

Oil accumulation in leaves directed by modification of fatty acid breakdown and lipid synthesis pathways

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Summary

Plant oils in the form of triacylglycerol (TAG) are used for food, industrial feedstock and biofuel manufacture. Although TAG is typically harvested from the fruit or seeds of oil crop species, plants can also accumulate small amounts of TAG in the leaves and other vegetative tissues. Here we show that leaf TAG levels can be increased significantly (10–20 fold) by blocking fatty acid breakdown, particularly during extended dark treatments or leaf senescence in the model plant *Arabidopsis*. Generation of a double mutant in fatty acid breakdown and diacylglycerol acyltransferase 1 (*DGAT1*) resulted in a severe vegetative growth phenotype suggesting that partitioning of fatty acids to TAG in leaves is carried out predominantly by this acyltransferase. *LEC2*, a seed development transcription factor involved in storage product accumulation, was ectopically expressed during senescence in the fatty acid breakdown mutant *COMATOSE* (*cts2*). This resulted in accumulation of seed oil type species of TAG in senescing tissue. Our data suggests that recycled membrane fatty acids can be re-directed to TAG by expressing the seed-programme in senescing tissue or by a block in fatty acid breakdown. This work raises the possibility of producing significant amounts of oil in vegetative tissues of biomass crops such as *Miscanthus*.

Keywords: biofuel, triacylglycerol, beta-oxidation, diacylglycerol acyltransferase 1, *LEC2*.

Introduction

Production of biodiesel from plant vegetable oil, along with bioethanol, is providing an increasing contribution to the world fuel economy (Dyer and Mullen, 2008). Further development of biofuel production is currently limited by crop yield and availability of suitable land (Durrett *et al.*, 2008). To expand production, research has centred on low input and low maintenance biomass for cellulosic digestion, seed-oil crops for marginal land and algal biofuel (Durrett *et al.*, 2008; Dyer and Mullen, 2008). The possibility of producing triacylglycerol (TAG) for biodiesel in leaves or other bulk vegetative tissues has only recently been considered (Durrett *et al.*, 2008). This could engender high biomass crops grown on marginal land, or in aquatic environments, with oil-production capacity. Fatty acids derived from these TAGs represent an excellent replacement feed-

stock for fossil-derived hydrocarbon fuels (Hill *et al.*, 2006).

Triacylglycerol is produced in considerable levels in some seeds (up to 50% DW) and this occurs primarily via *de novo* synthesis of fatty acids in the plastid (Murphy, 2001; Durrett *et al.*, 2008). Fatty acid chains are condensed with the glycerol backbone via the ER-based Kennedy pathway that terminates with a final rate-limiting step catalysed by *DGAT1*. This is an *sn-3*-specific acyltransferase that generates TAG from acyl-CoA and diacylglycerol (DAG) (Murphy, 2001; Durrett *et al.*, 2008; Dyer and Mullen, 2008). Biogenesis of seed oil bodies (oleosomes) initiates in specific ER domains leading to oil droplets encompassed by a phospholipid monolayer and embedded by a protein coat chiefly comprised of oleosins (Murphy, 2001; Durrett *et al.*, 2008). Oil body diameters are restricted to 0.1–2 μm by the oleosins (Siloto *et al.*, 2006),

which may also maintain oil body integrity during seed maturation, desiccation and imbibition (Murphy, 2001). In non-seed oilcrops such as olive and palm, oil droplets rather than oil bodies accumulate in the fleshy pericarp of the fruit. The lack of oleosins or equivalent proteins in the oil droplets means that there is no restraint on coalescence and droplets can reach a size of 30 μm during fruit maturation (Salas *et al.*, 2000).

A recent field survey of 302 angiosperm species in the north-central USA found that 24% have conspicuous cytosolic oil droplets in leaves, with usually one large oil droplet per mesophyll cell (Lersten *et al.*, 2006). Relatively high TAG levels have been reported in stem tissues of Scots Pine and the desert plant *Tetraena mongolica* (5 and 10% DW respectively) (Durrett *et al.*, 2008). A specific role for cytosolic leaf TAG has not been proven but participation in carbon storage and/or membrane lipid re-modelling has been envisaged (Murphy and Parker, 1984; Murphy, 2001; Kaup *et al.*, 2002; Lin and Oliver, 2008). For instance, turnover of membrane lipids in photosynthetic *Arabidopsis* leaves has been estimated to be 4% of total fatty acids per day (Bao *et al.*, 2000). In senescing leaves, plastidial 16:3 fatty acids are partitioned into TAG prior to further mobilization, and DGAT1 is thought to be instrumental in this process (Kaup *et al.*, 2002). Notably, over-expression of the *Arabidopsis* DGAT1 gene in tobacco leaves results in enhanced TAG accumulation (Bouvier-Navé *et al.*, 2001). Leaf TAG accumulation is also seen in *Arabidopsis* mutants disrupted in *TGD1*, which encodes a plastid lipid permease (Xu *et al.*, 2005).

The fact that transcriptional control plays a major part in regulating oil accumulation in seeds offers a further opportunity to engineer oil production in non-seed tissues. A number of transcription factor genes have been identified that play a key role in storage oil and protein accumulation in developing *Arabidopsis* seeds, including *LEC1* (Mu *et al.*, 2008), *LEC2* (Santos-Mendoza *et al.*, 2005), *FUS3* (Wang *et al.*, 2007) and *WR11* (Cernac and Benning, 2004). Ectopic expression of these genes in non-seed tissues results in the induction of the seed reserve accumulation programme to varying extents (Cernac and Benning, 2004; Santos-Mendoza *et al.*, 2005; Wang *et al.*, 2007; Mu *et al.*, 2008). Seed storage reserve accumulation has also been reported in non-seed tissues of *Arabidopsis* mutants disrupted in *PICKLE* or in both of the *HSL2* and *HSL1* genes whose products repress embryonic traits (Ogas *et al.*, 1999; Tsukagoshi *et al.*, 2007). In the majority of these reports the occurrence of TAG is dependent on the provision of carbohydrates (Cernac and Benning,

2004; Casson and Lindsey, 2006; Tsukagoshi *et al.*, 2007).

Triacylglycerol is mobilized to sucrose during seed germination through the action of TAG-lipases, peroxisomal fatty acid β -oxidation, the glyoxylate cycle and gluconeogenesis (Graham, 2008). Disruption of a number of genes involved in this process results in a block in TAG breakdown, defects in seed germination and an inability to establish photosynthetic competence (Graham, 2008). These genes include *COMATOSE* (*CTS*), a peroxisomal fatty acid ABC transporter (Zolman *et al.*, 2001; Footitt *et al.*, 2002), *ACX1* and *ACX2* (Pinfield-Wells *et al.*, 2005), encoding the acyl-CoA oxidase enzymes that carry out the first step of peroxisomal β -oxidation and *KAT2* (Germain *et al.*, 2001), encoding keto-acyl thiolase which carries out the final step to produce acetyl CoA. Perhaps surprisingly, when rescued on sucrose and transferred to soil, mutants in these genes have so far displayed relatively few phenotypic abnormalities for the rest of the plant life-cycle (Footitt *et al.* (2007a,b), Theodoulou *et al.* (2005). Here we describe the effect of extended dark treatment on fatty acid breakdown mutants, showing that this leads to accumulation of TAG in the leaf and severe phenotypic effects (either wilting and necrosis or advanced senescence). We also show that TAG accumulates in naturally senescing leaves of fatty acid breakdown mutants and, through the production of double mutants, implicate *DGAT1* in the partitioning of fatty acids into TAG. Finally, we show that ectopic expression of the *LEC2* seed-development transcription factor during senescence of *cts2* results in accumulation of TAG molecular species that are similar to those found in seed oil.

Results and discussion

Blocking fatty acid breakdown leads to TAG accumulation in leaves subjected to extended dark treatment

To establish the contribution of fatty acid breakdown to energy balance in leaves, fatty acid breakdown mutants were subjected to extended dark treatments to test starvation responses (Figure 1). Plants harbouring one of two *CTS* peroxisomal ABC transporter mutant alleles, *cts2* (Footitt *et al.*, 2002) or *pxa1* (Zolman *et al.*, 2001), displayed severe wilting progressing to necrosis after 2–3 days of extended dark (Figure 1a; *pxa1* not shown). This dramatic response was associated with rapid

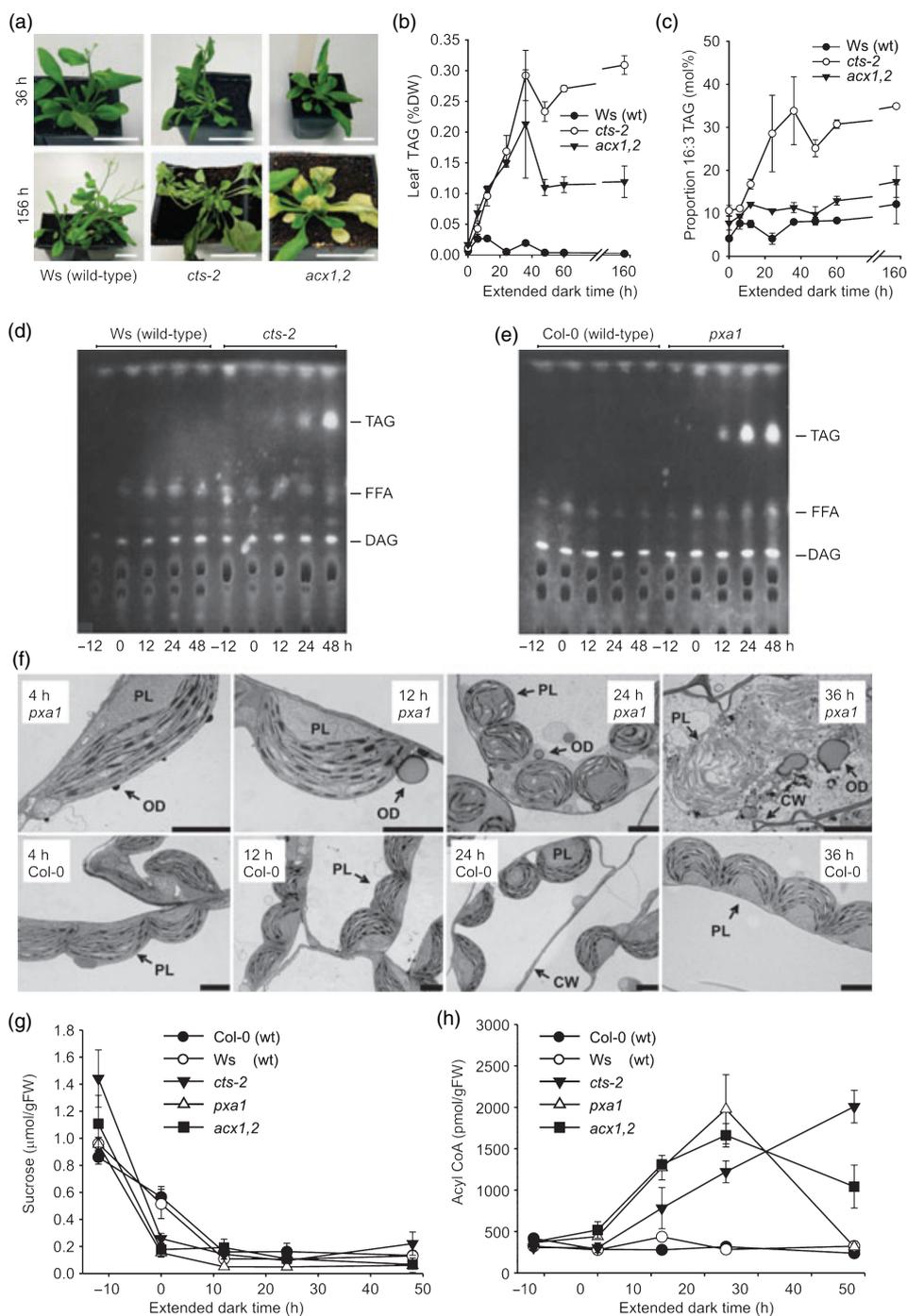


Figure 1 Dark treatment of *Arabidopsis* mutants blocked in fatty acid breakdown leads to TAG accumulation in leaves. Mutants were defective in the peroxisomal ABC-transporter *CTS* (*pxa1* or *cts-2* alleles) or acyl-CoA oxidase (*acx1,2* double mutant). Plants were grown in a 12 h light–dark cycle for 4 weeks and subject to extended dark at the end of the dark phase. (a) Appearance of mutants compared with wild-type control (Ws) at 36 h and 156 h extended dark (scale bar = 2 cm). Total leaf TAG content is shown (b) and also relative 16:3 TAG amount (c) during dark treatment as determined by LC-MS. Data are shown as mean \pm SE, $n = 3$. (d) and (e) Thin Layer Chromatographic analysis of total lipid extracts from leaves of *cts-2* (d) and *pxa1* (e) and their corresponding wild types in extended dark treatments. TAG accumulates in both mutant alleles of the peroxisomal ABC transporter but not in the wild type. (f) TEM indicating formation of oil droplets (OD) and changes in plastid (PL) morphology during stages of extended dark (indicated) in the *CTS* mutant (*pxa1*) compared with wild-type control (Col-0). CW = cell wall; scale bar = 2 μm. Sucrose levels (g) and acyl-CoA levels (h) are shown during normal dark phase (–12 to 0 h) and extended dark treatment for both *CTS* mutant alleles (*pxa1* and *cts-2*) and *acx1,2* compared with wild-type controls (Ws and Col-0). Data are shown as mean \pm SE, $n = 4$.

accumulation of leaf TAG to levels that were at least 10-fold that of the wild-type (Figure 1b). Wild-type plants showed little sign of stress even after 6 day dark treatment and rather than accumulating TAG, levels were actually depleted. A response similar to *cts-2* was observed with a keto-acyl thiolase mutant, *kat2* (Germain *et al.*, 2001) (not shown). However in a dark-treated acyl-CoA oxidase, *acx1,2* double mutant (Pinfield-Wells *et al.*, 2005), advanced senescence occurred (as determined by significant yellowing of the leaves) without wilting or necrosis (Figure 1a). Although TAG was also produced rapidly from the onset of extended dark it only attained half the levels observed in *cts-2* (Figure 1b). In *cts-2*, the proportion of plastid-derived 16:3 TAG (TAG species containing 16:3 fatty acids) increased from 10% at the onset of extended dark, to a maximum of about 35% after 24 h whereas in both *acx1,2* and wild-type, levels remained below 20% (Figure 1c). Thin layer chromatographic analysis (TLC) showed that TAGs accumulate in leaves of both *pxa1* and *cts-2* but not the corresponding wild types following dark treatment (Figure 1d,e), with a first appearance of TAG after 12 h extended dark.

Electron microscopy analysis of *pxa1* leaf tissue showed cytosolic oil droplets visible after only a few hours extended dark that ultimately formed large coalescing oil droplets by 36 h (Figure 1f). An alteration in plastid structure including disappearance of the outer and inner membrane by 36 h correlated with wilting of the plant (Figure 1f). Other organelles were not obviously altered in this analysis. Interestingly, dark treatment was associated with decreased sucrose content in all fatty acid breakdown mutants at the normal end of the night cycle ($t = 0$ time point) (Figure 1g). This suggested that lipid turnover in wild-type might contribute significantly to carbohydrate status in the absence of light and during normal diurnal cycling. Total acyl-CoA levels were elevated dramatically in the fatty acid breakdown mutants in extended dark (Figure 1h). CTS is normally involved in uptake of a range of substrates destined for peroxisomal metabolism, whereas ACX1 and ACX2 specifically catalyze long chain acyl-CoA substrates (Zolman *et al.*, 2001; Footitt *et al.*, 2002; Pinfield-Wells *et al.*, 2005; Hooks *et al.*, 2007; Graham, 2008). This distinction could explain the more extreme phenotype observed in the dark-treated CTS transporter mutants (e.g. wilting and necrosis in addition to TAG accumulation) in comparison to the *acx1,2* mutant (leaf yellowing and TAG accumulation, but no wilting and necrosis).

Blocking fatty acid breakdown leads to TAG accumulation in senescent leaves

To establish if a similar oil accumulation occurred under more natural conditions such as senescence, when fatty acid turnover is high, rosette leaves were harvested at different stages of senescence from plants on which 50% of siliques had turned yellow (Figure 2a). Elevated levels of TAG relative to wild-type were indeed apparent in senescing leaves from both *cts-2* and *pxa1* mutants with maximum TAG of 1–2% DW at stage 4 ($\geq 90\%$ yellow leaves) (Figure 2a). A similar accumulation of TAG was also observed in the *acx1,2* double mutant (not shown). The proportion of 16:3 TAG peaked later at stage 5 to stage 6 with loss of leaf turgidity, rising from 5% to 10–15% in both wild-type and mutant lines (Figure 2a). This later increase in 16:3 TAG is consistent with a delay in breakdown of ageing plastids (gerontoplasts) that is commonly observed during leaf senescence (Kaup *et al.*, 2002). The accumulation of TAG in senescing leaves differed from the production of TAG in *cts-2* and *pxa1* with extended dark treatment. In the latter case, the percentage of 16:3 TAG was much higher and TAG accumulation was associated with rapid plastid disruption (Figure 1c,f). Similar to the dark treated plants, TAG accumulation during senescence of *cts-2* plants resulted in the production of large, often coalescing cytosolic oil droplets (Figure 2b). Enlarged plastoglobuli characteristic of senescing leaves, were also observed in *cts-2* mutant cells but there was no significant difference in size and number of these compared with wild-type controls as determined by direct measurements of representative micrographs. This indicates that that the additional TAG seen in the leaves of mutant lines was not associated with enhanced plastoglobuli formation (Figure 2b). Notably, the total amount of TAG accumulating in senescing leaves was significantly higher (2–5 fold) than that of dark-treated leaves (compare Figures 1b and 2a).

Partitioning of fatty acids to TAG in leaves involves DGAT1

The *DGAT1* mutant SK353 (Col-0 ecotype) has previously been shown to be compromised in TAG accumulation during seed development, producing seed with 58–66% of TAG relative to wild type levels (Poirier *et al.*, 1999). The mutant was characterized further and found to have a splice-site mutation causing a frameshift upstream of a putative DAG-binding site motif (Zou *et al.*, 1999), suggesting that the gene encodes a non-functional

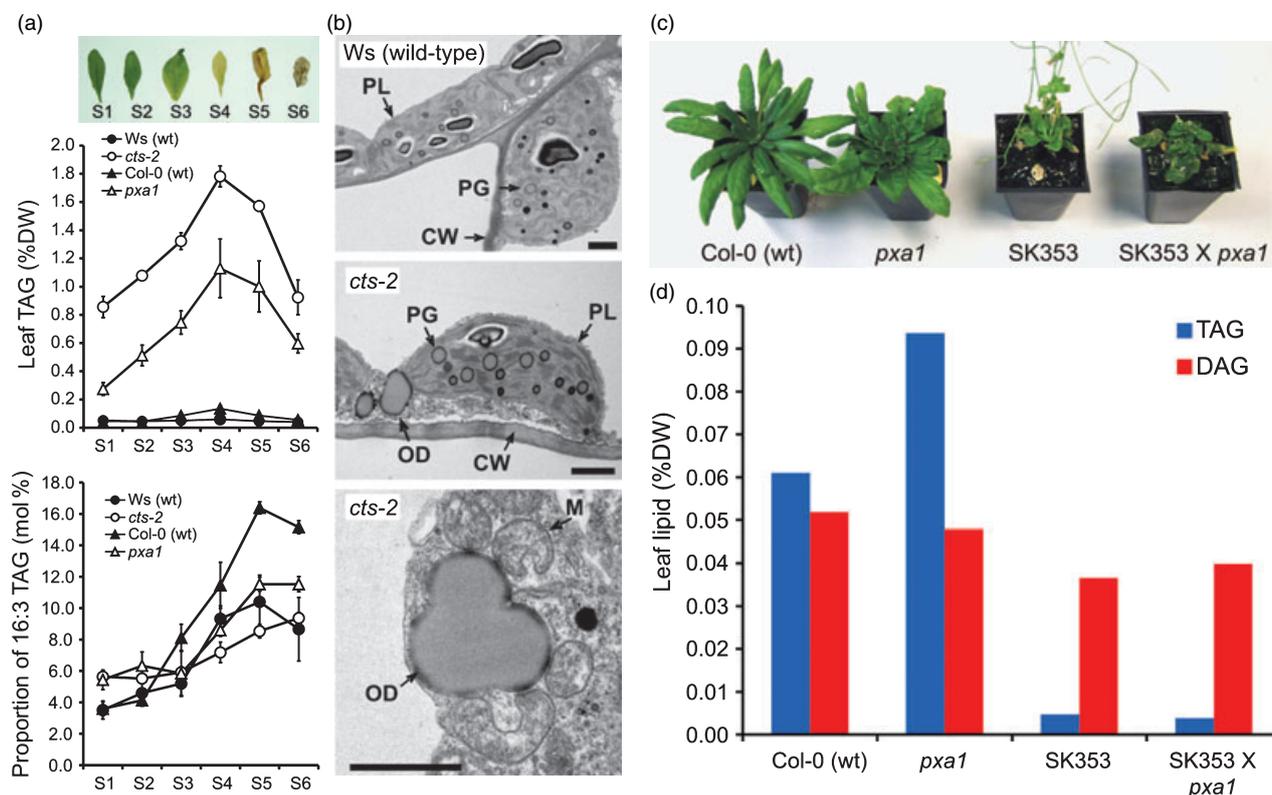


Figure 2 Senescing leaves of *Arabidopsis* mutants blocked in fatty acid breakdown accumulate TAG. The effect on foliar tissue because of mutations in fatty acid breakdown (a, b) and/or TAG synthesis (c) is shown. Mutants were defective in the *CTS* ABC-transporter (*pxa1* or *cts-2* alleles) and in *DGAT1* (SK353 allele). (a) Rosette leaves were harvested from plants with 50% yellowing siliques and leaves were graded according to senescence stage (S) (top panel): S1 (100% green); S2 ($\leq 10\%$ yellow); S3 (50% yellow); S4 ($\geq 90\%$ yellow); S5 (100% yellow with turgidity loss) and S6 (dry leaf). Accumulation of TAG in senescing leaves in *CTS* mutant lines (middle panel) and relative 16:3 TAG amount (lower panel) compared with wild-type control (Ws or Col-0) are shown. Data are shown as mean \pm SE, $n = 4$. (b) TEM analysis of stage 4 senescent leaves at maximum TAG production (S4, panel a) showing plastids (PL) with enlarged plastoglobuli (PG) in wild-type (Ws) and mutant (*cts-2*) cells (top and middle panels respectively). Large, often coalescing cytosolic oil droplets (OD) were also observed in *cts-2* mutant cells (middle and lower panels); CW = cell wall; scale bar = 1 μm . (c) Appearance of 4 week-old SK353 \times *CTS* (*pxa1*) double mutant compared with parent and wild-type (Col-0) lines grown in a 12 h light–dark regime. (d) TAG and DAG levels in total lipid extracts from young expanded leaves of the SK353 \times *pxa1* double mutant together with the single mutants and Col-0 wild-type (wt). Leaf lipids were extracted from young expanded leaves of 4 week-old plants (similar to those shown in Figure 2c) and separated by LC-MS to quantify TAG and DAG.

enzyme (Figure S1). SK353 also displayed an early flowering and senescence phenotype (Figure 2c). Notably, the SK353 mutant line accumulated significantly less TAG in young leaf tissues in comparison to either the wild-type (Col-0) or *pxa1* mutant lines, indicating that DGAT1 is a major factor in TAG synthesis in leaves as well as seeds (Figure 2d). The importance of DGAT1 in partitioning fatty acids to TAG in leaves of fatty acid breakdown mutants was assessed by crossing SK353 with *pxa1* (Figure 2c). In contrast to the parent lines, the phenotype of the double mutant was characterized by extreme retardation of growth, leaf thickening and yellow lesions. TAG production was impeded in the double mutant to the same extent as the SK353 parent (Figure 2d). Low TAG/DAG ratios are characteristic of *DGAT1* mutants (Poirier *et al.*, 1999) and consistent with this, the TAG/DAG ratios were

low in both SK353 and the double mutant whereas in wild-type and *pxa1* the ratios were above one. The severe growth phenotype in the double mutant (also observed in a cross of SK353 to *cts-2* and *kat2*, not shown) suggests that partitioning of fatty acids to TAG is essential for normal growth when fatty acid breakdown is impaired. The ability to partition fatty acids to TAG may represent an adaptive response that enables plant cells to avoid toxic effects of free fatty acids.

Ectopic expression of the seed development transcription factor, LEC2, results in TAG accumulation in senescing leaves

Ectopic expression of transcription factors responsible for the seed development programme can also lead to TAG

accumulation in non-seed tissues. TAG production in roots and unfertilized ovules has been reported in plants constitutively over-expressing *LEC2* (Stone *et al.*, 2008). This phenomenon has not been reported in senescing leaves however, which is the stage where most leaf TAG accumulates in the fatty acid breakdown mutants. We compared leaf TAG production in fatty acid breakdown mutants with that of previously published *LEC2* over-expression lines (Santos-Mendoza *et al.*, 2005). We first confirmed that dexamethasone (DEX) induction of *LEC2* activity resulted in TAG accumulation in the first true leaves of young seedlings on sucrose plates, as previously reported (Santos-Mendoza *et al.*, 2005) (Figure S2). Next we examined leaves of soil-grown plants following induction of *LEC2*. With young (3–4 week) plants on soil, this approach did not raise leaf TAG above wild-type control levels (data not shown). In fact enhanced leaf TAG accumulation was only observed once the plants had entered senescence (Figure 3a). Interestingly, the overall leaf TAG accumulation profile during senescence was highly reminiscent of the *cts-2/pxa1* mutants where peak levels also occurred at stage 4 ($\geq 90\%$ yellow leaves) (Figure 2a). The high proportion of seed-like TAG containing 20:1 (a fatty acid normally only found in seed oil) indicates that the ectopic expression of *LEC2* leads to induction of a seed-specific elongase in the senescing leaf tissues. EM analysis of stage 4 leaves showed cytosolic oil droplets in two independent transgenic lines and discrete non-coalescing droplets resembling oil bodies in developing embryos (Figure 3b). Number of plastoglobuli was highly variable in plastids of both wild type and transgenic lines. Very large starch grains were also present in plastids of senescing leaves ectopically expressing *LEC2* (Figure 3b). Interestingly, we observed similar accumulation of oil droplets and starch grains in young seedlings ectopically expressing *LEC2* when maintained on sucrose-containing media (Figure S2). Accumulation of starch as well as TAG in seedlings was also seen in a similar analysis of previously published *FUS3* inducible over-expression lines (Figure S2) (Wang *et al.*, 2007) and has been reported for the *turnip LEC1* allele and *pickle* repressor mutant (Ogas *et al.*, 1999; Casson and Lindsey, 2006). Significant changes in TAG accumulation in senescing leaves were not observed in the case of *FUS3* (data not shown).

Taken together, our data suggested that fatty acids from elevated turnover during senescence might be re-directed into TAG by ectopic expression of the seed-programme in leaves. It was not clear if the high level of seed-like TAGs was indicative of additional *de novo* syn-

thesis of fatty acids because of seed-programme induction or could be accounted for by the elongation of existing recycled fatty acids. Therefore, we considered if a block in fatty acid breakdown combined with the ectopic induction of the seed-programme could lead to further TAG accumulation in leaves.

To establish the interaction between fatty acid turnover and induction of the seed development programme, *cts-2* was crossed into the inducible *LEC2* over-expression line #31 (Figure 3c). The levels of TAG in senescing leaves of *cts-2* were approximately twofold that observed for senescing leaves ectopically expressing *LEC2* in a wild-type background. This indicated that of the two mechanisms, a block in fatty acid breakdown was the most effective at generating leaf TAG under our conditions. F_2 progeny that were homozygous for *cts-2* and carrying the *LEC2* inducible transgene, showed approximately the same levels of TAG accumulation as *cts-2* alone. This demonstrates that the two mechanisms for generating TAG in senescing leaves are non-additive, possibly because they are both utilizing fatty acids from membrane lipid turnover that would otherwise enter the β -oxidation pathway. The amount of 16:3 TAG was unchanged when *LEC2* was ectopically expressed in the *COMATOSE* background suggesting that turnover of plastid membrane lipids was unaffected by seed programme induction in senescing leaves.

Conclusions

In conclusion, these data clearly demonstrate that it is possible to engineer plants to accumulate TAG in foliar leaf tissues by two different mechanisms. We show that fatty acids that are normally catabolized can be used for TAG accumulation in leaves either by blocking fatty acid breakdown or introducing the seed-programme in senescing leaves. Metabolic engineering of the same mechanisms in crop plants could be achieved by inducing a block in fatty acid breakdown through gene silencing or by induction of the seed reserve accumulation programme using a senescence-regulated gene promoter. Such strategies would not compromise germination or plant development and could be particularly effective if designed to work in foliar material post-harvest.

The maximum levels of TAG observed were approximately 2% dry weight. Even this level could have a significant impact on the biofuels supply chain. For example, yields of up to 30 tonnes dry weight per hectare (t/ha) have been reported for the biomass crop *Miscanthus × giganteus* in Northern Europe and 99 t/ha for *Echinochloa*

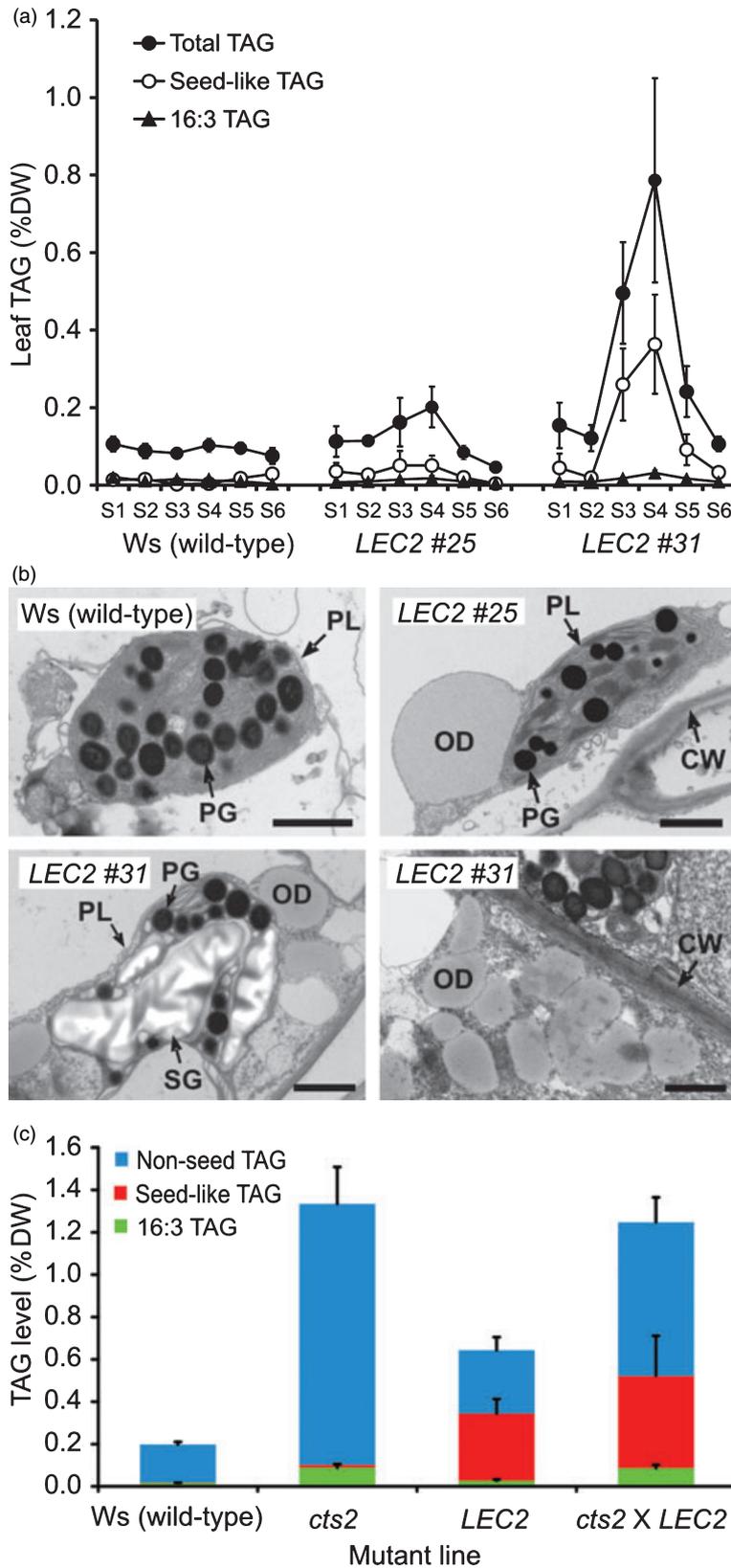


Figure 3 DEX-inducible *LEC2* over-expression also induces TAG accumulation in senescing leaves. (a) Total TAG accumulation in senescing leaves from two independent *LEC2* over-expression lines is shown along with wild-type control (Ws). Also shown are the 16:3 TAG (plastid origin) and seed-like TAG components (containing 20:1). Data are shown as mean \pm SE, $n = 3$. Plant material was sprayed with DEX at 50% silique yellowing, harvested 2 weeks after spraying and leaves were staged as for Figure 2a. (b) TEM analysis of senescing leaves at maximum TAG accumulation (S4) after 1 week DEX-induction showing plastids (PL) with prominent plastoglobuli (PG) in wild-type (Ws) and *LEC2* over-expressing lines. In addition, large, cytosolic oil droplets (OD) were also detected in *LEC2* over-expressing lines #25 (top right) and #31 (lower panels). SG = starch grain; CW = cell wall; scale bar = 1 μ m. (c) Leaf TAG levels in S4 senescent leaves (treated as above) from F_2 lines originating from a *cts-2* \times *LEC2* (*cts-2* line #31) cross that were homozygous for *cts-2* and carried the *LEC2* transgene ($n = 4$). TAG content was compared with parental lines (*LEC2* line #31, $n = 11$; *cts-2*, $n = 13$) and wild-type (Ws) controls ($n = 15$). Data are shown as mean \pm SE.

polystachya (another C_4 grass) in the Amazonian basin (Heaton *et al.*, 2008). If 2% of this dry mass were oil, this would translate to 0.6 and 2.0 t/ha respectively. The average dry weight yield derived from all published trials of *Miscanthus* is 22 t/ha (Heaton *et al.*, 2004), which would give 0.4 t/ha of oil. This compares favourably with world oilcrop yields (2005/2006) for rapeseed and soybean of 1.2 t/ha and 0.5 t/ha respectively (Durrett *et al.*, 2008). Additionally, the fact that *Miscanthus* is typically harvested after the growing season when it has senesced makes the proposition to generate oil in this particular species extremely feasible using the methods outlined herein. The fact that TAG accumulates in oil droplets in senescing tissue can also be seen as an advantage since it should be relatively easy to extract by mild mechanical methods, as is typically used for extraction of oil from the fruit pericarp of olive or palm (Salas *et al.*, 2000).

Increasing levels of TAG above 2% dry weight may be possible by further manipulation of both synthesis and breakdown. With respect to controlling breakdown it is possible that, as with TAG accumulation in seeds, TAG-lipases could be involved in re-cycling of fatty acids from TAG and compromising yield (Eastmond, 2006). If so, these lipases could be further targets for manipulation. With respect to increasing flux of carbon into TAG, it is interesting to note that fatty acid synthesis and β -oxidation rates are both elevated in the *Arabidopsis fatb* mutant, which is disrupted in a plastidial acyl-ACP thioesterase (Bonaventure *et al.*, 2004). Unfortunately, we found that *fatb* crossed with several fatty acid breakdown mutants (*cts-2*, *pxa1*, *kat2*) resulted in embryo lethal phenotypes (not shown). The possibility remains that *de novo* fatty acid synthesis could be increased by tissue-specific manipulation of specific targets such as *FATB*. Further refinement of the ectopic expression of the suite of already identified seed development transcription factors could also lead to further gains in foliar TAG. Finally, it may also be extremely useful to characterize the mechanism of TAG accumulation in species such as the desert plant *T. mongolica* (Durrett *et al.*, 2008) and use such knowledge to engineer higher oil production in target crops.

Experimental procedures

Plant materials

Seeds were surface sterilized and germinated in continuous light on plates containing 1/2 MS media, 1% (w/v) agar with 20 mM sucrose, where indicated. Seeds were then stratified for

72 h at 4 °C in the dark and transferred to a growth cabinet with 18 h light (70 $\mu\text{mol}/\text{m}^2/\text{s}$) at 20 °C. Where required, germination was induced by nicking the testa with a sterile needle. Fatty acid breakdown mutants were phenotypically identified by radical protrusion without further establishment in the absence of sucrose and transferred to sucrose plates. Lines carrying the DEX-inducible *LEC2:GR* transgene (#25 and 31, as described in Santos-Mendoza *et al.*, 2005) were initially selected with kanamycin and transferred to sucrose plates containing 10 μM DEX. Seedlings showing pronounced leaf embryo-like morphology (Santos-Mendoza *et al.*, 2005) were recovered on sucrose plates without DEX and then transferred to soil. DEX-inducible *FUS3* over-expression lines (#4, 5 and 14) were initially selected on hygromycin and treated as above (Wang *et al.*, 2007). In the dark treatment experiments, plants were grown in a Sanyo growth cabinet with a 12 h light (20 °C)/12 h dark (20 °C) cycle at 140 $\mu\text{mol}/\text{m}^2/\text{s}$. Dark treatment was initiated at the end of the diurnal dark period at 4 weeks. Senescence experiments were carried out on plants grown in a Percival Scientific growth chamber with 6 weeks of short days [8 h light (20 °C)/16 h dark (20 °C)] followed by 4 weeks of long days (16 h light/8 h dark) with humidity at 70% throughout and with light at 170 $\mu\text{mol}/\text{m}^2/\text{s}$. For *LEC2* or *FUS3*-induction experiments, plants were sprayed twice a week with 30 μM DEX for 2 weeks prior to harvest. Harvesting of senescent rosette leaves was carried out after approximately 50% of siliques showed yellowing.

Transmission electron microscopy

Leaf material was vacuum-infiltrated with 2.5% glutaraldehyde in 50 mM potassium phosphate (KPi) buffer with 2 h incubation on a rotor, followed by three 30 min wash steps with KPi. Tissues were post-fixed in 1% osmium tetroxide (OsO_4) for 1 h on ice followed by washes in KPi as above. Step-wise dehydration was performed in acetone dilutions (25%, 50%, 70%, 90% and 100%) with a 30 min incubation on a rotor followed by incubation in resin (Agar Scientific, Stansted, UK) dilutions (25%, 50%, 70%, and 100% twice). The leaf sections were embedded in the pre-polymerized resin at 70 °C. The samples were stained with 2% uranyl acetate and 0.5% lead citrate. Micrographs were taken with a Philips 410 transmission electron microscope.

Metabolite analyses

Neutral lipids were extracted from 5–10 mg freeze-dried leaf samples by incubation in 400 μL hot isopropanol (75 °C) for 15 min with 0.5 mg/mL ^{13}C tri-olein standard and cooled to RT. Hexane was added to achieve hexane/isopropanol (3 : 2 v/v) and incubated at RT for 30 min. Three further extractions in hexane/isopropanol (3 : 2 v/v) were carried out. Lipid was partitioned into hexane with 1/2 vol 6.7% sodium sulphate and dried down in HPLC tapered vials in a speedi-vac and reconstituted in 100 μL ethanol. Separation of TAG and DAG by LC-MS was carried out as described (Burgal *et al.*, 2008). For Thin Layer Chromatography, total lipids extracts were developed in the solvent system: Hexane : Diethylether : Acetic Acid (70 : 30 : 1 v/v). Lipids were

visualized by spraying plates with Fluorescein and exposing to UV light. Acyl-CoA analysis was carried out on 10 mg of fresh leaf material ground in liquid nitrogen. Separation procedures and preparation of standards were followed as described (Ishizaki *et al.*, 2005). Sucrose measurements were carried out on 10 mg of fresh leaf material homogenized in 0.5 mL 80% ethanol and incubated at RT for 1 h with intermittent vortexing. After centrifugation the pellet was dried under vacuum and resuspended in 0.2 mL water. Sucrose was then quantified using an enzymatic test kit (Boehringer Mannheim/Roche, R-Biopharm, Rhone Ltd, Glasgow, UK).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The SK353 mutant allele of *Arabidopsis* *DGAT1* is caused by a splice-site mutation that is predicted to generate a truncated protein lacking a putative DAG-binding motif.

Figure S2 Large starch grains and cytosolic oil bodies are associated with DEX-induced expression of *LEC2* and *FUS3* in seedlings.

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