1 **An inhibitor of oil body mobilisation in Arabidopsis**

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Materials and Methods	475		
Results and Discussion	1764		
Acknowledgements	112		

16 **Summary (184)**

An inhibitor of oil body mobilisation

- Fatty acid β-oxidation is an essential process in many aspects of plant development, and storage oil in the form of triacylglycerol (TAG) is an important food source for humans and animals, for biofuel and for industrial feedstocks. In this study we characterise the effects of a small molecule, diphenyl methylphosphonate, on oil mobilization in *Arabidopsis thaliana*.
- Confocal laser scanning microscopy, transmission electron microscopy and quantitative lipid profiling were used to examine the effects of diphenyl methylphosphonate treatment on seedlings.
- Diphenyl methyl phosphonate causes peroxisome clustering around oil bodies but does not affect morphology of other cellular organelles. We show that this molecule blocks the breakdown of pre-existing oil bodies resulting in retention of TAG and accumulation of acyl CoAs. The biochemical and phenotypic effects are consistent with a block in the early part of the β-oxidation pathway.
- Diphenyl methyl phosphonate appears to be a fairly specific inhibitor of TAG mobilisation in plants and whilst further work is required to identify the molecular target of the compound it should prove a useful tool to interrogate and manipulate these pathways in a controlled and reproducible manner.

Key words: Arabidiopsis; Peroxisome; β-oxidation; Oil body; Triacylglycerol; lipid metabolism, inhibitor

An inhibitor of oil body mobilisation

Introduction

β-oxidation is the major metabolic pathway by which fatty acids and fatty acid-like molecules are metabolised. In plants and yeasts, unlike mammals, β-oxidation is wholly compartmentalised in peroxisomes which contain a complete sequence of metabolic reactions to convert both saturated and unsaturated fatty acids to acetyl-CoA as well as metabolism of hormone precursors such as Indole butyric acid (IBA) and oxo phytodienoic acid (OPDA) (Goepfert & Poirier, 2007; Graham, 2008; Wiszniewski *et al.*, 2009; Hu *et al.*, 2012). In oil seeds, reserve lipids are stored primarily as triacylglycerols (TAGs) in oil bodies of the cotyledons and/or endosperm that are formed from the endoplasmic reticulum during seed development (Murphy, 2012).

Upon seed germination, TAG hydrolysis is carried out by oil body-located TAG lipases such as SDP1 and SDPL (Eastmond, 2006; Kelly *et al.*, 2011). (Fig. 1) Substrates for β-oxidation are transported into peroxisomes by the ABC transporter CTS/PXA1/PED3. Recent biochemical characterisation of the CTS protein has shown that it possesses an intrinsic thioesterase activity that cleaves acyl-CoAs during the transport cycle (De Marcos Lousa *et al.*, 2013), so presumably fatty acids are activated by cytosolic acyl CoA synthetases prior to transport and reactivated within the peroxisome by acyl CoA synthetases LACS 6 and 7 (Fulda *et al.*, 2004) which require ATP supplied by the peroxisome ATP transporters PNC1 and PNC2 (Arai *et al.*, 2008; Linka *et al.*, 2008) . LACS6 and 7 are specific for fatty acids and it is not known if other β- oxidation substrates are also accepted as CoA thioesters and cleaved upon transport. A peroxisomal

protein PXN that transports a range of substrates including NAD+ and CoA has been identified (Agrimi *et al.*, 2012; Bernhardt *et al.*, 2012) .

Once within peroxisomes, acyl-CoAs are oxidised by acyl-CoA oxidases (ACXs) 1-4 which exhibit overlapping chain length specificity ((Graham, 2008) and references therein) to produce a Δ^2-*trans* enoyl-CoA. The Δ^2-*trans* enoyl-CoA is the substrate for the multifunctional protein (MFP) which has 2-*trans* enoyl hydratase and 1-3-hydroxyacyl-CoA dehydrogenase activities. Arabidopsis contains two MFP genes: *MFP1*(*AIM1*), expressed at low level in seedlings (Richmond & Bleecker, 1999) and *MFP2* which encodes the major MFP activity in seedlings (Rylott *et al.*, 2006). The resulting 3-ketoacyl-CoA, is cleaved by 3-ketoacyl thiolase (PED1/KAT2) to liberate acetyl CoA, and an acyl-CoA two carbons shorter than the original molecule (Hayashi *et al.*, 1998; Germain *et al.*, 2001). The acetyl CoA produced by the thiolase reaction can enter the glyoxylate cycle and play a gluconeogenic role (Kunze *et al.*, 2006) or be converted to citrate by peroxisomal citrate synthase and exported to mitochondria for metabolism by the TCA cycle (Pracharoenwattana *et al.*, 2005).

Thus β-oxidation requires a concerted set of enzyme activities localised in peroxisomes, where disruption of any one of which can impact on oil mobilization. The genetic approach has been very valuable in identifying additional roles for β-oxidation beyond germination(Baker *et al.*, 2006), in dormancy breaking (Footitt *et al.*, 2002), auxin responses (Zolman *et al.*, 2001b), biosynthesis of jasmonates (Afitlhile *et al.*, 2005; Theodoulou *et al.*, 2005), fertility (Footitt *et al.*, 2007) and floral development (Richmond & Bleecker, 1999). Not all of these roles are yet fully understood.

An inhibitor of oil body mobilisation

An alternative, complementary, approach to genetics is to use inhibitors of specific enzyme steps. Whilst mutants are defective in specific steps, they may still exhibit pleiotropic effects. The MONODEHYDROASCORBATE REDUCTASE 4 (*sdp2*) mutant has a block in TAG mobilization because the SDP1 lipase is sensitive to inactivation by H_2O_2 which accumulates in this mutant (Eastmond, 2007) and the *chy1* mutant, defective in peroxisomal valine catabolism, is also defective in β-oxidation due to accumulation of toxic methacrylyl-CoA (Zolman *et al.*, 2001a). Inhibitors have the advantage of controlled application with respect to time and concentration, and can be applied to specific tissues or structures. Additionally, where multiple genes encode overlapping activities such as with the ACX family, multiple knockouts may be required to see a phenotype whereas application of a single inhibitor may inhibit all family members to a greater or lesser degree. A number of inhibitors of mammalian β-oxidation have been described (Schulz, 1983; Schulz, 1987) (Youssef *et al.*, 1994) but there do not appear to be any available inhibitors to study this process in plants, which differ from mammals in having an exclusively peroxisomal β-oxidation system.

In this study, we report on the identification and characterisation of a small molecule which blocks TAG mobilization in Arabidopsis seedlings, providing a valuable tool for the interrogation and manipulation of oil breakdown. This could be particularly useful in the investigation of tissue or developmental specific roles of oil breakdown, or in plant species where genetic analysis is not as facile as model plants like Arabidopsis.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana lines contained the following constructs, 35S:: GFP -MFP2-(Cutler *et al.*, 2000); 35S::CSY3-GFP (Pracharoenwattana *et al.*, 2005); 35S::ST-GFP (Saint-Jore *et al.*, 2002); 35S::GFP-HDEL (Batoko *et al.*, 2000); 35S::FABD2-GFP (Sheahan *et al.*, 2004); Oleosin-GFP (Wahlroos *et al.*, 2003); 35S::ATPβ-GFP (Logan & Leaver, 2000) Seeds were sterilised, stratified in darkness for 48 h at 4°C, and sown on ½ Murashige and Skoog (MS) media (Duchefa, Haarlem, Netherlands) 0.8% (w/v) plant agar for vertical growth, or 0.3% (w/v) plant agar for 24-well microplate growth. Diphenyl methylphosphonate (TCI Europe nv Zwijndrecht, Belgium), 25 mM stock solution in DMSO, was diluted as indicated with hand hot ½MS media. Sucrose was added to 20 mM when required. Light (16 h per day) seedlings were grown for six days (unless otherwise stated) at 23°C. Transplant assay: Stratified seeds were grown for seven days on ½MS containing 0.1% (v/v) DMSO or 25 μM diphenyl methylphosphonate then transferred to ½MS containing 0.1% (v/v) DMSO or 25 μM diphenyl methylphosphonate for a further seven days. IBA assay was as described (Dietrich *et al.*, 2009).

Microscopy

For confocal microscopy an upright laser scanning microscope (LSM 510; Zeiss, Jena, Germany) with a 40x or 63x oil immersion objective lens was used for imaging. All images were scanned under identical conditions (laser power, photomultiplier gain, pinhole diameter and zoom) in relation to the relevant controls. GFP was imaged with the 488nm line of an argon ion laser with a 505-530 band pass filter and Nile Red(Dietrich *et*

al., 2009) with a 543nm helium neon laser and 560 to 615 nm band pass filter. Post-acquisition image processing was done using the LSM 5 browser (Zeiss) and Adobe Photoshop 9.0 software (Adobe Systems, Mountain View, CA, USA).

Electron microscopy: Short sections of hypocotyls were fixed in 1% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 0.1M sodium cacodylate buffer, pH6.9, washed 3x in buffer and post fixed in 2% (w/v) aqueous osmium tetroxide for 90 min (Faso *et al.*, 2009). Samples were washed four times in water and subsequently block stained overnight in 1% aqueous uranyl acetate. Samples were dehydrated in acetone and embedded in TAAB low viscosity resin (TABB, Reading UK) and sectioned with a RMC PowerTome XL ultra-microtome. Post-staining was carried out in lead citrate for 5-10 min and sections were observed with a Hitachi H-7650 transmission electron microscope.

Fatty acid and acyl-CoA profiling

Stratified seeds were plated onto ½ MS media (0.8% (w/v) plant agar) containing 25 µM diphenyl methylphosphonate or 0.1% (v/v) DMSO and 20 mM sucrose. After five days in the light, hypocotyls and cotyledons were harvested for fatty acid profiling. Fatty acids and acyl-CoAs were profiled from the same extracts as described in (Larson & Graham, 2001) with the modifications described (Larson *et al.*, 2002). Lipid extraction and neutral lipid analysis by LC/MS/MS were performed as described (Burgal *et al.*, 2008).

Results & Discussion

Diphenyl methylphosphonate treated seedlings retain oil bodies but other organelles are unaffected

A small scale confocal laser scanning microscopy (CLSM)-based screen for compounds that altered peroxisome morphology and/or fluorescence intensity in an Arabidopsis line that expresses a peroxisomal targeted GFP reporter was carried out (Brown *et al.*, 2011). The 70 compounds used in the small scale screen originated from a primary screen for compounds that affected hypocotyl gravitropism (Surpin *et al.*, 2005) thus were known to be bioavailable and bioactive in plants. This report describes the detailed characterisation of diphenyl methyl phosphonate which was identified as causing peroxisome clustering.

Control six day old light grown seedlings have punctate peroxisomes that are distributed throughout the cytoplasm of hypocotyl cells, (Fig. 2a, c). When 25µM diphenyl methylphosphonate is present in the medium, clustering of peroxisomes around spherical bodies of varying size is observed (Fig. 2d-f). These bodies stain with the lipophilic dye Nile Red (Fig.2b, e, g) and contain oleosin, as revealed by oleosin-GFP fluorescence (Fig. 2h), identifying them as oil bodies. Co-localisation of the peroxisomal GFP reporter and the Nile Red stained oil bodies shows that the peroxisomes cluster around the oil bodies (Fig. 2f & g). Transmission electron microscopy shows this dramatic accumulation of multiple large oil bodies in hypocotyl cells in the presence of diphenyl methyl phosponate (Fig. 2i) and in the presence of diphenylmethyl phosphonate and 20 mM sucrose (Fig. 2j). Hypocotyl cells from control seedlings grown in the presence of sucrose but without diphenylmethyl phosphonate showed a large vacuole with just a thin

layer of cytoplasm and normal cellular organelles (Fig. 2k). The effect of the compound was compared in the presence and absence of sucrose to facilitate subsequent comparisons with mutants disrupted in β- oxidation that are dependent upon sucrose for post germinative growth.

Only oil bodies and peroxisomes are affected by diphenyl methyl phosphonate treatment. When seedlings expressing ST -GFP (Golgi marker; Fig. 3a, b), GFP-FABD2 (actin marker; Fig.3c, d) GFP-HDEL (ER marker Fig. 3e, f) or ATPβ-GFP (mitochondrial marker; Fig. 3g, h) were grown for six days on 25μM diphenyl methyl phosphonate there was no obvious difference in appearance between control (Fig 3a,c,e,f) and treated seedlings (Fig. 3b,d,g,h). Germination was not affected by the compound (data not shown), however, seedlings grown for four weeks in the presence of 25μM diphenyl methyl phosphonate were small pale and stunted (Fig. 3j, k) compared to control (Fig. 3i) seedlings.

Hypocotyl growth in the dark is compromised in the absence of sucrose but IBA conversion to IAA is not affected

When seedlings were grown in the dark in the presence of diphenyl methyl phosphonate a dose-dependent inhibition of hypocotyl growth was observed which was completely rescued by sucrose at concentrations of up to 5μM and partially rescued even at 50μM diphenyl methyl phosphonate (Fig 4a). Hypocotyl growth in the dark depends upon energy and carbon supplied by β-oxidation and many mutants defective in β-oxidation show a similar sucrose rescue phenotype (Pinfield-Wells *et al.*, 2005). Beta oxidation is

197 also required to convert indole butyric acid (IBA) to indole acetic acid (IAA) (Fig.1)
198 which results in stunting of roots and hypocotyl (Zolman *et al.*, 2000). Seedlings were
199 grown in the dark on sucrose in the presence of different concentrations of IBA and in the
200 presence or absence of diphenyl methyl phosphonate (Fig. 4b). Treated and untreated
201 seedlings at the same IBA concentration showed the same percentage of hypocotyl
202 shortening compared to the zero IBA treated and untreated controls, which were set to
203 100% (Fig 4b). IBA and fatty acids share some steps in β-oxidation (Fig. 1) such as
204 transport by CTS/PXA1, cleavage by KAT2/PED1 and the need for ATP and CoA
205 supplied by membrane transporters, but other steps are distinct. These include an as yet
206 unidentified acyl CoA synthetase(s), probably the acyl CoA oxidase/dehydrogenase IBR3
207 and IBA-specific enoyl CoA hydratases ECH2 and IBR10 (reviewed in (Hu *et al.*,
208 2012)). IBA metabolism is arguably a very sensitive test of steps in β oxidation that are
209 shared with fatty acids since the *pxa1-1* mutation is dominant for IBA resistance but
210 recessive for sucrose dependence of hypocotyl elongation (Zolman *et al.*, 2001b). Thus
211 the diphenyl methyl phosphonate target would appear to be specific to fatty acid
212 degradation.
213
214 Organophosphorus phosphonate containing compounds inhibit a wide range of enzymes
215 of the serine hydrolase class that possess a His/Asp/Ser catalytic triad, including acetyl
216 cholinesterase, serine proteases and lipases, where a reaction between the active site
217 serine and the phosphonate gives a covalent complex that mimics the transition state.
218 Different inhibitors show different specificities (Oskolkova & Hermetter, 2002; Salisbury
219 & Ellman, 2006). The clustering of peroxisomes around retained oil bodies seen in Fig 2

is reminiscent of the phenotype of the *sdp1* and *sdp2* mutants and SDP1 is inhibited by the inhibitor E600 (diethyl *p*-nitrophenyl phosphate) and diisopropyl fluorophosphate (Eastmond, 2006).

Quantitative lipid analysis of treated seedlings reveals retention of TAG and accumulation of acyl CoAs

To investigate the block in oil body mobilization, a comprehensive analysis of the lipid composition of five day old hypocotyl and cotyledon tissue from seedlings grown in the presence or absence of diphenyl methylphosphonate was carried out. Since oil bodies are retained in the hypocotyls of treated seedlings in the presence of sucrose (Fig. 1j), seedlings were grown in the presence of 20 mM sucrose in order to facilitate comparison with a large data set of fatty acid, acyl-CoA and TAG profiles of a range of β-oxidation mutants (Hernandez *et al.*, 2012).

Fig. 5a shows an overview of lipids in control and diphenyl methylphosphonate treated seedlings, compared with dry seed. Galactolipids, the major lipids of chloroplast membranes, are absent in dry seed but no difference is seen between control and treated seedlings. Diacylglycerols (DAGs) are slightly elevated in treated compared to control seedlings, but the most striking change is in the level of TAGs. As expected, five day old control seedlings had very low TAG levels (7% of dry seed) but diphenyl methylphosphonate-treated seedlings retained 64% of the TAG seen in dry seed. In treated seedlings the profile of acyl-CoAs show elevated levels of 20:0 and especially 20:1 compared to control (Fig. 5b), similar to a number of β-oxidation mutants (e.g

243 *pxa1(cts)*, *lacs6/lacs7 acx1/acx2* and *kat2*) but less like the TAG lipase *sdp1*mutant which
244 shows a significantly reduced level of acyl-CoAs in 2 day old seedlings compared to the
245 corresponding wild type (Eastmond, 2006) and a very slightly increased level of 20:1
246 CoA in 5 day old *sdp1* compared to wild type (Hernandez *et al.*, 2012).
247
248 A quantitative analysis of TAG species in control and treated seedlings and dry seed was
249 carried out (Fig. 5c) and shows that breakdown of all TAG species is compromised by
250 diphenyl methylphosphonate, but some species are more compromised than others and
251 some even show slightly increased levels in the treated seedlings compared to dry seed.
252 This is particularly true for 18:3-20:1-18:3, 20:1-18:2-18:3 and to a lesser extent for 18:3-
253 18:2-18:3, 20:1-20:1-18:3 18:3-18:2-22:1 and 18:3-18:3:18:3. This is reflected in the
254 higher levels of 18:3n3 18:2n6c and 20:1n9 in the treated seedlings (Fig 5d) and is similar
255 but not so extreme as seen in the *pxa1* mutant, but clearly different to the *acx1/acx2*
256 double mutant, which shows *increased* levels of TAG after five days (Hernandez *et al.*,
257 2012). Further, interference with CoA biosynthesis can be excluded since such mutants
258 have a reduced acyl-CoA pool (Rubio *et al.*, 2006). In contrast, mutants which block β-
259 oxidation directly or indirectly downstream of the TAG lipase accumulate acyl-CoAs and
260 oil bodies are retained, suggesting a feedback inhibition of lipolysis (Graham, 2008).
261 Inhibition of processes other than β-oxidation could impact the acyl-CoA pool, for
262 example inhibition of incorporation of acyl-CoAs into membrane lipids or cuticular wax,
263 resulting in feedback inhibition of the TAG lipase. However this is unlikely in this
264 instance as total monogalactosyl diacyl glycerol, the most abundant chloroplast lipid, is
265 unchanged in diphenyl methylphosphonate treated seedlings relative to control (Figure 5)

and the level of MGDG with two 18:3 acyl chains is increased relative to MGDG with one 16:3 and one 18:3 in the treated samples (not shown), suggesting membrane lipids are acting as a sink for excess 18 carbon acyl-CoAs. Indeed these acyl-CoAs do not show increased levels in treated versus control seedlings (Figure 5b).

Diphenyl methyl phosphonate treatment results in irreversible inhibition of oil body breakdown, not induction of oil body synthesis.

To determine whether the inhibitor blocked the mobilization of existing oil bodies or promoted their *de novo* synthesis, seedlings were grown in the absence (0.1% DMSO; Fig. 6a) or presence of 25 μM diphenyl methylphosphonate (Fig. 6b). After seven days, when oil bodies had been completely metabolised in the control and were retained in the treated seedlings, the seedlings were transplanted to media without (Fig 6c, f) or with (Fig. 6d, e) 25 μM diphenyl methylphosphonate. Transplantation of seven day old seedlings onto the compound did not induce the formation of oil bodies or lipid droplets (Fig. 6d). Oil bodies were retained in hypocotyls of treated seedlings after seven days on chemical-free media, suggesting an irreversible inhibition of TAG breakdown (Fig. 6f). In contrast to the *pxa1* mutant where post-germinative recycling of fatty acids into TAG occurs in seedlings (Hernandez *et al.*, 2012) and leaves (Slocombe *et al.*, 2009), the mode of action of the compound is not through promoting TAG synthesis.

In summary we have identified a compound, diphenyl methyl phosphonate, which appears to be a reasonably specific inhibitor of oil body mobilization during post germinative growth. The phenotypes show a number of similarities with the *sdp1* mutant

and the SDP1 lipase has been shown to be inhibited by diethyl *p*-nitrophenyl phosphate, but the TAG profiles differ and show more similarity to the *pxa1* mutant. We considered whether diphenyl methyl phosphonate might inhibit the thioesterase activity of the CTS protein but *in vitro* assays on insect expressed protein showed no inhibition of the activity and neither was the activity of the peroxisomal acyl CoA thioesterase ACH2 which has a Asp/Gln/Ser catalytic triad (Tilton *et al.*, 2004) affected (data not shown). It is possible that *in vivo* there is partial inhibition of more than one target. While further analysis is required to delimit the precise mode of action this compound will be a useful tool for investigating aspects of lipid breakdown. It has the advantage that it can be applied at different concentrations at any developmental stage allowing early effects of inhibition to be studied, or to any tissue allowing investigation of tissue specific effects. It should be useful in the study of lipid breakdown in less genetically tractable plant species.

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311 the effects of diphenyl methyl phosphonate on ATPase and thioesterase activities of CTS
312 and ACH2.
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Figure legends

Figure 1. Peroxisomal β-oxidation of fatty acids and indole butyric acid.

Intermediates are shown in black type, enzymes in blue and cofactors in red. Boxes within the membrane indicate transporter proteins. Metabolism of hydrogen peroxide produced by acyl CoA oxidases (ACX) 1-4 and NADH produced by multifunctional proteins MFP2 and AIM1 are omitted for clarity. Abbreviations TAG; triacyl glycerol, FA fatty acid; IBA indole butyric acid, IBA CoA indole butyryl CoA; IBE-CoA indole butenol CoA. I3HB CoA indole 3 hydroxybutenyl CoA I3KBCoA indole-3-keto-butenyl CoA; IAA CoA indole acetyl CoA

Figure 2: Diphenyl methylphosphonate treatment results in clustering of peroxisomes around lipid bodies.

GFP-PTS1 (peroxisomes) and Nile Red (lipid bodies) fluorescence in hypocotyl cells of seedlings grown for six days on ½MS medium (0.3% agar) containing either DMSO (0.1%) (**a**, **b & c**), or 25 µM diphenyl methylphosphonate (**d**, **e**, **f, g, h, i & j**). GFP-PTS1 fluorescence is shown in panels (**a**, **d** & **g**), Nile Red fluorescence is shown in panels (**b**, **e** & **g**) and merged images with bright-field background are panels (**c**, **f, g** & **h**). A higher magnification of GFP-PTS1 and Nile Red fluorescence in 6 day old hypocotyls grown on 25 µM diphenyl methylphosphonate can be seen in (**g**). Oleosin-GFP fluorescence in six day old hypocotyls grown on 25 µM diphenyl methylphosphonate is also localised to lipid bodies (**h**). Scale bar = 20 µm (**a**–**f**) and 10 µm (**g** & **h**). Transmission electron micrographs of oil bodies in hypocotyls grown on 25 µM diphenyl methylphosphonate without, (**i**) and with, (**j**) 20 mM sucrose. (**k**) shows hypocotyl from a seedling grown on 20 mM sucrose without diphenyl methylphosphonate. Scale bars 2 µm.

Figure 3: Diphenyl methylphosphonate effects on plant growth and other cellular organelles.

Fluorescent pattern of GFP-reporter proteins for Golgi, (sialyltransferase-signal anchor sequence-GFP; **a** & **b**) actin (GFP-FABD2; **c** & **d**); the ER (GFP-HDEL; **e** & **f**) and mitochondria (ATPβ-GFP; **g** & **h**). Images are of hypocotyl cells from six day old seedlings which have been grown on ½MS medium (0.3% agar), containing either 0.1% DMSO (**a**, **c**, **e & g**) or 25 µM diphenyl methylphosphonate (**b**, **d**, **f** & **h**). Scale bar = 20 µm (**a** & **b**), 20 µm (**c** & **d**) and 10 µm (**e f, g** & **h**). Arabidopsis seedlings grown for four weeks on ½MS medium containing either 0.1% DMSO (**i**); or 25 µM diphenyl methylphosphonate (**j, k) k** is a magnification of **j**. Scale bar = 5 mm.

An inhibitor of oil body mobilisation

519

520 **Figure 4: Diphenylmethyl phosphonate inhibits hypocotyl elongation in the absence**
521 **of sucrose but does not affect IBA metabolism**

522 Dark grown seedlings (n =16) grown in the presence (black bars) or absence (white bars)
523 of sucrose at the indicated concentration of diphenyl methyl phosphonate. Figure shows
524 the mean hypocotyl length +/-SE (**a**). Seedlings (n=60) were grown in the dark on 0.5%
525 sucrose for 5 days in the presence of a range of concentrations of IBA and with or
526 without 25 µM diphenylmethyl phosphonate as indicated. Results are standardized to
527 growth on media without IBA and are mean hypocotyl length +/-SE (**b**).

528

529 **Figure 5: Diphenyl methylphosphonate treated seedlings retain TAG and**
530 **accumulate acyl-CoAs.**

531 PTS1-GFP seedlings were grown on media containing 20 mM sucrose and 25 µM
532 diphenyl methylphosphonate or 20 mM sucrose only (control) for five days. The roots
533 were excised and the cotyledons and hypocotyls used for lipid analysis. (**a**) Overview of
534 lipid content. Dry seed refers to dry seed from the same batch of the PTS1-GFP line that
535 was used to grow the seedlings. (**b**) Comparison of acyl-CoA profile of treated and
536 control seedlings. (**c**) Graph showing a comparison of the level of different TAGs
537 between control and diphenyl methylphosphonate treated seedlings expressed as the
538 difference in amount of each TAG species between the five day old seedling and dry
539 seed. This shows that a few TAG species actually increase in treated seedlings. (**d**) Graph
540 showing the profile of fatty acids in control and diphenyl methylphosphonate treated
541 seedlings. Error bars show the standard deviation of five replicates

542

543 **Figure 6: Diphenyl methylphosphonate prevents mobilization of pre-existing lipid**
544 **bodies.**

545 PTS1-GFP seedlings were grown on media containing either 0.1% DMSO (**a**) or 25 µM
546 diphenyl methylphosphonate (**b**) for seven days before being transplanted to DMSO (**c,**
547 **f**) or 25 µM diphenyl methylphosphonate (**d, e**) . As seen by the GFP-PTS1
548 (peroxisomes) and Nile Red (lipid bodies) fluorescence in hypocotyl cells, diphenyl
549 methylphosphonate appears to prevent breakdown of oil bodies already present in the
550 seeds prior to germination (**f**), rather than promoting synthesis of new oil bodies (**d**).
551 Scale bar = 20 µm.

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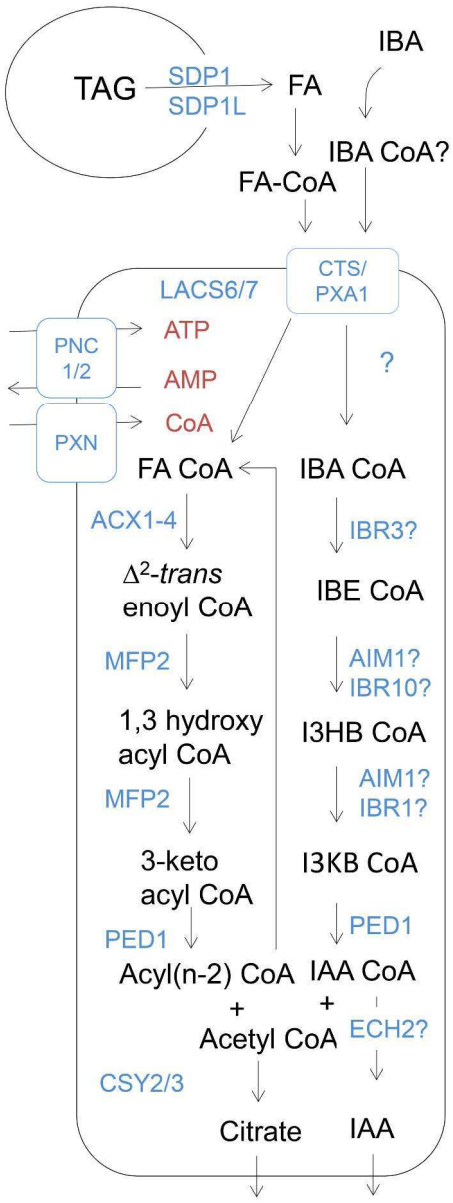

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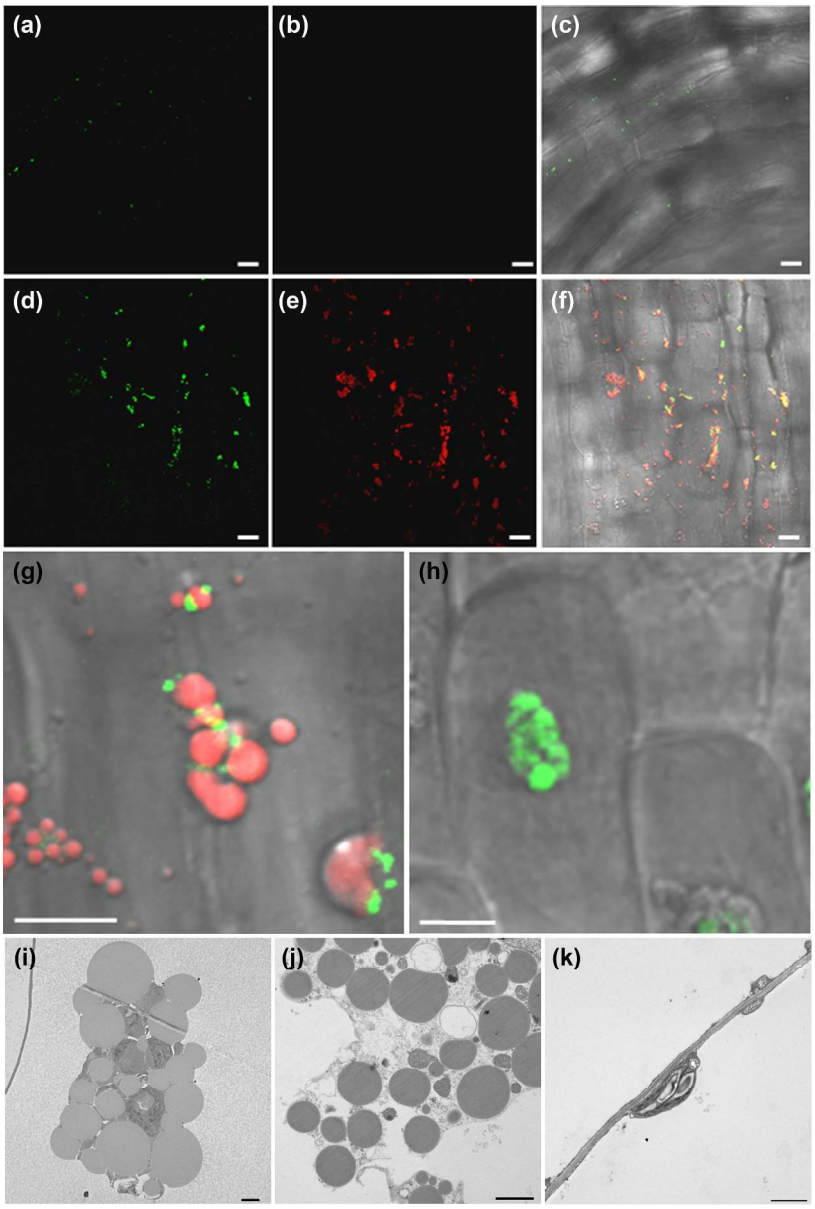

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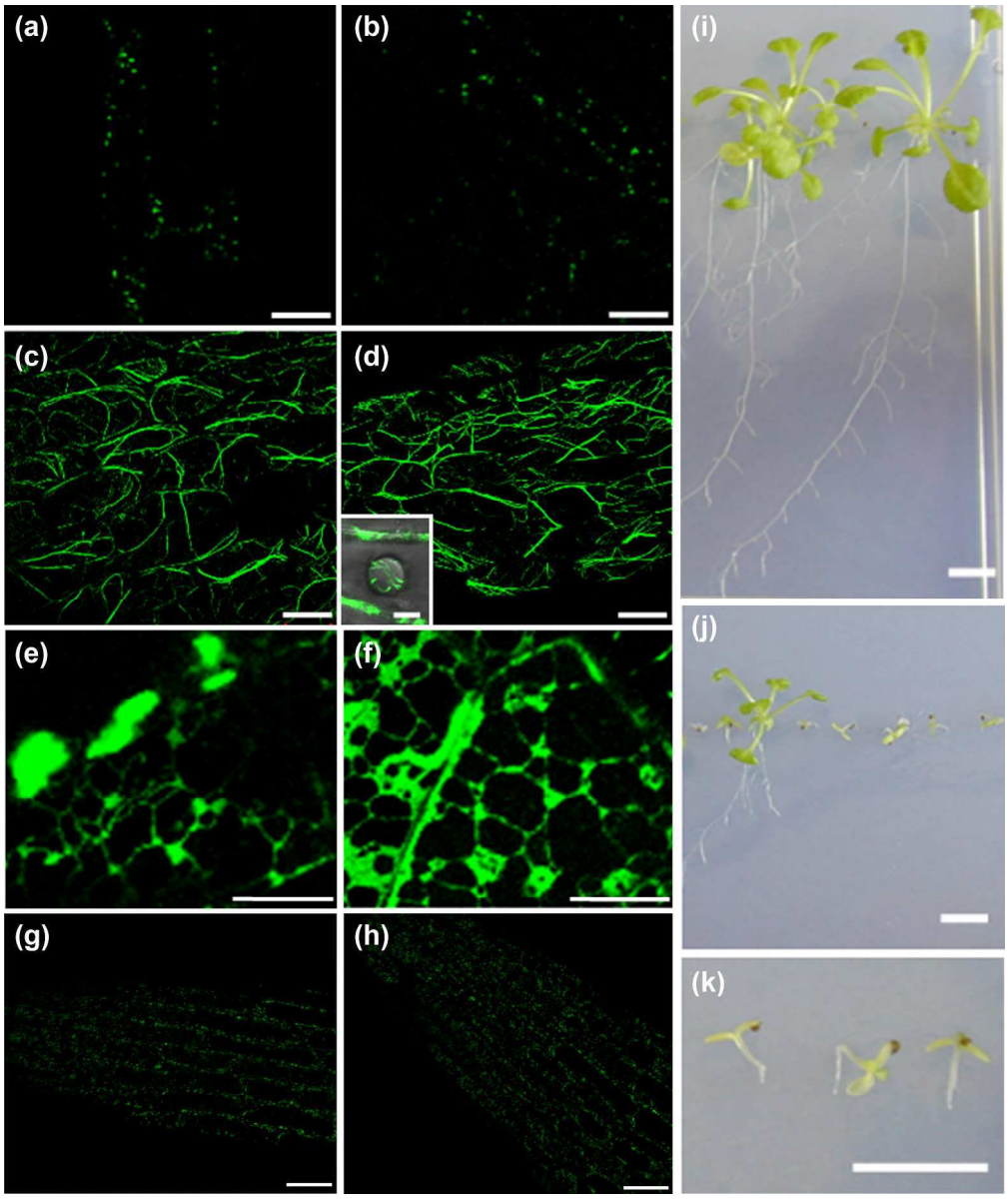

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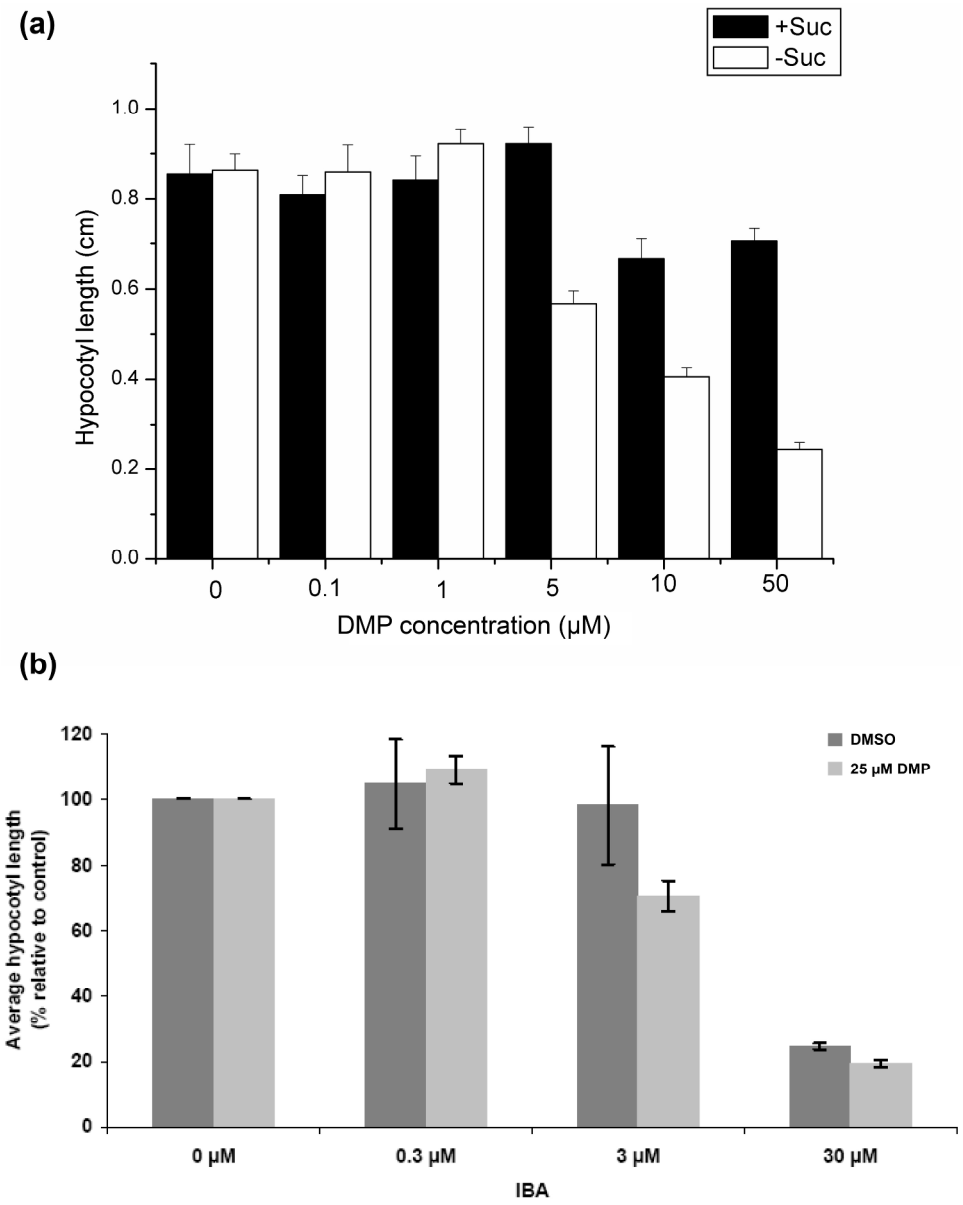

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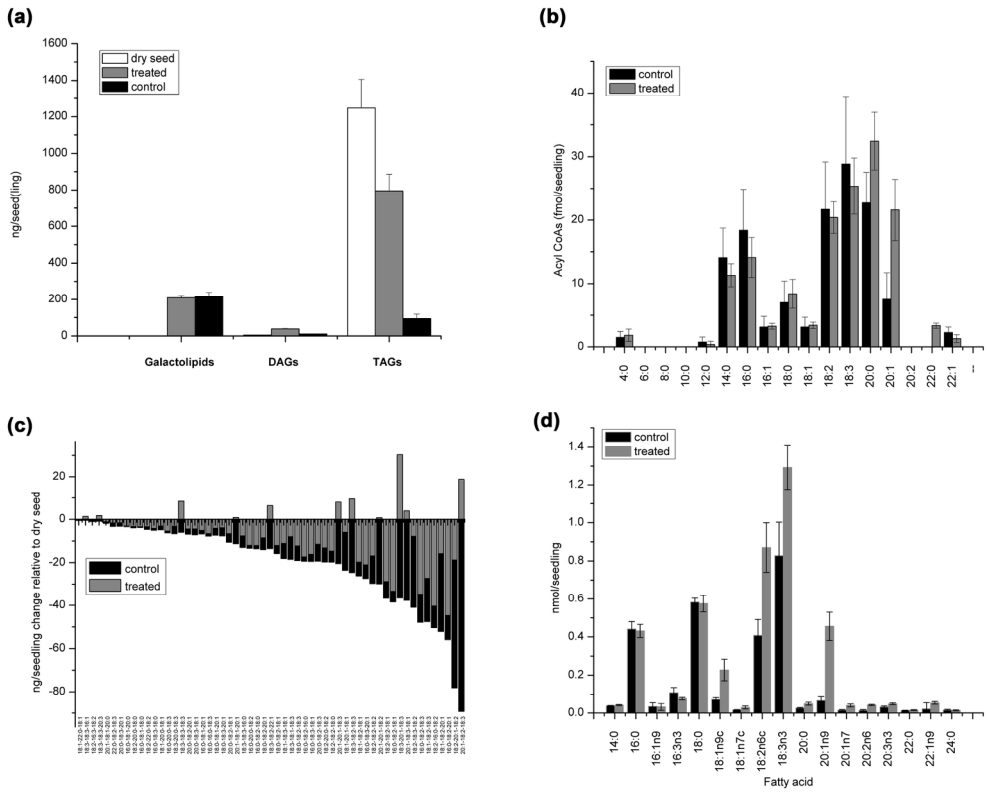

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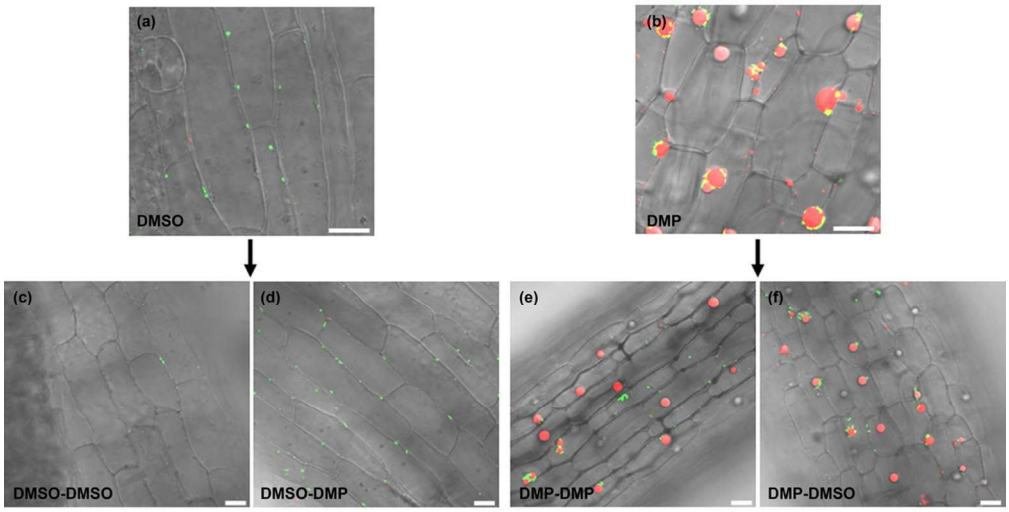

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