard molecular techniques (Sambrook et al., 1989). The 35S:GFP cassette from pAVA393 (von Arnim et al., 1998) was cloned into pGREENII (Hellens et al., 2000) using the *Hin*dIII and *Xba*I sites. SDP1 was then digested from pCR2.1 using *Nco*I and subcloned into the *Nco*I site of the modified pGREENII vector. One milligram of gold particles (1-μm diameter) was coated with 6 μg of vector DNA by ethanol precipitation. Seedlings were bombarded with ~50 μg of gold particles using a Helios gene gun system (Bio-Rad Laboratories) according to the manufacturer's instructions. GFP and oil body membrane lipase activity were detected after 24 h.

Phylogenetic Analysis

The patatin-like (Pfam01734) domains in various lipases were identified by querying the NCBI Conserved Domain Database (Marchler-Bauer et al., 2005). The amino acid sequences of the domains were aligned using ClustalX (version 1.83) (Thompson et al., 1997) and edited with BioEdit (version 5.0.9) (Hall, 1999). The alignment is shown in Supplemental Figure 1 online. A consensus phylogenetic tree was constructed with the neighbor-joining method using MEGA (version 2.1) (Kumar et al., 2001), and bootstrap values were determined from 1000 trials. The tree was drawn using TreeView (version 1.6.6.).

Accession Numbers

Sequence data for the genes (or proteins) from this article can be found in the GenBank data library under accession numbers AY136470 (SDP1), AK117921 (SDP1-like), BX813896 (ATGL-like), NP_014044 (TGL3), NP_013015 (TGL4), NP_014724 (TGL5), AY894804 (ATGL), NM_140466 (Brummer lipase), X01125 (Patatin), and AJ002596 (Patatin-like1).

Supplemental Data

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

Supplemental Figure 1. An Amino Acid Sequence Alignment of Patatin-Like Domains Found in Lipases from Animals, Plants, and Yeast.

Supplemental Table 1. Frequency of *sdp1* Germination.

Supplemental Table 2. The Activities of Enzymes Involved in the Conversion of Fatty Acids to Sugar in *sdp1* Seedlings.

Supplemental Table 3. New SSLP and CAPS Markers Used to Map *SDP1*.

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